Regulation of the innate immune phenotype in atherosclerosis:

navigating the inflammatory landscape

Helin Tercan

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Regulation of the innate immune phenotype in atherosclerosis: navigating the inflammatory landscape

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according to the decision of the Doctorate Board
to be defended in public on

Thursday, January 30, 2025
at 10:30 am

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Table of contents

Chapter 1:
General introduction and outline of thesis 9

Chapter 2:
Trained immunity: long-term adaptation in innate immune responses 23

Chapter 3
Clonal hematopoiesis is associated with cardiovascular events in patients with stable coronary artery disease 41

Chapter 4:
The association between clonal hematopoiesis driver mutations, immune cell function and the vasculometabolic complications of obesity 79

Chapter 5:
Association between DNMT3A-driven clonal hematopoiesis, trained immunity and immune cell function in obesity 117

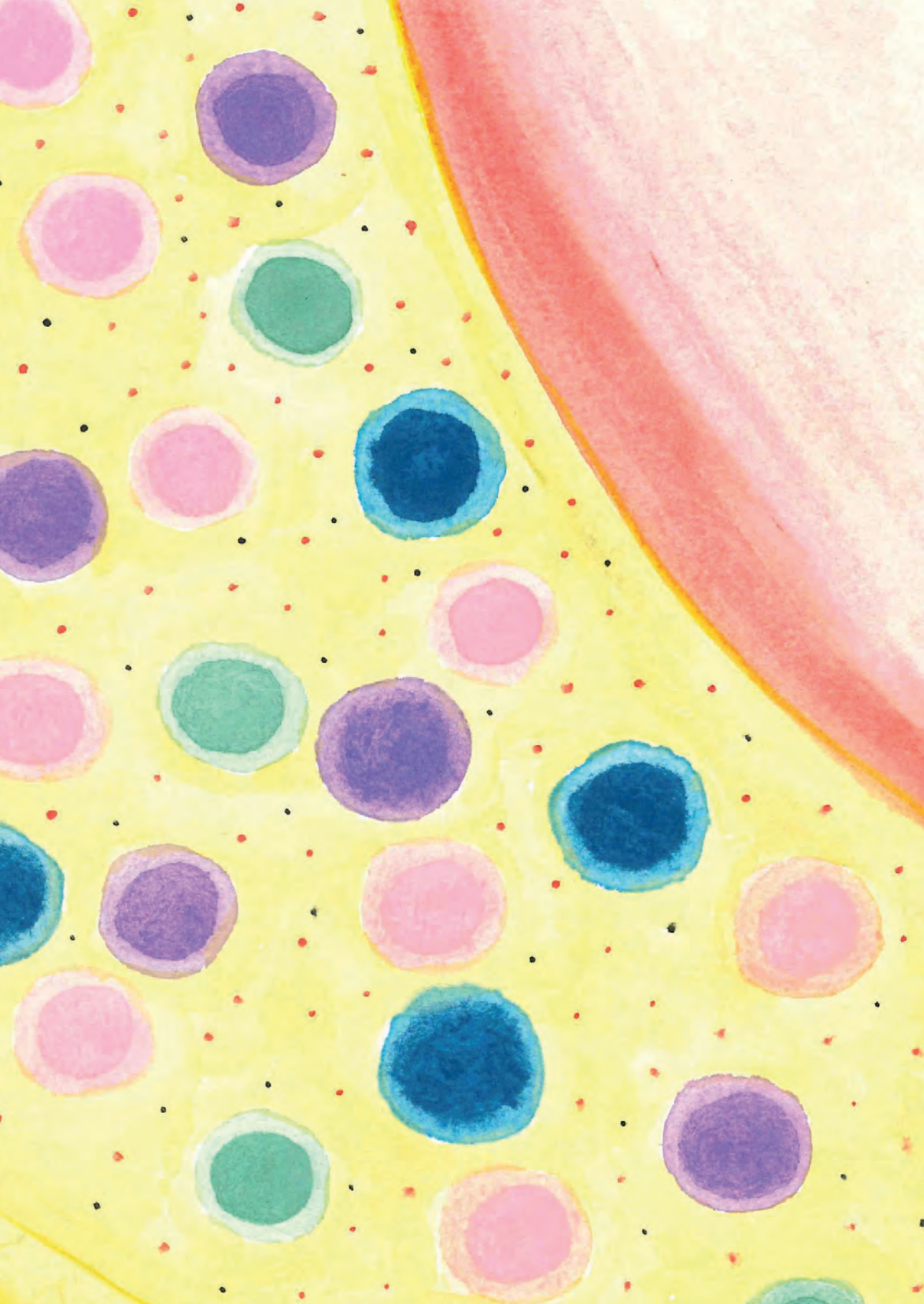
Chapter 6:
The Effect of Low-Dose Colchicine on the Phenotype and Function of Neutrophils and Monocytes in Patients with Chronic Coronary Artery Disease: a double-blind randomized placebo controlled cross-over study 147

Chapter 7:
Summary 197

Chapter 8:
General Discussion 203

Chapter 9:
Nederlandse samenvatting 217

Appendices 223



Chapter 1:

General introduction and outline of thesis

Atherosclerotic cardiovascular disease

The history of atherosclerosis predates our understanding of modern medicine. Despite prevailing knowledge that atherosclerosis is a modern disease associated with sedentary life style, it has been shown that in preindustrial populations, including one preagricultural hunter-gatherer group, atherosclerosis was a common malady[1]. A more modern misconception was that a middle-aged white man was the prime candidate to suffer from a heart attack. We now know that risk of developing atherosclerosis reaches beyond western populations and affects younger adults, women and people with diverse ethnic backgrounds[2]. Thus, addressing this global healthcare threat is now even more pressing.

Atherosclerotic cardiovascular disease (ASCVD) refers to diseases caused by atherosclerosis such as coronary artery disease, stroke and peripheral artery disease. Atherosclerosis is a lipid-driven pathophysiological process characterized by accumulation of low density lipoprotein (LDL) and remnant lipoproteins in the arterial wall. In addition to the well-established causal contribution of lipids to disease pathophysiology, chronic low-grade inflammation plays a major role in every stage of the disease[3]. Within atherosclerotic plaques, monocyte-derived macrophages are the most abundant immune cells, and they play important roles in initiation and progression of the plaques[4]. In addition, accumulating evidence points to pathogenic roles of neutrophils in atherogenesis[5,6]. The traditional risk factors for atherosclerosis include dyslipidemia, hypertension, obesity, diabetes, smoking and sedentary lifestyle. Although obesity and atherosclerosis are distinct conditions, they share common pathophysiological mechanisms. As inflammation is critical for all stages of atherosclerosis, it is also associated with obesity, type II diabetes and insulin resistance[7]. This strengthens the mechanistic link between obesity and atherosclerosis.

In the past years it has been established that these risk factors can induce long-term rewiring of the innate immune cell phenotype, which is termed ‘trained immunity’. Trained immunity could mediate, or contribute to, the pro-atherogenic effects of these traditional risk factors[8]. In addition, clonal hematopoiesis has recently been identified as a new immunological mechanism that can lead to immune cell activation and can contribute to atherogenesis[9]. In this thesis, I investigated these two novel immunological mechanisms in the context of atherosclerosis, and how they can interact.

Currently, prevention of ASCVD is largely restricted to pharmacological reduction of risk factors, such as hypercholesterolemia or hypertension. However, despite optimal risk factor reduction, a substantial residual cardiovascular risk remains in many patients. Recent large clinical trials showed that anti-inflammatory drugs, such as colchicine, can further reduce the residual risk[10]. How this treatment affects the innate immune system is not yet fully understood. Therefore, in Chapter 6, I studied in detail how colchicine treatment modulates monocyte and neutrophil phenotype and function.

Atherosclerotic plaque formation

Atherosclerotic plaque formation in the arteries starts with increased expression of adhesion molecules on endothelial cells, making them a sticky surface for leukocyte adhesion. In parallel, the permeability and composition of the endothelial layer becomes altered, enabling entry of apoB containing lipoproteins such as LDL. Within the plaque microenvironment, LDL is oxidized. The oxidized LDL (oxLDL) particles can further activate endothelial cells. Eventually, circulating monocytes attach to the activated endothelial cells, and enter the intimal space, where they differentiate into macrophages. In the early stages of atheroma formation, macrophages take up oxidized lipoproteins via scavenger receptors and become foam cells. In addition, macrophages secrete cytokines and chemokines, which further fuels plaque inflammation. As plaque formation progresses, smooth muscle cells migrate from the media to intima. Smooth muscle cells and foam cells die in the plaque and accumulate in the center of the plaque, termed the necrotic core. In advanced plaques, cholesterol crystals can be found in the necrotic core, contributing to activation of NLR family pyrin domain containing 3 (NLRP3) inflammasome in macrophages. Eventually, disruption of the plaque can occur by rupture or erosion, which triggers the formation of a thrombus in the arterial lumen which can completely occlude the artery. In addition to rupture of the plaque, superficial erosion can also precede thrombus formation. Erosion does not involve rupture of the plaque but involves superficial injury to the endothelium. This endothelial injury is largely induced by neutrophils and their extracellular traps (NETs)[2,11].

Innate immune cells in atherosclerosis

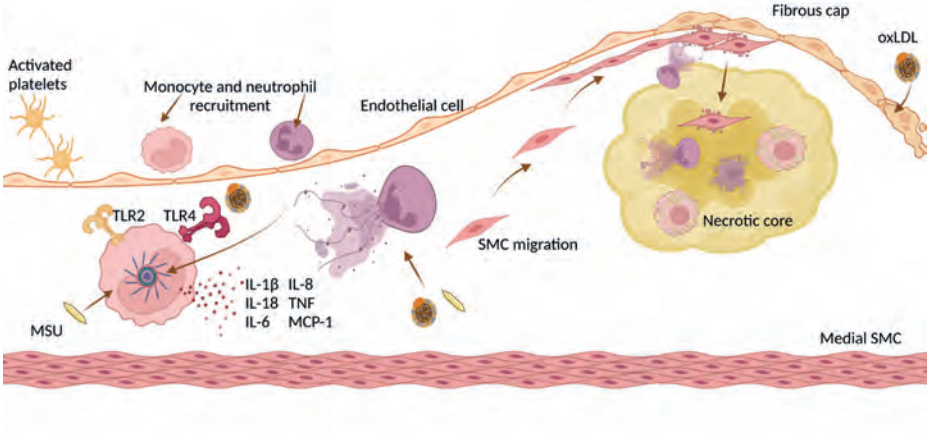


Figure 1: Schematic representation of the inflammatory events in atherosclerosis. Activated platelets promote recruitment of monocytes and neutrophils. Infiltrated monocytes and neutrophils contribute to inflammation by the secretion of proinflammatory molecules. Medial smooth muscle cells (SMC) migrate and accumulate around the fibrous cap. SMCs undergo cell death upon exposure to NETs. Figure created with BioRender.com (Agreement number: MC26UVKUKK).

Monocyte-derived macrophages are the chief producers of inflammatory cytokines and chemotactic molecules in the atherosclerotic plaque. Endogenous molecules such as oxLDL in the plaque can act as Damage Associated Molecular Patterns (DAMPs) and activate Pattern Recognition Receptors (PRRs) on monocytes and macrophages. oxLDL particles can bind to CD36 scavenger receptor on the membrane, leading to IL-6, IL-8, TNF and MCP-1 production via TLR2 and TLR4 activation[12,13]. Additionally, cholesterol or mono sodium urate (MSU) crystal induced NLRP3-inflammasome activation leads to IL-1β production by macrophages[14].

Possibly owing to their short life span, neutrophils have been largely overlooked in the context of atherosclerosis. In recent years this view has shifted, and neutrophils gained considerable attention. Neutrophils are excellent phagocytes, and are armed with granules packed with proteolytic

enzymes. They can produce large quantities of Reactive oxygen species (ROS) and expel their DNA content in the form of NETs, immobilizing pathogens. However, disturbances to tissue homeostasis can render neutrophils hyperactivated, contributing to thrombus formation and rupture[15].

Neutrophils are cardinal producers of several chemotactic proteins such as CCL2, cathepsin G, cathelicidin and α-defensins which induces monocyte influx to the area of inflammation[16–19]. Moreover, excessive NET formation can induce NLRP3 inflammasome activation and eventually IL-1β and IL-18 production by macrophages. Smooth muscle cells (SMCs) are also in contact with NETs and their decor of cytotoxic histone H4. This can lead to death of SMCs, and aggravation of SMC death can disrupt the fibrous cap[20].

The importance of neutrophils is also illustrated by the observation that the neutrophil counts are increased in ASCVD. Epidemiological studies show that increased neutrophil-to-lymphocyte ratio can predict future cardiovascular events. In fact, neutrophil counts alone are determinants of future cardiovascular events[21].

Clonal hematopoiesis

Circulating innate immune cells are derived from their hematopoietic progenitor cells in the bone marrow niche. Hematopoietic stem and progenitor cell (HSPC) metabolic activity can be measured using ^{18}F-fluorodeoxyglucose positron emission tomography/magnetic resonance imaging (^{18}F-FDG PET/MRI). Recent studies using this imaging modality showed activation of bone marrow already at early stages of atherosclerosis[22].

Bone marrow is a highly proliferative tissue, responsible for continuous production of immune cells. Proliferative tissues are prone to stochastic acquisition of somatic mutations at a higher rate. Most of these mutations will be eliminated given they do not provide a selective advantage. However, if a mutation confers a fitness or survival advantage to the hematopoietic cell that it arose in, it will lead to the clonal expansion of the HSC and its progeny. This process is termed clonal hematopoiesis[23].

Clonal hematopoiesis was first discovered while studying the non-random inactivation of the X chromosome in healthy woman. As a result of non-

random inactivation more than expected proportion of blood cells were found to share the same silencing pattern, suggesting that they are derived from the same clone[24]. In a supercentenarian woman approximately 65% of the peripheral blood cells were found to be derived from 2 related HSC clones[25]. More recently, it has been shown that mosaic loss-of Y chromosome in the hematopoietic lineage coincides with clonal hematopoiesis in men[26].

Clonal hematopoiesis driver mutations (CHDMs) are predominantly identified in a set of genes, the most common ones being DNMT3A, TET2, ASXL1, TP53, JAK2 and SF3B1. The driver mutation of clonal hematopoiesis might not be identified in some cases and is therefore attributed to neutral drift[23].

Originally, clonal hematopoiesis was thought to be solely linked with hematological malignancies. However, large epidemiological studies showed discrepancy between the number of cases of clonal hematopoiesis and the prevalence of hematological malignancies which led to the remarkable discovery of the link between clonal hematopoiesis and increased risk of cardiovascular disease[27,28]. This was observed particularly in association with clones with a variant allele frequency (VAF) of ≥2%. VAF indicates the size of the clone and the individuals harboring a clonal hematopoiesis mutation with a VAF of ≥2%, and without evidence of hematologic malignancy, dysplasia, or cytopenia, is defined as clonal hematopoiesis of indeterminate potential (CHIP). The CHIP criterion is initially determined by the limitations of the utilized sequencing methodology[23].

Even though many epidemiological studies show that the presence of clonal hematopoiesis predisposes to ASCVD, the exact mechanism and the direction of this association is not fully understood. This causal association is predominantly studied in TET2 and DNMT3A knockout mouse models. TET2 and DNMT3A are epigenetic modifier enzymes with opposing roles, yet the consequences of having TET2 or DNMT3A mutations are convergent on clonal hematopoiesis with a risk of cardiovascular disease. TET2 knockout mouse models of clonal hematopoiesis showed increased IL-1β production by plaque macrophages causally linking CH to atherosclerosis[29]. Similarly, loss of DNMT3A in murine myeloid cells accelerated atherosclerosis[30]. However, only a few studies in humans investigated how the presence of CH affect the leukocyte phenotype.

Trained immunity

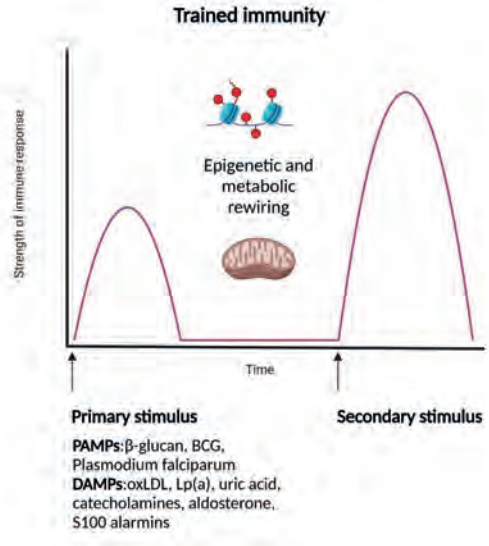


Figure 2: Schematic representation of the trained immune response. The primary stimuli can be various PAMPs and DAMPs, and the restimulation with an unrelated secondary stimulus evokes an increased immune response. Figure created with BioRender.com (Agreement number: JJ26UVPI2M).

In the past ten years, researchers from our laboratory and others have identified a novel immunological mechanism that can contribute to atherosclerosis development, which is called trained immunity. Trained immunity defines the memory characteristics of innate immune cells. Brief exposure to various PAMPs (β-glucan, BCG, Plasmodium falciparum) or DAMPs (oxLDL, Lp(a), uric acid, catecholamines, aldosterone and S100 alarmins) can induce an enhanced immune response to a subsequent unrelated secondary stimulus[31]. This persistent hyperresponsive phenotype is orchestrated by metabolic and epigenetic changes (Figure 2). In Chapter 2, I describe the specific metabolic and epigenetic pathways that drive trained immunity, and how trained immunity can contribute to atherosclerosis pathophysiology. It is crucial to emphasize that IL-1β is central to trained immunity and TET2 driven clonal hematopoiesis, possibly linking these two phenomena[32,33].

Anti-inflammatory therapies for atherosclerosis

As mentioned above, for a long time strategies to prevent ASCVD were only focused on risk factor reduction, such as statins for LDL cholesterol lowering and beta blockers or diuretics as antihypertensive[34]. Only in the last decade, driven by increased understanding of the key role of inflammation in atherogenesis, studies have also shown that anti-inflammatory drugs can lower CVD risk[11]. The CANTOS trial was the first phase III clinical trial that showed effectiveness of anti-inflammatory therapy in atherosclerosis by specifically targeting IL-1β with a monoclonal antibody (canakinumab). In patients with stable coronary artery disease, treatment with canakinumab (150 mg, 4 times a year) showed a 15% reduction in major adverse cardiovascular events (MACE). However, there was a statistically significant increase in infections in the group receiving canakinumab[35]. Therefore, the CANTOS trial emphasized the balance of benefit-to-risk ratio in anti-inflammatory therapies. Following the original CANTOS trial, a substudy showed that individuals with clonal hematopoiesis due to TET2 mutations responded better to canakinumab, emphasizing the association between TET2 mutations and elevated IL-1β production[36].

Other landmark trials investigated the effect of low-dose colchicine treatment. This ancient anti-inflammatory drug, extracted from the *Colchicum Autumnale*, has been on the market as early as 1500 Before Common Era, and it has been used in the treatment of gout and Familial Mediterranean Fever. This affordable and effective anti-inflammatory drug was repurposed for the treatment of coronary artery disease. Colchicine's main anti-inflammatory role stems from the disruption of microtubule assembly, thereby inhibiting key leukocyte functions such as chemotaxis, cytokine release, and phagocytosis[37].

The low-dose colchicine (LoDoCo) trial was the first study investigating daily use of colchicine at a low dose (0.5 mg/day) for the treatment of coronary artery disease. The LoDoCo study had a prospective randomized open-label, blinded end-point design, and reported a significant decrease in composite incidence of acute coronary syndrome, out-of-hospital cardiac arrest, or non-cardioembolic ischemic stroke[38]. Following the success of LoDoCo, two larger clinical trials, Colchicine Cardiovascular Outcomes Trial (COLCOT) and LoDoCo2, demonstrated the effectiveness of colchicine for reducing MACE in patients with recent acute coronary syndrome (30 days within event), and stable coronary artery disease (6 months after the event) respectively[39,40].

A substudy of the LoDoCo2 trial, conducted during the 30-day run-in period, included 174 patients. After 4 weeks of daily low-dose colchicine treatment, an untargeted proteomics assay showed a decrease in neutrophil degranulation-related biomarkers[41]. This finding highlights the relevance of modulation of inflammation driven by innate immune cells. Based on this, Chapter 6 of this thesis investigates the immune phenotype of neutrophils and monocytes upon colchicine or placebo treatment.

Aims and Outline of the Thesis

The aim of this thesis was to investigate how clonal hematopoiesis and trained immunity, as two newly identified immunological mechanisms, shape the immune phenotype of monocytes and neutrophils, the two protagonists of atherosclerosis, and how this relates to the development of atherosclerosis and ASCVD.

Chapter 2 outlines the epigenetic and metabolic pathways involved in the induction of trained immunity in relation to cardiovascular disease and beyond.

In **Chapter 3** we studied the association between CH and a first MACE in a cohort of patients with stable coronary artery disease. By using a targeted ultrasensitive smMIP sequencing approach we examined the effects of large clones (VAF≥2%), and small clones (VAF<2%). Specifically, we investigated the association between CH and innate immune phenotype and function.

In **Chapter 4** we investigated how CH relates to immune cell function, systemic inflammation and vasculometabolic functions in a cohort of individuals with obesity. Additionally, we studied the association of clonal hematopoiesis and immune parameters in a sex stratified manner.

Based on our findings in Chapter 4, in **Chapter 5** we selected individuals with DNMT3A mutations and individuals without CHDMs to characterize the innate immune phenotype and function, and we investigated whether CH affects the susceptibility to build trained immunity.

Lastly, in **Chapter 6** we examined in a double-blind randomized controlled trial in patients with a history of a myocardial infarction, how colchicine treatment affects monocyte and neutrophil phenotype and function.

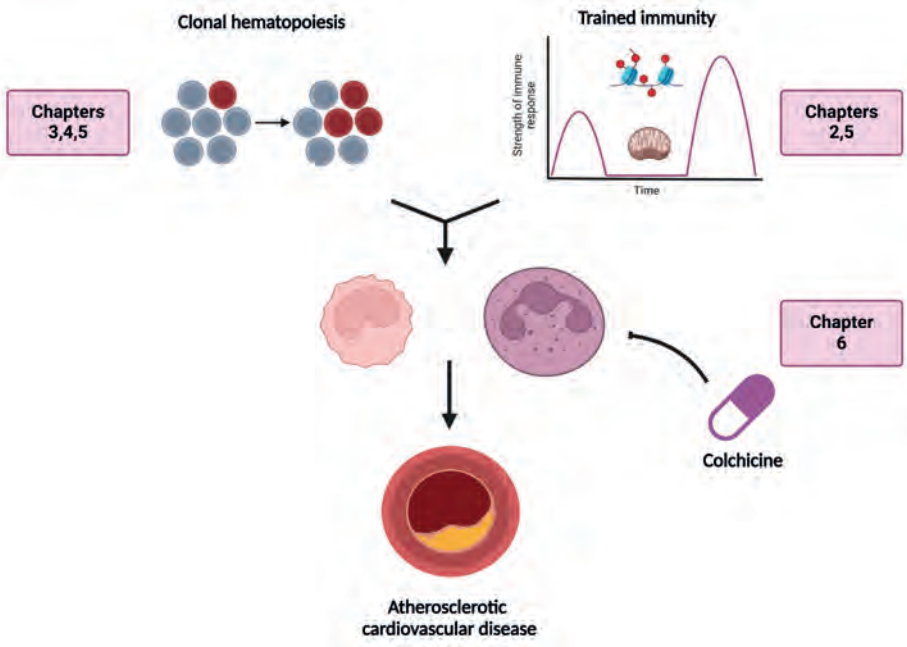


Figure 3: Diagram summarizing the contents of this thesis. Figure created with BioRender.com Agreement Number:AR26XV42NM

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1

Chapter 2:

Trained immunity: long-term adaptation in innate immune responses

Helin Tercan; Niels P. Riksen; Leo A.B. Joosten; Mihai G. Netea Siroon Bekkering*
*Corresponding author

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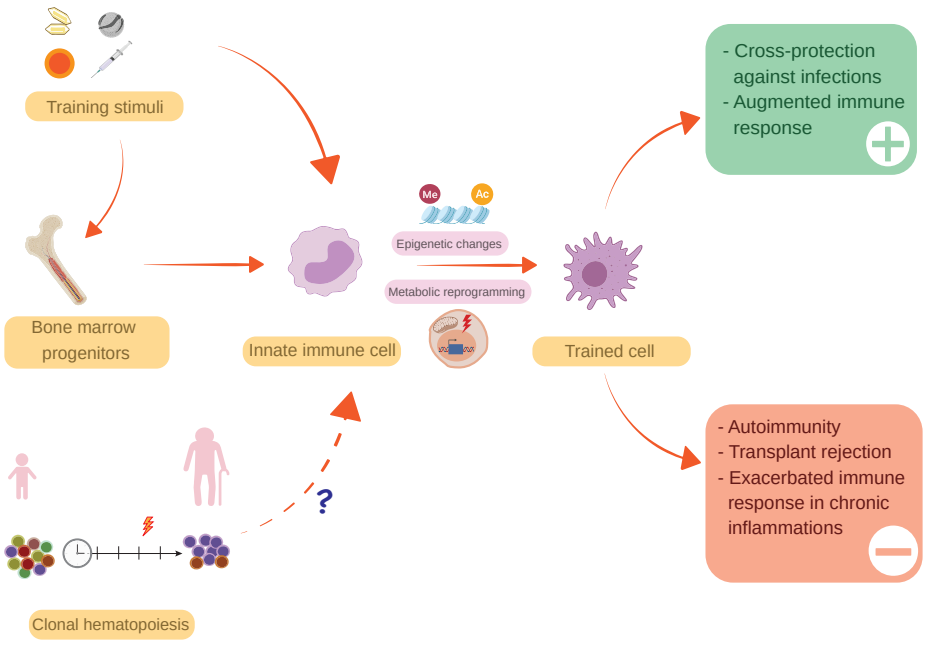

Graphical abstract – *Mechanisms and factors mediating trained immunity.* Microbial and self-derived intermediates can induce training in innate immune cells and bone marrow progenitors, leading to unique epigenetic and metabolic changes. A trained phenotype can result in favorable as well as maladaptive consequences. Clonal hematopoiesis is a potential mechanism contributing to trained immunity.

Highlights

- Trained immunity describes the memory characteristics of innate immune cells mediated by unique epigenetic changes and metabolic reprogramming
- Trained immunity can be induced by pathogenic and self-derived stimuli
- Trained phenotype can persist up to several months even a year due to training of HSPCs
- Trained immunity can provide beneficial effects such as cross-protection against infections and augmented immune response but also unfavorable outcomes such as autoimmunity, transplant rejection and exacerbated immune response in chronic inflammation

Key words: Trained immunity, innate immunity, epigenetics, metabolism

Abstract

Adaptive immune responses are characterized by antigen specificity and induction of life-long immunological memory. Recently, it has been reported that innate immune cells can also build immune memory characteristics, a process termed *trained immunity*. Trained immunity describes the persistent hyperresponsive phenotype that innate immune cells can develop after brief stimulation. Pathogenic stimuli such as microorganisms, but also endogenous molecules including uric acid, oxidized low-density lipoprotein (oxLDL) and catecholamines are capable of inducing memory in monocytes and macrophages. While trained immunity provides favorable cross-protection in the context of infectious diseases, the heightened immune response can be maladaptive in diseases driven by chronic systemic inflammation, such as atherosclerosis. Trained immunity is maintained by distinct epigenetic and metabolic mechanisms and persists for at least several months *in vivo* due to reprogramming of myeloid progenitor cells. Additionally, certain non-immune cells are also found to exhibit trained immunity characteristics. Thus, trained immunity presents an exciting framework to develop new approaches to vaccination, but also novel pharmacological targets in the treatment of inflammatory diseases.

Abbreviations

BCG:	Bacillus Calmette-Guérin
oxLDL:	oxidized low-density lipoprotein
IPL:	Immune-gene priming long noncoding RNA
CHIP:	Clonal hematopoiesis of indeterminate potential
H3K4me1:	monomethylation of histone 3 at lysine 4
H3K4me3:	trimethylation of histone 3 at lysine 4
H3K27ac:	acetylation of histone 3 at lysine 27

Background

Every living organism is in close contact with its environment, and is continuously exposed to various types of stressors and stimuli, including microorganisms. This exposure to potential pathogens necessitates defense mechanisms that are exerted at various levels, including a specialized system of organs and cells: the immune system[1,2]. The vertebrate immune system is divided into the innate and adaptive immunity, with the former being the evolutionarily ancient line of host defense. The innate immune system is capable of detecting and eliminating a variety of pathogens via activation of germline-encoded pattern-recognition receptors (PRRs) that can recognize evolutionarily conserved pathogenic structures[3,4]. Lifelong immunological memory is a hallmark of adaptive immunity that evolved in vertebrates[5,6]. This type of classical memory is sustained by the action of long-lived T and B lymphocytes, and recognizes specific antigens[7].

In the past decade the field of immunology witnessed a paradigm shift by studies showing that innate immune cells also exhibit *de facto* memory characteristics[8]. Innate immune cells such as monocytes, macrophages and NK cells briefly exposed to certain stimuli, manifest long-term enhanced response upon restimulation. Importantly, this increased responsiveness is generally nonspecific in relation to the original stimulus. This persistent functional reprogramming of innate immune cells is termed "trained immunity"[9]. The trained phenotype is maintained via characteristic epigenetic mechanisms which are accompanied and regulated by distinct metabolic changes[8]. Initial work on trained immunity focused on microorganisms and microbial products including *Candida albicans*, its cell wall component β-glucan, and the Bacillus Calmette-Guérin (BCG) vaccine[10]. Later, it was shown that also self-derived molecules such as uric acid, oxLDL, catecholamines, and hormones like aldosterone can induce distinct functional reprogramming of innate immune cells[11-17]. Importantly, the magnitude of the trained immunity response shows considerable heterogeneity, and depends on the specific stimulus (with microbial products generally inducing a stronger response than endogenous stimuli), and genetic variation in epigenetic and metabolic pathways[18,19].

Trained immunity in infectious diseases

Although the concept of trained immunity has recently been described in mammals, it recapitulates several previously defined memory characteristics of the innate immune system described earlier in various taxa. In studies

performed in the last half century, plants were shown to possess a non-specific defense mechanism, called "systemic acquired resistance", that confers long term protection against subsequent infections[20]. In addition, ample evidence indicates the presence of rudimentary memory characteristics in the innate immune system of invertebrates. Numerous studies performed with sponges, fruit flies and mosquitos indicate augmented immune responsiveness upon a second challenge with unrelated stimuli[21-23].

This phenomenon, and the potential to induce powerful protection against subsequent infections, is confirmed in studies in vertebrates as well. BCG vaccination is shown to be protective against a subsequent lethal systemic *C. albicans* infection in severe combined immunodeficiency (SCID) mice that lack adaptive immunity[24,25]. Likewise, administration of β-glucan confers non-specific protection against recurrent infections in mice[26,27]. The most compelling evidence in humans for nonspecific memory of innate immune cells stems from the significant decrease in overall childhood mortality rates after BCG vaccination, which cannot be solely explained by protection against tuberculosis[28]. The heterologous protection administered by BCG is further exemplified by protection against viremia induced by yellow fever vaccination in healthy adult subjects, as well as in experimental human malaria infection [29,30]. Based on this experimental and epidemiological evidence, clinical trials have been initiated recently to test the capacity of BCG vaccination to protect against SARS-CoV2 infection[31].

Trained immunity in chronic inflammatory diseases

Contrary to aforementioned protective effects of trained immunity in the setting of infections, inappropriate induction of trained immunity might actually be maladaptive in chronic inflammatory diseases in which cells of the innate immune system play a role in the pathophysiology of disease, e.g. atherosclerosis, gout, neurodegenerative disorders, and transplant rejection[32-34]. *In vitro* experiments show that monocytes can develop a trained immune phenotype after brief exposure to endogenous atherogenic substances, such as oxLDL and lipoprotein (a)[13,35]. Western-type diet contributes to development of obesity, type II diabetes and cardiovascular diseases. When fed a Western-type diet, myeloid cells in atherosclerosis-prone $Ldlr^{-/-}$ mice displayed characteristics of trained immunity which was dependent on activation of NLRP3 inflammasome[36]. The augmented cytokine production capacity that characterizes trained immunity, is also observed in patients with coronary atherosclerosis, familial hypercholesterolemia, and in patients with cerebral small vessel disease, an atherosclerosis-like disease affecting

the small vessels in the brain[37-39]. Hyperuricemia is another risk factor for atherosclerotic cardiovascular disease, which was recently shown to induce long-term proinflammatory activation of innate immune cells[11,12]. The topic of trained immunity in cardiovascular disease is further reviewed in detail by Flores-Gomez *et al*. in this issue of ATVB.

Maladaptive responses of trained immunity are also implicated in exacerbating several other inflammatory diseases. Trained immunity promotes neuropathology in mouse models of Alzheimer's disease[33]. Furthermore, inappropriate activation of the immune system is implicated in systemic autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus that could partially be explained by the contribution of trained immunity[40,41]. Lastly, the hyperactive monocytes isolated from patients with hyper-IgD syndrome, an autoinflammatory disorder, show profound similarities to monocytes trained *ex vivo* with β-glucan, which is due to accumulation of the metabolite mevalonate[15].

The mechanism of trained immunity opens new avenues in research to improve prevention and treatment in a wide array of inflammatory diseases. Therefore, in this review we explain the basics of trained immunity, how it is maintained despite the short lifespan of innate immune cells, detail its ramifications and propose means of harnessing its therapeutic potential.

Intracellular mechanisms mediating trained immunity

The molecular mechanisms of trained immunity are orchestrated by unique epigenetic and metabolic programs that are closely intertwined.

Epigenetic remodeling

Gene expression is tightly regulated at multiple layers by the action of transcription factors and regulatory elements such as promoters, enhancers and repressors. An additional level of regulation is ensured at the epigenetic level. Epigenetic reprogramming can occur at the level of DNA methylation, histone modifications or via the action of noncoding RNAs.

Several studies have identified genome-wide epigenetic signatures associated with trained immunity[42,43]. There are multiple epigenetic marks associated with trained immunity: an increase in markers of open chromatin such as trimethylation of histone 3 at lysine 4 (H3K4me3), a mark observed at the promoter regions of actively transcribed genes, H3K4me1 typically

found at enhancers and accompanied by acetylation of histone 3 at lysine 27 (H3K27ac)[43], and a decrease in histone marks depicting closed chromatin such as H3K9me2. These epigenetic marks are written and erased by histone-modifying enzymes. Research endeavors identified KDM5 histone demethylase and Set7 lysine methyltransferase to be epigenetic enzymes involved in the regulation of β-glucan induced trained immunity: KDM5 activity to erase H3K4me3 marks at the promoter regions was inhibited during induction of trained immunity, while Set7 was responsible for writing H3K4me1 marks at the enhancer regions of trained immunity genes[14,44].

Fanucchi *et al*. uncovered the immune-gene priming long noncoding RNA (IPL) UMLILO to be a key factor in regulation of gene expression upon β-glucan priming. This prototypical IPL functions at a topologically associated domain that clusters certain chemokine promoters (CXCL1, CXCL2, CXCL3 and IL-8) together, thereby ensuring their H3K4me3 signature inducing the trained phenotype[45]. The topic of epigenetic modulation of trained immunity by noncoding RNA warrants further investigation.

Studies on the role of DNA methylation in trained immunity are scarce. However there is some evidence that DNA methylation patterns could discriminate between "responders" and "non-responders" to BCG vaccination, with only "responders" showing persistent changes in DNA methylation patterns[46]. Additionally, *in vitro* DNA methylation patterns were studied in β-glucan-induced training and LPS-induced tolerance, and this revealed a role for DNA methylation only in tolerance and not in training[47]. Nonetheless, further research to elucidate the role of DNA methylation in trained immunity is required.

The role of epigenetics in immunity is further explained in the review by Lutgens and colleagues in this issue.

Metabolic adaptations

Cellular metabolism is a crucial determinant of physiological functioning of a cell. In addition to providing energy and building blocks for macromolecule synthesis, cellular metabolites have signaling function and regulate the immune response. It is of note that many intermediates of metabolic pathways can modulate the function of epigenetic enzymes by acting as substrates or cofactors, which provides an important coupling between epigenetic and metabolic processes[48]. In recent years, several metabolic pathways have been

shown to be essential for the induction of trained immunity, which has been the subject of recent reviews [14,42,49-51].

Analysis of the metabolome and transcriptome of β-glucan trained macrophages revealed upregulation of various metabolic pathways, such as glucose metabolism, glutaminolysis and cholesterol synthesis[14]. The upregulation of glycolysis is mediated by the activation of the mTOR pathway[42]. Although training with a high concentration of β-glucan is associated with a shift from oxidative phosphorylation to aerobic glycolysis, a lower concentration of β -glucan, as well as BCG, and oxLDL activate both glycolysis and oxidative phosphorylation [18,19,42]. Set7 lysine methyltransferase was recently identified as key regulator of the induction of oxidative phosphorylation[44]. Additionally, glutaminolysis is an essential metabolic pathway for trained immunity: glutamine replenishes the TCA cycle by its conversion into glutamate and α-ketoglutarate. The subsequent increase in fumarate induces inhibition of lysine demethylase KDM5 to induce trained immunity[14]. Further research revealed that intracellular accumulation of mevalonate, a metabolite in the early steps of cholesterol synthesis is essential for the induction of trained immunity via inducing H3K4me3 on IL6 and TNFA promoters. In addition, statins can prevent training *in vitro* by interfering with this pathway[15].

Itaconate is a key metabolite that is shown to regulate the delicate balance between innate immune tolerance and trained immunity by inducing metabolic alterations in macrophages[49]. Itaconate is derived from cis-aconitate by the action of immune-responsive gene 1 (*IRG1*). Induction of IRG1 has been associated with induction of immune tolerance and this is counterbalanced by training with β-glucan[50].

Finally, fatty acid synthesis is important in the inflammatory activation of macrophages. A recent study identified this pathway as critical for trained immunity induced by the adrenal hormone aldosterone, which is associated with cardiovascular disease. Aldosterone-trained macrophages *in vitro* did not display changes in glycolysis or oxygen consumption, yet were characterized by an upregulation of genes involved in fatty acid synthesis and pharmacological inhibition of this pathway during restimulation of the cells prevented the augmented cytokine production[16].

Trained immunity in hematopoietic stem and progenitor cells

A trained immune phenotype is widely shown in mature myeloid cells such as monocytes, macrophages and DCs, as well as lymphoid cells such as NK-cells, all of which have relatively short circulating half-lives, with the exception of macrophages in the tissues, which can survive for long periods. Yet, *in vivo*, circulating trained monocytes are present up to one year after BCG vaccination[25,52]. Therefore, the natural question arises: how can these cells maintain the trained phenotype for long periods despite their short half-life? The answer to this puzzling question might be provided by recent studies that show training on the level of hematopoietic stem and progenitor cells (HSPCs) in the bone marrow.

HSPCs sense and respond to the inflammatory stimuli in the bone marrow milieu[53]. Chavakis and colleagues demonstrated in mice that after β-glucan administration, hematopoietic stem cells (HSCs) show a trained phenotype marked by elevated cytokine production capacity and myeloid skewing[54]. Likewise, BCG evokes a comparable signature in mouse HSCs. Presence of BCG in the bone marrow led to a trained phenotype in HSCs and their derivative multipotent progenitors and bone marrow derived macrophages, conferring enhanced protection against subsequent *Mycobacterium tuberculosis* infection[55]. This was recently confirmed in a human study as well; in human volunteers that were vaccinated with BCG, trained immunity in the bone marrow was observed 90 days after BCG vaccination, leading to trained immunity in peripheral cells as well[56]. Additionally, Western type diet in *Ldlr*$^{-/-}$ mice induces training in myeloid progenitors via epigenetic and metabolic reprogramming, which remains even four weeks after switching to chow diet[36]. The topic of trained immunity in the bone marrow is further detailed by Chavakis *et al*. in this issue.

Expanding the concept of trained immunity

Trained immunity is not restricted to innate immune cells, and evidence is accumulating that long-term adaptation can develop following brief stimulation of various non-immune cells including vascular endothelial cells, vascular smooth muscle cells, fibroblasts, epithelial stem cells and microglia[57-60]. This concept has been recently proposed by Antonio Cassone and termed 'expanded trained immunity'[61].

During atherogenesis both vascular endothelial cells and smooth muscle play an active role. Vascular endothelial cells are known to secrete cytokines,

recognize PAMPs and DAMPs and be involved in antigen presentation and phagocytosis, thus acting as "conditional innate immune cells"[57]. Endothelial cells adopt a persistent inflammatory phenotype following brief exposure to high glucose concentrations[62]. Also, vascular smooth muscle cells are capable of building a sustained proinflammatory phenotype after brief exposure to oxLDL[58]. In addition, epithelial stem cells are shown to recollect the memory of previous insults and respond more rigorously to a secondary infection, in a process termed 'inflammatory memory' which resembles trained immunity. This action is sustained by AIM2 inflammasome activation and its downstream mediators caspase-1 and IL-1β[59]. Microglia function as macrophages in the central nervous system. Notably, microglia have been shown to respond to a second challenge stronger after initial stimulation with systemic administration of LPS[60].

A phenomenon that could potentially lead to development of trained immunity is clonal hematopoiesis of indeterminate potential (CHIP). It has been long thought that clonal hematopoiesis is exclusively responsible for development of hematological malignancies[63]. However, with the recent advancements in DNA sequencing technologies it is now clear that clonal hematopoiesis is much more widespread than initially thought. Mutations in more than 100 genes have been identified as drivers of clonal hematopoiesis. Notably, loss-of-function mutations in two critical epigenetic modifier enzymes, DNMT3A and TET2, are accounting for the biggest proportion of clonal hematopoiesis driver mutations[64]. Several clinical studies identify CHIP as a strong risk factor for coronary heart disease, especially atherosclerosis[65,66]. Further mechanistic studies revealed that this is, at least in part, due to the increased expression of inflammatory markers such as IL-6 and IL-1β in *TET2* deficient monocytes[67,68]. As IL-1β is a central factor in trained immunity, it is tempting to speculate that CHIP could predispose monocytes to trained immunity, but this warrants further investigation.

Clinical applications and future perspectives

In this brief overview we introduced trained immunity, a concept that challenges the dogma of immunological memory being attributed solely to the adaptive immune system. This *de facto* innate immune memory is elicited by distinctive epigenetic and metabolic programs. Trained immunity represents a great potential for clinical use as it is implicated not only in inducing cross-protection to certain infections, but also in the pathophysiology of chronic non-infectious inflammatory conditions, such as atherosclerosis or transplant

rejection. This opens new avenues for immunotherapy aimed at stimulating trained immunity to improve host defense against infections or repressing trained immunity to prevent the detrimental consequences of chronic inflammation (Figure 1). An example of exploiting trained immunity for the benefit of host defense with BCG vaccination has recently been proposed in the context of the SARS-CoV2-pandemic[69]. In addition, BCG vaccination is able to prevent against viral respiratory tract infections in the elderly[70]. In contrast, in the setting of atherosclerosis or other chronic inflammatory disorders, limiting trained immunity could have potential beneficial clinical effects. In this regard, the well-established metabolic and epigenetic programs that evoke trained immunity pose excellent therapeutic targets[71].

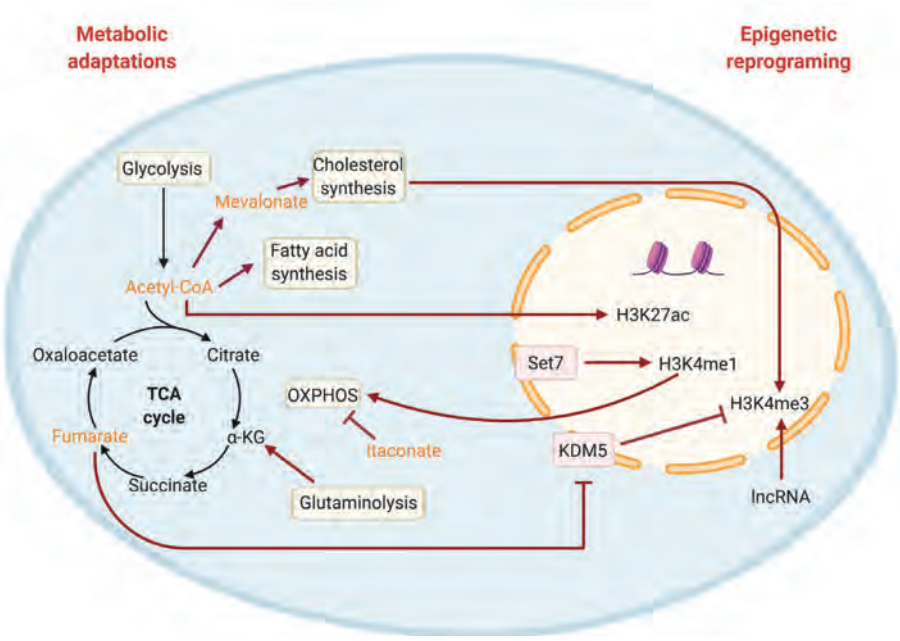

Figure 1: *Intracellular mechanisms orchestrating trained immunity*. Distinct metabolic pathways and epigenetic signatures involved in the induction of trained immunity are highlighted. Histone-modifying enzymes Set7 and KDM5 bridge between metabolic adaptations and epigenetic reprogramming.

Several existing drugs inhibit specific metabolic pathways that drive trained immunity, including inhibitors of the mTOR pathway (rapamycin), hydroxy methylglutaryl-CoA (HMG-CoA) inhibitors by preventing mevalonate synthesis, or NLRP3 inflammasome inhibitors[15,42]. In addition, the specific epigenetic enzymes that regulate the epigenetic reprogramming of trained cells provide attractive pharmacological targets. Several epigenetic drugs

are already being used in clinical practice in hematological and oncological disorders[71].Choosing a nanoparticle delivery approach that selectively targets these drugs to the specific innate immune cells (e.g. plaque macrophages) could improve the specificity of these drugs and prevent off-target effects [71].

In conclusion, trained immunity proves to be an indispensable element of host defense. Although we now know the role of certain metabolic and epigenetic pathways in trained immunity, many questions remain to be answered. Exciting avenues include identifying the spectrum of cells that can build immune memory, and unveiling the role of IPLs in regulating trained immunity. In addition, single cell sequencing advances would be beneficial to identify potential (monocyte) subsets that are particularly amenable to trained immunity. Lastly, the relation between trained immunity and other mechanisms of innate immune cell activation, such as clonal hematopoiesis or immune cell senescence warrants further investigation.

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Disclosures

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Chapter 3

Clonal hematopoiesis is associated with cardiovascular events in patients with stable coronary artery disease

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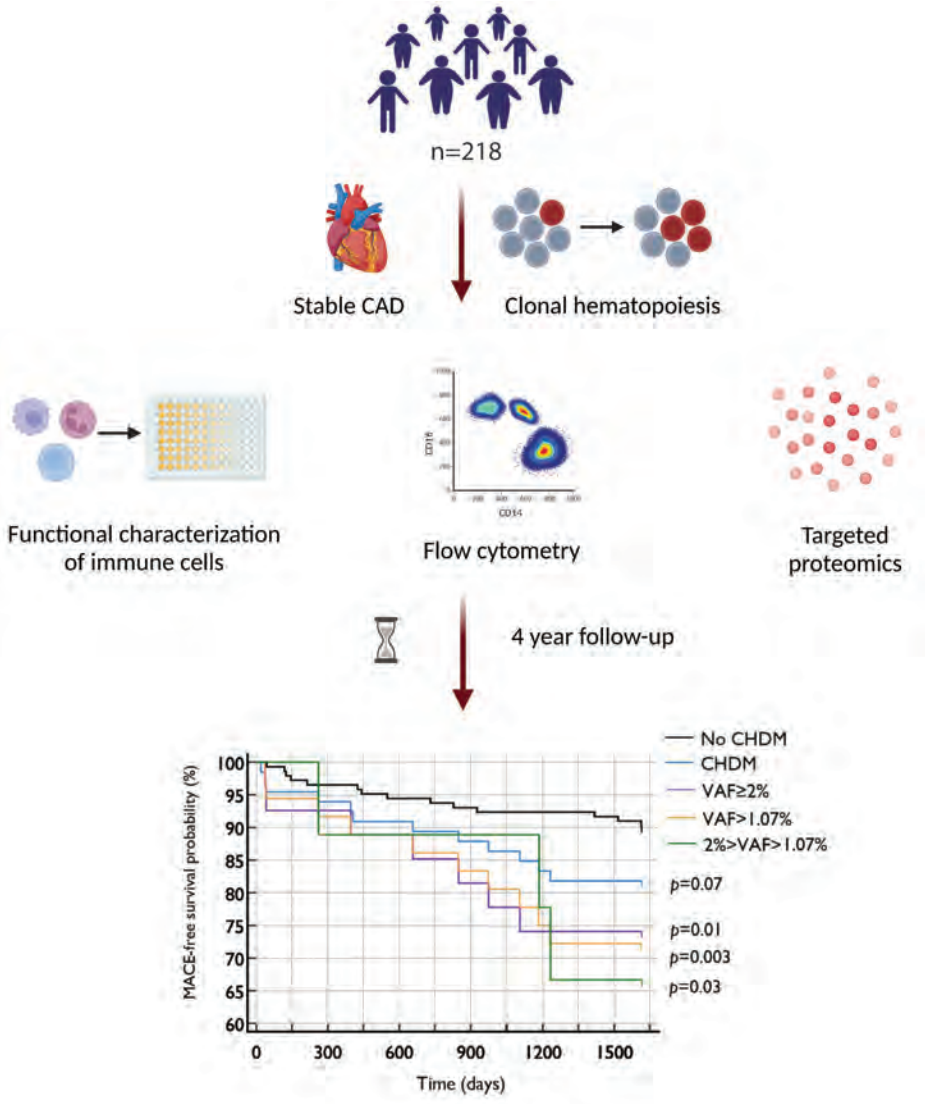
n=218
Stable CAD
Clonal hematopoiesis
Functional characterization of immune cells
Flow cytometry
Targeted proteomics
4 year follow-up
MACE-free survival probability (%)
100
95
90
85
80
75
70
65
60
0
300
600
900
1200
1500
Time (days)
No CHDM
CHDM
VAF≥2%
VAF>1.07%
2%>VAF>1.07%
p=0.07
p=0.01
p=0.003
p=0.03

Summary

Clonal hematopoiesis (CH) is a risk factor for atherosclerotic cardiovascular disease, but the impact of smaller clones, and the effect on inflammatory parameters is largely unknown. Using ultrasensitive single-molecule molecular inversion probe sequencing, we evaluated the association between CH and a first major adverse cardiovascular event (MACE) in patients with angiographically documented stable coronary artery disease (CAD) and no history of acute ischemic events. CH was associated with an increased rate of MACE at four years follow-up. The size of the clone predicted MACE at an optimal cut-off value of 1.07% variant allele frequency (VAF). Mutation carriers had no change in monocytes subsets or cytokine production capacity but had higher levels of circulating tissue factor, matrilysin, and proteinase-activated receptor-1. Our study identified CH driver mutations with a VAF as small as 1.07% as a residual cardiovascular risk factor and identified potential biomarkers and therapeutic targets for patients with stable CAD.

Introduction

Innate immune cells are critical for the development and destabilization of atherosclerotic plaques. Recently, clonal hematopoiesis was identified as risk factor for atherosclerotic coronary artery disease. Clonal hematopoiesis is defined as the process in which somatic mutations in hematopoietic stem cells in the bone marrow lead to expansion of leukocyte clones.[1]

The most frequent clonal hematopoiesis driver mutations (CHDM) involve mutations in the genes *DNMT3A* (DNA methyltransferase 3a), *TET2* (tet methylcytosine dioxygenase 2), *ASXL1* (ASXL transcriptional regulator 1), and *JAK2* (Janus kinase 2).[2] These mutations provide a selective proliferation or survival advantage to the hematopoietic stem cells in which they occur, which then differentiate and contribute disproportionately to the population of mature blood cells.[3-5] While the presence of these mutations is associated with a ten-fold increased risk of haematological malignancies, they also confer a higher risk for cardiovascular disease (CVD).[4,5] Individuals carrying such mutations at a variant allele frequency (VAF) of at least 2%, and in the absence of hematologic disease, are considered as having clonal hematopoiesis of indeterminate potential (CHIP).[1,6]

Several population-based studies have shown that CHIP is associated with a higher incidence of cardiovascular disease, including coronary artery disease (CAD) and stroke.[2,7] Recent large biobank studies using exome sequencing to characterize CHIP status showed that most CHIP gene mutations were found in *DNMT3A*, and they confirmed the association with incident cardiovascular disease,[8] including myocardial infarction, stroke, and peripheral arterial disease.[9] Kessler et al, however, did not find an association with *DNMT3A* mutations and CVD in a population based cohort.[8] One very recent large biobank study showed that CHIP was an independent predictor of recurrent adverse events and all-cause mortality in patients with established atherosclerotic CVD.[10] In smaller prospective studies using targeted sequencing of approximately 50 whole genes recurrently mutated in clonal hematopoiesis, the presence of CHDMs was also associated with prognosis in patients with established CVD. The presence of somatic *DNMT3A* and *TET2* mutations was associated with an increased incidence of death and major adverse cardiovascular events (MACE) in acute myocardial infarction survivors.[11] Moreover, CHIP presence was associated with worse short-term outcomes in patients presenting with cardiogenic shock complicating acute myocardial

infarction.[12] In patients that suffered an ischemic stroke, the presence of CHDMs with a VAF>1% was associated with an increased risk of recurrent stroke, myocardial infarction, and all-cause death.[13] However, whether CHDMs predict a first cardiovascular event in patients with stable CAD is unknown.

While most studies on clonal hematopoiesis and CVD are restricted to CHDMs with a VAF >2%,[2,11] or even 10%,[8] there is few evidence for an adverse cardiovascular effect of smaller clone sizes. In patients after stroke, CHDM with a VAF>1% are associated with recurrent MACE.[13] Also, for patients with chronic ischemic heart failure, clone sizes with a VAF < 2% are associated with worse outcomes at follow-up.[14]

In this study, we aimed to investigate whether the presence of CHDMs predict a first MACE in patients with angiographically proven stable coronary artery disease. To this end, we used an ultrasensitive single molecule molecular inversion probe-based technique, that only captures a subset of known CHDM loci, but with an ultra-high sensitivity which detects very low VAFs.[15]

Unique to our study is that we also assessed innate immune cell phenotype using flow cytometry and cytokine production capacity in all subjects, in addition to targeted proteomics of plasma to explore potential underlying mechanisms of how CHDMs predispose to CVD.

Results

The cohort included 218 patients with a mean age of 64.9 years and a mean body mass index of 29.3 kg/m^2. Men represented 68.3% of the population. Arterial hypertension was diagnosed in 90.8% of patients, while diabetes mellitus was present in 35.8% of them. The mean baseline left ventricular ejection fraction was 53.2%. Statin therapy was administered in 92.2% of the cohort. Paroxysmal or persistent atrial fibrillation was present in 12.8% of the patients. The baseline characteristics of the population are presented in Table 1.

Table 1. Baseline patient characteristics in the study cohort

PARAMETER	**n=218**
CLINICAL DATA	
Age (years), mean±SD	64.9±8.7
Males, n (%)	149 (68.3)
Females, n (%)	69 (31.6)
BMI (kg/m2), mean±SD	29.3±3.5
Waist (cm), mean±SD	101.9±11.6
Smokers, n (%)	19 (8.7)
Heart rate (beats/min), median (Q1-Q3)	68 (60-75)
Hypertension, n (%)	198 (90.8)
SBP (mmHg), mean±SD	137.9±22.4
DBP (mmHg), mean±SD	79.7±10.9
Diabetes, n (%)	78 (35.7)
LABORATORY DATA	
Glycemia (mg/dl), median (Q1-Q3)	109.1 (97.5-131.3)
Total cholesterol (mg/dl), mean±SD	167.6±46.6
LDL-cholesterol (mg/dl), mean±SD	91.4±33.8
HDL-cholesterol (mg/dl), mean±SD	42.9±12.7
Triglycerides (mg/dl), median (Q1-Q3)	151.2 (106.4-181.9)
Creatinine (mg/dl), median (Q1-Q3)	0.9 (0.8-1.1)
eGFR (ml/min/1.73m^2), median (Q1-Q3)	82.4 (68.1-100.7)
ECHOCARDIOGRAPHIC DATA	
LVEF (%), mean±SD	53.2±9.1
CONCOMITANT MEDICATION	
Aspirin, n (%)	181 (83)
P2Y12 inhibitor, n (%)	134 (61.4)
Anticoagulant (AVK/NOAC), n (%)	28 (12.8)

Table 1. Continued

CONCOMITANT MEDICATION	
Betablocker, n (%)	181 (83)
ACE inhibitor/ARB, n (%)	168 (77)
Calcium channel blocker, n (%)	64 (29.3)
Nitrate, n (%)	72 (33)
Statin, n (%)	201 (92.2)
Fibrate, n (%)	14 (6.4)
ATHEROSCLEROSIS SEVERITY	
SYNTAX Score I, median (Q1-Q3)	11 (7-21)
SYNTAX Score II – PCI, median (Q1-Q3)	29.5 (22.2-35.7)
SYNTAX Score II – CABG, median (Q1-Q3)	26.7 (20.3-33.4)
REVASCULARIZATION	
Complete, n (%)	67 (31.3)

ACE = angiotensin converting enzyme; ARB = angiotensin receptor blocker; AVK = antivitamin K; BMI = body mass index; CABG = coronary artery bypass graft; DBP = diastolic blood pressure; eGFR = estimated glomerular filtration rate; HDL-cholesterol = high density lipoprotein cholesterol; LDL-cholesterol = low density lipoprotein cholesterol; LVEF = left ventricular ejection fraction; n = number; NOAC = novel oral anticoagulant; PCI = percutaneous coronary intervention; Q1 = 1st quartile; Q3 = 3rd quartile; SBP – systolic blood pressure.Clonal hematopoiesis driver mutations

CHDMs were present in 33% of the cohort (72 patients), and 13.3% of the cohort (29 patients) met the criteria for CHIP. The prevalence of CHDM was higher in women then in men (41.6% vs. 26.7%, p=0.02). We identified CHDMs in 13 individual genes (Figure 1, Supplementary Table S1). The most frequent mutations were in *DNMT3A* (56.9%), *TET2* (11.1%), and *JAK2* (6.9%) (Figure 1A). *DNMT3A* was the most frequently mutated gene in patients older than 55 years, in both males and females (Figure 1, B and C).

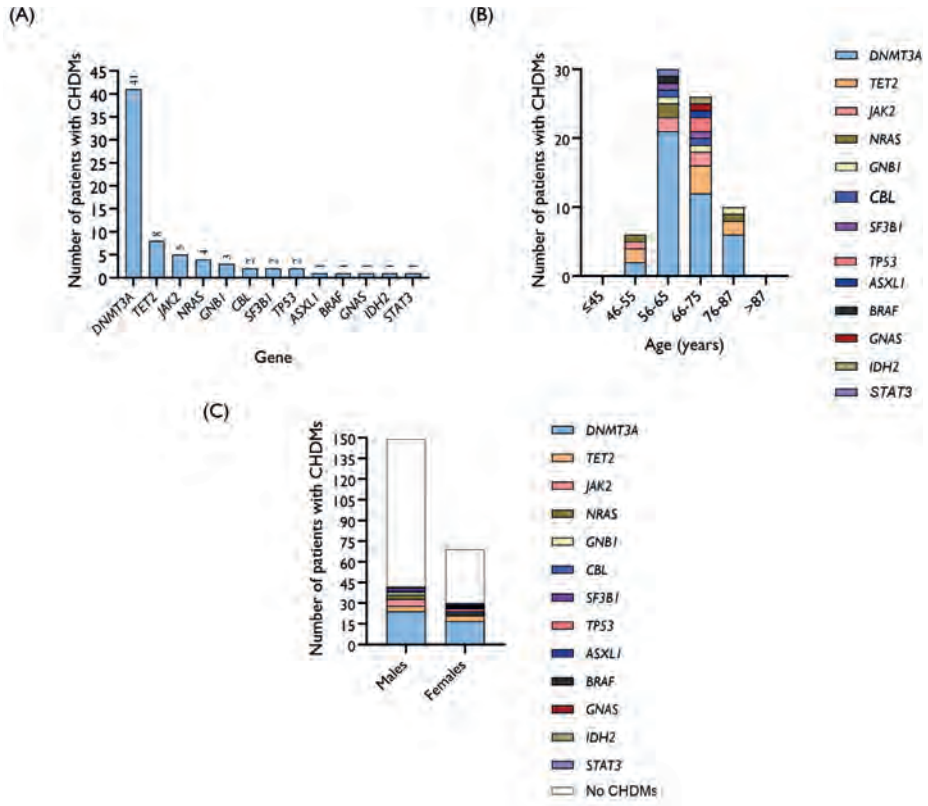

Figure 1. CHDMs in the study cohort. Panel (**A**) - The distribution of mutations in the cohort; Panel (**B**) - Number of individuals with CHDMs per age category; Panel (**C**) – Number of individuals with CHDMs per sex category. CHDM = clonal hematopoiesis driver mutations.

The association between CHDM and major adverse cardiovascular events

The median follow-up time was 4 (3.7 – 4.1) years. A MACE was recorded in 12.4% of the cohort (27 patients). During follow-up, 3.2% of patients developed an acute myocardial infarction and 3.7% developed an acute ischemic stroke. A history of atrial fibrillation was present in only one of the patients who developed ischemic stroke. Cardiovascular mortality reached 7.3%, while the rate of non-cardiovascular death was 3.7%. During follow-up, 8.1% of patients underwent myocardial revascularization by either PCI or coronary artery bypass graft in the setting of an acute coronary syndrome, while 9.5% of them underwent elective, planned myocardial revascularization procedures.

In univariate Cox proportional-hazards regression analysis, the presence of CHIP was strongly associated with MACE (HR 3.19; 95%CI 1.27 – 8.01; *p*=0.01). ROC curves analysis showed that in patients with CHDM, the VAF predicted MACE at an optimal cut-off value >1.07% (AUC 0.701; 95%CI 0.575 – 0.807; *p*=0.007) (Figure 2A). Also, the presence of a CHDM with VAF>1.07 was associated with an increased risk for MACE at follow up (HR 3.40; 95%CI 1.49 – 7.78; *p*=0.003).

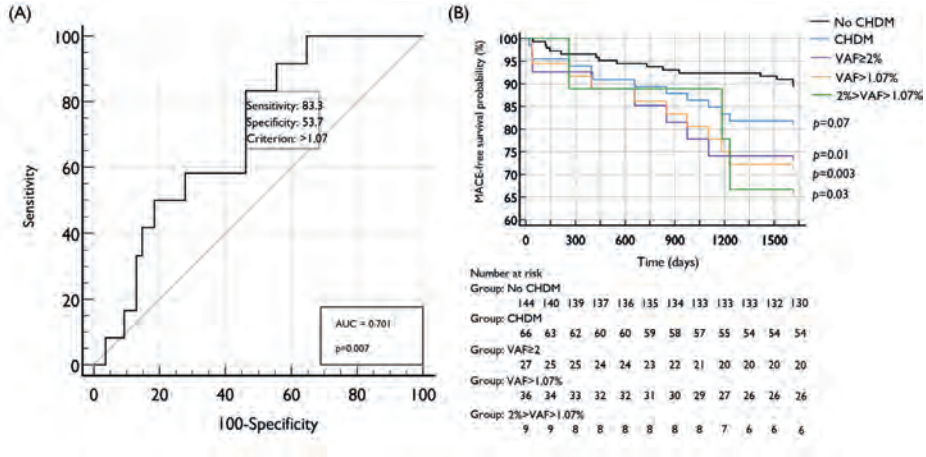

Figure 2. The association between CHDM and MACE. Panel (**A**) - ROC curve for the association between VAF and MACE at 4 years follow-up. VAF>1.07% predicts MACE with a Sensitivity of 83.3% (AUC 0.701; 95%CI 0.575 – 0.807; p = 0.007); Panel (**B**) – MACE-free survival according to the presence of CHDMs and the size of the clone. All comparisons are made to the group without CHDM. Patients who suffered a non-cardiovascular death were excluded from the analysis. AUC = area under the curve; CHDM = clonal hematopoiesis driver mutations; MACE = major adverse cardiovascular events; ROC = receiver operating characteristic; VAF = variant allele frequency.

A gene-specific analysis showed that the presence of *DNMT3A* mutations was associated with a higher rate of MACE as compared to the group without CHDMs, but the result did not reach statistical significance (HR 1.79; 95%CI 0.68–4.72; *p*=0.23). The presence of non-*DNMT3A* mutations was associated with a higher rate of ischemic events and cardiovascular mortality at follow-up (HR 2.46; 95%CI 0.93 – 6.49; *p*=0.06). The survival curves are presented in the Supplementary material file (Supplementary Figure S1).

There was also a significant association of smaller size clones (VAF between 1.07% and 2%) with ischemic events and cardiovascular mortality (HR 3.86; 95%CI 1.09 – 13.57; *p*=0.03). The survival curves are presented in Figure 2B.

Multivariable Cox proportional-hazards models showed that both CHIP and CHDM with VAF>1.07 were associated with MACE independent of conventional cardiovascular risk factors (age, sex, body mass index, smoking, systolic blood pressure, diabetes mellitus, LDL-cholesterol, and BNP value) (Supplementary Table S4).

Association of CHDM with baseline characteristics and cardiovascular risk factors

In our study cohort, there was no significant association between age and the presence of CHDM (p=0.19), nor between age and VAF (p=0.33). However, carriers of CHDMs with a VAF>1.07% were older as compared to patients without CHDMs (67.4±7.6 vs. 64.4±8.9 years, p=0.05).

We further explored whether the presence of CHDM or CHIP was associated with other demographic characteristics or with classical cardiovascular risk factors (Table 2). Female sex (p=0.02) and systolic blood pressure (p=0.02) were associated with the presence of CHDM. In multivariable logistic regression analysis, the association of both parameters with CHDM was independent of age (p=0.03 for each parameter). Elevated systolic blood pressure was also associated with CHIP (Table 2).

CHDM carriers had a higher clinical SYNTAX Score as compared to patients without mutations (p=0.02).

To explore potential mechanism of how the presence of CHDMs increases the risk for cardiovascular events, we assessed their correlation with circulating leukocyte numbers, and circulating monocyte subsets and function (cytokine production capacity).

There was no difference in leukocytes number and differentiation between patients with or without CHDM, except for a slightly higher number of eosinophils in patients with CHDM (p=0.01). In addition, there was no difference in monocyte subsets (classical, intermediate, non-classical), and no significant difference in CD11b expression (Supplementary Table S5).

We also investigated cytokine production capacity by ex-vivo stimulation of PBMCs with LPS, Pam3Cys and heat-killed *C. albicans*. Supplementary Figure S2 shows that 24 hours cytokine production was not significantly different between patients with or without CHDM or CHIP. Also, for the 72 hours

production of IL-17, IL-22, and IFN-gamma, there was no difference between the patients with and without a CHDM.

Exploring how CHDMs affect MACE: association with circulating proteins

To explore whether circulating proteins are associated with the presence of CHDMs and with MACE, we used targeted proteomics and assessed relative cardiovascular and inflammatory protein levels between patients with and without CDHM. Table 3 shows proteins that were higher in patients with CHDMs: plasma renin (REN) (p=0.002), leptin (LEP) (p=0.003), tissue factor (TF) (p=0.01), interleukin-18 (IL-18) (p=0.01), matrilysin (matrix metalloproteinase-7, MMP-7) (p=0.02), tyrosine-protein kinase Mer (MERTK) (p=0.02), monocyte chemotactic protein 2 (MCP-2) (p=0.02), thrombomodulin I (p=0.03), and AMBP (p=0.03). In patients with CHIP, only three proteins were significantly higher: IL-18 (p=0.009), IL-12B (p=0.03), and proteinase-activated receptor 1 (PAR-1) (p=0.04). Of these proteins, TF (p=0.006), MMP-7 (p=0.007), and PAR-1 (p=0.01) were also associated with MACE. Analysis of publicly available scRNAseq data confirmed increased expression of the tissue factor gene (F3) in unstimulated classical monocytes from subjects with *DNTM3A* mutations compared to non-CHDM controls (relative fold 1.14%, p-adj=1.45E-5).

Table 2. The association between baseline parameters, MACE, and the presence of CHDMs

PARAMETER	No CHDM (n=146)	CHDM (n=72)	*p*-value
CLINICAL DATA			
Age (years)	64.4±8.9	66.1±8.3	0.19
Female sex (%)	26.7	41.6	0.02
BMI (kg/m^2)	29.1±3.6	29.8±3.2	0.15
Waist (cm)	101.7±12.1	102.4±10.7	0.66
Smokers (%)	10.3	5.5	0.24
Heart rate (beats/min), median (Q1 – Q3)	68 (60 – 75)	69 (60–74.2)	0.66
SBP (mmHg)	135.5±19.3	142.6±27.1	0.02
DBP (mmHg)	79.8±11.2	79.7±10.3	0.97
Hypertension, %	90.4	91.6	0.76
Diabetes, %	36.3	34.7	0.82
LABORATORY DATA			
Total cholesterol (mg/dl)	165.2±41.1	172.4±56.0	0.56
LDL-cholesterol (mg/dl)	89.7±28.7	94.9±42.3	0.43
HDL-cholesterol (mg/dl)	43.2±13.6	42.4±10.6	0.68
Triglycerides (mg/dl), median (Q1 – Q3)	147.4 (105.4 – 172.2)	162.2 (107.6 – 204.7)	0.31
Glycemia (mg/dl)	122±41.9	122.6±41.0	0.68
Creatinine (mg/dl), median (Q1 – Q3)	0.9 (0.8 – 1.1)	0.90 (0.76 – 1.10)	0.98
eGFR (ml/min/1.73m^2), median (Q1 – Q3)	84.4 (69.5 – 100.5)	79.9 (62.0 – 102.6)	0.46
ECHOCARDIOGRAPHY			
LVEF (%)	53.1	53.2	0.80
MEDICATION, %			
Aspirin	80.8	87.5	0.25
P2Y12 inhibitor	57.5	69.4	0.10
Betablocker	82.8	83.3	0.93
ACE inhibitor/ARB	76.7	77.7	0.86
Statin	91.8	93.1	0.75
ATHEROSCLEROSIS SEVERITY			
Three-vessel disease, %	28.7	33.8	0.44
SYNTAX Score I, median (Q1 – Q3)	11.5 (7 – 22)	11 (7 - 17)	0.84
SYNTAX Score II – PCI, median (Q1 – Q3)	27.9 (21.1 – 33.6)	32.3 (25.3 – 37.3)	0.02
Complete revascularization, %	30.7	32.3	0.81
MACE, n (%)	14 (9.6)	13 (18.1)	0.07

Values are presented as mean± SD, unless otherwise stated. ACE = angiotensin converting enzyme; ARB = angiotensin receptor blocker; BMI = body mass index; CHDM = clonal hematopoiesis driver mutations; DBP = diastolic blood pressure; eGFR = estimated glomerular filtration rate; HDL-cholesterol = high density lipoprotein cholesterol; LDL-cholesterol = low density lipoprotein cholesterol;

VAF<2% (n=43)	*p*-value	VAF≥2% (n=29)	*p*-value
65.8±8.9	0.35	66.3±7.2	0.28
41.8	0.07	41.3	0.11
30.1±3.5	0.11	29.4±2.8	0.50
102.6±11.4	0.67	102.2±9.7	0.81
4.6	0.25	6.9	0.57
70 (60 - 75)	0.74	68 (61 - 70)	0.73
140.1±28.7	0.22	146.1±24.7	0.01
79.4±10.6	0.85	80.2±10.2	0.86
93.0	0.60	89.6	0.90
23.2	0.11	51.7	0.12
173.4±54.0	0.37	170.9±59.8	0.52
98.7±46.5	0.16	89.2±35.4	0.94
43.1±11.4	0.67	41.4±9.5	0.51
155.8 (110.8 – 200.7)	0.64	170.8 (101.4 – 233.4)	0.23
118.1±36.4	0.72	129.4±46.8	0.39
0.9 (0.7 – 1.1)	0.85	0.8 (0.7 – 1.1)	0.92
79.5 (61.9 – 97.6)	0.32	80.4 (59.0 – 106.7)	0.96
53.4	0.84	52.9	0.87
88.3	0.25	86.2	0.49
67.4	0.24	72.4	0.13
79.0	0.57	89.6	0.36
81.4	0.51	72.4	0.62
90.7	0.83	96.5	0.41
34.8	0.43	32.1	0.71
12 (7 – 17)	0.87	9.5 (7.2 – 19.5)	0.88
33.3 (28.0 – 37.3)	0.01	30.5 (23.1 – 41.1)	0.32
30.2	0.94	35.7	0.60
6 (13.9)	0.41	7 (24.1)	0.02

MACE = major adverse cardiovascular events; n = number; PCI = percutaneous coronary intervention; Q1 = 1st quartile; Q3 = 3rd quartile; SBP = systolic blood pressure. Exploring how CHDMs affect MACE: association with leukocytes, and monocyte subsets and function

Table 3. The association between proteomic biomarkers and the presence of clonal hematopoiesis driver mutations

PARAMETER	No CHDM (n=146)	CHDM (n=72)	*p*-value	VAF<2% (n=43)	*p*-value	VAF≥2% (n=29)	*p*-value
MERTK	6.6 (6.3 – 6.8)	6.7 (6.4 – 6.9)	0.04	6.8 (6.5 – 7.0)	0.008	6.6 (6.3 – 6.8)	0.86
REN	6.9 (6.2 – 7.5)	7.2 (6.9 – 7.8)	0.005	7.3 (6.9 – 7.8)	0.003	7.1 (6.5 – 7.9)	0.21
AMBP	7.9 (7.8 – 8.0)	8.0 (7.8 – 8.1)	0.02	8.0 (7.8 – 8.1)	0.11	8.0 (7.8 – 8.2)	0.06
TF	6.1 (5.8 – 6.3)	6.2 (6.0 – 6.3)	0.02	6.1 (6.0 – 6.3)	0.06	6.2 (5.9 – 6.4)	0.10
LEP	6.7 (6.0 – 7.2)	7.0 (6.2 – 7.8)	0.004	7.0 (6.5 – 7.7)	0.01	7.0 (6.0 – 8.1)	0.07
TM	9.8 (9.6 – 10.1)	9.9 (9.7 – 10.1)	0.02	9.9 (9.7 – 10.2)	0.07	9.9 (9.7 – 10.1)	0.11
IL-18	8.9 (8.6 – 9.3)	9.1 (8.8 – 9.5)	0.01	9.1 (8.7 – 9.4)	0.19	9.2 (9.0 – 9.5)	0.007
IL-12B	6.0 (5.5 – 6.4)	6.2 (5.6 – 6.5)	0.16	5.9 (5.6 – 6.3)	0.85	6.3 (5.8 – 7.0)	0.02
PAR-1	8.8 (8.6 – 9.2)	9.0 (8.8 – 9.4)	0.03	9.0 (8.7 – 9.3)	0.28	9.1 (8.8 – 9.6)	0.02

Values are presented as median and interquartile range (Q1 – Q3).
All comparisons are made to the group without CHDM.
AMBP = protein AMBP; IL = interleukin; CHDM = clonal hematopoiesis driver mutations; LEP = leptin; MERTK = tyrosine-protein kinase Mer; PAR-1 = proteinase-activated receptor 1; Q1 = 1st quartile; Q3 = 3rd quartile; REN = renin; TF = tissue factor; TM = thrombomodulin; VAF – variant allele frequency.

Discussion

The results of our study add several insights to the growing body of evidence that clonal hematopoiesis contributes to atherosclerotic cardiovascular disease. First, we showed that an inexpensive smMIP-based sequencing technique for CHDMs can be used for the prediction of a first MACE in patients with symptomatic stable CAD and angiographically documented coronary atherosclerosis. Although this technique only covers a selection of known CHDM hotspots (including the entire *DNMT3A* gene), it can reliably detect CHDMs up to a VAF of >0.1%. Using this approach, we showed that the presence of CHDMs with a VAF >1.07% is associated with ischemic events and cardiovascular mortality. Secondly, we explored potential mechanisms that link CHDMs with cardiovascular disease by assessing their association with monocyte phenotype and circulating inflammatory proteins, which is unique compared to previous studies. There was no difference in monocyte subsets in patients with or without CHDMs, nor was there a significant difference in the cytokine production capacity of PBMCs. The concentrations of several circulating proteins were significantly associated with CHDMs and CHIP, with TF, MMP-7 (matrilysin), and PAR-1 also being predictive of MACE.

While previous studies have linked CHIP to lower survival in patients with acute myocardial infarction, or ischemic heart failure,[11,16] or with recurrent adverse events and all-cause mortality in patients with established atherosclerotic CVD,[10] this study is the first to demonstrate that CHDMs are associated with a first ischemic event and cardiovascular mortality in patients with stable CAD. All patients received secondary preventive treatment with antithrombotic therapy and statins (92.2%). Hence, our study shows that clonal hematopoiesis contributes to the residual cardiovascular risk in these patients.

A VAF higher than 2% has been commonly used as cut-off to define CHIP,[6,11,16-18] and in this study we showed that CHIP was significantly associated with MACE independent of demographic and clinical factors. The cut-off of 2% was earlier chosen mainly based on limited sensitivity of whole genome sequencing techniques to detect smaller clone sizes. Only few studies showed that CHDMs with lower VAF can also have cardiovascular consequences. Arends et al showed in patients after ischemic stroke, that the presence of CHDMs with a VAF≥1% was associated with a higher risk for future cardiovascular events.[13] A recent report established an optimized cut-off value for *DNMT3A* and *TET2* mutations for prediction of all-cause death in patients with heart failure

of 1.15% and 0.73%, respectively.[14] Also in our study, further ROC analysis revealed that CHDMs predict the occurrence of acute ischemic events and cardiovascular death when the VAF is >1.07%. Because of the relatively small sample size and of the subsequent low number of events per patient group, this result should be considered as hypothesis generating and should be further validated in larger cohorts.

The conventional strategy of CHDM identification is though whole-exome sequencing, which is expensive and can yield incidental findings. To circumvent this, we used an inexpensive smMIP based method for targeted enrichment of CHDM hotspots that can identify CHDMs reliably up to a VAF of 0.1%. Using 300 probes, we covered CHIP/clonal hematopoiesis-related hotspots in 24 genes, including the entire *DNMT3A* gene since this is the gene with the most CHDMs. In the other genes, we only cover a minority of all CHDMs that have been discovered up to date. Despite this limited coverage, our approach strongly predicts the occurrence of ischemic events and cardiovascular mortality in our patient cohort.

The strength of our study is that we assessed in detail circulating immune cell phenotype and function as well as circulating inflammatory proteins in all 218 patients, which enabled us to explore potential mechanisms that link CHDM presence with MACE. Current insights are mainly derived from experimental animal studies focussing on clonal hematopoiesis-related mutations in specific genes. Mice with 10% *TET2*-deficient bone marrow cells develop accelerated atherosclerosis because *TET2*-deficient macrophages exhibit an increase in NLRP3 inflammasome-mediated interleukin-1β secretion.[19] In a macrophage cell line complete inactivation of *TET2* promoted gene expression of IL-1β and IL-6, whereas *DNMT3A* inactivation increased the expression of IL6, Cxcl1 and 2.[20] There are only few data in humans that explored these mechanisms. First, Abplanalp et al. used single cell RNA sequencing of PBMCs in six patients with heart failure harbouring *DNMT3A* CHDMs and showed that the circulating monocytes and T cells of these patients demonstrated a significantly higher expression of inflammatory genes, while the authors did not investigate cytokine production.[21] Interestingly, the inflammatory transcriptional changes associated with *DNMT3A* mutations occurred in a much larger population of cells than the population harbouring the specific mutation. A potential explanation for this finding is that cells with CHDM indirectly modulate the function of non-CHDM-containing immune cells, either in bone marrow or in circulation. In addition, in individuals from the UK Biobank, genetically reduced

IL-6 signalling abrogated the increased cardiovascular risk associated with CHIP, suggesting that increased IL-6 signalling mediates this increased risk.[22] While this finding was recently validated by the same group of authors in a larger sample of patients,[23] another study using the same whole exome sequencing data from the UK Biobank did not confirm the role of IL-6 inhibition in reducing the risk of cardiovascular disease among CHIP carriers.[8] These discrepancies were attributed to the different filtering strategies applied for CHIP in the two studies,[23] the discordant results underlining the challenges of using whole exome sequencing data to identify true clonal hematopoiesis from artifacts in large population biobanks.[23] Importantly, in addition to experimental studies showing direct effects of experimental clonal hematopoiesis on atherosclerosis, there are also indications for reversed causality.[24] Heyde et al used mathematical modelling and experimental studies in mice to illustrate that the presence of atherosclerosis itself accelerates clonal hematopoiesis by increased proliferation rates of bone marrow progenitor cells.[24] Exacerbated expansion of progenitor cells with somatic CHDM also occurs in the context of obesity-induced inflammation in mice.[25] Similarly, a large prospective cohort study in obese individuals showed that clone sizes increased with age in individuals with obesity, but not in those who underwent bariatric surgery.[26] This suggests that clonal hematopoiesis and atherosclerosis can reinforce each other in a vicious cycle.[27]

We and others have shown that isolated PBMCs,[28,29] and their bone marrow progenitors[30] from patients with established coronary artery disease are characterized by an increased cytokine production capacity. We hypothesized that in our cohort, PBMCs from patients with CHDM or with CHIP are characterized by a higher cytokine production capacity. Although point estimates of all pro-inflammatory cytokines were higher in patients with CHIP, mainly for IL-6 and IL-1β, this did not reach statistical significance. This suggests that in our cohort the adverse cardiovascular effect of CHDMs is not due to increased overall PBMC cytokine production. These findings do not exclude, however, that there is heterogeneity in cytokine production capacity of specific immune cell types (such as the monocytes), with a higher capacity in those cells containing the CHDM, which is not reflected in a higher overall PBMC cytokine production. In addition, our results do not exclude increased cytokine production capacity of monocytes with a *TET2* mutation since we only identified few *TET2* CHDMs. Future studies using single cell sequencing and stimulation assays of purified cell populations are necessary to explore this in more detail.

In addition, we showed that the concentrations of several circulating proteins were higher in patients with CHDM, three of these (TF, MMP-7, and PAR-1) being predictive of MACE. TF is a potent initiator of the coagulation cascade and is present in blood following plaque rupture.[31] During the early stages of atherosclerosis, cytokines induce the expression of TF in monocytes, while at later stages, TF is also detected in macrophages, endothelial cells, smooth muscle cells, as well as in the necrotic core of plaques.[32,33] TF is also found in the circulating monocytes of patients with acute myocardial infarction,[33] and it was associated with an adverse prognosis in patients with unstable angina.[34] Analysis of previously published scRNAseq data from patients with *DNMT3A* CHDMs and control subjects revealed increased TF expression in classical monocytes,[21] suggesting that increased monocyte TF production could contribute to the higher circulating TF concentration in these patients. PAR family of proteins are expressed on platelets and contribute to inflammatory signalling by activation and cleavage through thrombin,[35] leading to increased circulating PAR-1 levels. Previous studies showed an increased expression of PAR-1 in the hearts of patients with ischemic and idiopathic dilated cardiomyopathy,[36] while animal studies have shown that its deficiency was associated with a reduced mRNA expression of proinflammatory and profibrotic markers.[37] PAR-1 is also expressed on monocytes and its expression increases after LPS stimulation.[38] In *ApoE*-/- mice, drugs targeting PAR-1 signalling attenuate atherosclerosis formation.[39] Therefore, a possible explanation for the development of acute ischemic events in patients with CHDM could be related to the occurrence of vulnerable plaques, or to rapidly progressive atherosclerosis on the background of chronic systemic inflammation. Finally, MMP-7 concentration was higher in patients with CHDM and was associated with an increased risk of ischemic events and cardiovascular mortality. Breakdown of the extracellular matrix of atherosclerotic plaques is considered an important event that triggers plaque destabilisation, and circulating MMP-7 concentrations have been associated with cardiovascular event rate in patients with carotid stenosis.[40] In carotid plaques, MMP-7 was localized to macrophages, and in primary monocytes in vitro MMP-7 expression could be increased by stimulation with TNF, in the presence of hypoxia and oxLDL.[41] The higher concentration of circulating TF, PAR-1, and MMP-7 in patients with CHDM and stable CAD highlights the potential role of these proteins as biomarkers or possible therapeutic targets. Future studies are warranted to confirm these findings and show causality. Another interesting observation is the higher systolic blood pressure in patients with CHDM, that was also previously reported.[14] The higher plasma renin concentration in these patients

suggests involvement of the renin-angiotensin-aldosterone system, but additional studies are warranted to further explore the mechanisms and origin of TF, PAR-1, MMP-7, and renin upregulation in the context of CHDMs.

In our study, guideline-based secondary prevention measures were implemented. Two-thirds of the participants had a history of elective myocardial revascularization, and more than 90% of them were on statin therapy. This comes to emphasize the role of immune cells in the residual risk in patients with stable CAD. Validation of these results in larger, prospective studies is needed to establish their value for the clinical practice.

In conclusion, we showed that smMIP based detection of a limited set of CHDMs in patients with symptomatic stable CAD is a strong biomarker associated with an increased rate of a first ischemic event and cardiovascular mortality at follow-up. This could not be explained by overall differences in monocyte subsets or activation or overall PBMC cytokine production capacity. Mutation carriers had increased circulating concentrations of TF, MMP-7, and PAR-1 through a mechanism that needs further exploration. We provide evidence on the role of clonal hematopoiesis as a residual cardiovascular risk factor and identify potentialbiomarkers and therapeutic targets for patients with stable coronary artery disease.

Limitations of study

The main limitation of our study is the relatively small sample size, which led to a limited statistical power when stratifying the cohort according to the size of the clones Similarly, the analysis restricted to the CHDMs in *DNMT3A* should be considered exploratory because of the smaller sample size. However, the selection of a well-defined, homogenous cohort of patients with symptomatic stable CAD, angiographically documented coronary atherosclerosis, and without a history of acute cardiovascular events allowed us to obtain consistent results. In addition, the identification of CHDMs with a highly sensitive method allowed for the detection of relatively small clone sizes, and a higher sensitivity to detect known CHDMs/CHIP mutations compared to standard approaches including exome sequencing. A disadvantage of our targeted sequencing approach was that we only detected a part of the CHDMs in genes other than *DNMT3A.* When we initially designed our smMIPs, we specifically targeted loci containing the majority of CHDMs known at that time.[15] A comparison however

of these loci with all currently reported CHDMs in the UK Biobank shows that this approach identifies less than 50% of all CHDMs in non-DNMT3A genes such as *TET2* and *ASXL1*. Therefore, our data preclude any strong conclusions with regard to specific non-*DNMT3A* CHDMs, and our conclusions are only robust for *DNMT3A* CHDMs, which are the most abundant CHDMs and which have recently been unequivocally demonstrated to cause atherosclerosis in experimental models.[42] Nonetheless, to eliminate false positives, we performed an independent identification of our candidate CHDMs in other publicly available datasets. This ensured the validity of candidate CHDMs identified in this study. Future studies using targeting smMIP sequencing should expand the loci to include at least the entire *TET2* gene.

Our study included patients with established CAD. Since we did not include a control group of healthy individuals, we cannot estimate whether the CHDMs prevalence in our group is higher. Comparisons with existing literature is troublesome because of differences in sequencing strategies. We recently used the similar smMIP sequencing approach to detect CHDMs in an otherwise healthy group of individuals with a BMI >27 kg/m2.[43] The average age was 67 years. We detected a CHDM in 29% of all individuals, and CHIP in 11%. This is comparable to the frequencies detected in our current cohort. However, differences in BMI and geographical region (Romania versus the Netherlands) preclude any firm conclusions about this comparison.

Authors contributions

M.I.D., and H.T. contributed to the conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing of the original draft, reviewing and editing, visualization, supervision, project administration.

A.B.T., S.B., L.A.B.J., M.G.N., R.C.V.D., and A.H. contributed to the methodology, validation, formal analysis, resources, data curation, writing, reviewing, and editing.

N.P.R., and A.C.I contributed to the conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing of the original draft, reviewing, and editing, visualization, supervision, project administration, and funding acquisition.

M.I.D., H.T., N.P.R., and A.C.I. have directly accessed and verified the underlying data reported in the manuscript.

Inclusion and diversity statement

We support inclusive, diverse, and equitable conduct of research.

Star methods

Resource availability

Lead contact: Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Professor Niels P. Riksen (niels.riksen@radboudumc.nl).

Materials availability: This study did not generate new unique reagents.

Data availability:

- Data of gene regions sequenced, identified CHDMs and the probes used in this study is available in Supplementary Tables S1, S2, and S3.
- The proteomics results have been publicly deposited at PeptideAtlas (see KRT)
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

Code availability

- This manuscript used previously developed CHMIP-RsCh-PIPELINE pipeline to identify CHDMs. The code used in the pipeline is publicly available on GitHub (https://github.com/RosanneVanDeuren/CHMIP-RsCh-PIPELINE/tree/main).

Study participant details

This was a prospective, observational, single-centre cohort study. Between May 2017 and September 2018, 1020 consecutive patients with symptomatic stable CAD were screened for inclusion. The inclusion criteria were the detection of inducible myocardial ischemia at the treadmill or imaging stress testing and the presence of at least one coronary atherosclerotic stenosis on angiography. Coronary stenosis evaluation was performed by quantitative coronary angiography (Inturis, Philips Medical Systems, Eindhoven, The Netherlands) by two independent senior interventional cardiologists. All lesions with more than 90% diameter stenosis were considered significant.[44] An intermediate coronary lesion was defined as a luminal narrowing with a diameter stenosis ≥30% but ≤90%.[45]

The exclusion criteria consisted of any documented history of acute cardiovascular events.

The research protocol was approved by the Institution's Ethics Committee on research on humans (approval number 385/2017), and all patients gave written informed consent. All the procedures followed were in accordance with institutional guidelines. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Method details

The classic cardiovascular risk factors were recorded in all patients.

To evaluate the extent of coronary atherosclerosis, SYNTAX Score I was calculated by a senior interventional cardiologist based on the most recent coronary angiography. SYNTAX Score II was calculated by combining the anatomic and clinical prognosis variables (http://syntaxscore.org/calculator/syntaxscore/frameset.htm).

Blood sampling

Total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured in fasting plasma using a Roche cobas c501 chemistry analyser. Low-density lipoprotein (LDL) cholesterol was calculated with the Friedewald formula. Total blood cell counts were determined with an automated Sysmex-XN 1000 haematology analyser (Sysmex, Hamburg, Germany).

Identification of candidate Clonal Hematopoiesis Driver Mutations (CHDMs)

CHDMs were identified in DNA obtained from whole blood by ultrasensitive single-molecule molecular inversion probe (smMIP) sequencing.[15,46,47] In brief, a total of 300 MIP probes were designed to cover the majority of well-known CHIP/CH-related hotspots in 24 genes, including *ASXL1*, *TET2*, and *DNMT3A*. The probes were designed in 2017, at the beginning of patient enrolment, based on the existing literature data at that time, and completely cover the *DNMT3A* gene (Supplementary material file – Tables S1, S2, S3). For each sample two technical Polymerase Chain Reaction (PCR) replicates were sequenced, after which two independent data processing strategies were applied to identify CHDMs. Variants were further filtered by a targeted quality control workflow, in which final variant allele frequencies were calculated using samtools mpileup.[48] The average coverage for all individuals over the entire panel was 2,986x. Identified candidate CHDMs were validated in publicly available data sets. In the case of patients with multiple CHDMs, the CHDM with the highest VAF was included in the analysis.

Individuals with CHDMs were then divided into categories VAF<2% and VAF≥2% based on the arbitrary clinical cut-off for CHIP in accordance with the current literature.

Peripheral blood mononuclear cells isolation and stimulation

Non-fasting blood was collected in EDTA vacutainer tubes. Sample processing occurred within 2 hours. Plasma and serum were stored at -80°C until further use.

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (GE Healthcare, Chicago, USA) density gradient centrifugation for half an hour. PBMCs were washed by centrifugation with cold phosphate saline buffer and concentrated in Roswell Park Memorial Institute-1640 (RPMI) cell culture medium (Lonza, Basel, Switzerland) supplemented with 2 mmol/L glutamine (Gibco, Dublin, Ireland), 1 mmol/L pyruvate (Gibco, Dublin, Ireland) and 50 ug/mL gentamicin (Merck, Darmstadt, Germany).

Cells were counted using an EVE automatic cell counter (VWR, West Chester, USA). At least 20 million PBMCs were stored in freezing medium at -80°C and used for flow cytometry analysis.

To evaluate the cytokine production capacity, $1x10^5$ PBMCs per well were seeded in round-bottom 96-well plates (Eppendorf, Hamburg, Germany), and incubated with supplemented RPMI cell culture medium. Subsequently, cells were stimulated for 24h with 1 ng/mL lipopolysaccharide (LPS) (from Escherichia coli serotype 055:B5 (Sigma-Aldrich, St. Louis, MO, USA)), or 10 µg/mL Pam3Cys (P3C) (L2000, EMC micro-collections, Tubingen, Germany).[49] To measure adaptive immune response, PBMCs were stimulated for 7 days in RPMI, with $1x10^6$/mL Heat-killed (HK) Candida albicans conidia (UC820 strain) supplemented with 10% human pooled serum (Sigma Aldrich, Saint Louis, USA).

After the 24h and 7 days incubation period, supernatants were collected after plate centrifugation and stored at -80°C until cytokine measurements with commercial enzyme-linked immunosorbent assay kits (TNF, IL-6, IL-1β, IL-17 and IL-22 (Duoset, R&D Systems, MN, USA); IFN-γ (PeliKine Compact, Sanquin, Amsterdam, the Netherlands)), following the instructions of the manufacturer.

Flow cytometry

Stored PBMCs were first washed with phosphate saline buffer. Cells were then washed two times with cell wash solution (BD, Franklin Lakes, USA) and centrifuged at 500xG at room temperature for 5 minutes. Over the remaining cell pellet, 10 µL of each CD14-phycoerythrin, CD16-fluorescein isothiocyanate and CD11b-allophycocyanin anti-human/mouse antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) were added and mixed with 250 µL of cell wash solution to avoid cell clumping. After a 30 minutes incubation at room temperature, one washing step with cell wash solution was performed and the pellet was re-suspended in 500 µL of cell wash buffer. The samples were analysed using a BD FACS Canto II Flow cytometer (BD, Franklin Lakes, NJ, USA). Monocytes were selected based on FSC/SSC and CD14/CD16 expression and monocyte subsets were identified in the CD14/CD16 plot as percentage of gated according to current guidelines.[50,51] Each subtype or the total monocyte population were then analysed for the presence of CD11b marker (Percentage positive and Median Fluorescent Intensity on positive cells). Data were analysed with FlowJo software v10.8 (Becton Dickinson). Single stains and unstained samples were used for compensation.

Proteome analysis by proximity extension assays technology

All EDTA plasma samples were shipped to Olink Proteomics AB (Uppsala, Sweden) for analysis. Using proximity extension assays (PEA) technology,[52] the levels of 177 inflammatory proteins from the Olink Cardiovascular II and Inflammatory panels were measured (Supplementary Table S6).[53] Oligonucleotide-labelled antibody probe pairs bind to their targeted protein in each sample. When the two probes are brought in close proximity, the oligonucleotides will hybridize in a pair-wise manner.[53] To adjust for intra- and inter-run variation, data is quality controlled and normalized using an internal extension control and an inter-plate control (https://www.olink.com/resources-support/document-download-center/; accessed August 2020).[53] The final data is presented in Normalized Protein eXpression (NPX) values, which is an arbitrary unit on a log2-scale.[53] A high NPX value corresponds to a higher protein concentration.[53]

Follow-up

Patient follow-up was performed after four years. The endpoint consisted of the first major adverse cardiovascular event (MACE) and was the composite of cardiovascular death, acute myocardial infarction, or acute ischemic stroke. Information regarding outcomes was obtained from interviews with the

patients or their relatives, from patient charts and discharge documents, from the hospital's electronic database, or from primary care physician records. Data regarding vital status was available in all patients from the electronic records of the national insurance company.

Analysis of publicly available scRNAseq data

Publicly available single cell RNAsequencing data from patients with heart failure with or without *DNMT3A* driver mutations were used.[21] Data from classical, intermediate, and non-classical monocytes were overlaid with findings from our own proteomics and prediction models to confirm gene expression differences in patients with *DNMT3A* CHDMs.

Quantification and statistical analysis

Data distribution was assessed using Kolmogorov–Smirnov and D'Agostino tests. Quantitative continuous data were summarized as mean ± standard deviation (SD) whenever data followed the normal distribution; otherwise, median, and interquartile range (Q1–Q3, where Q1 = first quartile and Q3 = third quartile) were used. Groups were compared with Student t-test or Mann-Whitney U-test, as appropriate. Categorical data were presented as counts and proportions and compared with Chi-square test, or Fisher's exact test, as appropriate.

Univariable Cox proportional-hazards regression was used to evaluate the association between variables of interest and MACE. To evaluate MACE-free survival, patients who suffered a non-cardiovascular death were excluded from the analysis. The hazards ratio (HR), along with 95% confidence intervals (Cis) and p-values, were computed for each regression. For proteomic data, HR per 1 SD increase in each protein levels were computed. Multivariable Cox proportional-hazards regression analysis was used to identify variables independently associated with outcomes. The parameters that were statistically significant in the univariate analysis, namely sex and systolic blood pressure, together with age, were included in the multivariable models.

Receiver-operating characteristic (ROC) curves were constructed to evaluate the accuracy of VAF in predicting MACE. The area under the ROC curves (AUC) was determined as a scalar measure of performance. The Youden index was used to identify the ideal cut-off values from the ROC curves.

Kaplan-Meier curves were constructed for survival analysis. The log rank test was applied for comparison of event-free survival analysis.

Adjustment for multiple comparisons was performed. A Benjamini-Hochberg adjusted significance level was used, at a controlled false discovery rate (FDR) of 0.25 (p-value<0.042).

Statistical analysis was performed with GraphPad Prism version 9.3.1 for Windows (GraphPad Software, CA, USA) and MedCalc (v 20.019, MedCalc Software, Ostend, Belgium). All reported *p*-values are two-sided.

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Supplementary material

Table of contents:

Table S1 (available on journal website). List of 24 genes sequenced and known clonal hematopoiesis hotspots, related to Figure 1.

Table S2 (available on journal website). smMIP probes, related to Figure 1.

Table S3 (available on journal website). Variant characteristics of identified candidate clonal hematopoiesis driver mutations in this study, related to Figure 1.

Table S4. The relation between the size of the clone and the risk of MACE, related to Table 2.

Table S5. The association between leukocytes, and monocyte subsets, and the presence of CHDMs, related to Table 2.

Table S6 (available on journal website). The list of all measured proteomic biomarkers, related to Table 3.

Figure S1. MACE-free survival according to the presence of DNMT3A or non-DNMT3A gene mutations, related to Figure 2.

Figure S2. The cytokine production capacity (median and interquartile range) in the patient groups without CHDM and with CHDM/CHIP, related to Table 2.

Table S4. The relation between the size of the clone and the risk of MACE, related Table 2

	MODEL							
	I		II		III		IV	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
CHIP	3.19(1.27 – 8.01)	0.01	3.50(1.32 – 9.21)	0.01	3.61 (1.34 – 9.69)	0.01	3.31(1.19 – 9.18)	0.02
VAF>1.07	3.40(1.49 – 7.78)	0.003	2.87(1.21 – 6.84)	0.01	2.83(1.16 – 6.92)	0.02	3.20(1.24 – 8.24)	0.01

Model I = Univariable model
Model II = Model I adjusted for age, sex, and systolic blood pressure
Model III = Model II adjusted for body mass index, smoking, diabetes mellitus, and LDL-cholesterol
Model IV = Model III adjusted for BNP
95% CI = 95% confidence interval; CHIP = clonal hematopoiesis of indeterminate potential; HR = hazard ratio; MACE = major adverse cardiovascular events; VAF = variant allele frequency.

Table S5. The association between leukocytes, and monocyte subsets, and the presence of CHDMs, related to Table 2

PARAMETER	No CHDM (n=146)	CHDM (n=72)	*p*-value	VAF<2% (n=43)	*p*-value	VAF≥2% (n=29)	*p*-value
LEUKOCYTES							
WBC (/mm^3), mean±SD	7630±1795	7806±2331	0.54	7863±2103	0.47	7721±2670	0.81
Neutrophiles (%), median (Q1 – Q3)	61.9 (56.7 – 61.9)	60.6 (55.2 – 66.1)	0.34	61.4 (55 – 66.3)	0.20	60.6 (54.7 – 66)	0.94
Lymphocytes (%), median (Q1 – Q3)	27.3 (21.6 – 32.1)	27.5 (22.5 – 33.7)	0.49	27.2 (22.5 – 33.2)	0.54	29.1 (21.6 – 34.5)	0.66
Monocytes (%), median (Q1 – Q3)	7.9 (6.8 – 9.2)	8.2 (6.5 – 9.2)	0.91	8.5 (6.4 – 9.6)	0.83	7.5 (6.6 – 8.7)	0.30
Basophils (%), median (Q1 – Q3)	0.6 (0.4 – 0.8)	0.6 (0.5 – 0.8)	0.13	0.6 (0.5 – 0.9)	0.56	0.6 (0.5 – 0.7)	0.56
Eosinophils (%), median (Q1 – Q3)	1.8 (0.9 – 2.6)	2.3 (1.2 – 3.5)	0.01	2.7 (1.4 – 3.7)	0.005	1.7 (1.1 – 3.5)	0.49
NLR, median (Q1 – Q3)	2.3 (1.7 – 3.0)	2.1 (1.6 – 2.9)	0.81	2.1 (1.7 – 2.8)	0.44	2.0 (1.5 – 3.1)	0.47
MONOCYTE SUBSETS							
Classical monocytes (%), median (Q1 – Q3)	85.9 (79.4 – 90.6)	84.9 (77.0 – 89.6)	0.62	84.5 (76.7 – 89.5)	0.43	86.8 (76.9 – 89.9)	0.89
Intermediate monocytes (%), median (Q1 – Q3)	7.6 (4.9 – 10.9)	8.5 (5.6 – 11.4)	0.31	9.2 (5.8 – 12)	0.13	6.5 (5.3 – 11.1)	0.88
Non-classical monocytes (%), median (Q1 – Q3)	4.9 (3.4 – 7.8)	4.6 (2.9 – 9.2)	0.55	4.4 (3.4 – 10.4)	0.73	4.9 (2.3 – 8.8)	0.55
CD11b+ classical monocytes (%), median (Q1 – Q3)	83.7 (62.4 – 93.4)	74.5 (52.7 – 92.5)	0.11	76.2 (60 – 92.5)	0.35	71 (38.6 – 94.9)	0.11
CD11b+ intermediate monocytes (%), median (Q1 – Q3)	81.8 (68.5 – 93.1)	76.7 (55.4 – 91.3)	0.16	78.9 (63 – 91.9)	0.64	70.5 (48.6 – 90.7)	0.06
CD11b+ non-classical monocytes (%), median (Q1 – Q3)	39.9 (23.2 – 59.9)	30.4 (18.3 – 58.3)	0.10	26.8 (13.7 – 55.6)	0.07	33.2 (19.7 – 62.5)	0.56

All comparisons are made to the group without CHDM. CD = cluster of differentiation; n = number; NLR = neutrophils/lymphocytes ratio; Q1 = 1st quartile; Q3 = 3rd quartile; SD = standard deviation; WBC = white blood cells.

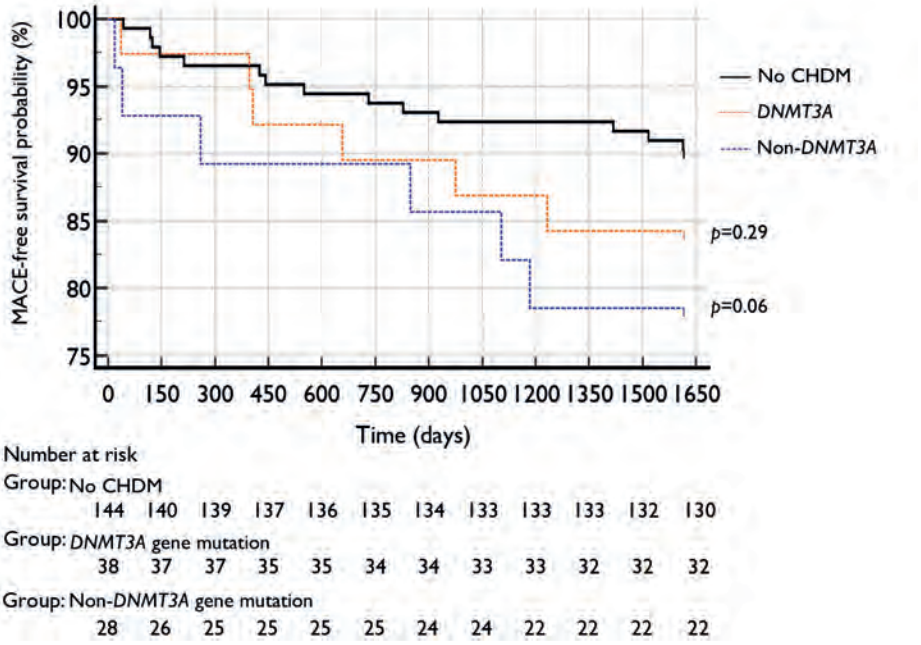

Figure S1. MACE-free survival according to the presence of DNMT3A or non-DNMT3A gene mutations, related to Figure 2. The comparison is made to the group without CHDMs. Patients who suffered a non-cardiovascular death were excluded from the analysis. CHDM = clonal hematopoiesis driver mutations; MACE = major adverse cardiovascular events.

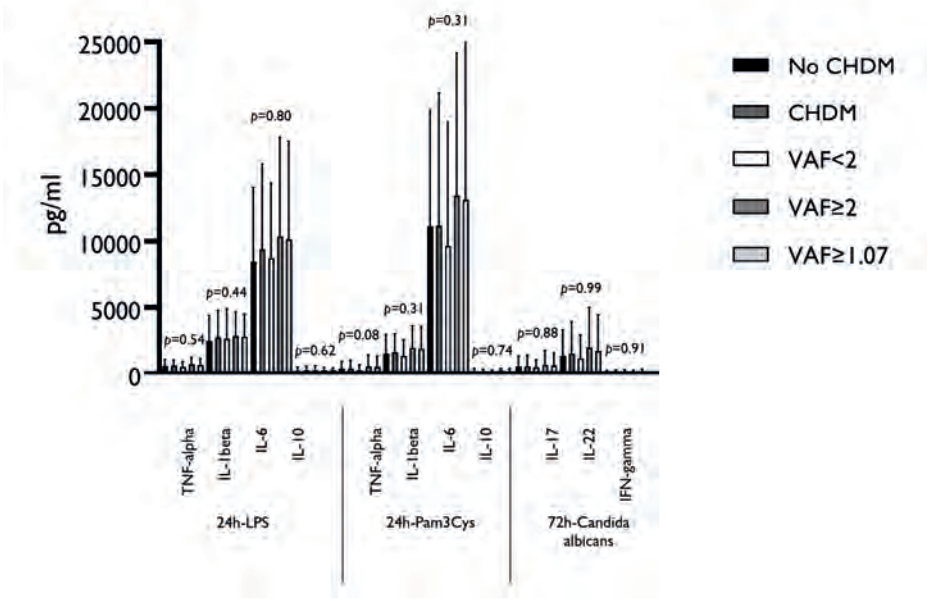

Figure S2. The cytokine production capacity (mean with standard deviation) in the patient groups without CHDM and with CHDM/CHIP, related to Table 2. The reported p-values are for the difference between patients with CHIP (VAF≥2%) and patients with no mutations. CHDM = clonal hematopoiesis driver mutations; CHIP = clonal hematopoiesis of indeterminate potential; IFN = interferon; IL = interleukin; TNF = tumour necrosis factor; VAF = variant allele frequency.

Chapter 4:

The association between clonal hematopoiesis driver mutations, immune cell function and the vasculometabolic complications of obesity

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Abstract

Background: Obesity is accompanied by dysregulated inflammation, which can contribute to vasculometabolic complications, including metabolic syndrome and atherosclerosis. Recently, clonal hematopoiesis of indeterminate potential (CHIP) has emerged as a risk factor for cardiovascular diseases. We aimed to determine how CHIP related to immune cell function, systemic inflammation, and vasculometabolic complications in obese individuals.

Methods and Results: 297 individuals with overweight and obesity, between the ages of 54 and 81, were recruited in a cross-sectional study. Clonal Hematopoiesis Driver Mutations (CHDMs) were identified with an ultrasensitive targeted assay. Assesment of carotid artery atherosclerosis was performed with ultrasound. Detailed immunological parameters, including cytokine production capacity of peripheral blood mononuclear cells (PBMCs), and targeted plasma proteomics analysis, were studied. Adipose tissue inflammation was determined in subcutaneous fat biopsies. Individuals with CHIP had higher concentrations of circulating IL-6. Total number of leukocytes and neutrophils were higher in individuals with CHIP. In contrast, *ex vivo* cytokine production capacity of PBMCs was significantly lower in individuals with CHIP. Sex stratified analysis showed that men with CHDMs had significantly higher leukocyte and neutrophil counts and *ex vivo* cytokine production capacity was lower in women with CHDMs. Surprisingly, the presence of atherosclerotic plaques was significantly lower in individuals with CHDMs. There was no relation between CHIP and metabolic syndrome.

Conclusions: In individuals with overweight or obesity, CHDMs are not associated with vasculometabolic complications, but rather with a lower presence of carotid plaques. CHDMs associate with increased circulating inflammatory markers and leukocyte numbers, but a lower PBMC cytokine production capacity.

Keywords: cross-sectional study, sex-stratified, obesity, clonal hematopoiesis, immunological parameters, carotid atherosclerotic plaque, adipose tissue

Research Perspective

What is new?

- In a cohort of subjects with overweight or obesity, using an ultrasensitive targeted assay, we detected Clonal Hematopoiesis Driver Mutations (CHDMs) in 28% of subjects, and Clonal Hematopoiesis of Indeterminate Potential (CHIP) in 11%.
- The presence of CHDMs was associated with higher circulating leukocytes, neutrophils, and interleukine-6, but with a lower ex vivo PBMC cytokine production capacity.
- CHDM presence was not associated with metabolic syndrome or insulin resistance.
- CHDM presence was associated with a lower presence of carotid atherosclerotic plaques.
- What questions should be addressed next?
- To investigate how CHDM presence affects monocyte versus macrophage cytokine production, both in the unstimulated and stimulated conditions.
- To investigate in detail how CHDMs differentially affect the initiation, progression, and destabilization of atherosclerotic plaques, in particular in the presence of obesity.

Non-standard Abbreviations and Acronyms

AI:	Augmentation Index
CHDM:	Clonal Hematopoiesis Driver Mutation
CHIP:	Clonal Hematopoiesis of Indeterminate Potential
DNMT3A:	DNA methyltransferase 3a
NLR:	Neutrophil-to-lymphocyte ratio
PWV:	Pulse Wave Velocity
TET2:	tet methylcytosine dioxygenase 2
VAF:	Variant Allele Frequency

Introduction

The prevalence of overweight and obesity has increased globally in the past decades. This poses a great risk for cardiometabolic diseases, and a significant burden on the healthcare system[1]. Obesity is associated with the development of atherosclerosis, the leading cause of cardiovascular diseases (CVD), via metabolic dysregulation and chronic low-grade inflammation. Furthermore, obesity can induce activation of innate immune cells, such as monocytes, which are critical in the development of atherosclerotic CVD[2]. In patients with established atherosclerotic CVD, isolated circulating monocytes are characterized by a hyperresponsive phenotype[3-5]. In the arterial wall, monocyte-derived macrophages play a key role in the pathophysiology of atherosclerosis[6]. Similarly, monocyte-derived macrophages in the adipose tissue are important regulators of inflammation and insuline resistance[7,8].

Innate immune cell function is influenced by many factors, and one recently described mechanism is clonal hematopoiesis. Somatic mutations are common in highly proliferative tissues, including the hematopoietic stem and progenitor cells in the bone marrow. If, by chance, one of these mutations confers a selective survival or fitness advantage, it leads to the clonal expansion of that cell[9]. Recent advances in sequencing technologies demonstrated that clonal hematopoiesis is common among the aging population[10]. Clonal hematopoiesis driver mutations (CHDMs) are linked with an increased risk for hematological malignancies[11,12], but multiple epidemiological studies also show that presence of CHDMs is associated with an increased risk of atherosclerotic CVD[13].

Common somatically mutated genes include *DNMT3A (*DNA methyltransferase 3a*)*, *TET2* (tet methylcytosine dioxygenase 2), *ASXL1* (ASXL transcriptional regulator 1), *JAK2* (Janus kinase 2) and *TP5*3 (tumor protein p53)[9]. Clonal hematopoiesis of indeterminate potential (CHIP) is defined as the presence of CHDMs with a variant allele frequency (VAF) ≥2% without clinical diagnosis of a hematological malignancy[11]. This cutoff is predominantly determined by the limitations of standard sequencing methods, including whole-exome sequencing, frequently used to identify CHDMs. Recently, the 2% cutoff has been challenged, underscoring the potential clinical relevance of driver mutations with lower VAFs[14].

A few experimental pre-clinical studies suggest that CHDMs are involved in the metabolic and cardiovascular complications of obesity. For example,

experimental studies in mice showed that TET2 driven clonal hematopoiesis is causal to age- and obesity-related insulin resistance. This is mediated by the activation of innate immune cells, in particular plaque macrophages, via NLRP3-inflammasome driven IL-1β production[15,16]. Furthermore, single cell RNA sequencing of unstimulated human monocytes from patients with heart failure revealed higher expression of inflammatory genes in individuals harboring DNMT3A mutations. In the general population, the presence of CHDM is associated with higher waist-to-hip ratio[17]. There are also indications for reverse causality, with atherosclerosis-associated inflammation accelerating clonal hematopoiesis in experimental mouse models[18]. Using the exact same sequencing technique and gene panel as in our study, Andersson-Assarsson *et al.*, recently reported an increase in time in obese individuals, but not in those who underwent bariatric surgery[19].

Here, we propose that clonal hematopoiesis is an important driver of inflammation in patients with obesity, predominantly associated with older age, and of the subsequent development of metabolic syndrome and atherosclerosis. We hypothesize that this is mediated by hyperinflammatory responsiveness of monocyte-derived macrophages with CHDMs in the arterial wall and in the adipose tissue, which is also reflected by hyperresponsiveness in circulating monocytes. To this end, we identified a panel of candidate CHDMs with an ultrasensitive assay in a cohort of 297 human subjects with overweight and obesity. We associated CHDMs to parameters of metabolic and atherosclerotic complications, to parameters of systemic inflammation, as well as to the innate immune cell phenotype and function and adipose tissue inflammation. Since the regulation of inflammation in relation to metabolic syndrome is highly sex-specific, we performed all analyses stratified by sex[20]. Lastly, we performed a DNMT3A-specific analysis as this gene harboured the most CHDMs in our cohort and it was shown to play a causal role in driving atherosclerosis in murine models[21].

Methods

Study subjects and clinical measurements

This cross-sectional single center cohort study was part of the Human Functional Genomics Project (Human Functional Genomics Project), a large scale project including various cohorts designed to understand the effects of human genetic variation and diversity of gut microbiome on the immune

system during health and disease. "The 300 Obesity(OB) cohort" is one of the cohorts belonging to the Human Functional Genomics Project, specifically aimed at understanding the role of inflammation in individuals with obesity. A cohort of 302 individuals ("the 300 OB cohort") with a BMI> 27 kg/m^2, between the ages of 54 and 82, consisting mostly of Western European ancestry, was recruited in the Radboud university medical center from 2014 to 2016. The research protocol was approved by the Radboud University Ethical Committee (nr. 46846.091.13), and all subjects gave written informed consent. The study protocol was performed in accordance with the 1975 Declaration of Helsinki.

For an extensive description of the cohort and measurements please see ter Horst *et al.*, 2020[20].

Lipid lowering medication was stopped four weeks before measurements. Blood samples were drawn between 8:30 and 12:00 in the morning after an overnight fast. The blood samples were collected into EDTA vacutainers and the following laboratory procedures took place immediately after collection. Total blood cell counts were performed in fresh EDTA blood samples using a Sysmex Hematoanalyzer XE5000. Plasma samples were stored at -80°C until further measurement. Blood glucose, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were determined by standard laboratory protocols. Systolic and diastolic blood pressure were measured after 30 minutes of supine rest.

Metabolic syndrome was defined according to the National Cholesterol Education Program ATP III criteria[22]. Participants having at least 3 of the following characteristics were determined to have metabolic syndrome:

1. Abdominal obesity: Waist circumference ≥ 102cm in men, or ≥ 88cm in women.
2. Triglycerides: serum TG ≥ 150 mg/dL (1.7 mmol/L), or treatment with lipid-lowering drugs.
3. HDL Cholesterol: serum HDL-C < 40 mg/dL (1 mmol/L) in men, or < 50 mg/dL (1.3 mmol/L) in women.
4. Blood pressure: ≥ 130/85 mmHg, or treatment for hypertension.
5. Fasting plasma glucose: ≥ 100 mg/dL (5.6 mmol/L), or treatment for elevated blood glucose.

Insulin resistance was determined according to the Homeostasis Model Assessment (HOMA) model[23].

Cardiovascular measurements

Carotid artery measurements were performed by ultrasound, as described previously[24]. Carotid intima-medial thickness (IMT) was determined in the proximal 1 cm straight portion of the carotid artery at 90°, 120° and 180° angles for 6 heart beats. The presence and thickness of plaques were assessed in the common carotid, internal carotid, or external carotid artery or at the carotid bulbus. A focal thickening of the carotid wall of at least 1.5x IMT or an IMT>1.5 mm was termed as plaque presence. Pulse Wave Velocity (PWV) and Augmentation Index adjusted for heart rate (AI) were measured to assess arterial stiffness by SphygmoCor (ATCOR Medical) under standard operating protocol.

Adipose tissue analysis

Detailed methodology regarding adipose tissue measurements were previously described[20]. Briefly, subcutaneous abdominal adipose tissue samples were obtained by needle biopsies under local anesthesia(biopsy needle 14g, 100 Sterican, B/Braun, Ref: 4665473). The biopsies were performed 6 to 10 cm lateral to the umbilicus in the right lower quadrant.

Adipocyte size, presence of macrophages, and crown-like structures (CLS) were determined by fluorescent microscopy (Zeiss Axiphot) utilizing Hematoxylin and Eosin (H&E) in combination with CD68 staining. From 4 microscopic fields of view, adipocyte cell diameters were measured and indicated as area and Feretmin (Feret minimal diameter: the minimal diameter of each cell). Adipose tissue sections were stained with a CD68-monoclonal antibody (Serotec, Oxford) to identify macrophages. The percentage of macrophages was determined as the total number of macrophages divided by the total number of adipocytes from 15 random microscopic fields of view. A CLS was defined as an adipocyte surrounded by at least 3 macrophages[25,26].

Additionally, total RNA was isolated from adipose tissue samples by Trizol (Invitrogen) extraction, followed by cDNA library preparation (iScript cDNA synthesis kit, Bio-Rad). mRNA expression levels were determined by real-time PCR and normalized to housekeeping gene (Ribosomal Protein L37a, RPL37A) expression. Primer sequences can be found in Supplementary table 1 (Table S1).

Identification of CHDMs

CHDMs were identified in whole blood by an ultra-sensitive assay, as previously described[19,27]. In short, 300 single-molecule molecular inversion probes (smMIP) were designed against a selection of well-known hotspots of a panel of 24 clonal hematopoiesis driver genes (Table S2). The *DNMT3A* gene, which contains the most driver mutations, was entirely covered[28]. We used the smMIP probe panel designed in 2017 [29], that was also used in recent studies[19,27] (Table S3). For each sample two technical PCR replicates were run, thereafter two independent data processing strategies and a quality control step were performed. Variant allele frequencies were calculated using samtools mpileup[30] (Table S4).

All identified CHDMs were validated in publically available datasets. For individuals with more than one CHDM, the CHDM with the highest VAF was included in the analysis. We divided all identified CHDMs into: "Low VAF" for CHDMs with a VAF<2% and "High VAF" for CHDMs with a VAF≥2% in accordance with the current CHIP cutoff.

From the 302 individuals of the cohort, whole blood samples of 3 individuals could not be obtained, and the samples from 2 individuals did not pass the quality control for sequencing. Therefore, 5 individuals were completely excluded from this study, corresponding to 297 individuals for the final analysis.

PBMC isolation and *ex vivo* stimulation

Peripheral Blood Mononuclear Cells (PBMCs) were isolated with differential density centrifugation over Ficoll-Paque(GE Healthcare). PBMCs were then washed thrice by centrifugation with phosphate saline buffer. Isolated PBMCs were resuspended in Dutch modified Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 50 µg/mL gentamicin (Centrafarm), 2 mM GlutaMAX and 1 mM pyruvate (Life Technologies). $0.5x10^6$ cells per well were stimulated for 24 hours in 96-wells round-bottom plates (Greiner) at 37 °C and 5% CO_2. Stimuli used include RPMI as negative control, Lipopolysaccharide (LPS) (Sigma-Aldrich, E. coli serotype 055:B5, further purified as described[31]) at low (1 ng/ml) and high concentrations (100 ng/ml) and Pam3Cys (1 ug/ml) (EMC microcollections, L2000). Supernatants were collected after 24 hours and stored at -20°C until measurements were performed.

LPS and Pam3Cys are agonists of Toll-like receptor (TLR) 4 and TLR2 respectively. These TLRs are relevant receptors in the context of atherosclerosis

and adipose tissue inflammation since several DAMPs and PAMPs in these microenvironments stimulate these receptors[32]. In addition, these TLR agonists are chosen based on our previous work in which we showed that LPS and Pam3Cys stimulation of PBMCs can accurately identify monocytes with a hyperinflammatory ("trained") phenotype[33,34]. We capture the response of monocytes by measuring monocyte-specific cytokines that can be released upon 24 hour stimulation when adaptive immune response cannot be initiated yet.

Cytokine measurements

Cytokine concentrations upon stimulation of PBMCs and in plasma were measured with commercially available Enzyme-linked Immunosorbent Assay (ELISA) kits according to instructions supplied by the manufacturer. For detailed information on the ELISA kits used please refer to Table S5.

Proteomic profiling

The concentrations of 177 inflammatory proteins from the Olink Cardiovascular II and Inflammatory panels were measured using the previously described proximity extension assays (PEA) technology (Olink Bioscience AB, Uppsala, Sweden)[35]. Quality control was ensured using internal extension control and inter-plate control. The data is presented in Normalized Protein eXpression (NPX) values, an arbitrary unit on log2 scale.

Statistics

Distribution of data was assessed with Shapiro-Wilk test. Normally distributed data is shown as mean ± standard deviation. If the data did not follow a normal distribution, it is shown as median and interquartile ranges. Each group (All CHDM, High VAF, and Low VAF) was independently compared to the subjects without CHDM ('No CHDM group') with Student's t-test or Mann-Whitney U test when appropriate. Spearman correlation was used to determine the association between VAF and various clinical and immunological parameters. The same statistical methodology was applied for the sex specific analyses. $P<0.05$ is considered statistically significant and is indicated with an asterisk in tables. All statistical analyses were performed in R version 4.1.1 (R Core Team). Part of the data is publically available on the website of Human Functional Genomics Project (HFGP (bbmri.nl)), the rest is available upon reasonable request to the HFGP committee.

Results

The study population included 297 individuals with a median age of 67 years (interquartile 1 and 3, 63-71 years) and BMI of 30 kg/m^2 (28-32 kg/m^2). Men represented 55% of the cohort. 54% of the cohort met the NCEP ATP III criteria of Metabolic Syndrome, and approximately half of the participants had carotid atherosclerotic plaques as measured by ultrasound. Hypertension was diagnosed in 60% of the study population, while diabetes was present in 12%. Median total cholesterol concentration was 6.3 mmol/l and triglyceride concentration was 1.61 mmol/l. 27% of the cohort used lipid lowering drugs. Detailed baseline characteristics of the study population is presented in Table 1.

CHDM prevalence and characteristics

110 candidate CHDMs were identified in 85 individuals; 62 individuals (21% of the cohort) carried a single CHDM and 23 individuals (8%) had more than one CHDM (Fig 1A). The VAF of all CHDMs ranged from 0.01% to 34.5%, with a mean of 3.3% and median of 1.1%.

In 33 individuals, we identified CHIP (i.e. CHDM with a VAF≥2%), and 52 individuals had CHDMs with VAF<2%. We identified CHDMs in 11 individual genes. Mutations in *DNMT3A* (73%) and *TET2* (7%) genes were the most common in the entire cohort (Fig 1A). Additionally, *DNMT3A* mutations were the most common across all age groups (Fig 1B).

Relation between CHDM and clinical characteristics

We did not observe statistically significant differences in baseline characteristics between subjects with and without CHDMs, apart from the finding that individuals with CHDMs with VAF<2% presented with higher heart rate (Table 2). Although there was no significant association between sex and presence of CHDMs, we observed a trend towards more women in the All CHDM group (p=0.054). Sex-specific analysis revealed that men with CHIP were significantly older than those without CHDMs and men with CHDMs with VAF<2% had significantly higher heart rate (Table S6). In women, there were no differences in baseline characteristics in any of the groups (Table S7). We did not observe a correlation between presence or size of the clones and any of the other baseline parameters. When restricting the analyses only to CDHMs in DNMT3A, we observed significantly more women among the carriers of CHDMs in this gene (data not shown).

Table 1: Baseline characteristics of the study cohort.

Parameter	n=297
Clinical Data	Median (IQR1-IQR3) or %
Sex (male. %)	55%
Age (years)	67 (63-71)
BMI (kg/m2)	30 (28-32)
Waist to hip ratio	1 (0.9-1)
Systolic blood pressure (mmHg)	130 (119-139)
Diastolic blood pressure (mmHg)	80 (74-86)
Heart rate (beats/minute)	61 (55-67)
Hypertension (%)	60%
Plaque presence	53%
Diabetes mellitus (%)	12%
Metabolic syndrome. NCEP ATP III criteria. (%)	54%
Pack years	16.1 (8.5-31)
Laboratory data	
Creatinine (µmol/l)	80 (67-91)
Glucose (mmol/l)	5.4 (5-6)
Urate (mmol/l)	0.4 (0.3-0.4)
Total cholesterol (mmol/l)	6.3 (5.6-7)
Triglycerides (mmol/l)	1.6 (1.3-2.1)
HDL cholesterol (mmol/l)	1.3 (1.1-1.5)
Non-HDL cholesterol (mmol/l)	4.9 (4.2-5.7)
LDL cholesterol (mmol/l)	4.1 (3.5-4.7)
Concomitant medication	
Antihypertensive drugs (%)	45%
Antidiabetic drugs (%)	9%
Lipid lowering drugs (%)	27%

Data is shown as median (interquarile range 1- interquartile range 3) and percentage (%) where appropriate. BMI: body mass index, HDL cholesterol: high density lipoprotein cholesterol, LDL cholesterol: low density lipoprotein cholesterol, NCEP ATP III (National Cholesterol Education Program Adult Treatment Panel III)

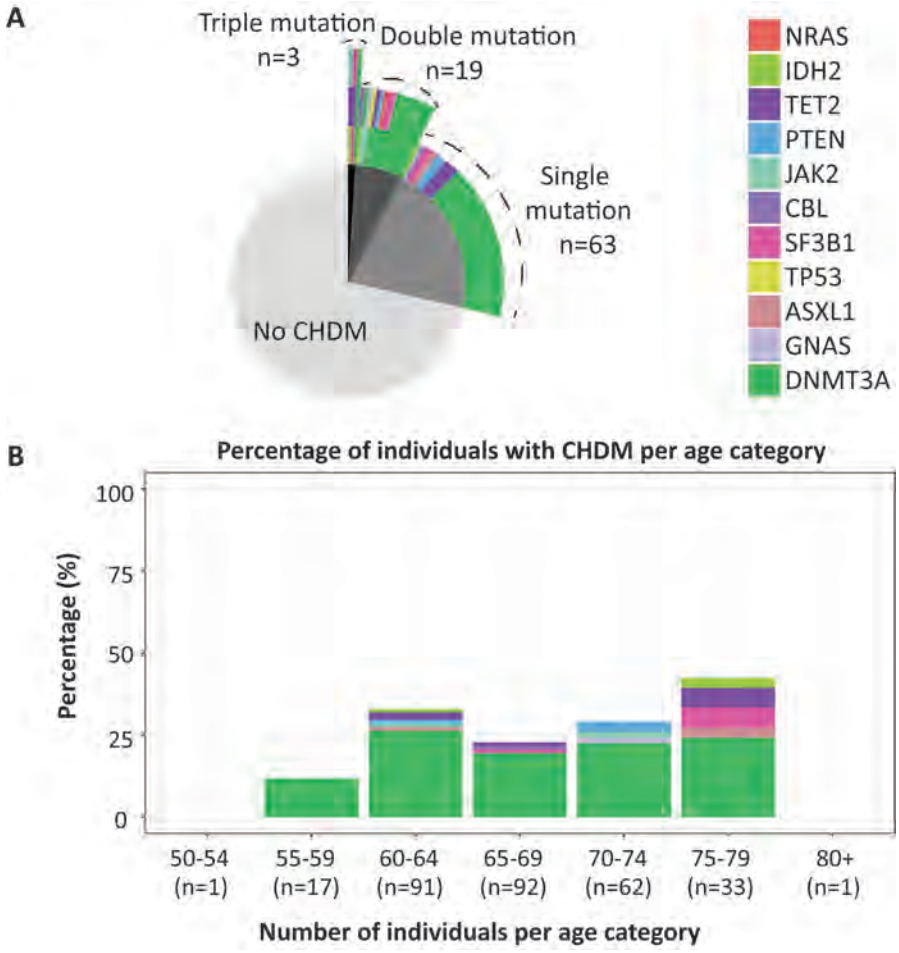

Figure 1: Characterization of candidate CHDMs identified in our study. **A**) Pie chart indicating number of individuals without CHDMs, with a single mutation, double mutations and triple mutations. Top rings indicate genes affected from the mutations. For more than one mutation carrying individuals second and third mutations are displayed as multiple rings on top of each other. **B**) Percentage of individuals with CHDMs per age category and gene affected. Age range of the entire cohort is depicted, "n" refers to total number of individuals in that age category.

Table 2: Baseline characteristics according to CHDM status.

	No CHDM (n=212)	All CHDM (n=85)	High VAF (n=33)	Low VAF (n=52)	Correlation with VAF
Age (years)	66 (63-70)	68 (63-72)	67 (63-74)	68 (63-71)	0,1
Sex (% Male)	58	46	45	46	
Weight (kg)	88 (82-97)	89 (81-99)	89 (80-98)	87 (82-99)	-0,1
Height (cm)	172 (165-178)	171 (164-176)	172 (164-176)	171 (164-177)	-0,1
BMI (kg/m^2)	30 (28-32)	30 (28-32)	30 (28-32)	30 (28-32)	0
Creatinine (µmol/L)	81 (67-92)	77 (68-89)	76 (70-88)	78 (67-89)	-0,2
Glucose (mmol/L)	5.4 (5-6)	5.4 (5-6)	5 (5-6)	5.4 (5-6)	0,004
Total cholesterol (mmol/L)	6 (6-7)	6 (5-7)	6 (5-7)	6 (6-7)	-0,1
Triglycerides (mmol/L)	1.6 (1.3-2.2)	1.5 (1.3-2)	1.5 (1.3-2)	1.5 (1.3-2)	-0,023
Heart rate (beats/min)	61 (55-67)	64 (58-71)	62 (59-67)	66 (57-73) *	-0,2
Antihypertensives (%)	47	40	52	33	
Lipid lowering drugs (%)	28	25	18	29	
Antidiabetic drugs (%)	9	7	6	8	

BMI: body mass index

All data are given as median (interquartile ranges 1-3). *indicates p<0.05 compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Association of CHDM with parameters of metabolic dysregulation

We investigated the association between presence of CHDMs and presence of metabolic syndrome and its individual components, and parameters related to insulin resistance and diabetes mellitus type 2. We did not observe a higher prevalence of metabolic syndrome, its individual components or diabetes in individuals with clonal hematopoiesis (Table 3).

Table 3: Parameters of metabolic dysregulation (metabolic syndrome and diabetes) according to CHDM status.

	No CHDM (n=212)	All CHDM (n=85)	High VAF (n=33)	Low VAF (n=52)	Correlation with VAF
Diabetes mellitus(%)	14	9	9	10	
Metabolic syndrome (%)	55	52	55	50	
Liver fat (mg/cm^3)	0.058 (0.024-0.12)	0.062 (0.029-0.16)	0.059 (0.034-0.14)	0.066 (0.017-0.17)	-0,01
Waist circumference (cm)	106 (100-110)	105 (100-112)	106 (100-110)	104 (100-113)	-0,09
Hip circumference (cm)	110 (106-114)	111 (107-114)	109 (106-114)	111 (109-114)	-0,09
HDL cholesterol (mmol/l)	1.27 (1.1-1.51)	1.32 (1.14-1.51)	1.32 (1.11-1.51)	1.33 (1.14-1.52)	0,02
LDL cholesterol (mmol/l)	4.15 (3.57-4.72)	4.06 (3.29-4.64)	4.04 (3.18-4.58)	4.07 (3.36-4.81)	-0,07
Systolic blood pressure (mmHg)	129 (118-138)	130 (123-140)	129 (123-140)	131 (123-140)	-0,05
Diastolic blood pressure (mmHg)	80 (74-85)	80 (72-87)	76 (72-87)	80 (72-87)	-0,08
HOMA-IR	7 (4-11)	7 (4-16)	5 (4-12)	8 (4-16)	-0,13

HDL cholesterol: high density lipoprotein cholesterol, LDL cholesterol: low density lipoprotein cholesterol, HOMA-IR: Homeostatic Model Assessment of Insulin resistance, mmHg: millimeter of mercury

All data are given as median (interquartile ranges 1-3). *indicates $p<0.05$ compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Association between adipose tissue inflammation and presence of CHDMs

Adipose tissue is an important site for generation of various cytokines and adipokines, and is strongly associated with the development of cardiometabolic complications of obesity. Therefore, we characterized adipose tissue biopsies by immunohistochemistry and qPCR and explored whether adipose tissue inflammation is more severe in individuals with CHDMs.

Although we did not identify a higher number of CD68$^+$ macrophages by immunohistochemistry, we observed significantly higher expression of CD68, adiponectin and TNF in individuals with CHDMs with VAF<2% measured with qPCR (Table S8).

Relationship between CHDM and parameters of atherosclerosis

To explore whether the presence of CHDM correlates with the presence of atherosclerosis in subjects with obesity, we investigated IMT and carotid plaque presence and characteristics in relation to CHDM. We found that the presence of carotid plaques was lower in individuals with any CHDM and CHDMs with VAF<2% compared to individuals without CHDMs. The same results were obtained for only the CHDMs in the DNMT3A gene (data not shown). Individuals with CHIP mutations had a significantly higher augmentation index (AI) suggestive of an increased systemic arterial stiffness (Table 4).

Table 4: Carotid IMT (cIMT) and parameters of carotid plaques and measures of arterial stiffness according to CHDM status of the participants.

	No CHDM (n=212)	All CHDM (n=85)	High VAF (n=33)	Low VAF (n=52)	Correlation with VAF
cIMT (µm)	791 (703-884)	753 (691-859)	815 (713-882)	745 (692-832)	0,11
Carotid plaque presence (%)	59	38 *	48	31 *	
Number of plaques	1 (1-2)	1 (1-2)	2 (1-2)	1 (1-2)	0,16
Max plaque thickness (mm)	2.2 (1.8-2.8)	2.65 (1.98-3.2)	2.75 (2-3.2)	2.55 (1.87-2.97)	0,17
Max stenosis (%)	0.29 (0.23-0.39)	0.3 (0.25-0.39)	0.37 (0.27-0.4)	0.29 (0.22-0.34)	0,25
PWV (m/s)	9.2 (8.4-10.7)	9.4 (8.1-10.4)	9.9 (8.2-10.4)	9.3 (8.1-10.9)	-0,03
Augmentation index (%)	25.7 (20.5-30.3)	27.5 (21.6-33.3)	29.9 (24.6-34.8)*	25.3 (19-32.1)	0,31
History of CVD (%)	16	14	15	13	

PWV: Pulse wave velocity, CVD: Cardiovascular disease
All data are given as median (interquartile ranges 1-3). *indicates p<0.05 compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Leukocyte number and function and presence of CHDMs

The absolute number of leukocytes and thrombocytes were measured in whole blood. We identified a significantly higher number of total leukocytes, and specifically neutrophils, in individuals with CHDMs and in CHIP carriers. DNMT3A CHDM carriers also had significantly higher number of total leukocytes (data not shown). There were no differences in monocyte or lymphocyte counts (Table 5). Sex-specific analysis revealed men with CHIP had significantly higher

neutrophil-to-lymphocyte ratio (Table S9). In contrast, there was no difference in any leukocyte count in women (Table S10).

Table 5: Leukocyte numbers and differentiation, and thrombocyte numbers separated according to CHDM status of the participants.

	No CHDM (n=212)	All CHDM (n=85)	High VAF (n=33)	Low VAF (n=52)	Correlation with VAF
Leukocytes 10^9/l	5.7 (5-6.6)	6.1 (5.3-7.4) *	6.2 (5.5-7.6) *	5.9 (5.3-7.4)	0,15
Neutrophils 10^9/l	3.2 (2.6-3.7)	3.4 (2.7-4.4) *	3.6 (2.9-4.5) *	3.2 (2.7-4.3)	0,13
Lymphocytes 10^9/l	1.8 (1.5-2.2)	1.9 (1.6-2.3)	1.9 (1.6-2.2)	1.9 (1.6-2.3)	0,1
Monocytes 10^9/l	0.5 (0.4-0.6)	0.5 (0.4-0.6)	0.5 (0.4-0.6)	0.5 (0.4-0.6)	0,1
Eosinophils 10^9/l	0.16 (0.09-0.21)	0.15 (0.1-0.29)	0.13 (0.09-0.29)	0.16 (0.11-0.22)	0,002
Basophils 10^9/l	0.03 (0.02-0.04)	0.03 (0.02-0.04)	0.03 (0.02-0.05)	0.03 (0.02-0.04)	-0,03
Thrombocytes 10^9/l	225 (194-262)	231 (196-266)	226 (203-281)	231 (196-264)	0,18
NLR	1.72 (1.31-2.18)	1.8 (1.41-2.25)	1.81 (1.5-2.42)	1.78 (1.32-2.08)	0,021

NLR: Neutrophil to lymphocyte ratio
All data are given as median (interquartile ranges 1-3). *indicates $p<0.05$ compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Ex vivo cytokine production capacity of PBMCs can be used as a measure of inflammatory responsiveness, and this has been shown to be higher in patients with established coronary heart disease[5,36]. Therefore, we characterized proinflammatory cytokine production capacity of PBMCs upon stimulation with Pam3Cys and LPS at different concentrations. Interestingly, we observed significantly lower production of IL-1β upon Pam3Cys stimulation in individuals with all CHDMs and CHDMs with VAF<2. Likewise, stimulation with LPS (both concentrations) led to significantly lower production of IL-6 in individuals with CHDMs, and CHIP carriers had significantly lower IL-6 upon stimulation with high concentration of LPS. Lastly, stimulation with Pam3Cys resulted in significantly less IL-6 production in all groups compared to individuals without any CHDMs (Table 6). Interestingly, the lower production of these cytokines in individuals with CHDMs was solely seen in women and not in men (Tables S11 and S12). When restricting the analyses to CHDMs in DNMT3A, we observed

similar lower *ex vivo* cytokine production capacity seen in the entire cohort (data not shown).

Table 6: *Ex vivo* cytokine production capacity of PBMCs separated according to CHDM status of the participants.

	No CHDM (n=212)	All CHDM (n=85)	High VAF (n=33)	Low VAF (n=52)	Correlation with VAF
LPS (1 ng/ml) IL-1β	1075 (599-1936)	998 (564-1710)	1036 (564-2013)	905 (561-1399)	0,05
LPS (100 ng/ml) IL-1β	2476 (1544-3713)	2210 (1245-3375)	2703 (1280-3274)	1823 (1240-3396)	0,01
Pam3Cys (1 µg/ml) IL-1β	280 (111-640)	174 (77-474) *	163 (53-569)	180 (82-424) *	-0,004
LPS (1 ng/ml) IL-6	5750 (3278-8968)	4850 (2969-6547) *	4464 (2989-6547)	5318 (2963-6449)	-0,06
LPS (100 ng/ml) IL-6	8322 (5206-12536)	7167 (4279-10797) *	6062 (3606-9402) *	7410 (4611-11114)	-0,09
Pam3Cys (1 µg/ml) IL-6	3975 (1759-6545)	2010 (1021-4548) *	2010 (811-4548) *	2034 (1364-4627) *	-0,05

LPS: Lipopolysaccharide, IL: interleukin, IL-1β and IL-6 concentration units are given in pg/ml
All data are given as median (interquartile ranges 1-3). *indicates $p<0.05$ compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

The relation between CHDMs and circulating cytokines and adipokines

To determine the association between systemic inflammation and presence of CHDMs we measured a selection of circulating cytokines and adipokines in plasma. We observed significantly higher concentrations of circulating IL-6 in individuals with CHIP. We identified a trend towards higher circulating IL-1β concentration in individuals with CHDMs with VAF<2%, although this did not reach statistical significance ($p=0.053$). We did not observe statistically significant differences in hsCRP concentrations. Additionally, individuals with CHDMs with VAF<2% had significantly higher concentrations of resistin in circulation (Table 7). We did not observe associations for any other circulating marker. There was no sex specific association between circulating markers and the presence of CHDMs (Tables S13 and S14).

To further investigate the association between circulating proteins and CHDM status, we performed a targeted proteomics approach with Olink panels Cardiovascular II and Inflammation. Table S15 shows the list of proteins that were significantly different in individuals with CHDMs. However, significance

was lost after correction for multiple testing. Figure S1 shows volcano plots indicating all proteins that were significantly higher or lower in the participants with CHDMs.

Table 7: Circulating cytokine and adipokine concentrations separated according to CHDM status of the participants.

	No CHDM (n=212)	All CHDM (n=85)	High VAF (n=33)	Low VAF (n=52)	Correlation with VAF
IL-6 (pg/ml)	2.4 (1.7-3.4)	2.6 (1.7-4.1)	2.9 (1.9-4.4) *	2.4 (1.6-3.4)	0,18
IL-1β (pg/ml)	0.06 (0.06-0.11)	0.06 (0.06-0.12)	0.06 (0.06-0.13)	0.07 (0.06-0.11)^	-0,048
IL-18 (pg/ml)	304 (227-490)	288 (216-484)	320 (221-455)	285 (210-505)	0,024
IL-18bp (ng/ml)	17.2 (14.1-21)	16.1 (13.3-19.2)	16 (13-17.8)	16.2 (14.1-20)	-0,14
hsCRP (µg/ml)	1.9 (0.9-3.3)	2 (1.2-3.3)	1.9 (0.9-3.9)	2 (1.4-3.3)	0,083
AAT (mg/ml)	0.9 (0.6-1.7)	0.9 (0.6-1.4)	0.9 (0.6-1.4)	0.9 (0.6-1.4)	0,019
Resistin (ng/ml)	10.6 (8.2-13.5)	11.5 (9.2-15)	11.6 (8.7-13.1)	11.4 (9.5-16.2) *	-0,095
Leptin (ng/ml)	15.8 (9.7-29.5)	18.1 (11.1-35.5)	17.2 (9.3-25.5)	19.1 (11.6-36.6)	-0,028
Adiponectin (µg/ml)	4.3 (2.8-6)	4.3 (3.1-5.7)	4.3 (3.4-5.2)	4.3 (3.1-6.4)	0,15

IL: interleukin, IL-18bp: IL-18 binding protein, CRP: C-reactive protein, AAT: Alpha-1 antitrypsin, ^: p= 0.053
All data are given as median (interquartile ranges 1-3). *indicates $p<0.05$ compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Discussion

In this cross-sectional study of older individuals with overweight or obesity, we investigated the presence of clonal hematopoiesis driver mutations in relation to a wide range of clinical and immunological parameters. We hypothesized that the presence of CHDMs predisposes to the development of metabolic syndrome and atherosclerosis, and that this is mediated by immune cell activation and systemic inflammation. Approximately 28% of the individuals had a CHDM. We showed that individuals with CHDMs had higher numbers of leukocytes and neutrophils, and a higher circulating IL-6 concentration. In contrast, the *ex vivo* cytokine production capacity of PBMCs

from individuals with CHDMs appeared to be lower compared to those without. On a clinical level, the presence of CHDMs of CHIP was not associated with the presence of metabolic syndrome or atherosclerosis. Rather, the presence of carotid plaques was significantly lower in individuals with CHDMs. Lastly, we identified that several of the associations with CHDMs were sex specific.

Acquisition of somatic mutations is a hallmark of aging, and obesity is known to accelerate the pace of aging[37]. Systemic inflammation and activation of the immune system drives the development of metabolic and atherosclerotic complications in obesity[7]. Thus, clonal hematopoiesis may be pivotal in the association between obesity and atherosclerotic cardiovascular disease in aging individuals.

Several epidemiological studies identified the most common clonal hematopoiesis driver mutations to be in *DNMT3A* and *TET2* genes[10,13]. While a VAF≥2% has been used traditionally to distinguish CHIP, recent advances in sequencing methodologies with superior sequencing depth have revealed that also smaller clone sizes can be associated with cardiovascular disease[38]. Recent work by Assmus *et al.* argued for mutation-specific cutoff values, 1.15% for DNMT3A and 0.73% for TET2, to predict all-cause mortality in heart failure patients[14]. Given the relevance of clones with smaller sizes, we included them in our analysis.

We observed a trend towards more women having CHDMs in this population. This finding prompted us to perform a sex-specific analysis. Men with CHIP were significantly older than those without CHDMs. However, women with or without CHIP were the same age. As women on average live longer than men, the clone growth might be delayed. Additionally, it has been shown that men generally have shorter telomere lengths, a trait associated with clonal hematopoiesis[39,40].

We hypothesized that in individuals with overweight and obesity, CHDMs would predispose to metabolic syndrome and to atherosclerosis due to increased monocytes responsiveness and increased systemic inflammation. We observed higher leukocyte and neutrophil numbers in individuals with CHDMs, in particular when the VAF was ≥2% (i.e. in the presence of CHIP). In addition, the circulating IL-6 concentration was higher in individuals with CHIP, confirming previous findings[41]. A previous genetic study showed that the increased CVD risk associated with CHIP is abrogated in individuals with

a genetically determined reduced IL-6 signaling[42], although this was not confirmed in a larger study[28]. In contrast to the higher circulating IL-6, we did not find a higher cytokine production capacity in PBMCs of individuals with CHDMs; we rather observed a lower production capacity for IL-6 and IL-1β in women with CHDMs. This contrasts the experimental finding that TET2-deficient mouse macrophages had increased cytokine production capacity[16]. Furthermore, single cell RNA sequencing of unstimulated human monocytes from patients with heart failure revealed increased expression of inflammatory genes in individuals harboring *DNMT3A* mutations[38]. These discrepant findings might indicate a differential effect of CHDMs on the inflammatory phenotype of monocytes versus macrophages or on baseline inflammatory gene expression versus protein production upon stimulation. A comparable differential inflammatory phenotype was observed between monocytes and macrophages of patients with primary aldosteronism, with a higher stimulated cytokine response in macrophages but not in monocytes[24]. One potential explanation is that the circulating PBMCs might have developed tolerance by the continuous exposure to higher concentrations of cytokines, thus impairing the *ex vivo* cytokine production capacity upon stimulation. Interestingly, the lower cytokine production capacity in the presence of CHDMs is exclusively observed in women in the sex stratified analysis.

In contrast to our hypothesis, we did not observe more atherosclerosis in individuals with CHDMs, as assessed with carotid ultrasound measuring to IMT and atherosclerotic plaques. Surprisingly, the presence of carotid plaques was even significantly lower in these individuals. Of note, the effect of CHDMs or CHIP on non-symptomatic atherosclerotic plaques has never been studied before. Various studies showed a strong association of CHIP with the occurrence of cardiovascular events, which are mostly triggered by destabilization of atherosclerotic plaques and subsequent thrombus formation[10,14]. Thus, our data suggest that clonal hematopoiesis does not facilitate the initial development of atherosclerotic plaques, but rather the destabilisation of these plaques. In line with this, atherosclerotic plaque development starts as early as in the second or third decade of life while clonal hematopoiesis is primarily seen in individuals older than 55[6,11].

Finally, there was no association between CHDMs or CHIP and markers of metabolic complications of obesity, including metabolic syndrome and its individual components, liver fat, and insulin resistance. Similarly, we did not observe increased adipose tissue inflammation in individuals with CHDMs or CHIP.

DNMT3A mutations are unequivocally the most common drivers of clonal hematopoiesis identified in several cohorts including ours. Recently, mechanistic studies in mice identified DNMT3A mutations to be causally linked to atherosclerosis[21]. Therefore, we also performed all analyses restricted to the CHDMs in DNMT3As. We recapiculated the majority of our findings from the main cohort, with an even stronger lower *ex vivo* cytokine production capacity, as well as lower carotid plaque presence. There were significantly more women among DNMT3A-carriers, as previously observed[43]. Absolute number of leukocytes was significantly higher in DNMT3-carriers.

A limitation of our study is the limited sample size of our cohort. This prevented us from studying the effects of individual genes harboring clonal hematopoiesis driver mutations. A second potential limitation is the sequencing approach utilized in identification of CHDMs. While the use of single-molecule molecular inversion probes allowed for ultrasensitive detection of small clones, the probes were designed against the majority of well-known CH hotspots, except for *DNMT3A* which was covered entirely. Thus, we cannot exclude the possibility that unknown drivers may be located outside the targets included in our assay. Furthermore, our cohort mainly consists of individuals with Western European origin. Therefore, our findings might not be generalized to diverse ethnic backgrounds.

A significant advantage of our study is that we performed extensive phenotyping of metabolic and atherosclerotic clinical parameters, as well as inflammatory and immune parameters. To the best of our knowledge, this study is the first to explore the association between CHDMs and the presence of nonsymptomatic atherosclerotic plaques and immune cell function in individuals with overweight or obesity. In addition, the use of single-molecule molecular inversion probes allowed us to identify CHDMs with a very low variant allele frequency.

In conclusion, we showed that in individuals with overweight or obesity, the presence of CHDMs is associated with higher circulating leukocyte and neutrophil numbers, and higher IL-6 concentration, yet with an impaired cytokine production capacity of isolated PBMCs in females. As multiple factors are analyzed in each section we cannot entirely eliminate potential false positive findings. Therefore, our findings need to be validated in independent cohorts, preferrentially with increased cohort size. Additionally, studies combining the differential assessment of monocyte and macrophage phenotypes, at both

unstimulated and stimulated states are needed to understand our observation of lower PBMC-cytokine production capacity in CHDM carriers. Furthermore, we found no association between CHDMs and the presence of metabolic syndrome or carotid atherosclerotic plaques, supporting the concept that clonal hematopoiesis does not affect atherosclerosis formation *per se*, but might trigger plaque destabilization and the subsequent occurrence of cardiovascular events.

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Disclosures

The authors have nothing to disclose.

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Supplementary material: Table of contents

Supplementary table 1: Adipose tissue samples real-time PCR primer sequences
Supplementary table 2 (available on journal website)**:** List of 24 genes sequenced and known clonal hematopoiesis hotspots
Supplementary table 3 (available on journal website)**:** single-molecule Molecular Inversion Probes (smMIP)
Supplementary table 4 (available on journal website)**:** Variant characteristics of candidate clonal hematopoiesis driver mutations in this study
Supplementary table 5: ELISA kits
Supplementary table 6: Baseline characteristics of men separated according to CHDM status
Supplementary table 7: Baseline characteristics of women separated according to CHDM status
Supplementary table 8: The association between adipose tissue inflammation and CHDM status of the entire study cohort
Supplementary table 9: Leukocyte numbers and differentiation, and thrombocyte numbers according to CHDM status of men
Supplementary table 10: Leukocyte numbers and differentiation, and thrombocyte numbers according to CHDM status of women
Supplementary table 11: *Ex vivo* cytokine production capacity of PBMCs separated according to CHDM status of men
Supplementary table 12: *Ex vivo* cytokine production capacity of PBMCs separated according to CHDM status of women
Supplementary table 13: Circulating cytokines and adipokines in plasma separated according to CHDM status of the men
Supplementary table 14: Circulating cytokines and adipokines in plasma separated according to CHDM status of women
Supplementary table 15: The association between targeted proteomic biomarkers and CHDM status of the entire study cohort
Supplementary figure 1: Targeted proteomics

Supplementary table 1: Adipose tissue samples real-time PCR primer sequences

Gene	Forward primer	Reverse Primer
Adiponectin	ATCGGTGAAACCGGAGTACC	GCATGTTGGGGATAGTAACGTAA
CD68	GCTGGCTGTGCTTTTCTCG	GTCACCGTGAAGGATGGCA
CD68(2)	CTTCTCTCATTCCCCTATGGACA	GAAGGACACATTGTACTCCACC
IL-18	GGCCTCTATTTGAAGATATGACTGATT	CCTCTAGGCTGGCTATCTTTATACATACT
IL-18bp	ATGAGACACAACTGGACACCA	GCCAGGTCACTTCCAATGC
IL-18Rβ	CCACAGTTACTTGGAGAGGCTTAAA	GGCATGTGGTAGCGCATTT
IL-1 α	ATCATGTAAGCTATGGCCCACT	CTTCCCGTTGGTTGCTACTAC
IL-1Ra	GCCTCCGCAGTCACCTAAT	TCCCAGATTCTGAAGGCTTG
IL-37	CAGCCTCTGCGGAGAAAGGAAGT	GTTTCTCCTTCTTCAGCTGAAGGGATGGAT
Leptin	GGTTGCAAGGCCCAAGAA	ACATAGAAAAGATAGGGCCAAAGC
MCP-1	CCAGTCACCTGCTGTTATAAC	TGGAATCCTGAACCCACTTCT
RPL37A	TAATACGACTCACTATAGGCTTTCTGGGCTC	TCTTCATGCAGGAACCACAG
TNF	CTCTTCTGCCTGCTGCACTTTG	ATGGGCTACAGGCTTGTCACTC

Supplementary table 5: ELISA kits

Description	Manufacturer	Manufacturer ID	Antibody registry
Human IL-1β ELISA kit	R&D systems	DY201	AB_2848158
Human IL-6 ELISA kit	Sanquin	M9316	AB_10851499
Human TNF- α ELISA kit	R&D systems	DY210	AB_2848160
Human IL-22 ELISA kit	R&D systems	DY782	AB_2928043
Human IL-17 ELISA kit	R&D systems	DY317	AB_2928042
Human IFNγ ELISA kit	Sanquin	M9333	AB_2934300
Human IL-1Ra ELISA kit	R&D systems	DRA00B	AB_2916104
Human Resistin ELISA kit	R&D systems	DY1359	AB_2893494
Human Leptin ELISA kit	R&D systems	DY398	AB_2861156
Human Adiponectin ELISA kit	R&D systems	DY1065	AB_2861158
Human AAT ELISA kit	R&D systems	DY1268	AB_2934301
Human IL-1Ra ELISA kit	R&D systems	DY280	AB_2934302
Human IL-18BP ELISA kit	R&D sistems	DBP180	AB_2934303
Human hsCRP ELISA kit (Plasma)	R&D systems	DY1707	AB_2928088
Human IL-18 ELISA kit	Simple plex (Biotechne / R&D)	SPCKB-PS-000501	
Human IL-6 ELISA kit (Plasma)	Simple plex (Biotechne / R&D)	SPCKB-PS-000190	
Human VEGF ELISA kit (Plasma)	Simple plex (Biotechne / R&D)	SPCKB-PS-000330	Multi-analyte cartridges
Human IL-1β (plasma)	Simple plex (Biotechne / R&D)	SPCKB-PS-000216	no antibody registry ID

Supplementary table 6: Baseline characteristics of men separated according to CHDM status

	No CHDM (n=124)	All CHDM (n=39)	High VAF (n=15)	Low VAF (n=24)	Cor. VAF
Age (years)	66 (62-70)	67 (63-71)	72 (67-77)*	65 (63-69)	0.33
BMI (kg/m2)	30 (28.2-32)	30.3 (28.3-32.2)	30 (28.9-30.6)	30.8 (28.2-32.8)	-0.18
Creatinine (µmol/L)	87 (80-95)	88 (81-93)	88 (84-93)	86 (79-93)	-0.067
Glucose (mmol/L)	5.5 (5.1-6)	5.4 (5-5.9)	5.4 (5.2-5.6)	5.5 (5-6.2)	-0.16
Total cholesterol (mmol/L)	5.9 (5.3-6.8)	6 (4.8-6.7)	5.3 (4.7-6.4)	6.1 (4.9-6.8)	-0.1
Triglycerides (mmol/L)	1.7 (1.2-2.2)	1.5 (1.3-1.9)	1.4 (1.4-1.8)	1.5 (1.3-2)	0.0081
Heart rate	61 (54-67)	64 (57-72)	61 (52-64)	67 (61-75)*	-0.39
Antihypertensives (%)	49	39	47	33	
Lipid lowering drugs (%)	33	23	13	29	
Antidiabetic drugs (%)	12	5		8	

BMI: body mass index. All data are given as median (interquartile ranges 1-3). *indicates p<0.05 compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Supplementary table 7: Baseline characteristics of women separated according to CHDM status

	No CHDM (n=88)	All CHDM (n=46)	High VAF (n=18)	Low VAF (n=28)	Cor. VAF
Age (years)	67 (64-71)	68 (63-74)	64 (63-71)	68 (65-74)	-0.22
BMI (kg/m2)	30 (28.6-31.9)	29.6 (28.2-31.9)	30.4 (28-32.5)	29.4 (28.4-31.2)	0.1
Creatinine (µmol/L)	67 (63-76)	70 (63-76)	70 (65-72)	72 (61-80)	-0.21
Glucose (mmol/L)	5.4 (5-5.9)	5.4 (4.9-6)	5.4 (5-6.3)	5.2 (5-6)	0.2
Total cholesterol (mmol/L)	6.6 (6-7.3)	6.4 (5.7-7)	6.7 (5.8-6.9)	6.4 (5.8-7.2)	-0.1
Triglycerides (mmol/L)	1.6 (1.3-2.1)	1.5 (1.3-2.2)	1.5 (1.3-2.1)	1.5 (1.3-2.3)	-0.087
Heart rate	62 (57-68)	63.5 (59-69)	65 (59-72)	63 (53-67)	0.025
Antihypertensives (%)	44	41	56	32	
Lipid lowering drugs (%)	21	26	22	29	
Antidiabetic drugs (%)	6	9	11	7	

BMI: body mass index. All data are given as median (interquartile ranges 1-3). *indicates p<0.05 compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Supplementary table 8: The association between adipose tissue inflammation and CHDM status of the entire study cohort

	No CHDM (n=212)	All CHDM (n=85)	High VAF (n=33)	Low VAF (n=52)	Cor. VAF
no. CLS/fields	0.06 (0-0.12)	0.06 (0-0.12)	0.07 (0-0.1)	0.06 (0-0.12)	0.06
no. Adipocytes/ fields	20.2 (18.1-22.8)	19.7 (17.7-23.9)	19.8 (18.1-23)	19.7 (17.3-25.6)	0.11
no. CD68/fields	2.3 (1.6-3.2)	2.2 (1.7-3.3)	2.1 (1.8-3.2)	2.2 (1.6-3.3)	0.096
% CD68	11.2 (8.2-15.8)	11.5 (8.9-15.8)	11 (9.3-14.7)	11.5 (8-16.3)	0.071
Area (Median)	2250.21 (1844.29-2718)	2234 (1853.3-2696.5)	2224.5 (1929.7-2601.1)	2238.5 (1829.7-2776)	0.035
Feretmin (Median)	47.2 (42.1-52.5)	47.1 (43.2-51.4)	47.4 (43.5-51.2)	47.1 (42.3-51.6)	0.042
Area (Mean)	3199.1 (2729-3779.4)	3282.1 (2761.9-3698.2)	3257.1 (2853.4-3576.5)	3285.5 (2738.8-3858.3)	-0.0013
SQ leptin	0.06 (0.04-0.1)	0.07 (0.05-0.1)	0.07 (0.05-0.1)	0.07 (0.05-0.2)	0.057
SQ IL-18Rβ	0.06 (0.02-0.2)	0.07 (0.03-0.2)	0.04 (0.03-0.2)	0.1 (0.03-0.3)	-0.014
SQ IL-1 α	0.05 (0.01-0.14)	0.07 (0.02-0.2)	0.04 (0.02-0.1)	0.1 (0.03-0.3)	-0.01
SQ MCP1	0.06 (0.04-0.09)	0.06 (0.04-0.1)	0.05 (0.04-0.07)	0.07 (0.04-0.1)	-0.14
SQ IL-37	0.04 (0.009-0.1)	0.05 (0.02-0.2)	0.03 (0.02-0.09)	0.09 (0.01-0.24)	-0.014
SQ CD68	0.07 (0.03-0.14)	0.08 (0.03-0.18)	0.05 (0.03-0.1)	0.11 (0.05-0.23)*	-0.11
SQ CD68(2)	0.05 (0.02-0.17)	0.07 (0.02-0.23)	0.04 (0.02-0.1)	0.11 (0.03-0.27)	-0.051
SQ Adiponectin	0.08 (0.04-0.14)	0.09 (0.04-0.18)	0.06 (0.04-0.12)	0.1 (0.06-0.22)*	-0.16
SQ TNF	0.04 (0.01-0.15)	0.05 (0.02-0.18)	0.03 (0.02-0.11)	0.1 (0.02-0.25)*	-0.026
SQ IL-18	0.19 (0.099-0.37)	0.16 (0.096-0.27)	0.16 (0.1-0.24)	0.18 (0.09-0.29)	0.07
SQ IL-18Bp	0.03 (0.005-0.36)	0.04 (0.01-0.54)	0.09 (0.01-0.89)	0.04 (0.01-0.33)	0.04
SQ IL-1RA	0.04 (0.02-0.11)	0.04 (0.01-0.09)	0.03 (0.02-0.07)	0.04 (0.01-0.18)	-0.038

CLS: Crown-like structure, SQ: starting quantity, CD68 gene expression measured with 2 primers
All data are given as median (interquartile ranges 1-3). *indicates $p<0.05$ compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Supplementary table 9: Leukocyte numbers and differentiation, and thrombocyte numbers according to CHDM status of men

	No CHDM (n=124)	All CHDM (n=39)	High VAF (n=15)	Low VAF (n=24)	Cor. VAF
Leukocytes 10^9/l	6 (5-6.7)	6.3 (5.7-7.4)*	6.4 (5.9-7.5)	6.2 (5.6-7.4)	0.18
Neutrophils 10^9/l	3.2 (2.7-3.8)	3.6 (2.9-4.8)*	4 (3.4-4.7)*	3.4 (2.7-4.7)	0.2
Lymphocytes 10^9/l	1.8 (1.5-2.2)	1.8 (1.6-2.1)	1.7 (1.4-2)	1.9 (1.6-2.3)	0.013
Monocytes 10^9/l	0.5 (0.4-0.6)	0.6 (0.5-0.7)	0.6 (0.5-0.7)	0.5 (0.5-0.7)	0.028
Eosinophils 10^9/l	0.2 (0.1-0.2)	0.2 (0.1-0.3)	0.1 (0.1-0.3)	0.2 (0.1-0.3)	-0.02
Basophils 10^9/l	0.03 (0.02-0.04)	0.03 (0.03-0.05)*	0.03 (0.03-0.05)*	0.03 (0.02-0.04)	0.07
Thrombocytes 10^9/l	217 (188-244)	208 (193-254)	215 (199-272)	206 (190-246)	0.31
NLR	1.8 (1.4-2.3)	2 (1.6-2.5)	2.3 (1.9-2.7)*	1.8 (1.2-2.4)	0.18

NLR: Neutrophil to lymphocyte ratio
All data are given as median (interquartile ranges 1-3). *indicates p<0.05 compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Supplementary table 10: Leukocyte numbers and differentiation, and thrombocyte numbers according to CHDM status of women

	No CHDM (n=88)	All CHDM (n=46)	High VAF (n=18)	Low VAF (n=28)	Cor. VAF
Leukocytes 10^9/l	5.5 (5-6.3)	5.9 (5-7.2)	6.2 (5-7.3)	5.8 (5.1-6.9)	0.18
Neutrophils 10^9/l	3.1 (2.6-3.6)	3.2 (2.6-3.9)	3.2 (2.5-3.9)	3.1 (2.7-3.9)	0.14
Lymphocytes 10^9/l	1.9 (1.5-2.3)	2 (1.6-2.3)	2.1 (1.7-2.3)	1.9 (1.5-2.3)	0.2
Monocytes 10^9/l	0.4 (0.4-0.5)	0.5 (0.4-0.6)	0.5 (0.4-0.6)	0.4 (0.4-0.5)	0.27
Eosinophils 10^9/l	0.1 (0.1-0.2)	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.2 (0.1-0.2)	0.035
Basophils 10^9/l	0.03 (0.02-0.04)	0.03 (0.02-0.04)	0.03 (0.02-0.04)	0.03 (0.02-0.04)	-0.046
Thrombocytes 10^9/l	247 (216-283)	247 (215-275)	247 (205-280)	248 (226-272)	-0.024
NLR	1.5 (1.3-2)	1.7 (1.3-2)	1.5 (1.3-1.8)	1.8 (1.4-2)	-0.13

NLR: Neutrophil to lymphocyte ratio
All data are given as median (interquartile ranges 1-3). *indicates p<0.05 compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Supplementary table 11: *Ex vivo* cytokine production capacity of PBMCs separated according to CHDM status of men

	No CHDM (n=124)	All CHDM (n=39)	High VAF (n=15)	Low VAF (n=24)	Cor. VAF
LPS (10 ng/ml) IL-1β	1029.7 (543.3-1732)	1116.9 (589-1657.3)	1258.9 (602.1-1637.)	1099.07 (597.29-1630.78)	-0.0086
LPS (100 ng/ml) IL-1β	2351.1 (1475.1-3770)	2809.6 (1554.1-4038.1)	2894.8 (2320.3-4038.1)	2491.5 (1466.8-4080.9)	-0.024
Pam3Cys IL-1β	280.3 (112.6-630.6)	291 (113.9-597.3)	287 (79-647)	315 (135.1-475.9)	0.023
LPS (10 ng/ml) IL-6	5367.8 (2940-8598.88)	5441.3 (4307.7-7309.4)	5342.8 (4307.65-6801.7)	6014.2 (4204.9-7363)	-0.18
LPS (100 ng/ml) IL-6	8103.6 (4800.8-12236.7)	8383.8 (5730.1-12896.95)	7827.2 (5580.3-13232.5)	8455.7 (6762-12011.8)	-0.14
Pam3Cys IL-6	3750.2 (1590.5-6675.8)	3654.1 (1602.85-5778.6)	3381.5 (1100.6-6721.7)	3744.9 (1998.4-5465.7)	-0.13

LPS: Lipopolysaccharide, IL: interleukin, IL-1β and IL-6 concentration units are given in pg/ml
All data are given as median (interquartile ranges 1-3). *indicates $p<0.05$ compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Supplementary table 12: *Ex vivo* cytokine production capacity of PBMCs separated according to CHDM status of women

	No CHDM (n=88)	All CHDM (n=46)	High VAF (n=18)	Low VAF (n=28)	Cor. VAF
LPS (10 ng/ml) IL-1β	1194.1 (677.5-2056.9)	832.2 (515.1-1682.4)	957.3 (450.4-2448.2)	790.1 (533.9-1306.8)	0.12
LPS (100 ng/ml) IL-1β	2548.5 (1575.3-3638.6)	1823.3 (1103.3-2950.5) *	2054.4 (992.7-3010.1)	1724.3 (1231.2-2833.3)	0.11
Pam3Cys IL-1β	291.4 (110.6-671.6)	127.04 (64.9-309.92) *	135.4 (55.9-397.1)	104.3 (67.9-277.2)*	0.093
LPS (10 ng/ml) IL-6	6107.8 (3837.8-9388.1)	4407.8 (2468-5910.9)*	4407.8 (2616.3-6051.7)*	4627 (2444.8-5766.8)*	0.098
LPS (100 ng/ml) IL-6	8344.9 (5445.7-13132.3)	6349.7 (3609.9-8306.8)	5121.7 (3502-7412)*	6921.65 (4114.38-10786.5)	0.041
Pam3Cys IL-6	4185.7 (2125.9-6202.9)	1569.8 (811.1-2579.2)*	1670.3 (684.9-3184.6)*	1569.7 (828-2256.4)*	0.25

LPS: Lipopolysaccharide, IL: interleukin, IL-1β and IL-6 concentration units are given in pg/ml
All data are given as median (interquartile ranges 1-3). *indicates $p<0.05$ compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient

Supplementary table 13: Circulating cytokines and adipokines in plasma separated according to CHDM status of men

	No CHDM (n=124)	All CHDM (n=39)	High VAF (n=24)	Low VAF (n=15)	Cor. VAF
IL-6 (pg/ml)	2.3 (1.6-3.3)	2.8 (1.8-4.6)	2.9 (2.1-4.6)	2.7 (1.7-4)	0.052
IL-1β (pg/ml)	0.06 (0.06-0.1)	0.06 (0.06-0.11)	0.06 (0.06-0.01)	0.08 (0.06-0.11)	-0.1
IL-18 (pg/ml)	313 (222.7-515)	300.9 (224.5-510.2)	277.7 (224.5-349.2)	341 (232.5-628.4)	-0.17
IL-18bpa (ng/ml)	17.1 (14.4-20)	16.4 (13.6-20.2)	16.5 (13-17.7)	16.2 (14.1-21.2)	-0.2
hsCRP (µg/ml)	1.4 (0.9-3)	1.9 (1.1-3.1)	1.9 (1-3.7)	1.9 (1.3-2.8)	0.033
AAT (mg/ml)	1 (0.6-1.7)	0.9 (0.6-1.4)	0.9 (0.7-1.1)	0.87 (0.61-1.44)	0.12
Resistin (ng/ml)	10.3 (8-12.8)	11.5 (9.3-15.2)	11.8 (10-14.9)	11.3 (9.2-15.9)	-0.0066
Leptin (ng/ml)	10.2 (7-15)	12.3 (7.5-17)	8.5 (6.9-16.1)	13.3 (8.4-21.3)	-0.22
Adiponectin (µg/ml)	3.1 (2.2-4.5)	3.3 (2.7-4.3)	3.7 (3-4.2)	3.1 (2.3-4.4)	0.092

IL: interleukin, IL-18bp: IL-18 binding protein, CRP: C-reactive protein, AAT: Alpha-1 antitrypsin All data are given as median (interquartile ranges 1-3). *indicates $p<0.05$ compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Supplementary table 14: Circulating cytokines and adipokines in plasma separated according to CHDM status of women

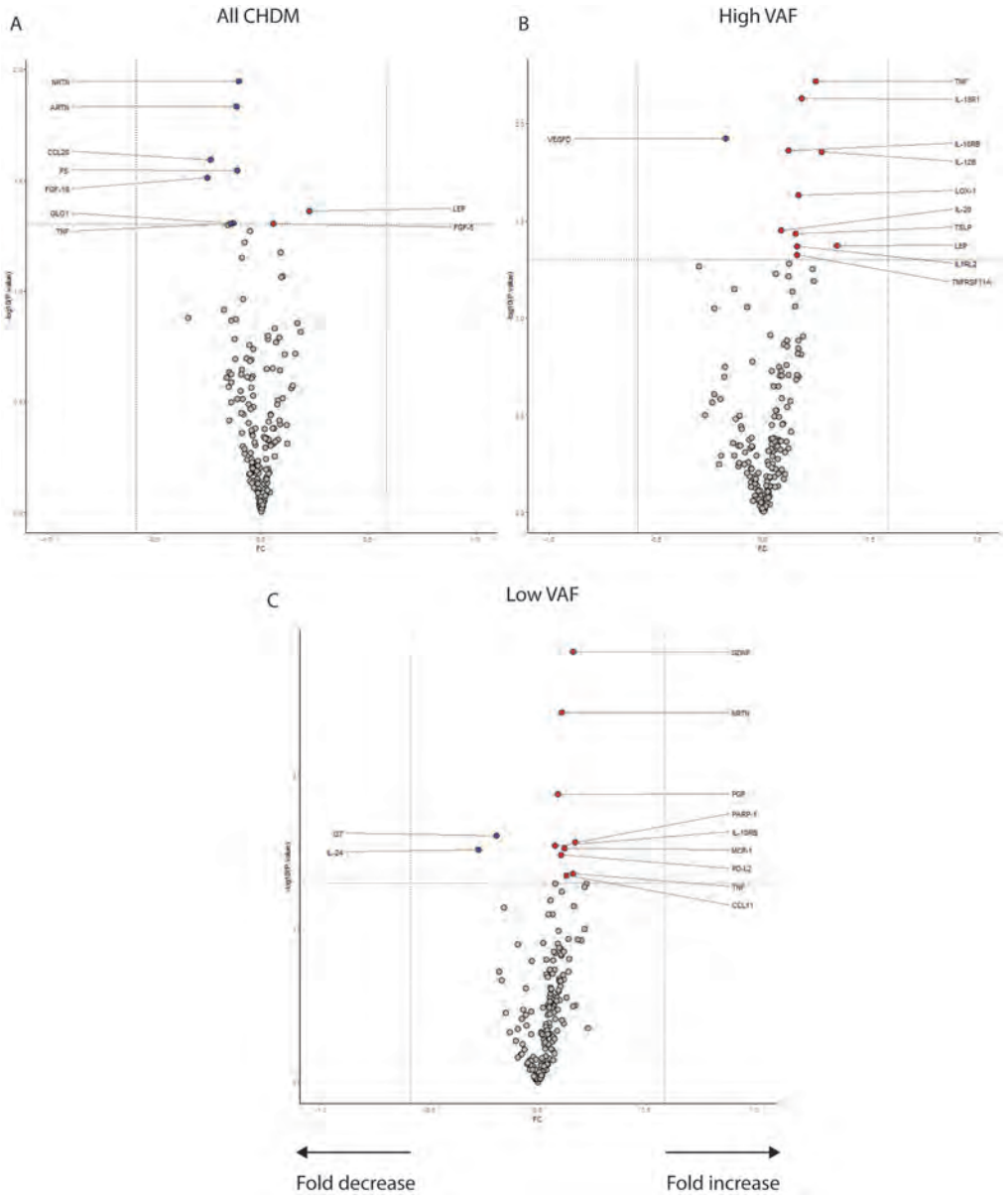
	No CHDM (n=88)	All CHDM (n=46)	High VAF (n=18)	Low VAF (n=28)	Cor. VAF
IL-6 (pg/ml)	2.7 (1.9-3.4)	2.6 (1.7-4.1)	3.3 (2-4.4)	2.2 (1.5-3)	0.36
IL-1β (pg/ml)	0.06 (0.06-0.11)	0.06 (0.06-0.13)	0.06 (0.06-0.14)	0.07 (0.06-0.11)	0.013
IL-18 (pg/ml)	300.1 (234.1-478.8)	283.7 (204.2-469.9)	411.3 (222.3-489.9)	252.5 (200-379)	0.27
IL-18bpa (ng/ml)	17.5 (14-21.4)	15.8 (13.5-19)	14.9 (12.8-18.7)	16.2 (14.2-19.2)	-0.098
hsCRP (µg/ml)	2.7 (1.1-4.6)	2.1 (1.3-3.7)	2 (0.9-3.7)	2.2 (1.5-3.8)	0.13
AAT (mg/ml)	0.9 (0.6-1.9)	0.9 (0.6-1.3)	0.8 (0.6-1.6)	0.9 (0.6-1.1)	-0.037
Resistin (ng/ml)	10.8 (8.6-15.3)	11.5 (9-14.4)	11.4 (8.3-13)	12.8 (10-16.2)	-0.18
Leptin (ng/ml)	31.5 (21.7-48.1)	27.5 (17.8-51)	25.4 (18.8-47.1)	28.8 (17.5-52.7)	-0.056
Adiponectin (µg/ml)	6 (4.6-7.3)	5.1 (4.2-8)	4.9 (4.2-6.8)	6 (4.2-8)	-0.046

IL: interleukin, IL-18bp: IL-18 binding protein, CRP: C-reactive protein, AAT: Alpha-1 antitrypsin All data are given as median (interquartile ranges 1-3). *indicates $p<0.05$ compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.

Supplementary table 15: The association between targeted proteomic biomarkers and CHDM status of the entire study cohort

	No CHDM (n=212)	All CHDM (n=85)	High VAF (n=33)	Low VAF (n=52)	Cor. VAF
SLAMF7	3.6 (3.3-4.1)	3.5 (3.2-3.9)	3.6 (3.3-4)	3.4 (3-3.7)*	0.22
IL1RL2	4.5 (4.3-4.8)	4.6 (4.4-4.9)*	4.6 (4.3-4.8)	4.6 (4.4-4.9)	0.15
IL-27	6.5 (6.3-6.7)	6.4 (6.1-6.7)*	6.3 (6.1-6.7)	6.4 (6.1-6.6)	0.021
GH	8 (6.5-9.4)	7.3 (6.3-9.3)	7.9 (6.3-9.9)	7.2 (6.2-8.7)*	0.23
GLO1	5.6 (5.4-5.9)	5.5 (5.1-5.8)	5.4 (5.1-5.8)*	5.6 (5.2-5.9)	-0.21
AMBP	7.9 (7.9-8)	8 (7.8-8.1)	7.9 (7.8-8)	8 (7.9-8.1)*	-0.073
CCL3	6.7 (6.3-7)	6.7 (6.1-7)	6.4 (6.1-6.7)*	6.7 (6.4-7.1)	-0.26
TNFRSF13B	10 (9.8-10.3)	9.9 (9.7-10.1)*	10 (9.8-10.2)	9.9 (9.7-10.1)*	0.042
LEP	6.8 (6.2-7.4)	7.1 (6.5-7.5)*	7.1 (6.4-7.4)	7.2 (6.5-7.6)*	0.012
NEMO	5.8 (5.2-6.4)	5.7 (5-6.2)	5.5 (5-5.8)*	5.9 (5-6.4)	-0.21
VEGFD	8.1 (7.9-8.3)	8 (7.8-8.2)	8.1 (7.9-8.4)	8 (7.8-8.1)*	0.33
AXIN1	2.7 (2.3-3.2)	2.6 (2-3.2)	2.3 (2-3)*	2.7 (2.1-3.5)	-0.16
TSLP	0.6 (0.4-0.8)	0.6 (0.4-0.8)	0.5 (0.3-0.7)*	0.6 (0.4-0.8)	-0.14
MMP-1	9.7 (9-10.4)	9.6 (9-10.2)	9.4 (8.9-9.7)*	9.8 (9.2-10.5)	-0.23
CCL3.1	5.1 (4.9-5.5)	5.1 (4.8-5.4)	4.9 (4.8-5.2)*	5.2 (4.8-5.7)	-0.22
FGF-19	8.3 (7.7-8.9)	8 (7.4-8.5)*	8.1 (7.5-8.6)	7.9 (7.3-8.5)*	0.081
ST1A1	2.9 (2.3-3.5)	2.8 (2.1-3.4)	2.4 (2-3.1)*	2.9 (2.1-3.5)	-0.17

All data are given as median (interquartile ranges 1-3). *indicates $p<0.05$ compared to No CHDM group. Correlation is indicated with Spearman correlation coefficient.



Supplementary figure 1: Volcano plots depicting increase and decrease in NPX (normalized protein expression) values of OLINK biomarkers measured in plasma with targeted proteomics. Differential expression of A) All CHDM, B) High VAF (VAF≥2%) C) Low VAF (VAF<2%) groups compared to No CHDM group.

Chapter 5:

Association between DNMT3A-driven clonal hematopoiesis, trained immunity and immune cell function in obesity

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Abstract

Trained immunity and clonal hematopoiesis are two newly identified immunological phenomena that contribute to the pathophysiology of atherosclerotic cardiovascular disease. These two phenomena share some convergent molecular mechanisms, such as IL-1β being a central regulator and involvement of epigenetic enzymes. Therefore, we hypothesize that presence of clonal hematopoiesis driver mutations (CHDMs) can predispose to an increased capacity to build trained immunity. We previously characterized how the presence of CHDMs relates to immune cell function and vasculometabolic complications in a cohort of older individuals with overweight and obesity. From this cohort we now selected 17 individuals with CH due to DNMT3A mutations and 15 without any known CHDMs. We performed in depth immune characterization via flow cytometry, functional assays with monocytes and neutrophils, and we measured the capacity to build trained immunity using β-glucan and oxLDL as stimuli. We corroborated our previous findings of lower *ex vivo* cytokine production capacity of PBMCs from individuals with DNMT3A mutations. Importantly, presence of DNMT3A CHDMs associated with higher trained immunity response. Moreover, we demonstrated that individuals with DNMT3A mutations were characterized with higher $CD10^{+}$ mature neutrophils and a lower neutrophil MPO release upon TLR2 stimulation. In conclusion, presence of DNMT3A CHDMs is associated with increased susceptibility to build a hyperresponsive trained monocyte phenotype. The exact molecular mechanisms behind this phenomena requires further investigation.

Keywords

Clonal hematopoiesis, trained immunity, atherosclerosis, obesity, immune cells

Introduction

Ageing gradually alters the composition and the function of the immune system. Adaptive immune response becomes impaired, marked by restricted T cell receptor repertoire and reduced antibody diversity[1]. Whereas innate immune cells display a heightened proinflammatory response on the baseline. Age-associated inflammation, also known as inflammaging, can lead to plethora of pathologies[2]. Clonal hematopoiesis is one of such age-associated phenomena[3].

Clonal hematopoiesis is defined as clonal expansion of leukocytes due to somatic mutations in hematopoietic stem cells. These somatic mutations can confer a certain survival or fitness advantage, leading to clonal expansion of leukocytes with this mutation. The presence of a clonal hematopoiesis driver mutation (CHDM) in circulating leukocytes with a variant allele frequency (VAF) ≥2%, and without evidence of hematological malignancy, dysplasia, or cytopenia is defined as Clonal Hematopoiesis of Indeterminate Potential (CHIP)[4]. Many epidemiolocal studies have established that CHIP is associated with an increased risk of atherosclerotic cardiovascular disease (ASCVD)[5], although we have previously reported that it is not associated with the presence of asymptomatic atherosclerotic plaques *per se*[6]. Recent work with increased sequencing depth showed that also CHDMs with VAF<2% can have clinical significance[7]. Mutations in the *DNMT3A* gene are the leading CHDMs in most cohorts, followed by TET2 and ASXL1 mutations[8].

Clonal hematopoiesis is a risk factor for obesity-associated metabolic complications and for ASCVD[9]. Despite strong evidence for a correlation between clonal hematopoiesis and ASCVD, the causal mechanisms are not fully understood. TET2 deficient mouse models have been shown to display accelerated atherosclerosis, marked by increased NLRP3-inflammasome driven IL-1β production by macrophages[10]. A more recent study showed a convergent phenotype of atherosclerosis in DNMT3A deficient mice, with increased intracellular pro-IL-1β expression measured with flow cytometry[11]. Single cell RNA sequencing of isolated monocytes of six patients with heart failure and CHIP due to DNMT3A CHDMs revealed a proinflammatory RNA expression profile at baseline[12]. In contrast, we recently observed a lower cytokine production capacity of isolated PBMCs from individuals with obesity and CHIP, after *ex vivo* exposure to LPS, compared to individuals with obesity and without CHDMs[6].

In the current study, we aim to explore how CHIP due to mutations in DNMT3A affects the phenotype of innate immune cells in more detail. In particular, we focus on trained immunity and on neutrophil function, as two novel protagonists of atherosclerosis pathophysiology. Trained immunity describes the persistent functional hyperresponsive phenotype of innate immune cells after brief stimulation to micro-organisms or to atherogenic stimuli, such as oxLDL[13]. A key driver of trained immunity, at least in the context of oxLDL[14], and of β-glucan[15] is IL-1β. Recent murine studies provided strong proof that trained immunity can accelerate atherosclerosis development, in the setting of hyperglycemia[16], intermittent high-fat diet[17], and post myocardial infarction[18]. Given the knowledge that CHDMs are mainly mutations in epigenetic enzymes and epigenetic reprogramming is the central mechanism of trained immunity, and cells with CHDMs produce more IL-1β, which can induce trained immunity, we hypothesize that the presence of CHDMs is associated with an increased potential to develop a trained immunity phenotype.

A second aim of this study is to explore the effects of CHDMs on neutrophil function. Most studies on CHIP focused on monocytes with only a few studies suggesting that CHDMs in TET2[19] and JAK2[20] can also affect neutrophil function. We recently showed that individuals with obesity and CHDMs have higher absolute neutrophil counts[6]. Accumulating evidence highlights the role of neutrophils in ASCVD[21,22]. Despite their short lifespan, neutrophils can also retain memory phenotype in their bone marrow progenitor cells[23].

We previously identified CHDMs in a cohort of older individuals with overweight and obesity[6]. For the current study we included 17 individuals with DNMT3A mutations and 15 without any known CHDMs, matched on age, sex and BMI. We studied the immune phenotype of PBMCs, purified monocytes and neutrophils from these individuals in detail by characterizing surface marker expression by flow cytometry, leukocyte differentiation, and cytokine production capacity upon *ex vivo* stimulation. And lastly, we performed trained immunity experiments on purified monocytes.

Methods

Study design and population

Participants were selected from the "300-Obese (OB)" cohort based on presence or absence of DNMT3A mutations in the blood that was collected

between 2014 and 2016, for simplicity we refer to this time point as 2016 sequencing. We also included the six individuals with a CHDM in TET2. We repeated DNA sequencing in 2023. Since the TET2 CHDM could not be replicated in three individuals and because our smMIP assay only covers a small part of TET2[6,7], we decided to only focus on DNMT3A CHDMs in the current paper. For an overview of the CHDMs in 2016 and 2023, and how we defined the final groups, please refer to **Table 1** and Consort Diagram in **Figure 1**. For detailed description of the 300 OB cohort and the comprehensive characterization of CHDMs please refer to Tercan *et al*[6]. Comparable distribution of BMI, age and sex was considered when selecting the individuals. 32 individuals with a median BMI of 30 kg/m^2, between the ages of 66 and 84, consisting of Western European ancestry, were recruited in the Radboud university medical center in 2021. The research protocol was approved by the Radboud University Ethical Committee (NL72552.091.20; 2020-6135), and all subjects gave written informed consent. The study protocol was performed in accordance with the 1975 Declaration of Helsinki.

For each participant venous blood was collected between 8-9 am into BD Vacutainer® K2EDTA (10 ml) tubes. All laboratory procedures were performed immediately following the blood collection. When possible, patients with and without DNMT3A mutations were included in the same day.

Blood sampling and chemistry parameters

2 EDTA vacutainer tubes were centrifuged for 10 minutes at 2749 g at RT to collect plasma. Plasma samples were then stored at −80˚C until measurement.

Whole blood composition was assessed by a Sysmex-XN 450 hematology analyzer. Plasma hs-CRP, creatinine, total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride levels were determined by standard laboratory procedures. Low-density lipoprotein (LDL) cholesterol levels were calculated with the Friedewald equation.

Identification of CHDMs

The first DNA samples were collected between 2014 and 2016 to identify presence of CHDMs. As mentioned above, individuals were selected for the current study based on these results. In the current study, we repeated DNA sequencing in 2023. CHDMs were identified in whole blood by the same ultra-sensitive assay as in the initial cross-sectional 300 OB cohort, as previously described[6,24].

Briefly, 300 single-molecule molecular inversion probes (smMIP) were designed to cover a selection of well-known hotspots of a panel of 24 clonal hematopoiesis driver genes, including the entire DNMT3A gene. As the DNMT3A gene contains the most driver mutations and is shown to be causally linked to atherosclerosis in mouse models[11], we only focused on CHDMs in this particular gene.

For each sample two technical polymerase chain reaction (PCR) replicates were run, thereafter two independent data processing strategies and a quality control step were performed. Variant allele frequencies were calculated using samtools mpileup[25].

Isolation of cells from peripheral blood

Peripheral Blood Mononuclear Cell (PBMC) isolation

PBMC isolation was performed with differential density centrifugation over Ficoll-Paque (GE Healthcare).

Briefly, blood was diluted in Phosphate Buffered Saline (PBS) (Gibco) and layered on Ficoll-Paque PLUS (Cytiva) density gradient centrifugation for 30 minutes at 615g (no brakes, RT).

The PBMC fraction enriched with plasma was collected and washed with PBS containing 0.1% human pooled serum and 1 mM UltraPure EDTA (0.5 M, pH 8, Life Technologies) at 190g for 15 min, RT, to separate the platelet rich plasma (PRP) from the PBMCs. The pellet containing PBMCs were then washed twice with cold PBS and resuspended in RPMI 1640 Dutch-modified culture medium supplemented with 1 mM pyruvate (Invitrogen), 2 mM glutamine (Invitrogen), 50 µg/mL gentamicin (Centrafarm).

The cell counts and PBMC composition were performed with the Sysmex-XN 450 hematology analyzer.

PRP collection and isolation of platelets

The PRP was collected from the plasma supernatant subsequently after Ficoll gradient separation. Collected PRP was diluted in PBS containing 0.1% human pooled serum and 1 mM UltraPure EDTA (0.5 M, pH 8, Life Technologies) and centrifuged at 190g for 15min. The supernatant containing PRP was collected and centrifuged at 2500g, 5minutes, 4°C and gently resuspended in previously

described RPMI 1640 Dutch-modified culture medium. Platelet count was measured with the Sysmex – XN 450 hematology analyzer.

Monocyte isolation

Part of the PBMC fraction was used to isolate monocytes. $CD14^+$ monocytes were isolated by negative selection by MACS pan-monocyte isolation kit (Miltenyi Biotec) according to manufacturer's protocol. The purity and count were assessed by the Sysmex- XN 450 hematology analyzer.

Polymorphonuclear (PMN) cell isolation

After removing the PBMC fraction, neutrophil isolation was performed by hypotonic lysis. Briefly, leftover cells (PMNs and erythrocytes) were incubated with hypotonic lysis buffer (155 mM NH4Cl, 10 mM KHCO3) for 15 and 10 minutes on ice. Afterwards, PMNs were washed twice in PBS and resuspended in RPMI 1640 medium without phenol red (Gibco, 32404014) supplemented with 50µg/mL gentamicin (Centrafarm), 2mM glutamax (Gibco), and 1mM pyruvate (Gibco). Neutrophils were counted with Sysmex Hematoanalyzer and brought to 5×10^6 cells/ml concentration for the following experiments.

Stimulation assays

PBMC stimulation

500.000 PBMCs per well were stimulated in duplicate with RPMI, LPS (10 ng/ml) (Sigma-Aldrich, E. coli serotype 055:B5, further purified as described[26]), Monosodium urate (MSU) crystals (300ug/ml), Pam3CYSK4 (Pam3Cys) 10 µg/mL (L2000, EMC Microcollections) for 24 hours in 96-wells round-bottom plates (Greiner) at 37 °C and 5% CO2. Supernatants were collected after 24 hours and stored at -80°C until measurements were performed.

7-day PBMC stimulation was performed with 500.000 cells per well with 10% human pooled serum with RPMI, LPS and PHA, without changing medium in 96-wells round-bottom plates (Greiner) at 37 °C and 5% CO_2. Supernatants were collected after 7 days and stored at -80°C until measurements were performed.

Trained immunity assays

We used our previously published protocol of *in vitro* trained immunity assay[27]. Briefly, 100.000 monocytes per well were seeded to flat-bottom 96-wells plate for 1 hour at 37 °C and 5% CO2. After 1-hour, non-attached monocytes

were removed during the collection of supernatants. Monocytes were trained with BCG SSI (750 ug/ml), β-glucan (2 ug/ml), and oxLDL 10 (ng/ml) in the presence of 10% human pooled serum (HPS) for 24h at 37 °C and 5% CO_2. After 24 hours and 3 days media was refreshed to RPMI with 10% HPS. On day 6 the trained monocytes were restimulated with LPS (10 ng/ml) or Pam3Cys (10 ug/ml) for 24 hours. Supernatants were collected and stored at -80°C until measurements were performed.

Neutrophil stimulation

500.000 neutrophils per well were stimulated in duplicate in flat bottom 96-well plates (Corning, NY, USA) for 4 hours with culture medium only (as control), LPS (1 µg/ml), MSU (300 µg/ml), LPS and MSU (1 µg/ml and 300 µg/ml respectively), Pam3Cys (10 µg/ml), Nigericin (1 µM), Ethanol (dissolving agent for Nigericin) and Phorbol-12-myristate-13-acetate (PMA, Sigma) (50 nM) at 37°C with 5% CO_2. After 4 hours, the plates were centrifuged for 8 minutes at 350g, and the supernatants were stored at -80°C until measurement.

ROS assay

ROS production of neutrophils was determined by a luminol (5-amino-2,3, dihydro-1,4-phtalazinedione)-based luminescence assay. To opaque flat-bottom 96-well plates (Corning, NY, USA) 200.000 neutrophils per well were added and stimulated in quadruplicate with serum-opsonized zymosan, PMA, and culture medium as control. Chemiluminescence was measured at 142 second intervals for 1 hour at 37°C in a BioTek Synergy HTreader. The integral of relative luminescence units per second (RLU/sec) was measured.

NETosis assays

NOX-dependent NET formation

200.000 neutrophils per well were seeded to flat-bottom 96-well plates (Corning, NY, USA) at 37°C for 20 minutes. After attachment of neutrophils, the supernatants were removed. The neutrophils were stimulated in quadruplicate with Nigericin (1 µM), Ethanol, PMA (50 µM) or culture medium as control for 3 hours at 37°C, 5% CO_2.

NOX-independent NET formation

100.000.000 platelets were either kept unstimulated or activated with 156uM Thrombin Receptor Activator Peptide 6 (TRAP6, Sigma) for 30 minutes at 37°C,

5% CO_2 in round bottom non-stick tubes (corning 352063, Fisher Scientific). In parallel, 200.000 neutrophils per well were attached to flat-bottom 96-well plates (Corning, NY, USA) at 37°C for 20 min. The neutrophils were stimulated in quadruplicate with unstimulated platelets, TRAP6-stimulated platelets or culture medium as control for 1 hour at 37°C, 5% CO_2.

After both assays, the neutrophils were washed twice with warm PBS and NETs were treated by partial digestion in culture medium supplemented with 5 U/ml micrococcal nuclease (MNase, Worthington biochemical corporation) 20 min at 37°C, 5% CO_2. MNase was inactivated by brief vortexing, and the neutrophils were pelleted by centrifugation. The supernatant containing partially digested NETs were kept at -80°C until measurement.

Measurement of DNA concentration in NETs with Sytox Orange

The DNA concentrations in the NET formation assay MNase-treated supernatants (NOX-dependent and NOX-independent formation assay) were quantified by adding 5 mM Sytox Orange Nucleic Acid Stain (Life Technologies) solution to undiluted sample. Fluorescence was measured with excitation and emission of 530/560nm using the BioTek Synergy HT multi-reader. All the measurements were done in duplicate.

Circulating plasma protein and cytokine measurements

Cytokine and neutrophil granule-associated protein concentrations upon stimulation of PBMCs, monocytes and neutrophils, as well as circulating hsCRP were measured with commercially available Enzyme-linked Immunosorbent Assay (ELISA) kits according to instructions supplied by the manufacturer. For detailed information on the ELISA kits used please refer to **Supplementary Table 1**.

Flow cytometry

Flow cytometric analyses were performed on EDTA whole blood samples. 2 panels were used to characterize monocyte and neutrophil phenotypes.

Red blood cell lysis was performed with BD Pharm Lyse buffer treatment for 15 minutes at room temperature (RT) in the dark. After washing with FACS buffer (1% BSA in PBS with 2mM EDTA) the pellet was resuspended in 100 µl of FACS Buffer and incubated with 10 µl Human TruStain FcX (Biolegend, San Diego, CA, USA) Fc block for 10 minutes. Cells were independently stained with the following two panels. For monocyte analysis, cells were stained using the

following anti-human fluorochrome-conjugated antibodies: CD45, CD3, CD19, CD56, CD14, CD16, HLA-DR, CD11b, CD11c, CD41, CCR2, CCR5. For neutrophil analysis, the following anti-human fluorochrome-conjugated antibodies were used: CD45, a lineage cocktail containing CD3, CD56, CD19, CD20 and CD14, CD123, CD15, CD16, CD35, HLA-DR, CD62L, CD49d, CD10 and CD11b. 50 µl of cells were incubated with 50 ul of the either antibody mix for 30 min at RT in the dark. Then the cells were washed and resuspended in FACS buffer. Helix NiR viability dye was added to the neutrophil panel for 15 min at RT in the dark. Cell populations and expression of markers were measured using a CytoFlex cytometer (Beckman Coulter, Brea, USA) which underwent daily quality control to correct for variation in laser settings. Flow Minus One controls were assessed for all markers for which MFI was assessed to set the correct gates. For a full overview of used antibodies see **Supplementary Table 2**.

Manual gating of the monocyte panel was performed by selecting for single cells and CD45+ immune cells, B cells, T cells and NK cells were removed based on the expression of CD19, CD3 and CD56. CD19-CD3-CD56- cells were used for monocyte gating; monocytes were identified based on the expression of CD14 and CD16 and HLA-DR. Monocyte subsets (classical, intermediate and nonclassical) were determined based on CD14 and CD16 expression and the exact gates were put based on HLA-DR and CCR5 expression (highest on intermediate monocytes), and CCR2 expression (highest on nonclassical monocytes) (**Supplementary Figure 1**).

Manual gating of the neutrophil panel was performed by removing doublets and debris and selecting for live granulocytes among CD45+ cells. Then, neutrophils were identified based on CD15 and CD16 surface expression. After excluding eosinophils and basophils, we characterized the maturation and activation level of neutrophils by CD49d, CD10, CD66b, HLA-DR, CD16 and CD62L (**Supplementary Figure 2**)

Statistical analysis

Distribution of data was assessed with the Shapiro-Wilk test. If the data did not follow a normal distribution, it is shown as median and interquartile range. Mann-Whitney U test was used to compare DNMT3A and No CHDM groups. $P<0.05$ is considered statistically significant and is indicated with an asterisk in tables. Statistical analyses were performed by using Graphpad version 9 and SPSS.

Results

Baseline characteristics did not differ among groups

Initially we included 36 individuals, 15 of whom had a DNMT3A CHDM, 15 without CHDMs and 6 with TET2 CHDM based on 2016 sequencing. However, based on the 2023 sequencing, one of the individuals without CHDMs gained a DNMT3A mutation, and for one individual there were not enough reads to confirm the absence of mutations. Two of the TET2 CHDMs (with very small VAFs, i.e. ~0.01) could not be repeated, therefore those individuals were moved to No CHDM, and one individual with TET2 mutation developed a DNMT3A mutation. Thus, we excluded the remaining 3 individuals with TET2 mutations. And the final groups in the current study included 15 individuals who did not have any known CHDMs and 17 had DNMT3A mutations. For the visual representation of inclusion please refer to **Figure 1**. The baseline characteristics of the participants are listed in **Table 1**. The median age was 75 years and BMI of 30 kg/m^2. All participants had Western European ancestry and 47% were male. There were no statistical differences in the baseline characteristics of the two groups.

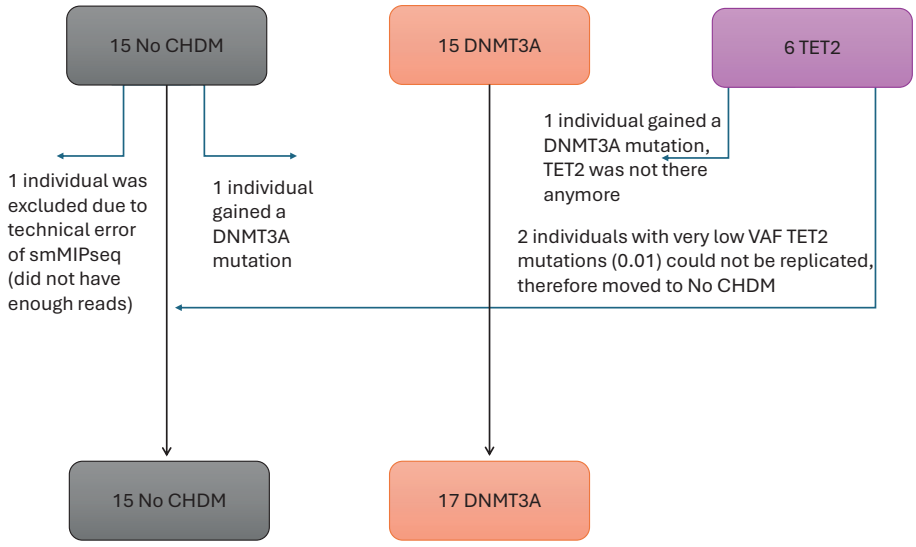

Figure 1: Consort diagram depicting the inclusion of individuals based on CHDMs identified in 2016 and 2023, final groups(No CHDM, DNMT3A) are included in the current study.

Table 1: Baseline characteristics

	No CHDM (n=15)	DNMT3A (n=17)	Total (n=32)
Age (year)	76 (75-77)	73 (69-78)	75 (72-77)
Sex (male, %)	47	47	47
Ancestry (Western European, %)	100	100	100
BMI (kg/m^2)	30 (29-31)	30 (28-32)	30 (29-32)
Waist circumference (cm)	108 (103-111)	115 (108-117)	110 (106-117)
Systolic blood pressure (mmHg)	148 (139-157)	144 (142-152)	144 (140-154)
Diastolic blood pressure (mmHg)	84 (72-88)	76 (72-82)	78 (72-86)
Heart rate (beats per minute)	64 (60-74)	72 (60-72)	72 (60-73)
Packyears (year)	14 (10-28)	17 (7-20)	145 (8-24)
Diabetes Mellitus (Type II, %)	7	6	6
Lipid lowering drug use (%)	70	80	75
Total cholesterol (mmol/l)	5.2 (4.8-5.9)	4.9 (4.6-6)	5.1 (4.7-6)
LDL cholesterol (mmol/l)	3.1 (2.7-3.6)	3.1 (2.5-3.8)	3.1 (2.6-3.8)
Triglycerides (mmol/l)	1.3 (1.1-1.6)	1.5 (1.1-1.7)	1.4 (1.1-1.6)
HDL cholesterol (mmol/l)	1.4 (1.3-1.7)	1.3 (1.2-1.5)	1.3 (1.2-1.6)
Non-HDL cholesterol (mmol/l)	3.8 (3.2-4.4)	3.7 (3.2-4.3)	3.8 (3.2-4.4)
Creatinine (umol/l)	81 (69-89)	79 (68-93)	80 (68-90)
eGFR (ml/min/1.73 m^2)	72 (67-78)	73 (69-80)	73 (68-78)
Glucose (mmol/l)	5.4 (5.2-6.2)	5.6 (4.9-6.2)	5.5 (5.2-6.2)
Hba1c (mmol/ mol)	40 (40-42)	38 (37-42)	40 (38-42)

Data shown as median (interquartile range 1-3) and percentage (%) where appropriate. BMI indicates body mass index; HDL, high-density lipoprotein; Hba1c Hemoglobin A1c

Majority of the DNMT3A mutations persisted in time

We identified DNMT3A CHDMs in 17 individuals. 2 of these mutations were newly identified in individuals that previously did not have CHDMs. Of the previously identified clone trajectories 8 clones grew, 2 remained stable and 7 shrank in size, based on the VAFs measured in 2016 and 2023. Clone characteristics and dynamics of the mutations are shown in **Table 2**.

DNMT3A mutations did not affect the leukocyte composition

In order to study if the presence of DNMT3A mutations alter the circulating leukocyte composition we measured leukocyte number and differentiation. Absolute counts and percentages of circulating leukocytes did not differ among individuals with DNMT3A mutations or without CHDMs (**Figure 2A-B**). Also, there was no difference in hsCRP, an indicator of systemic inflammation (data not shown).

Table 2: Clone characteristics

Position	Coding DNA annotation	Protein amino acid annotation	Amino acid change	VAF 2016	VAF 2023
25457243	c.2644C>T	p.Arg882Cys	R -> C (882)	18.2	25.7
25463182	c.2311C>T	p.Arg771Ter	R -> [STOP] (771)	11.4	6.9
25457161	c.2726T>G	p.Phe909Cys	F -> C (909)	4.8	6.1
25462020	c.2387G>T	p.Gly796Val	G -> V (796)	22.9	22.3
25464537	c.1976G>A	p.Arg659His	R -> H (659)	3.3	5.0
25463521	c.2161A>T	p.Lys721Ter	K -> [STOP] (721)	34.5	45.0
25457242	c.2645G>A	p.Arg882His	R -> H (882)	7.8	5.9
25467083	c.1792C>T	p.Arg598Ter	R -> [STOP] (598)	3.5	5.5
25463289	c.2204A>G	p.Tyr735Cys	Y -> C (735)	6.5	4.7
25457242	c.2645G>A	p.Arg882His	R -> H (882)	11.3	10.6
25457246	c.2641delA	p.Ser881Alafs*25	[STOP] AA 905	5.1	7.8
25470498	c.976C>T	p.Arg326Cys	R -> C (326)	16.7	14.0
25463181	c.2312G>A	p.Arg771Gln	R -> Q (771)	2.8	3.5
25463554	c.2128T>A	p.Cys710Ser	C -> S (710)	6.0	3.6
25468919	c.1444G>T	p.Glu482Ter	E -> [STOP] (482)	3.3	3.3
25463568	c.2114T>C	p.Ile705Thr	I -> T (705)	6.1	4.4
25457242	c.2645G>A	p.Arg882His	R -> H (882)	0	0.6

Monocyte subsets or activation status did not differ in individuals with DNMT3A mutations

To investigate whether DNMT3A mutations were associated with changes in the monocyte phenotype and subsets we performed multicolor flow cytometry. We did not observe any difference in the percentages of classical, intermediate, or non-classical monocytes among DNMT3A carriers or individuals without CHDMs (**Figure 2C**).

In total monocytes as well as subsets, there were no difference in activation markers HLA-DR, CCR2, CCR5, CD11b, CD11c and CD41, both with regard to the percentage of cells with these markers, as well as for MFI, between individuals without CHDMs and with DNMT3A mutations (**Figure 2D**).

The effect of DNMT3A mutations on PBMC *ex vivo* cytokine production capacity

To assess whether monocytes are functionally distinct in individuals with DNMT3A mutations or without CHDMs we stimulated isolated PBMCs *ex vivo* for 24 hours with various PRR ligands, as previously done for the total cohort of

297 individuals[6]. For the majority of the cytokines, there was a trend for lower cytokine production in individuals with DNMT3A mutations, in line with our previous observations in the entire 300 OB cohort, although this did not reach statistical significance, apart from IL1RA production upon MSU stimulation, that was significantly lower in individuals with DNMT3A mutations (**Figure 3D**).

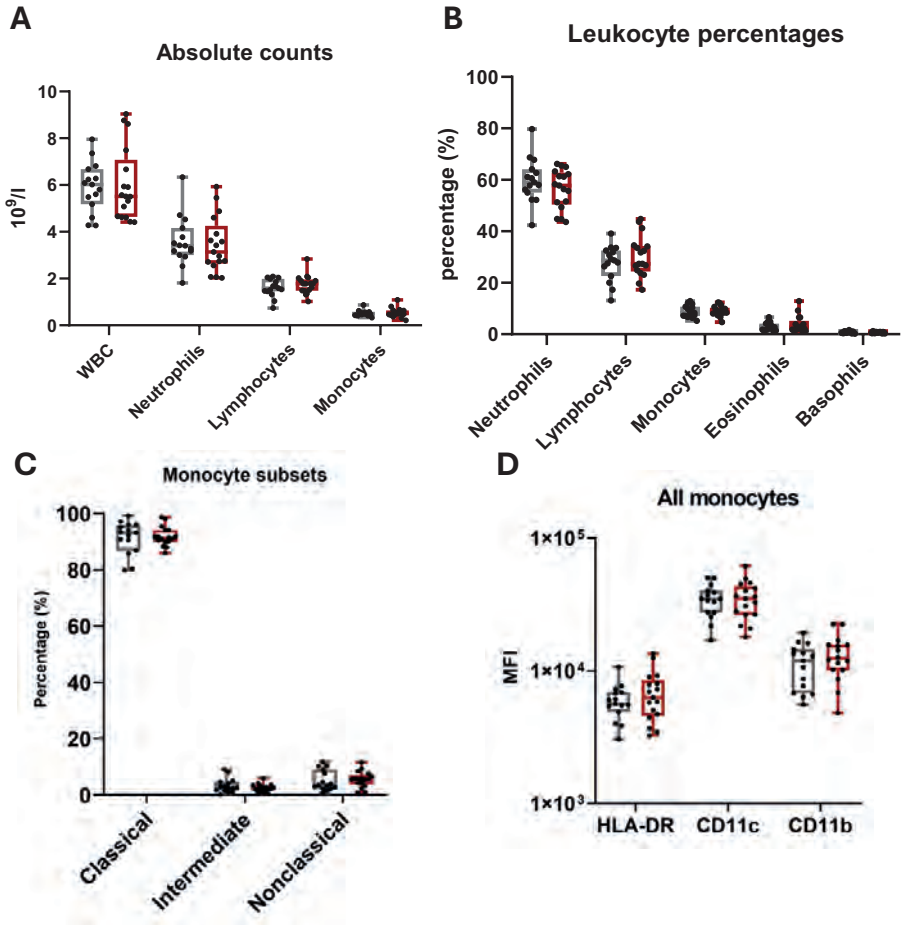

Figure 2: Leukocyte counts and monocyte phenotype, No CHDM: Gray, DNMT3A: Red.

Absolute counts and percentages of monocytes measured with Sysmex Hematoanalyzer. Percentages of monocyte subsets and some key activation markers measured with multicolor flow cytometry. WBC: White Blood Cell, HLA: Human Leukocyte Antigen DR isotype, CD: Cluster of Differentiation

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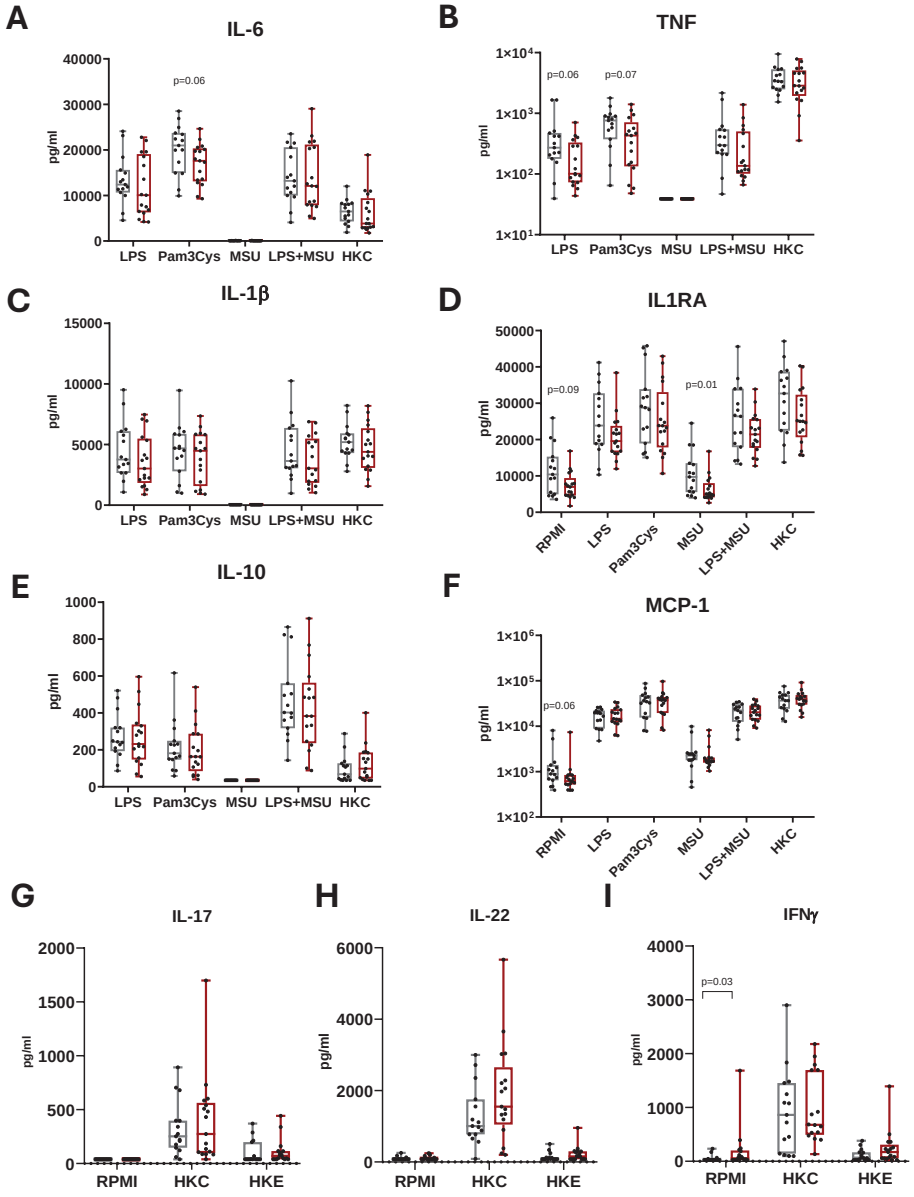

Figure 3: *Ex vivo* cytokine production capacity of PBMCs, No CHDM: Gray, DNMT3A: Red.

Panels A-F demonstrate cytokine production upon 24-hour stimulation of PBMCs with the following stimuli LPS (Lipopolysaccharide), Pam3Cys (Pam3CYSK4), MSU (Mono sodium urate), HKC (Heat-killed Candida albicans). Panels G-I demonstrate cytokine production upon 7-day stimulation of PBMCs with HKC (Heat-killed Candida albicans) and HKE (Heat-killed Escherichia coli). IL: Interleukin, TNF: Tumor necrosis factor, MCP-1: Monocyte Chemoattractant Protein-1, IFN γ: Interferon gamma

The effect of DNMT3A mutations on trained immunity

We subsequently set out to test our hypothesis that trained immunity induction is augmented in monocytes from individuals with DNMT3A CHDMs. We induced trained immunity by exposing isolated monocytes for 24 hours to three different triggers of trained immunity, β-glucan, BCG and oxLDL. After 6 days of differentiation into macrophages we restimulated the cells with LPS and Pam3Cys and measured cytokine production.

The trained immunity capacity is calculated as a fold change cytokine production over the background control (RPMI). For detailed investigation of the monocyte cytokine production and trained immunity capacity we show both raw cytokine production values as well as the fold change. Similar to the PBMC *ex vivo* cytokine production capacity, there was a general trend for lower cytokine production in the macrophages in individuals with DNMT3A mutations (**Figure 4A, C, E**). Particularly when monocytes were untrained (i.e. only exposed to RPMI) and restimulated with TLR ligands there was a trend for lower cytokine production (**Figure 4A** TNF production of LPS restimulated RPMI, p=0.07; **Figure 4E** IL1RA production of Pam3Cys restimulated RPMI, p=0.04).

In contrast, the capacity to build trained immunity, calculated as fold change in cytokine production after 24 hour exposure to the training stimuli, compared to the un-trained control situation, was higher in individuals with DNMT3A mutations for several cytokines and training stimuli (**Figure 4**). Specifically, β-glucan (p=0.01) and oxLDL (p=0.1) training induced higher TNF fold change after LPS restimulation (**Figure 4B**); β-glucan training resulted in a higher IL-6 fold change after Pam3Cys restimulation (p=0.08) (**Figure 4D**); and β-glucan training induced higher IL1RA fold change with both LPS (p=0.08) and Pam3Cys (p=0.01) restimulation (**Figure 4F**).

DNMT3A driven CH did not associate with changes in the adaptive immune response

It has been shown that clonal hematopoiesis predominantly skews the HSPCs towards myelopoiesis. Thus, the effects of CHDMs on lymphocyte function are less well characterized. To provide an in-depth immune characterization, we measured some key adaptive cytokines; IL-17, IL-22 and IFNy upon stimulation with heat-killed *C. albicans* and *E. coli* for 7 days (**Figure 3G-I**). Except for higher IFNy production on the baseline for individuals with DNMT3A mutations, we did not observe any statistically significant differences among the groups (**Figure 3I**).

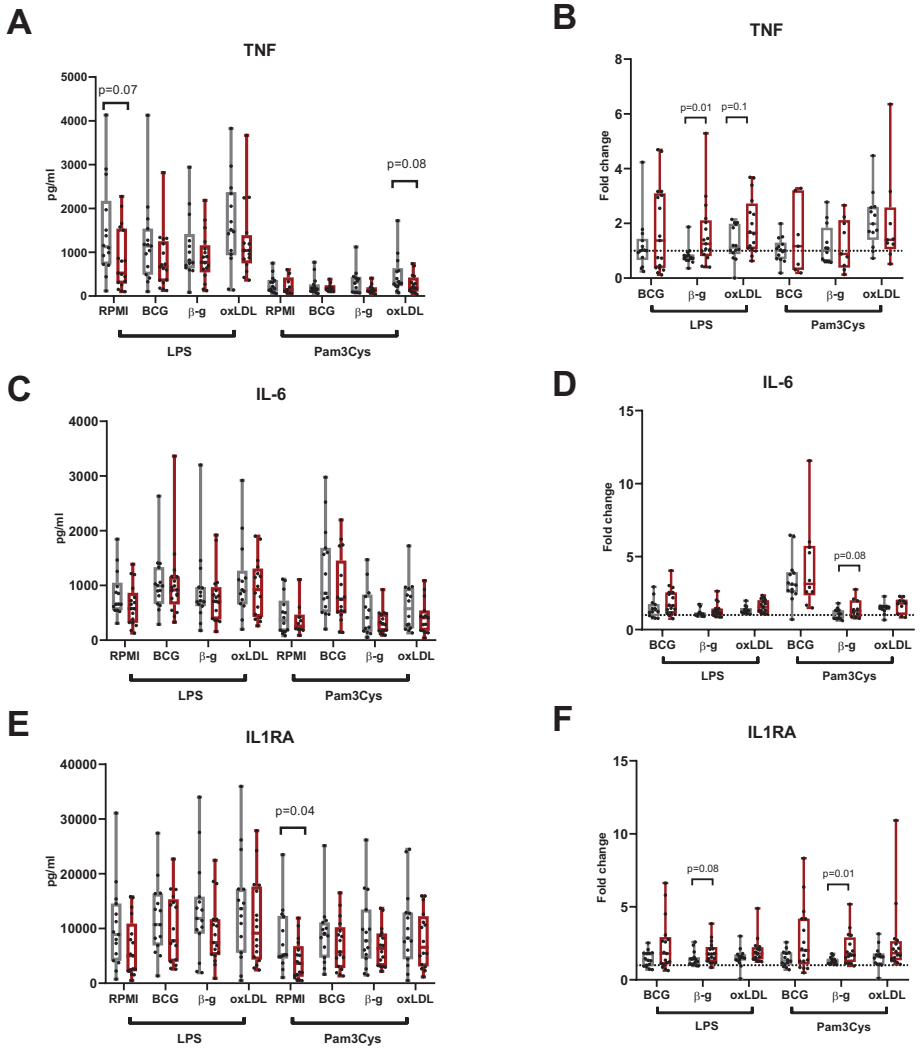

Figure 4: Monocyte training. No CHDM: Gray, DNMT3A: Red.

Graphs on the left column (A, C, E) show raw values of cytokine production capacity with training stimuli BCG (Bacillus Calmette–Guérin SSI), β-g (β-glucan) , oxLDL (oxidized low-density lipoprotein), and RPMI as background control restimulated with either LPS or Pam3Cys. Graphs on the right column (B, D, F) show fold change cytokine production over RPMI. Dashed line at fold change 1.

The effects of DNMT3A mutations on neutrophil phenotype and function

We phenotypically characterized neutrophils from whole blood with a multicolor flow cytometry approach. We assessed a panel of maturation and activation markers. Neutrophils from individuals with DNMT3A mutations were characterized by a higher expression of the CD10 maturation marker, without changes in the percentage of cells expressing CD10 (**Table 3**). We observed that individuals with DNMT3A mutations had higher percentage of neutrophils expressing HLA-DR, although this did not reach statistical significance (p=0.1). There were no differences in expression of the other surface markers.

Table 3: Neutrophil flow cytometric characterization

FCM Mature PMN	No CHDM (n=15)		DNMT3A (n=17)		
	Mean	**SD**	**Mean**	**SD**	**p-value**
% of neutrophils	99.7	0.2	99.7	0.2	0.8
CD10 MFI	63287	19245	77250	21588	**0.04**
CD10 %	98.0	1.5	98.7	1.0	0.2
CD66b MFI	15127	3628	16815	4805	0.4
CD15 MFI	36551	9582	43853	17703	0.3
CD11b %	1.0	0.0	1.0	0.0	>0.9
CD11b MFI	14333	5005	19002	7518	0.1
CD35 %	100	1	100	1	0.6
CD35 MFI	12735	4681	14021	5956	0.7
CD62 %	99.7	0.2	99.3	0.9	**0.01**
CD62L MFI	61391	25071	62173	21816	0.9
HLA-DR %	32.5	37.1	60.3	39.4	0.1
HLA-DR %	3699	3228	3730	3118	0.3

We assessed the degranulation capacity of neutrophils based on primary (MPO, NE) and secondary granule (s100A8/9, NGAL) contents with ELISA. Neutrophils from individuals with DNMT3A mutations had significantly lower MPO release upon TLR2 signaling via Pam3Cys stimulation. We did not identify differences in the concentrations of any other granule marker (**Table 4**).

Neutrophils can expel their DNA in the form of extracellular traps, which contributes to atherosclerosis development and plaque destabilization. In addition to the DNA content, these neutrophil extracellular traps (NETs) contain nuclear, cytoplasmic and granular proteins[22]. We quantified the amount

of DNA released during the NOX-dependent and –independent NET formation assays with a Sytox based assay. The presence of DNMT3A mutations was not associated with changes in the concentration of DNA released during NET formation (data not shown).

Table 4: Neutrophil *ex vivo* degranulation capacity

	PMN 4h	No CHDM (n=15)		DNMT3A (n=17)		p-value
		Mean	SD	Mean	SD	
NGAL(ng/ml)	RPMI	11.0	4.6	10.1	4.6	0.65
	LPS	26.2	11.7	24.2	14.4	0.65
	Pam3Cys	62.9	23.0	58.1	17.1	0.65
	MSU	23.8	10.6	23.1	20.6	0.14
	LPS+MSU	29.5	10.2	32.0	24.8	0.18
	Nigericin	48.5	17.6	44.0	23.8	0.22
	Ethanol	20.3	13.8	14.0	8.6	**0.05**
	PMA	198.5	44.5	206.6	37.5	0.97
MPO(ng/ml)	RPMI	142.9	91.2	130.1	91.8	0.68
	LPS	208.7	146.8	159.5	90.7	0.41
	Pam3Cys	144.9	80.5	85.3	37.1	**0.02**
	MSU	241.2	117.2	225.8	159.4	0.39
	LPS+MSU	291.9	128.8	277.3	177.1	0.6
	Nigericin	455.7	151.8	398.4	132.0	0.28
	Ethanol	190.5	134.9	134.9	113.8	0.18
	PMA	596.8	292.2	612.5	347.5	0.82
S100A8/9(ng/ml)	RPMI	291.8	117.3	254.1	99.1	0.37
	LPS+MSU	1395.7	604.5	1100.9	502.9	0.2
	Pam3Cys	1931.6	866.9	1530.5	350.9	0.16
	MSU	1846.2	679.4	1927.3	1422.8	0.37
	LPS+MSU	1839.0	563.9	1776.3	1159.0	0.26
	Nigericin	48.5	17.6	44.0	23.8	0.22
	Ethanol	1528.9	674.5	1124.6	511.2	**0.04**
	PMA	8080.0	2278.6	7441.5	2026.9	0.55
IL-8 (pg/ml)	RPMI	23.5	0	23.5	0	>0.99
	LPS	31.3	17.9	24.3	3.4	0.09
	PMA	191.6	81.7	241.8	156.8	0.5

An important function of neutrophils is production of reactive oxygen species (ROS), involved in antimicrobial host defense and inflammation. We measured ROS production capacity of neutrophils upon stimulation with opsonized zymosan and PMA. There were no statistically significant changes in the ROS production capacity of neutrophils from individuals without CHDMs or DNMT3A mutations (data not shown).

Discussion

In the present study, we aimed to explore two potential immunological mechanisms that could contribute to the increased cardiovascular risk in patients with clonal hematopoiesis due to CHDMs in the *DNMT3A* gene. First, we showed that the presence of a DNMT3A CHDM is associated with an increased trained immunity response. Secondly, we explored in detail how DNMT3A CHDMs affected neutrophil phenotype and function, and showed that individuals with DNMT3A mutations had more $CD10^+$ mature neutrophils and lower MPO release after TLR2 stimulation. These findings offer exciting new immunological pathways that can contribute to the link between CHIP and CVD.

We focused specifically on DNMT3A CHDMs, since mutations in the DNMT3A gene are the leading drivers of clonal hematopoiesis, they are associated with ASCVD[11], and because of sequencing covered the entire DNMT3A gene, in contrast to the other genes involved in CH. We selected individuals from our 300 OB study, based on the sequencing results from 2016, and repeated this sequencing in 2023[6]. Clone size of TET2-driven CH is shown to grow exponentially with age, whereas DNMT3A clone growth dynamics are slower and mostly stable[28]. Approximately 60% of the clone trajectories identified in our cohort either grew or remained static.

One of the two main aims of this study was to explore the hypothesis that CH is associated with an increased tendency to develop trained immunity. Trained immunity refers to the immunological phenomenon that innate immune cells, such as monocytes, can build a long-term hyperinflammatory phenotype after brief stimulation, e.g. with micro-organisms, but also with endogenous atherogenic molecules, such as oxLDL[29] , high glucose concentrations[16], or catecholamines[30]. This is established through rewiring of key metabolic pathways, and through epigenetic reprogramming[13]. Accumulating experimental evidence irrefutably showed that trained immunity can accelerate the development

of atherosclerosis[16,18]. We argued that *DNMT3A*-related CH could modulate the susceptibility to mount trained immunity responses for two reasons. First, *DNMT3A* encodes for a histone methyltransferase and trained immunity is regulated by epigenetic processes, including histone methylation, and DNA methylation[31,32]. Secondly, it is known that IL-1β signaling is involved in the development of the trained phenotype, at least for trained immunity induced by oxLDL and by β-glucan[14,15]. Because there are indications that DNMT3A deficiency increases IL-1β expression[11,12], this could be another mechanism linking CH and trained immunity.

We first recapitulated our previous findings from the 300 OB cohort of a lower *ex vivo* cytokine production capacity of PBMCs from individuals with DNMT3A mutations[6]. Interestingly, a recent single cell RNA sequencing study in unstimulated monocytes from individuals with heart failure revealed a higher proinflammatory cytokine expression at baseline[12].

In line with our hypothesis, monocytes from individuals with DNMT3A mutations were more amenable to be trained by β-glucan, which reached significance for TNF production upon TLR4 restimulation and IL1RA production upon TLR2 restimulation. Also, there was a trend for higher TNF production in LPS restimulated oxLDL trained monocytes however this did not reach statistical significance (p=0.1). This could partially be explained by the fact that oxLDL is not a stimulus as potent as β-glucan. In conclusion, baseline cytokine production capacity of monocytes from individuals with DNMT3A was lower, whereas capacity to build trained immunity was higher compared to individuals without CHDMs. This finding nicely fits the framework predicting trained immunity responses that was recently described in a cohort of 323 healthy individuals that were trained with BCG vaccination *in vivo*[33]. The authors showed that 213 individuals had a trained immunity response, whereas 78 individuals were categorized as non-responders. Interestingly, individuals with the strongest BCG-induced trained immunity responses, were characterized by low cytokine production at baseline (i.e. before BCG administration) and low chromatin accessibility at genes involved in innate immunity[33]. The lower baseline cytokine production capacity in our study in individuals with DNMT3A CHDMs could potentially be due to intrinsic effects of the DNMT3A mutation on cytokine production, or could be by the fact that individuals with CHDMs already have elevated levels of systemic inflammation as we previously shown[6], that itself induces a state of immune-tolerance. Future studies are needed to investigate the epigenetic landscape of the

DNMT3A mutated cells to understand how this affects trained immunity. In addition, single cell sequencing studies should be performed to investigate the individual cellular effects of DNMT3A CHDMs on cytokine production and on trained immunity.

The second main aim of our study was to investigate in detail how DNMT3A CHDMs affect the phenotype and function and neutrophils. There is accumulating evidence that neutrophils are important regulators of atherosclerosis development[21,22]. In addition, we recently showed that the atherosclerosis accelerating effect of high fat diet-induced trained immunity is dependent on neutrophil activation. Up to now, information about how CHDMs affect neutrophil function is scarce, and mainly stems from CHDMs in TET2 and JAK[19,20]. We did not find relevant effects on neutrophil surface marker expression or protein, NET, and ROS release, except a higher expression of CD10. $CD10^-$ immature neutrophils have been found to associate with increased inflammatory markers and cardiac damage in patients with acute myocardial infarction[34].

In a TET2 mutant mouse model, neutrophils were shown to have increased primary granule content marked by MPO and NE quantity[19]. In contrast, our findings illustrate that MPO quantity upon Pam3Cys stimulation was lower in the neutrophils from individuals with DNMT3A mutations. This could hint to a gene specific effect in clonal hematopoiesis and should be investigated in future studies. The same study demonstrated that the NET architecture from TET2 mutant neutrophils was altered. We measured the amount of DNA released during NOX-dependent and –independent NET formation. The released DNA concentrations were comparable among the two groups, alas we were not able to characterize the NET architecture.

A limitation of our study is the small sample size, which prevented us from reaching robust conclusions for some immunological parameters. However, we selected these individuals from a larger cohort in which we previously also demonstrated a lower cytokine production capacity in individuals with CHDM, showing robust internal validity. A second limitation is that our cohort consisted of individuals with Western European ancestry, thus our findings cannot be extended to diverse ethnic groups.

A strength of our study is the in-depth immune characterization including phenotypic and functional characterization of monocytes and neutrophils,

and adaptive immune responses. In addition to the extensive immunological characterization, we strived for a balanced distribution of sex, age and BMI between groups to minimize potential confounding factors. To the best of our knowledge, this is the first study that investigated the association between trained immunity and clonal hematopoiesis in individuals with obesity.

In conclusion, we show that in individuals with obesity, DNMT3A mutations are associated with *lower ex vivo* cytokine production capacity and a heightened susceptibility to develop a trained immunity phenotype in response to stimuli. Future studies are necessary to unravel the underlying mechanism of this association and the consequences for the development of atherosclerotic CVD.

Acknowledgements
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Disclosure of interest
HT, HB, BCC, SB, NR, NPR, RCvD, AH, and LABJ have no disclosures. MGN is scientific founder of TTxD and Lemba Therapeutics.

Data availability
The anonymized data underlying this study is available upon reasonable request to the corresponding author.

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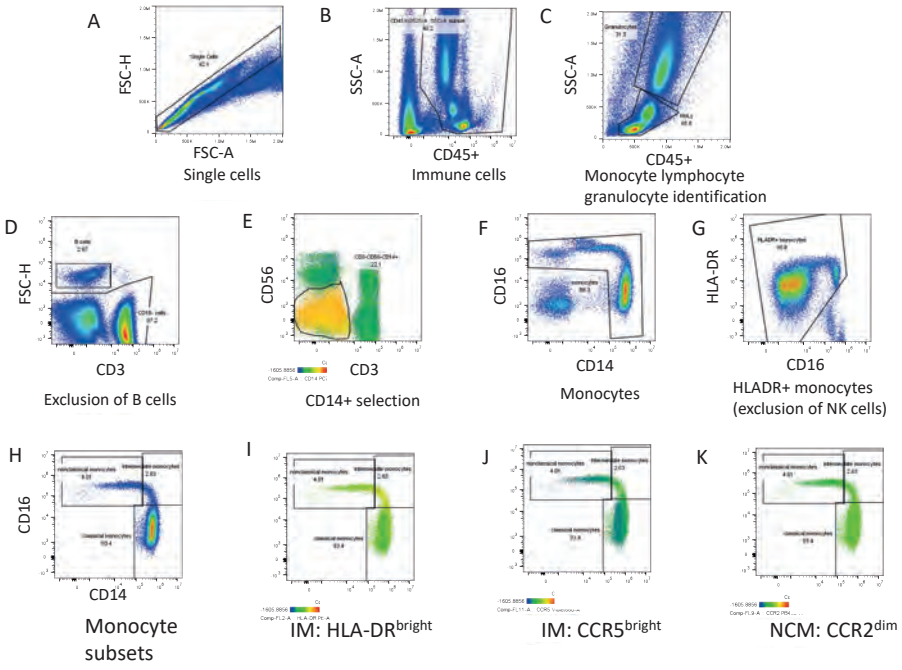
Supplementary Information

Supplementary Table 1: ELISA kits used in this study

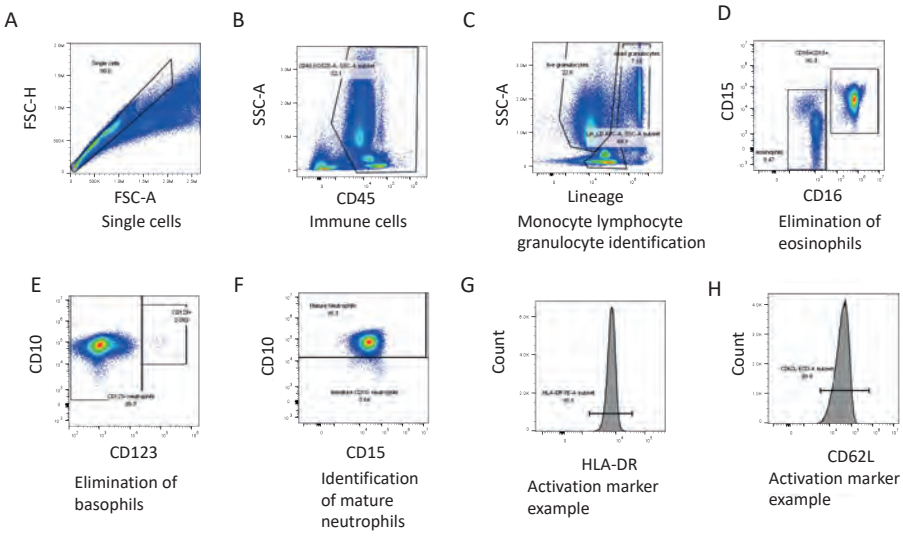
Product	Product number	Manufacturer
Human IL-1β DuoSet ELISA	DY201	Bio-Techne/R&D
Human IL-1RA DuoSet ELISA	DY280	Bio-Techne/R&D
Human IL-6 DuoSet ELISA	DY206	Bio-Techne/R&D
Human IL-10 DuoSet ELISA	DY217B	Bio-Techne/R&D
Human TNF DuoSet ELISA	DY210	Bio-Techne/R&D
Human hsCRP ELISA	DY1707	Bio-Techne/R&D
Human IL-8 DuoSet ELISA	DY208	Bio-Techne/R&D
Human Neutrophil Elastase/ELA2 DuoSet ELISA	DY9167-05	Bio-Techne/R&D
Human S100A8/S100A9 Heterodimer DuoSet ELISA	DY8226-05	Bio-Techne/R&D
Human Myeloperoxidase DuoSet ELISA	DY3174	Bio-Techne/R&D
Human Lipocalin-2/NGAL DuoSet ELISA	DY1757	Bio-Techne/R&D

Supplementary Table 2: Antibodies used for flow cytometry

Antibodies	Fluorochrome	Clone	Company	Identifyer
Anti-human CD16	FITC	3G8	Biolegend	Cat# 302006 RRID AB_314206
Anti-human HLA-DR	PE	immu-357	Beckman Coulter	Cat# IM1639U RRID AB_2876782
Anti-human CD62L	PEDazzle584	DREG-56	Biolegend	Cat# 304842 RRID AB_2565874
Anti-human CD49d	PECy5.5	9F10	Biolegend	Cat# 304312 RRID AB_10641699
Anti-human CD10	PC7	HI10a	Biolegend	Cat# 312213 RRID AB_2146549
Anti-human lineage cocktail (CD3, CD14, CD19, CD20, CD56)	APC	UCHT1; HCD14; HIB19; 2H7; HCD56	Biolegend	Cat# 348703 RRID: N/A
Anti-human CD66b	APC-700	G10F5	Biolegend	Cat# 305114 RRID AB_2566038
Anti-human CD15	APC-Cy7	MEM-166	Biolegend	Cat# 323047 RRID AB_2750189
Anti-human CD123	BV421	6H6	Biolegend	Cat# 306018 RRID AB_10962571
Anti-human CD45	BV510	HI30	Biolegend	Cat# 304036 RRID AB_2561940
Anti-human CD35	BV650	E11	BD Bioscience	Cat# 744277 RRID AB_2742115
Anti-human CD11b	BV785	ICRF44	Biolegend	Cat# 301346 RRID AB_2563794
Anti-human CD11c	PEDazzle584	BU15	Biolegend	Cat# 337227 RRID AB_2564548
Anti-human CD3	PC5.5	UCHT1	Biolegend	Cat#300410 RRID AB_314064
Anti-human CD14	PC7	61D3	eBioscience	Cat#25-0149 RRID AB_1582276,
Anti-human CD56	APC	N901	Beckman Coulter	Cat# IM2474, RRID AB_130791
Anti-human CD19	AF700	HIB19	Biolegend	Cat: 302226, RRID AB_493751
Anti-human CD41	APC-Cy7	HIP8	Biolegend	Cat: 303716, RRID AB_10897646
Anti-human CCR2	BV421	48607	BD Biosciences	Cat: 564067, RRID AB_2738573
Anti-human CCR5	BV650	3A9	BD Biosciences	Cat: 564999, RRID: AB_2739037
Brilliant stain buffer	-	-	BD Bioscience	Cat: 563794, RRID: N/A
Helix NP™ NIR	-	-	Biolegend	Cat# 425301, RRID, N/A



Supplementary Figure 1: Gating strategy defining monocyte population using flow cytometry. Manual gating strategy for monocyte subsets: single cells (A) and CD45+ immune cells (B). Gating for monocytes, lymphocytes and granulocytes based on forward and side scatter (C). We excluded B-cells based on high expression of CD19 (D). Thereafter, monocytes were identified based on the expression of CD14 and CD16 (E-F) HLA-DR+ monocytes were selected to eliminate NK cells (G). Monocyte subsets (classical, intermediate and nonclassical) were determined based on CD14 and CD16 expression and the exact gates were put based on HLA-DR and CCR5 expression (highest on intermediate monocytes), and CCR2 expression (highest on nonclassical monocytes) (H-K).



Supplementary Figure 2: Gating strategy for defining neutrophil population using flow cytometry. Manual gating strategy for neutrophil sub-analysis: gating for (A) single cells and (B) CD45+. Then granulocytes were selected based FSC/SSC (C). Eosinophils and basophils were eliminated based on low CD16 expression, and high CD123 expression respectively (D-E). Mature neutrophils were determined based on CD10 expression. (F) Neutrophils were further analyzed for their median fluorescent intensity of activation markers HLA-DR (G) and CD62L (H)

Chapter 6:

The Effect of Low-Dose Colchicine on the Phenotype and Function of Neutrophils and Monocytes in Patients with Chronic Coronary Artery Disease: a double-blind randomized placebo controlled cross-over study

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Clinical Pharmacology & Therapeutics, 2024, 10.1002/cpt.3394

Study highlights

What is the current knowledge on the topic?

Despite optimal treatment of cardiovascular risk factors, many patients remain to have a high residual risk of atherosclerotic cardiovascular disease (ASCVD). Recent landmark trials showed that low-dose colchicine (0.5 mg/day) treatment provides significant benefit in reducing major cardiovascular events in patients with ASCVD, and based these results, colchicine is now included in guidelines as Class IIb recommendation to further lower ASCVD in secondary prevention setting. However, the exact mechanism of action that confers this risk reduction remains elusive.

What question did this study address?

We hypothesized that an in-depth characterization of the innate immune function in a randomized controlled cross-over trial in patients with a history of myocardial infarction in the advised dose of 0.5 mg/day improves our understanding of the mechanisms of action of colchicine.

What does this study add to our knowledge?

Our main finding is that treatment with colchicine predominantly affect neutrophils, in contrast to monocytes, with a reduction in CD62L expression, and NGAL release, and changes in the expression of various genes with immunomodulatory roles.

How might this change clinical pharmacology or translational science?

Accumulating evidence points to the pathogenic role of neutrophils in ASCVD, and genetically determined high neutrophil counts predispose to ASCVD occurrence. Our findings suggests that colchicine treatment might be particularly effective in patients with high neutrophil counts or high CD62L expression at baseline. Therefore, our findings could improve patient selection for colchicine treatment, and promote the development of more specific immunomodulatory drugs in the future.

Abstract

Recent landmark trials showed that colchicine provides a substantial benefit in reducing major cardiovascular events in patients with coronary artery disease. Yet, its exact mechanism of action is still poorly understood. This study aimed to unravel the effect of colchicine on monocyte and neutrophil

A randomized double-blind placebo-controlled cross-over intervention study was executed in patients with a history of myocardial infarction. In neutrophils, colchicine treatment decreased CD62L expression and NGAL release upon *ex vivo* stimulation, and increased PMA-induced ROS production. The effects of colchicine on monocytes were limited to a decrease in HLA-DR expression on the intermediate and nonclassical monocytes. Also, on the level of RNA expression, colchicine did not affect monocyte phenotype, while affecting various immunomodulating genes in neutrophils.

Overall, our study suggests that treatment with colchicine affects neutrophil function, particularly by reducing neutrophil recruitment, lowering concentrations of NGAL and by changing the expression of various genes with immunomodulatory potential, whereas the effect on monocytes is limited.

Keywords: colchicine, coronary artery disease, atherosclerosis, monocytes, neutrophils

Introduction

Atherosclerosis is a chronic low-grade inflammatory disease of the arterial wall in which monocyte-derived macrophages are the most abundant immune cells.[1] Accumulating evidence also points to neutrophils as contributors to atherosclerotic disease.[2] Recent landmark trials showed that the immunomodulating drugs canakinumab and colchicine improve cardiovascular outcomes in patients post myocardial infarction and in patients with chronic coronary artery disease (CAD).[3-5]

Colchicine is an ancient anti-inflammatory drug derived from the *Colchicum autumnale* plant and has been used for decades for the treatment of gout and familial Mediterranean fever.[6] Its exact mechanisms of action are not fully understood. Experimental *in vitro* studies showed that colchicine inhibits tubulin and microtubule assembly, and attenuates nucleotide-binding, leucine-rich repeat, and pyrin-domain-containing 3 (NLRP3) inflammasome-mediated crystal induced inflammation.[7-9] A recent proteomics sub-study of the low-dose colchicine 2 (LoDoCo2) trial reported that colchicine mainly lowered circulating proteins that are derived from neutrophils.[10] Previous *in vitro* and experimental *in vivo* studies on the effect of colchicine on monocyte function show inconsistent results. A single administration of 1.0 mg of colchicine in acute coronary syndrome (ACS) patients reduced monocyte intracellular and secreted levels of interleukin (IL)-1β. In the plasma of these ACS patients, IL-1β, IL-18 and IL-6 were not altered by colchicine.[11] In contrast, a single administration of 1.5 mg of colchicine reduced absolute coronary sinus plasma concentrations of IL-1β and IL-6 in ACS patients. Also, the coronary sinus – arterial gradient or 'trans-coronary gradient' (a measure of intracardiac production) of IL-1β, IL-18 and IL-6 was reduced upon colchicine treatment in ACS patients. [12] With regard to the effect of colchicine on neutrophils, data are limited. In a recent study in mice, Weng *et al*. showed that colchicine does not directly modulate myeloid cell function, but only via indirect hepatic mechanisms involving the release of hepatokines.[13]

It is unknown how colchicine, in the low dose given for cardiovascular risk reduction, affects neutrophil and monocyte phenotype and function, in patients with CAD. Therefore, in this study, we investigated this in a randomized double-blind placebo-controlled intervention study with a cross-over design in patients with a history of myocardial infarction.

Methods

Study design

This study is a single-center, randomized double-blind placebo-controlled intervention study, with a cross-over after one month of treatment between 0.5 mg colchicine once daily and placebo, or vice versa. A cross-over design was chosen to avoid heterogeneity due to differences in baseline medication (e.g., statins), severity in the baseline inflammatory profile and the severity of atherosclerosis of each patient. Between cross-over, there was a wash-out period of two weeks in which study medication was terminated to avoid a carry-over effect.[14] This study was approved by the Medical Ethics Committee of the Radboud University Medical Centre, Nijmegen, The Netherlands (NL73042.091.20). The study was conducted in compliance with the principles of the Declaration of Helsinki. The trial was registered at NTR-new NL8582.

Study population

Patients 18 to 80 years of age were eligible if they had suffered a type 1 myocardial infarction in the past, were without cardiac complaints and stable for at least three months and provided written informed consent. Patients were not eligible if they used CYP3A4 or P-glycoprotein inhibitors, if they had moderate to severe renal impairment (a serum creatinine >150µmol/l or estimated glomerular filtration rate (eGFR) <50mL/min/1.73m2), suffered from moderate to severe hepatic disease, pre-existing chronic gastro-intestinal complaints, or malignant disease in past five years. Furthermore, patients were not eligible if they had an elevated inflammatory profile as evidenced by a high-sensitivity C-reactive protein (hs-CRP) >10mg/l, chronic or recent (<1 month) infections, recent hospital admission or surgery with general anesthesia (<3 months), suffered from auto-immune or inflammatory disease, used immunosuppressants or anti-inflammatory drugs or were vaccinated < 1 month ago. Women who were pregnant, breast feeding or considering pregnancy and men considering conceiving were also not eligible.

Randomization and study flow

After obtaining informed consent, blood was drawn via venous puncture to measure blood count, renal function and hs-CRP and assess participant eligibility. The following visit, participants were randomized between two groups: one group started treatment with colchicine 0.5mg once daily for one month, while the other group received placebo. After a washout period of minimal 2 weeks participants were crossed over between the groups

and received placebo or colchicine 0.5mg daily for one month accordingly. Blood was drawn via venous puncture directly before and at the end of each treatment. For a schematic overview of the study design, see **Figure 1**.

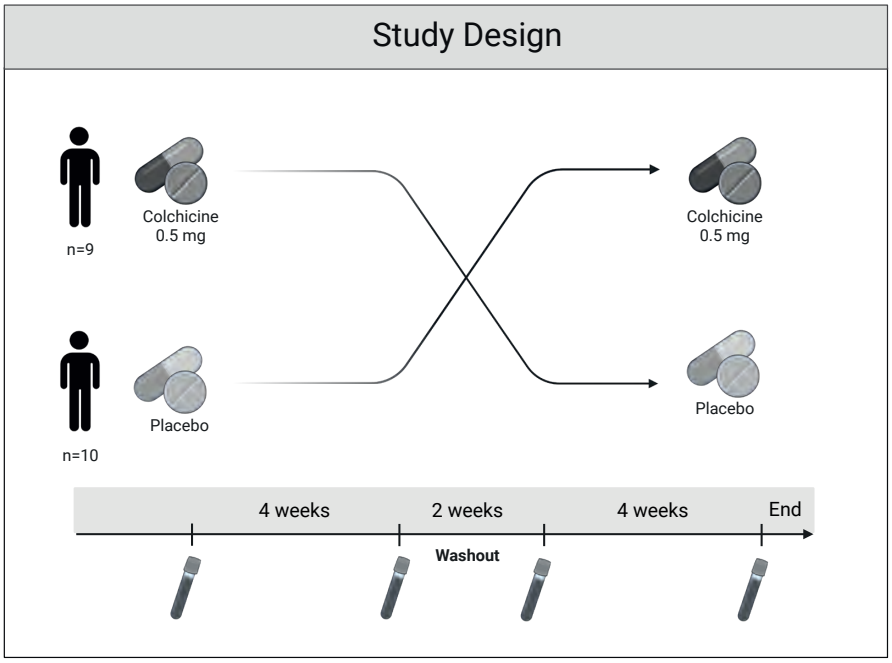


Figure 1: Study design. The figure shows the cross-over design of the study.

This study was executed during the COVID-19 pandemic and some participants were eligible to receive a COVID-19 vaccination during the course of the trial. To avoid that this COVID-19 vaccination affected the results of the study, the washout period between visit 3 and visit 4 was extended for some participants to allow for a minimum of one month between vaccination and visit 4.

Blood sampling and chemistry parameters

Blood was collected into BD Vacutainer® K2EDTA (10 ml) and BD Vacutainer® Lithium heparin (6 ml). At each timepoint 40 ml EDTA and 6 ml lithium heparin venous blood was taken. Within 4 hours of collection, two EDTA vacutainers (20ml) and the lithium heparin vacutainer were centrifuged for 10 minutes at 3800 rpm at room temperature to collect the plasma, which was stored at -80°C. Whole blood composition and cell counts were measured with Sysmex- XN 450 hematology analyzer. Plasma hs-CRP, creatinine, total

cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride levels were analyzed with commercially available enzymatic methods. Low-density lipoprotein (LDL) cholesterol levels were calculated with the Friedewald equation.

At the indicated time points we isolated whole blood, PBMCs, monocytes, and neutrophils for flow cytometry, functional assays, and RNA sequencing as described in the supplementary methods.

Statistical analysis

Due to the exploratory nature of this study, we did not perform a formal sample size calculation.

For each measurement, the change from baseline (delta) within each period (i.e. t2 – t1 and t4 - t3) was calculated and used as the outcome measure. Differences in the deltas between the two treatment options were analyzed using a mixed-effects linear regression with restricted maximum likelihood. A p-value of < 0.05 was considered statistically significant. In this model, period and treatment were specified as fixed effects, and participants were specified as a random effect.

Results

Baseline characteristics

We included 22 patients. During the study, two participants were excluded due to withdrawal of informed consent, and development of a bursitis in the left shoulder, respectively. In the last 4-week treatment period, one participant developed a viral airway infection and was also excluded from analysis. In total, 19 participants were included and analyzed in this study. **Figure 2** depicts the consort diagram of the study.

The baseline characteristics and blood measurements are shown in **Table 1**. The mean age was 62.3 years, and most participants were male (86%). The average time since prior myocardial infarction was 13.2 months and all participants had previously undergone coronary artery revascularization. Most participants were taking statins (95%).

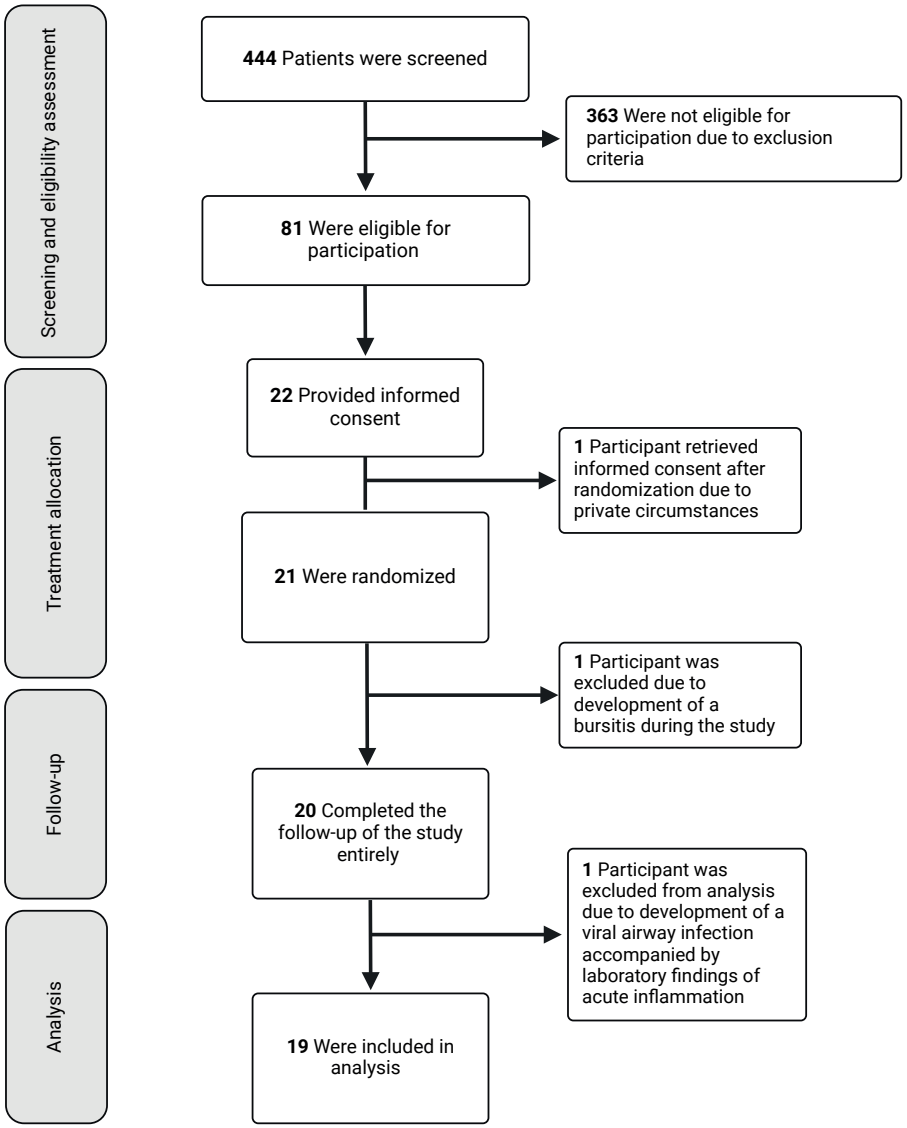


Figure 2: Consort diagram. The figure shows the selection of subjects and reasons for exclusion.

Table 1: Baseline characteristics of the study population.

		Study population (n=19)	
Demographics			
Age – years ± SD		62.3	± 7.4
Sex - no. (%)	Female	2	(11)
Cardiovascular risk factors			
BMI, mean ± SD		28.3	± 5.3
Hypertension - no. (%)		7	(37)
Current smoker - no. (%)		1	(5)
Diabetes mellitus - no. (%)		3	(16)
	Insulin dependent	2	(11)
Cardiovascular history			
Time since prior MI – months, mean ± SD		13.2	± 4.9
Prior coronary revascularization - no. (%)		19	(100)
Coronary artery bypass grafting - no. (%)		3	(16)
Percutaneous coronary intervention - no. (%)		18	(95)
Cardiovascular medication use			
Single antiplatelet therapy - no. (%)		8	(42)
Dual antiplatelet therapy - no. (%)		11	(58)
Anticoagulant - no. (%)		0	(0)
Statin - no. (%)		18	(95)
High-intensity statin - no. (%)		14	(74)
Renin angiotensin inhibitor - no. (%)		11	(58)
Beta-blocker - no. (%)		13	(68)
Blood measurements at enrollment			
Hemoglobin - mmol/L, mean ± SD		9.0	± 0.9
Leukocytes - 10^9/L, mean ± SD		6.8	± 1.4
Thrombocytes - 10^9/L, mean ± SD		238	± 49.1
Creatinin - µmol/L, mean ± SD		83	± 12.2
eGFR - ml/min/1.73m^2, mean ± SD		81	± 10.2
Triglycerides - mmol/L, mean ± SD		1.6	± 1.1
Total cholesterol - mmol/L, mean ± SD		3.5	± 0.5
Low-density lipoprotein cholesterol - mmol/L, mean ± SD		1.6	± 0.6
High-density lipoprotein cholesterol - mmol/L, mean ± SD		1.2	± 0.3
High sensitivity C-reactive protein - mg/L, mean ± SD		1.8	± 1.3

Abbreviations: BMI = body mass index; MI = myocardial infarction; SD = standard deviation.

Side effects and Adverse Events

During the 4-week treatment period with colchicine, three participants experienced adverse events. An overview of all adverse events is provided in **Table S1**.

Colchicine treatment does not alter cytokine production in a whole blood setup

We stimulated whole blood to examine the overall effect on all immune cells. We measured the production of key innate immune cytokines, chemokines, and neutrophil-associated markers upon stimulation. However, we did not find statistically significant differences between colchicine and placebo treated participants (**Table S2**).

Colchicine does not affect circulating immune cell numbers

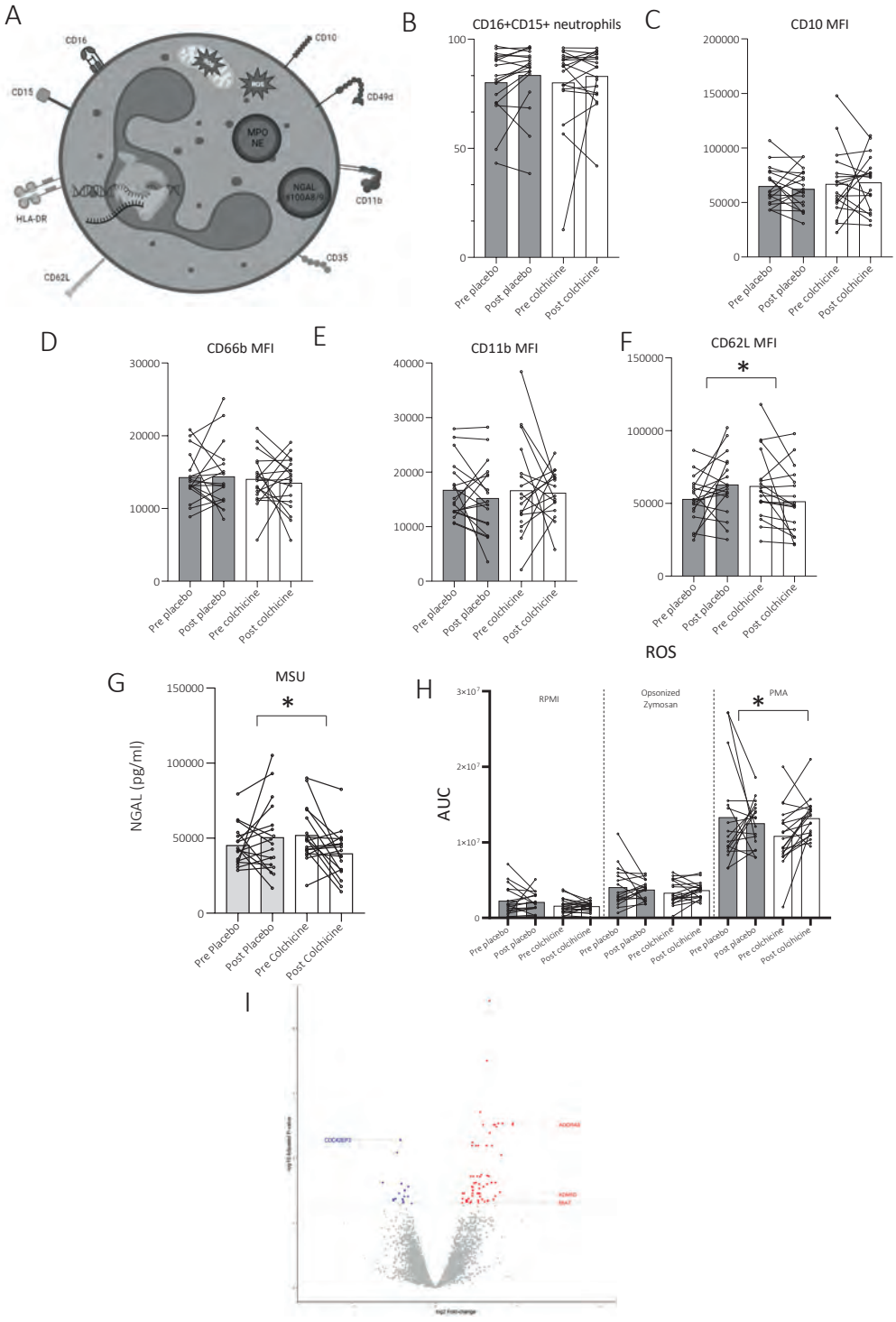
Colchicine treatment did not significantly alter the absolute or relative counts of the different leukocytes (**Table S3**). Although there was a trend towards a lower neutrophil-lymphocyte ratio upon colchicine treatment, this did not reach statistical significance (p=0.073).

The effect of colchicine on neutrophil phenotype and function

Colchicine decreases CD62L expression on neutrophils

We characterized the phenotype of neutrophils from fresh whole blood by flow cytometry. CD62L expression was significantly decreased after 1 month of colchicine treatment, both on mature and immature neutrophils. There were no changes in the other surface markers (**Table S4 and Figure 3F**).

> Figure 3: The effect of colchicine on neutrophil phenotype and function. **(A)** Markers used for characterization of maturation and activation level of neutrophils. No differences were found in expression of CD16/CD15 **(B)**, CD10 **(C)**, CD66b **(D)** and CD11b **(E)** markers. Expression of CD62L expression was significantly decreased upon 1 month of colchicine treatment **(F)**. NGAL – a granule associated protein – was significantly decreased in neutrophils stimulated with MSU crystals upon colchicine treatment **(G)**. ROS production capacity of neutrophils was also measured. Stimulation with PMA increased ROS production upon colchicine treatment, showing that colchicine treatment did not impair ROS production of neutrophils **(H).** CDC42EP3 (Cell division control protein 42 effector protein 3) was downregulated, ADORA3(Adenosine A3 Receptor) and MIAT(Myocardial Infarction Associated Transcript) were upregulated **(I)**. Abbreviations: CD = cluster of differentiation; MFI = median fluorescence intensity; HLA-DR = Human Leukocyte Antigen – DR isotype; CDC42EP3 = cell division control protein 42 effector protein; 3) ADORA3 = Adenosine A3 Receptor; MIAT = Myocardial Infarction Associated Transcript.


A
CD16
CD10
CD15
CD49d
MPO
NE
NGAL
#100A8/9
CD11b
HLA-DR
CD35
CD62L
B
CD16+CD15+ neutrophils
100
50
0
Pre placebo
Post placebo
Pre colchicine
Post colchicine
C
CD10 MFI
200000
150000
100000
50000
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Pre placebo
Post placebo
Pre colchicine
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NGAL (pg/ml)
150000
100000
50000
0
Pre Placebo
Post Placebo
Pre Colchicine
Post Colchicine
ROS
H
RPMI
Opsonized Zymosan
PMA
*
AUC
3×10⁷
2×10⁷
1×10⁷
0
Pre placebo
Post placebo
Pre colchicine
Post colchicine
I

The effect of colchicine on degranulation capacity of isolated neutrophils

To assess the degranulation capacity of isolated neutrophils, we stimulated purified neutrophils *ex vivo* for 4 hours with various Pattern Recognition Receptor (PRR) ligands before and after each treatment period and measured the concentrations of 4 granule-associated proteins: Myeloperoxidase and Neutrophil Elastase found in the primary granules, S100A8/9 heterodimer and Neutrophil Gelatinase-associated Lipocalin (NGAL) found in secondary granules. Colchicine treatment significantly reduced the MSU-induced NGAL release (**Figure 3G**). Most of the other granule associated protein concentrations were reduced after colchicine treatment, however none of these reached statistical significance (**Table S5**).

We measured neutrophil-associated granular proteins in plasma as well, and we observed no differences by colchicine treatment. (**Table S6**).

The effect of colchicine on NET formation and ROS production

We quantified the amount of DNA released during NOX-dependent and -independent NET formation assays. We did not find any differences in the amount of DNA released in either assay or various stimuli used comparing placebo and colchicine treated neutrophils (**Table S7**).

We measured total ROS production for 1 hour from neutrophils stimulated *ex vivo* with serum opsonized Zymosan and PMA by a luminol based assay. ROS production upon PMA stimulation was increased after 4 weeks of colchicine treatment (**Figure 3H**).

The transcriptomic profile of neutrophils changes upon colchicine treatment

Finally, we characterized RNA expression of unstimulated isolated neutrophils before and after colchicine treatment. To this end, we performed bulk RNA sequencing of purified neutrophils from randomly selected 8 patients before and after colchicine treatment. We identified 80 DEGs, of which 64 genes were upregulated and 16 were downregulated after colchicine treatment. Pathway analysis on DEGs did not reveal significant enrichment in neutrophil-inflammation specific pathways (**Figure S4**). Among the DEGs, CDC42EP3 (Cell division control protein 42 effector protein 3) was downregulated, ADORA3 (Adenosine A3 Receptor) and MIAT (Myocardial Infarction Associated Transcript), and Lysine-specific demethylase 5D (KDM5D) were upregulated (**Figure 3I**).

The effect of colchicine on monocytes and PBMCs

Colchicine does not affect circulating monocyte subset populations and their activation state.

We performed flow cytometry to investigate the immunophenotype of monocytes after colchicine treatment using both unbiased as well as biased methods. No differences were observed in the percentage of classical, intermediate, or non-classical monocytes (**Figure 4A**). Assessment of activation markers HLA-DR, CCR2, CCR5, CD11b, CD11c and CD41 showed that colchicine treatment did not affect the expression of these markers in total monocytes (**Table S8**), although in intermediate and in nonclassical monocytes HLA-DR MFI values were significantly reduced upon colchicine treatment. (**Figure 4B**).

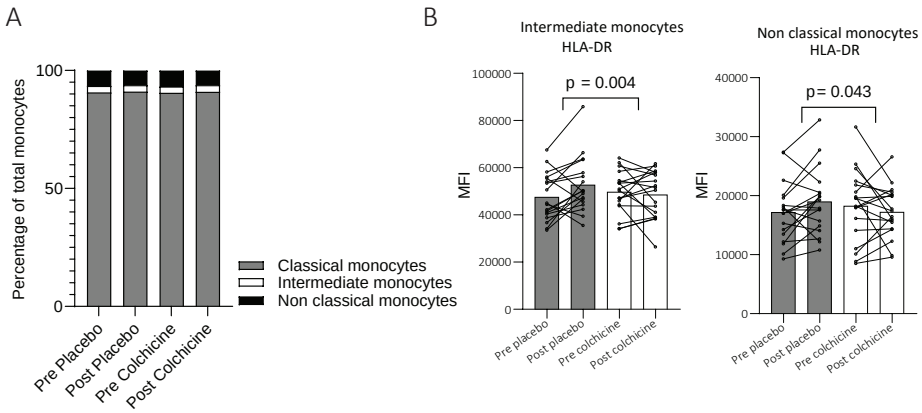

Figure 4: Monocyte subsets and HLA-DR expression (A) Monocyte subsets (classical, non-classical and intermediate monocytes) before and after treatment with either placebo or low-dose colchicine (n=19). Data are presented as stacked bar graph showing the percentages. (B) HLA-DR expression in intermediate and non-classical monocytes was decreased upon colchicine treatment. Data are presented as bar graphs showing Median Flurorescence Intensity (MFI). MFI = median fluorescence intensity; HLA-DR = Human Leukocyte Antigen – DR isotype

We further employed an unsupervised clustering analysis on the monocyte panel. Different immune cell populations and monocyte subsets were defined based on marker expression, and we further confirmed these populations by manual gating. We visually investigated changes before and after each treatment period and did not observe any changes in the lymphocyte or monocyte populations. However, we did observe changes in the granulocyte population, which were further analyzed in detail using a neutrophil specific panel of flow cytometry markers as described above (**Figure S3, Figure 3A-F**).

Colchicine treatment does not alter cytokine production capacity of PBMC

To investigate whether PBMCs are functionally different during colchicine treatment, we stimulated PBMCs *ex vivo* with Toll-like receptor 4 and 2 ligands (LPS and Pam3Cys) as well as NLRP3 inflammasome activators (MSU crystals and Nigericin) for 24 hours and measured cytokine production of TNF, IL-1β, IL-6, IL-1Ra and IL-10. We did not observe any significant differences, apart from an increase in TNF, IL-1β and IL-10 production upon MSU stimulation (**Table S9**).

Colchicine does not affect RNA expression of unstimulated or LPS-stimulated monocytes

There were no significant differences in gene expression profiles of unstimulated or LPS stimulated monocytes before versus after treatment with colchicine (**Figure S5**). However, when we compared the differentially expressed genes (DEGs) by LPS, we identified 939 unique LPS-induced DEGs before colchicine treatment and 827 unique genes after the colchicine treatment. In other words, in the monocytes isolated before colchicine treatment, LPS changed the expression of 939 genes which were not significantly changed by LPS in the monocytes isolated after colchicine treatment. We decided to focus on the genes that showed an opposite fold change before and after colchicine treatment. This yielded 35 LPS-induced DEGs before colchicine treatment, which showed an opposite direction of expression after colchicine treatment. Similarly, we identified 36 LPS-induced DEGs after colchicine treatment that showed opposite direction of expression before colchicine treatment (**Figure S5B**). Pathway analysis on these unique DEGs with opposite fold changes did not reveal enrichment of specific inflammatory pathways.

Discussion

In the present randomized double-blind placebo-controlled cross-over intervention study, we examined in detail the effects of 4 weeks of low-dose colchicine treatment on neutrophil and monocyte phenotype and function in patients with a history of myocardial infarction. We show that colchicine treatment alters specific effector functions of neutrophils, marked by decreased CD62L expression and lower NGAL release upon *ex vivo* stimulation, and higher PMA-induced ROS production. In contrast, the effects on monocytes were limited to a decrease in HLA-DR expression on the intermediate and nonclassical monocytes. Also, on the level of RNA expression, colchicine did

not affect monocyte phenotype, while affecting various immunomodulating genes in neutrophils.

Despite state-of-the-art risk factor treatment, the incidence of atherosclerotic cardiovascular disease (ASCVD) remains substantial in many patients. Based on recent trial evidence, the broad anti-inflammatory drug colchicine is now included in guidelines as Class IIb recommendation to further lower ASCVD in secondary prevention setting.[15] The exact mechanism of action that drives this risk reduction, however, is still incompletely understood. We hypothesized that an extensive functional and transcriptomic assessment of innate immune function in a randomized cross-over trial in patients after myocardial infarction in the advised dose of 0.5 mg/day would unveil new mechanisms that could help to improve patient selection and the development of more selective immunomodulatory drugs in the future.

The main finding of our study is that colchicine mainly affects neutrophil phenotype and function, in contrast to monocyte function. Flow cytometry analysis revealed that CD62L expression was significantly decreased after 4 weeks of low-dose colchicine treatment, both on mature and immature neutrophils. L-selectin is crucial for neutrophil attachment to the vasculature and homing to inflammatory sites.[16] Consequently, our results suggest that colchicine might inhibit neutrophil recruitment to the atherosclerotic plaque. In addition, we showed that MSU-induced NGAL concentrations decreased after colchicine treatment. NGAL, is a protein found in the secondary granules of neutrophils that regulates the proteolytic activity of MMP9, and its concentration in plasma has been associated with vulnerable plaques in patients with carotid artery stenosis.[17] However, neither NGAL nor other granule proteins were altered in plasma. It is important to note that even though neutrophils are the main producers of these proteins, other cell types such as monocytes can also produce some NGAL, MPO and s100A8/9.[18-20]

ROS production capacity is an important function of neutrophils, which can enhance oxidized lipid formation and activation of endothelium, contributing to atherogenesis. While colchicine treatment did not change ROS production capacity of neutrophils upon opsonized zymosan stimulation, PMA-induced ROS production unexpectedly increased after colchicine treatment. This contradicts previous research that observed that *ex vivo* exposure to colchicine lowered ROS production in MSU and PMA stimulated neutrophils respectively.[21] The effect

of colchicine on MSU stimulated neutrophil ROS production should be topic of future studies.

Formation of NETs is an important function of neutrophils which facilitates trapping extracellular pathogens as well as wound healing. However, NETs can also contribute to atherosclerosis pathophysiology. We assessed the NET formation capacity of neutrophils with NOX-dependent and independent NET-inducing stimuli. NOX-dependent NET formation capacity remained unaltered after either of the treatment periods.

Bulk RNA sequencing of purified unstimulated neutrophils revealed 80 DEGs by colchicine treatment, these included several genes with known immunomodulatory functions. The adenosine A3 receptor gene (ADORA3) expression was upregulated by colchicine. The ADORA3 is expressed on neutrophils and regulates several important neutrophil functions, including reducing neutrophil adherence to coronary endothelium, and promoting cardiac recovery after reperfusion.[22, 23] Therefore, upregulation of the neutrophil ADORA3 could contribute to the beneficial cardiovascular effects of colchicine. Also, the Myocardial Infarction Associated Transcript (MIAT), was upregulated by colchicine. MIAT encodes for a spliced long non-coding RNA and altered expression of this locus has been reported to be associated with a susceptibility to myocardial infarction [24]. Its specific role in neutrophils is unknown. Another gene that showed a significant upregulation by colchicine treatment is the lysine demethylase KDM5D. KDM5 is a key enzyme in the development of innate immune memory in monocytes, which is called trained immunity.[25] Accumulating evidence points to a role for trained immunity in the pathophysiology of atherosclerosis.[26] Brief exposure of monocytes to β-glucan, which is a potent inducer of trained immunity, leads to a downregulation of KDM5 activity, which results in enrichment of the activating histone 3 modification lysine 4 trimethylation (H3K4me3). Therefore, the increased expression of KDM5D might revert the histone modifications that drive trained immunity, which slows atherosclerosis progression. Finally, the expression of CDC42EP3, a gene involved in actin reorganization was decreased by colchicine, indicating the effects of colchicine on the cytoskeleton rearrangements, a process critical to neutrophil function.[27]

To our surprise, colchicine treatment only induced minimal effects on monocytes. Flow cytometric characterization of monocytes revealed reduced HLA-DR expression on intermediate and non-classical monocytes.

Various studies report that mainly the number of intermediate monocytes is associated with ASCVD, and this subset is primarily responsible for inflammatory cytokine production.[28, 29] Membrane HLA-DR is responsible for antigen presentation of monocytes to T cells, a decrease in HLA-DR surface expression is regarded as sign of immunosuppression.

PBMCs isolated from patients with coronary artery disease are characterized by augmented *ex vivo* cytokine production capacity.[30, 31] In the current study, this *ex vivo* cytokine production capacity of PBMCs was mostly unaltered by colchicine treatment. In the colchicine-treated condition, some cytokines after exposure to MSU were higher than in the control condition, but this is not clinically relevant since the MSU condition only was a negative control for the MSU + LPS condition, and indeed in itself did not increase cytokine production. Similarly, bulk RNA sequencing showed no difference induced by colchicine in RNA expression of both unstimulated and LPS stimulated monocytes. When we compared the differentially expressed genes in response to LPS exposure in the monocytes before and after colchicine treatment, we did observe that colchicine significantly changed the direction of RNA expression by LPS for 35-36 genes, but these did not include specific inflammation related pathways.

Overall, our results regarding the peripheral blood monocytes only show minor changes in monocyte phenotype and no effect on cytokine production capacity. Similarly, in atherosclerotic mice, colchicine had no effect on circulating monocytes or monocyte subsets.[32, 33] Yet, colchicine treatment did reduce the numbers of inflammatory monocytes and macrophages in aortic plaques by mitigating myeloid cell uptake from the blood. Moreover, downregulation of leukocyte adhesion molecules and chemokine receptors on blood monocytes was observed upon colchicine treatment.[33], which contrasts with our findings in human monocytes of patients with coronary artery disease. Nonetheless, we cannot rule out the possibility that the phenotype or function of plaque macrophages is altered by colchicine treatment.

Our study has a few limitations including limited sample size. Secondly, our cohort mostly consists of individuals with Western European ancestry, and we could not achieve balanced distribution of sex. Therefore, our results need to be validated in larger cohorts with diverse ethnic backgrounds and equal number of men and women.

Our results carry the potential to advance the anti-inflammatory treatment of patients with cardiovascular diseases. Our main finding is that the strongest effects of colchicine are on neutrophils, in contrast to monocytes, with a reduction in CD62L expression, and NGAL release, and changes in the expression of various genes with immunomodulatory potential. This is particularly relevant since accumulating evidence points to the pathogenic role of neutrophils in ASCVD.[2] Genetically determined high neutrophil counts predispose to ASCVD occurrence.[34] This suggests that colchicine might be particularly effective in patients with high neutrophil counts or high CD62L expression at baseline. In addition to future patient stratified clinical studies with colchicine, our results trigger further experimental studies on how adenosine signaling and histone methylation processes determine the adverse cardiovascular effects of neutrophils. These studies might help to develop more specific drugs that target these pathways for cardiovascular risk reduction.

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Supplementary data

Supplementary data are available in the additional 'supplementary tables figures methods file.'

Declarations

Disclosure of interest

JHC reports membership in advisory boards with Amgen and AstraZeneca. All other authors declared no competing interests for this work.

Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

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Ethical approval

This study was approved by the Medical Ethics Committee of the Radboud University Medical Centre, Nijmegen, The Netherlands (NL73042.091.20). The study was conducted in compliance with the principles of the Declaration of Helsinki.

Pre-registered clinical trial number

The trial was registered at NTR-new NL8582.

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Supplementary Material Table of Contents

Supplementary Tables

Table S1: Adverse events
Table S2: Cytokine production upon whole blood stimulation.
Table S3: Overview of white blood cell composition during placebo and colchicine treatment.
Table S4: Flow cytometric characterization of neutrophils from whole blood.
Table S5: Neutrophil granule-associated protein release upon stimulation.
Table S6: Circulating neutrophil granule-associated proteins in plasma.
Table S7: DNA release during NET formation.
Table S8: Effect of low-dose colchicine on monocyte subsets and activation status.
Table S9: Cytokine production of PBMCs upon stimulation with various stimuli.
Table S10: Flow cytometry markers.
Table S11: ELISA assays.

Supplementary Figures

Figure S1: Gating strategy for defining neutrophil population using flow cytometry.
Figure S2: Gating strategy defining monocyte population using flow cytometry.
Figure S3: Unbiased analysis of the monocyte flow cytometry panel.
Figure S4: Neutrophil RNA expression upon colchicine treatment.
Figure S5: Transcriptional response to colchicine treatment on monocytes before and after 4 hours RPMI or LPS stimulation.

Supplementary Methods

Table S1: Overview of adverse events.

	Adverse event	N	Related to study medication	Action
Before randomization	bursitis left shoulder	1	no	exclusion and replacement by other participant
	hematoma elbow	1	no	continue study
	chalazion eyelid	1	no	continue study
During colchicine treatment	high glucose levels	1	no	continue study
	backpain	1	no	continue study
	viral airway infection	1	no	exclusion from analysis
	dental procedure	2	no	continue study
	pain knee, arthrosis	1	no	continue study
During placebo treatment	headache and subconjunctival hemorrhage	1	no	continue study
	malfunctioning insulin pump	1	no	continue study
	chest pain, acute coronary syndrome ruled out	1	no	continue study
During wash-out period	fall from bicycle, rib contusion and hematoma	1	no	continue study

Table S2: Cytokine production upon whole blood stimulation.

Cytokine	Stimuli	Pre placebo (mean± SD)	Post placebo (mean± SD)	Pre colchicine (mean± SD)	Post colchicine (mean± SD)	p-value
IL-1β (pg/ml)	RPMI	41 ± 7	40 ± 3	40 ± 4	40 ± 3	
	LPS	1522 ± 546	1562 ± 617	1733 ± 953	1634 ± 576	0.55
	PHA	505 ± 241	570 ± 374	550 ± 423	551 ± 322	0.17
IL-1Ra (pg/ml)	RPMI	445 ± 222	440 ± 176	447 ± 247	422 ± 140	
	LPS	4523 ± 1696	4954 ± 2193	5164 ± 1827	4669 ± 2004	0.17
	PHA	4185 ± 1463	4726 ± 1853	4667 ± 1390	4488 ± 1620	0.66
IL-6 (pg/ml)	RPMI	469 ± 0	469 ± 0	469 ± 0	469 ± 0	
	LPS	8961 ± 2404	9058 ± 2871	10459 ± 3603	9010 ± 3275	0.17
	PHA	8871 ± 4649	8507 ± 3781	8837 ± 4197	8010 ± 4241	0.42
IL-8 (pg/ml)	RPMI	318 ± 22	328 ± 57	313 ± 0	322 ± 41	
	LPS	1816 ± 795	1950 ± 1038	1828 ± 1083	1955 ± 1122	0.84
	PHA	4034 ± 1430	4230 ± 2196	4674 ± 3929	4822 ± 3298	0.22
S100A8/9 (ng/ml)	RPMI	921 ± 341	871 ± 287	1018 ± 355	946 ± 354	0.78
	LPS	310 ± 93	325 ± 169	337 ± 184	373 ± 284	0.79
	PHA	440 ± 192	433 ± 152	452 ± 225	446 ± 167	0.87

Data are presented as mean ± SD. *P-value <0.05 was considered significant.
Abbreviations: IL-1β = interleukin-1 beta; IL1-Ra = interleukin-1 receptor antagonist protein; IL-6 = interleukin-6; IL-8 = interleukin-8; S100A8/9 = S100 calcium-binding protein A8/9 heterodimer; RPMI = Roswell Park Memorial Institute culture medium; LPS = lipopolysaccharide; PHA = phytohemagglutinin.

Table S3: Overview of white blood cell composition during placebo and colchicine treatment.

	Pre placebo (mean ± SD)	Post placebo (mean ± SD)	Pre colchicine (mean ± SD)	Post colchicine (mean ± SD)	p-value
White blood cell count (10^9/l)	6.5 ± 1.1	6.2 ± 1.0	6.4 ± 1.0	6.0 ± 0.8	0.88
Neutrophil count (10^9/l)	3.9 ± 0.9	3.7 ± 0.7	3.9 ± 0.8	3.4 ± 0.7	0.69
Neutrophil (%)	58.7 ± 6.7	58.8 ± 6.7	60.6 ± 6.2	57.1 ± 6.5	0.09
Lymphocyte count (10^9/l)	1.8 ± 0.5	1.7 ± 0.5	1.7 ± 0.4	1.7 ± 0.4	0.16
Lymphocyte (%)	27.8 ± 7.0	27.9 ± 6.5	26.6 ± 5.8	29.1 ± 5.7	0.13
Monocyte count (10^9/l)	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.20
Monocyte (%)	9.0 ± 1.7	8.7 ± 1.5	8.5 ± 1.5	8.9 ± 2.0	0.20
Platelet count (10^9/l)	230 ± 55.0	224 ± 50.1	219 ± 49.8	223 ± 47.0	0.17
Neutrophil lymphocyte ratio (NLR)	2.3 ± 0.9	2.3 ± 0.8	2.4 ± 0.8	2.1 ± 0.7	0.07

Data are presented as mean ± SD. *P-value <0.05 was considered significant.

Table S4: Flow cytometric characterization of neutrophils from whole blood.

		Pre placebo (mean± SD)	Post placebo (mean ± SD)	Pre colchicine (mean ± SD)	Post colchicine (mean ± SD)	p-value
Live granulocytes	**CD16+CD15+ (%)**	80 ± 15	83 ± 15	80 ± 20	83 ± 13	0.93
All neutrophils	**CD16 (MFI)**	548500	552030	538789	571111	0.29
Immature neutrophils	**CD66b (MFI)**	15663 ± 5016	14112 ± 5457	14758 ± 6088	15621 ± 6295	0.30
	CD15 (MFI)	44570 ± 11260	42959 ± 12781	43223 ± 17453	45977 ± 13537	0.37
	CD11b (%)	100 ± 0	99 ± 5	98 ± 9	93 ± 23	0.65
	CD11b (MFI)	11659 ± 5132	9772 ± 5183	10591 ± 6962	10523 ± 4983	0.34
	CD35 (%)	95 ± 4	92 ± 7	89 ± 18	82 ± 31	0.73
	CD35 (MFI)	6656 ± 2068	6068 ± 2829	5796 ± 2989	5951 ± 2910	0.44
	CD62L (%)	94 ± 5	92 ± 8	95 ± 5	94 ± 4	0.48
	CD62L (MFI)	46141 ± 17064	59782 ± 26157	52023 ± 24502	47199 ± 17677	<0.0001
	HLA-DR (%)	56 ± 34	42 ± 39	39 ± 31	46 ± 33	0.04
	HLA-DR (MFI)	3137 ± 5605	4657 ± 8399	1574 ± 742	3074 ± 5648	0.96
Mature neutrophils	**CD66b (MFI)**	14263 ± 3284	14372 ± 4429	14022 ± 3544	13490 ± 3575	0.75
	CD15 (MFI)	42197 ± 12050	45597 ± 13882	41767 ± 12231	43158 ± 13463	0.56
	CD11b (%)	100 ± 0	100 ± 1	100 ± 1	90 ± 28	0.18
	CD11b (MFI)	16682 ± 5383	15198 ± 6545	16611 ± 8759	14639 ± 6094	0.89
	CD35 (%)	100 ± 0	99 ± 1	98 ± 6	89 ± 31	0.24
	CD35 (MFI)	12316 ± 3150	11693 ± 4824	12086 ± 5241	11273 ± 5919	0.99
	CD62L (%)	99 ± 1	99 ± 1	100 ± 1	99 ± 1	0.12
	CD62L (MFI)	52834 ± 16151	62780 ± 20950	61751 ± 23482	51205 ± 22027	<0.0001
	HLA-DR (%)	51 ± 37	40 ± 41	32 ± 31	36 ± 30	0.12
	HLA-DR (MFI)	4412 ± 5864	6439 ± 8303	2797 ± 2056	4252 ± 6283	0.79

Data are presented as mean ± SD.*P-value <0.05 was considered significant.
Abbreviations: CD = cluster of differentiation; MFI = median fluorescence intensity; HLA-DR = Human Leukocyte Antigen – DR isotype.

Table S5: Neutrophil granule-associated protein release upon stimulation.

Granule	Stimuli	Pre placebo (mean ± SD)	Post placebo (mean ± SD)	Pre colchicine (mean ± SD)	Post colchicine (mean ± SD)	p-value
Myeloperoxidase (ng/ml)	RPMI	359 ± 151	498 ± 304	383 ± 150	363 ± 124	0.08
	LPS	363 ± 176	509 ± 400	371 ± 157	369 ± 160	0.19
	Pam3Cys	248 ± 146	301 ± 168	245 ± 142	241 ± 104	0.29
	MSU	330 ± 191	309 ± 170	387 ± 165	316 ± 134	0.43
	LPS+MSU	352 ± 147	381 ± 160	393 ± 184	341 ± 132	0.21
	Nigericin	601 ± 225	667 ± 372	581 ± 155	587 ± 196	0.56
	Ethanol	329 ± 130	351 ± 205	379 ± 208	301 ± 128	0.14
	PMA	463 ± 170	521 ± 213	420 ± 172	519 ± 189	0.53
Neutrophil Gelatinase Associated Lipocalin (ng/ml)	RPMI	42 ± 14	45 ± 18	40 ± 13	38 ± 13	0.51
	LPS	59 ± 24	69 ± 30	57 ± 20	59 ± 20	0.34
	Pam3Cys	108 ± 43	111 ± 29	103 ± 40	105 ± 37	0.93
	MSU	45 ± 13	50 ± 24	52 ± 18	40 ± 16	0.024*
	LPS+MSU	63 ± 15	67 ± 19	61 ± 15	59 ± 18	0.38
	Nigericin	66 ± 35	73 ± 32	65 ± 28	62 ± 27	0.26
	Ethanol	38 ± 13	41 ± 15	40 ± 14	34 ± 10	0.13
	PMA	198 ± 93	235 ± 55	230 ± 86	249 ± 53	0.47

Table S5: Continued

Granule	Stimuli	Pre placebo (mean ± SD)	Post placebo (mean ± SD)	Pre colchicine (mean ± SD)	Post colchicine (mean ± SD)	p-value
S100A8/9 (ng/ml)	RPMI	2415 ± 602	2617 ± 910	2483 ± 651	2416 ± 679	0.48
	LPS	2038 ± 525	2331 ± 929	2142 ± 476	2151 ± 568	0.39
	Pam3Cys	2321 ± 684	2648 ± 754	2293 ± 585	2344 ± 547	0.29
	MSU	3147 ± 628	3245 ± 1165	3372 ± 912	3113 ± 1011	0.44
	LPS+MSU	2748 ± 989	2657 ± 1076	2841 ± 1006	2811 ± 1093	0.79
	Nigericin	2914 ± 957	3256 ± 1067	2985 ± 1188	2939 ± 990	0.28
	Ethanol	2265 ± 565	2497 ± 767	2336 ± 764	2201 ± 500	0.15
	PMA	5013 ± 2396	5579 ± 1244	5444 ± 3161	6141 ± 1823	0.86
Neutrophil Elastase (ng/ml)	RPMI	52 ± 32	64 ± 31	59 ± 47	62 ± 29	0.57
	LPS	66 ± 26	81 ± 55	73 ± 40	69 ± 21	0.26
	Pam3Cys	61 ± 20	69 ± 35	52 ± 16	65 ± 26	0.79
	MSU	62 ± 52	58 ± 33	55 ± 27	48 ± 22	0.84
	LPS+MSU	80 ± 51	69 ± 27	73 ± 26	65 ± 21	0.61
	Nigericin	91 ± 23	92 ± 32	84 ± 26	80 ± 20	0.57
	Ethanol	29 ± 49	27 ± 32	22 ± 26	19 ± 24	0.99
	PMA	79 ± 25	105 ± 56	75 ± 25	101 ± 36	0.99

Data are presented as mean ± SD. *P-value <0.05 was considered significant.

Abbreviations: S100A8/9 = S100 calcium-binding protein A8/9 heterodimer; RPMI = Roswell Park Memorial Institute culture medium; LPS = lipopolysaccharide; Pam3Cys = Pam3CYSK4; MSU = Monosodium urate (MSU) crystals; PMA = Phorbol-12-myristate-13-acetate.

Table S6: Circulating neutrophil granule-associated proteins in plasma.

	Pre placebo (mean± SD)	Post placebo (mean± SD)	Pre colchicine (mean ± SD)	Post colchicine (mean± SD)	p-value
MPO (ng/ml)	28.1 ± 8.4	28.9 ± 7.8	30.0 ± 7.2	27.5 ± 8.0	0.44
NE (ng/ml)	15.6 ± 4.2	17.0 ± 4.6	17.0 ± 3.7	16.5 ± 4.0	0.80
S100A8/9 (ng/ml)	435.5 ± 269.2	344.7 ± 153.2	363.1 ± 167.4	337.0 ± 156.1	0.31
NGAL (ng/ml)	78.1 ± 22.0	90.3 ± 33.7	87.2 ± 2.,1	75.4 ± 18.5	0.14

Data are presented as mean ± SD. *P-value <0.05 was considered significant.
Abbreviations: MPO = myeloperoxidase, NE = neutrophil elastase, NGAL = Neutrophil Gelatinase Associated Lipocalin.

Table S7: DNA release during NET formation.

		Pre placebo (mean± SD)	Post placebo (mean ± SD)	Pre colchicine (mean ± SD)	Post colchicine (mean ± SD)	p-value
Stimuli NOX dependent	RPMI (ng/ml)	349 ± 138	320 ± 116	353 ± 136	339 ± 128	0.81
	Nigericin (ng/ml)	500 ± 222	429 ± 182	511 ± 190	430 ± 231	0.91
	Ethanol (ng/ml)	416 ± 140	379 ± 147	396 ± 134	354 ± 185	0.92
	PMA (ng/ml)	478 ± 202	409 ± 161	469 ± 248	469 ± 270	0.81
Stimuli NOX independent	RPMI (ng/ml)	304 ± 118	189 ± 80	271 ± 101	239 ± 99	0.13
	Unstimulated platelets (ng/ml)	385 ± 112	288 ± 86	337 ± 88	319 ± 102	0.09
	TRAP6-stimulated platelets (ng/ml)	332 ± 105	263 ± 86	290 ± 84	332 ± 91	0.01

Data are presented as mean ± SD. *P-value <0.05 was considered significant.
Abbreviations: RPMI = Roswell Park Memorial Institute culture medium; PMA = Phorbol-12-myristate-13-acetate.

Table S8: Effect of low-dose colchicine on monocyte subsets and activation status.

		Pre placebo (mean ± SD)	Post placebo (mean ± SD)	Pre colchicine (mean ± SD)	Post colchicine (mean ± SD)	p-value
Monocytes	**CD45 positive (%)**	12.3 ± 2.9	10.8 ± 2.9	11.5 ± 1.9	11.7 ± 3.4	0.117
	HLADR (MFI)	7038 ± 2341	7237 ± 2612	7209 ± 2538	6792 ± 2131	0.225
	CCR2 (%)	91.6 ± 3.8	91.8 ± 2.9	91.1 ± 3.8	91.7 ± 2.2	0.670
	CCR2 (MFI)	8674 ± 2403	8359 ± 2505	7013 ± 3527	8700 ± 2847	0.083
	CCR5 (%)	14.9 ± 8.2	12.3 ± 6.4	16.7 ± 6.0	16.5 ± 8.3	0.363
	CCR5 (MFI)	1563 ± 291	1572 ± 363	1563 ± 264	1638 ± 288	0.573
	CD11b (%)	95.6 ± 2.5	96.2 ± 2.3	95.9 ± 3.5	96.2 ± 1.3	0.763
	CD11b (MFI)	12545 ± 3842	11585 ± 2735	11210 ± 4518	11008 ± 2950	0.607
	CD11c (%)	99.9 ± 0.1	99.9 ± 0.1	99.9 ± 0.1	99.9 ± 0.1	0.631
	CD11c (MFI)	33506 ± 5994	33031 ± 6734	30104 ± 9995	30769 ± 7762	0.674
	CD41(%)	2.8 ± 1.2	2.6 ± 1.1	2.7 ± 1.2	2.9 ± 1.3	0.224
	CD41 (MFI)	109089 ± 22628	116289 ± 16497	111855 ± 15739	119296 ± 14185	0.934
Classical monocytes (CM)	**CM (%)**	90.7 ± 4.3	91.0 ± 3.3	90.6 ± 3.7	91.0 ± 2.6	0.890
	HLA-DR (MFI)	6512 ± 2243	6661 ± 2432	6632 ± 2398	6213 ± 2051	0.196
	CCR2 (%)	98.6 ± 1.3	98.6 ± 1.1	97.9 ± 3.2	98.7 ± 1.0	0.171
	CCR2 (MFI)	8736 ± 2410	8412 ± 2508	7062 ± 3544	8757 ± 2865	0.083
	CCR5 (%)	16.1 ± 8.5	12.9 ± 5.6	17.4 ± 6.1	15.9 ± 6.4	0.429
	CCR5 (MFI)	1439 ± 228	1443 ± 362	1516 ± 338	1591 ± 258	0.625
	CD11b (%)	99.5 ± 0.4	99.5 ± 0.3	99.4 ± 0.4	99.4 ± 0.3	0.727
	CD11b (MFI)	12603 ± 3900	11652 ± 2800	11282 ± 4585	11056 ± 2989	0.627
	CD11c (%)	99.9 ± 0.2	99.9 ± 0.0	99.9 ± 0.1	99.9 ± 0.1	0.122
	CD11c (MFI)	31739 ± 5522	31183 ± 6251	28371 ± 9424	29028 ± 7253	0.650
	CD41 (%)	2.7 ± 1.2	2.6 ± 1.1	2.7 ± 1.1	2.9 ± 1.3	0.267
	CD41 (MFI)	108774 ± 23184	116220 ± 16822	110975 ± 15907	119031 ± 14211	0.901

Table S8: Continued

		Pre placebo (mean ± SD)	Post placebo (mean ± SD)	Pre colchicine (mean ± SD)	Post colchicine (mean ± SD)	p-value
Intermediate monocytes (IM)	**IM (%)**	2.8 ± 1.0	2.8 ± 1.2	2.6 ± 1.2	2.8 ± 1.2	0.528
	HLA-DR (MFI)	47553 ± 10012	52672 ± 11788	49713 ± 9026	48571 ± 9995	0.004*
	CCR2 (%)	60.1 ± 14.0	54.2 ± 12.1	64.0 ± 19.9	58.7 ± 18.5	0.909
	CCR2 (MFI)	4640 ± 1612	4826 ± 1539	3772 ± 1994	4655 ± 2037	0.232
	CCR5 (%)	50.9 ± 21.3	43.1 ± 20.2	51.7 ± 16.1	48.0 ± 18.3	0.315
	CCR5 (MFI)	1959 ± 561	2119 ± 745	2120 ± 693	2188 ± 625	0.616
	CD11b (%)	99.2 ± 1.3	99.8 ± 0.3	99.6 ± 0.6	99.5 ± 0.8	0.064
	CD11b (MFI)	17298 ± 6153	16689 ± 2929	16078 ± 5040	16163 ± 3328	0.708
	CD11c (%)	99.8 ± 0.0	99.9 ± 0.1	99.8 ± 0.6	99.9 ± 0.1	0.300
	CD11c (MFI)	96412 ± 17058	96512 ± 14409	92731 ± 16822	97328 ± 20458	0.526
	CD41 (%)	3.0 ± 1.3	2.7 ± 1.0	2.9 ± 1.3	3.0 ± 1.2	0.359
	CD41 (MFI)	113286 ± 23771	119959 ± 22348	113365 ± 19131	125272 ± 15924	0.467

Table S8: Continued

		Pre placebo (mean ± SD)	Post placebo (mean ± SD)	Pre colchicine (mean ± SD)	Post colchicine (mean ± SD)	p-value
Non classical monocytes (NCM)	**NCM (%)**	6.5 ± 3.5	6.2 ± 2.8	6.7 ± 2.9	6.2 ± 1.8	0.694
	HLA-DR (MFI)	17179 ± 5110	18985 ± 5628	18247 ± 6115	17214 ± 4359	0.043*
	CCR2 (%)	0.7 ± 0.4	1.0 ± 1.2	1.1 ± 1.3	0.9 ± 0.6	0.213
	CCR2 (MFI)	4228 ± 2475	5015 ± 3245	4599 ± 1390	4074 ± 1464	0.228
	CCR5 (%)	6.3 ± 2.7	7.0 ± 3.6	7.7 ± 4.3	6.2 ± 2.8	0.069
	CCR5 (MFI)	1272 ± 205	1338 ± 290	1468 ± 592	1467 ± 295	0.598
	CD11b (%)	58.6 ± 18.4	55.0 ± 11.3	62.5 ± 15.4	54.0 ± 10.8	0.188
	CD11b (MFI)	4824 ± 1625	5164 ± 1306	4859 ± 1958	5291 ± 1348	0.791
	CD11c (%)	99.3 ± 0.8	99.2 ± 0.7	99.4 ± 0.5	99.2 ± 1.2	0.757
	CD11c (MFI)	90221 ± 15721	93545 ± 12415	91206 ± 14205	97753 ± 15071	0.508
	CD41 (%)	2.9 ± 1.2	2.6 ± 1.1	2.9 ± 1.2	3.1 ± 1.3	0.193
	CD41 (MFI)	117873 ± 20500	123628 ± 19593	116846 ± 14988	124873 ± 16080	0.732

Data are presented as mean ± SD. For each measurement, the change from baseline score (delta) within each period (i.e. post placebo – pre placebo and post colchicine – pre colchicine) was calculated and used as the outcome measure. Differences in the deltas between the two treatment options were analyzed using a mixed-effects linear regression with restricted maximum likelihood. In this model, period and treatment were specified as fixed effects, and participants were specified as a random effect. *P-value <0.05 was considered significant.

Abbreviations: CCR2 = C-C chemokine receptor type 2; CCR5 = C-C chemokine receptor type 5; CD = cluster of differentiation; HLA-DR = Human Leukocyte Antigen – DR isotype; MFI = median fluorescent intensity.

Table S9: Cytokine production of PBMCs upon stimulation with various stimuli.

Cytokine	Stimuli	Pre placebo (mean ± SD)	Post placebo (mean ± SD)	Pre colchicine (mean ± SD)	Post colchicine (mean ± SD)	p-value
TNF (pg/ml)	**RPMI**	39 ± 0	39 ± 0	39 ± 1	39 ± 0	N/A
	LPS	845 ± 682	712 ± 518	917 ± 751	604 ± 350	0.36
	MSU	82 ± 75	50 ± 30	77 ± 93	92 ± 103	0.005*
	LPS+MSU	984 ± 764	898 ± 652	1152 ± 869	824 ± 553	0.26
	Pam3Cys	907 ± 611	855 ± 562	947 ± 524	888 ± 493	0.89
	Nigericin	204 ± 284	172 ± 205	226 ± 326	224 ± 243	0.74
IL-1β (pg/ml)	**RPMI**	39 ± 0	41 ± 8	39 ± 2	39 ± 0	N/A
	LPS	5609 ± 2948	5944 ± 2794	6724 ± 4282	5060 ± 1826	0.09
	MSU	122 ± 149	66 ± 74	114 ± 154	171 ± 257	0.001*
	LPS+MSU	5741 ± 2428	5998 ± 1833	7124 ± 4476	5587 ± 1585	0.09
	Pam3Cys	4750 ± 1955	4618 ± 1631	4764 ± 1573	4430 ± 1482	0.54
	Nigericin	626 ± 1071	338 ± 514	456 ± 777	519 ± 768	0.24
IL-6 (pg/ml)	**RPMI**	46 ± 0	61 ± 46	48 ± 6	46 ± 0	N/A
	LPS	11108 ± 4688	14684 ± 4713	12243 ± 4389	13515 ± 3885	0.12
	MSU	489 ± 583	398 ± 464	438 ± 524	476 ± 685	0.62
	LPS+MSU	15320 ± 5734	17306 ± 4306	14892 ± 4068	15878 ± 5326	0.53
	Pam3Cys	18763 ± 8003	18844 ± 7199	20376 ± 7403	18641 ± 6704	0.17
	Nigericin	770 ± 949	733 ± 1042	603 ± 745	662 ± 1189	0.80

Table S9: Continued

Cytokine	Stimuli	Pre placebo (mean ± SD)	Post placebo (mean ± SD)	Pre colchicine (mean ± SD)	Post colchicine (mean ± SD)	p-value
IL-1Ra (pg/ml)	**RPMI**	11299 ± 7587	10735 ± 7201	12979 ± 8227	9394 ± 4190	0.10
	LPS	24124 ± 9127	25061 ± 10543	25747 ± 10003	23928 ± 8402	0.12
	MSU	11213 ± 7116	9752 ± 5527	11696 ± 6976	9279 ± 5471	0.47
	LPS+MSU	24551 ± 9360	24739 ± 9949	25584 ± 11060	22805 ± 7840	0.10
	Pam3Cys	22670 ± 12226	20997 ± 8068	20777 ± 7791	20955 ± 8337	0.52
	Nigericin	957 ± 494	1016 ± 619	944 ± 382	836 ± 242	0.25
IL-10 (pg/ml)	**RPMI**	35 ± 0	35 ± 0	35 ± 0	35 ± 0	N/A
	LPS	762 ± 514	751 ± 477	792 ± 781	704 ± 506	0.44
	MSU	39 ± 9	36 ± 3	36 ± 6	38 ± 8	0.009*
	LPS+MSU	973 ± 580	943 ± 581	911 ± 793	837 ± 601	0.71
	Pam3Cys	271 ± 159	260 ± 146	268 ± 183	250 ± 185	0.79
	Nigericin	36 ± 3	36 ± 3	35 ± 2	35 ± 3	0.41

Data are presented as mean ± SD. *P-value <0.05 was considered significant.

Abbreviations: TNF = tumor necrosis factor; IL-1β = interleukin-1 beta; IL1-Ra = interleukin-1 receptor antagonist protein; IL-6 = interleukin-6; IL-10 = interleukin-10; RPMI = Roswell Park Memorial Institute culture medium; LPS = lipopolysaccharide; MSU = Monosodium urate (MSU) crystals; Pam3Cys = Pam3CYSK4.

Table S10: Flow cytometry markers.

Antibodies	Fluorochrome	Clone	Company	Identifyer
Anti-human CD16	FITC	3G8	Biolegend	Cat# 302006 RRID AB_314206
Anti-human HLA-DR	PE	immu-357	Beckman Coulter	Cat# IM1639U RRID AB_2876782
Anti-human CD62L	PEDazzle584	DREG-56	Biolegend	Cat# 304842 RRID AB_2565874
Anti-human CD49d	PECy5.5	9F10	Biolegend	Cat# 304312 RRID AB_10641699
Anti-human CD10	PC7	HI10a	Biolegend	Cat# 312213 RRID AB_2146549
Anti-human lineage cocktail (CD3, CD14, CD19, CD20, CD56)	APC	UCHT1; HCD14; HIB19; 2H7; HCD56	Biolegend	Cat# 348703 RRID: N/A
Anti-human CD66b	APC-700	G10F5	Biolegend	Cat# 305114 RRID AB_2566038
Anti-human CD15	APC-Cy7	MEM-166	Biolegend	Cat# 323047 RRID AB_2750189
Anti-human CD123	BV421	6H6	Biolegend	Cat# 306018 RRID AB_10962571
Anti-human CD45	BV510	HI30	Biolegend	Cat# 304036 RRID AB_2561940
Anti-human CD35	BV650	E11	BD Bioscience	Cat# 744277 RRID AB_2742115
Anti-human CD11b	BV785	ICRF44	Biolegend	Cat# 301346 RRID AB_2563794
Anti-human CD11c	PEDazzle584	BU15	Biolegend	AB_2564548 (BioLegend Cat. No. 337227)
Anti-human CD3	PC5.5	UCHT1	Biolegend	AB_314064 (BioLegend Cat. No. 300410)
Anti-human CD14	PC7	61D3	eBioscience	AB_1582276, cat25-0149
Anti-human CD56	APC	N901	Beckman Coulter	IM2474, RRID, AB_130791
Anti-human CD19	AF700	HIB19	Biolegend	Cat: 302226, AB_493751
Anti-human CD41	APC-Cy7	HIP8	Biolegend	Cat: 303716, AB_10897646
Anti-human CCR2	BV421	48607	BD Biosciences	Cat: 564067, RRID AB_2738573
Anti-human CCR5	BV650	3A9	BD Biosciences	Cat: 564999, RRID: AB_2739037
Brilliant stain buffer	-	-	BD Bioscience	Cat: 563794, RRID: N/A
Helix NP™ NIR	-	-	Biolegend	Cat# 425301, RRID, N/A

Table S11: ELISA assays.

Product	Product number	Manufacturer
Human IL-1β DuoSet ELISA	DY201	Bio-Techne/R&D
Human IL-1RA DuoSet ELISA	DY280	Bio-Techne/R&D
Human IL-6 DuoSet ELISA	DY206	Bio-Techne/R&D
Human IL-10 DuoSet ELISA	DY217B	Bio-Techne/R&D
Human TNF DuoSet ELISA	DY210	Bio-Techne/R&D
Human hsCRP ELISA	DY1707	Bio-Techne/R&D
Human IL-8 DuoSet ELISA	DY208	Bio-Techne/R&D
Human Neutrophil Elastase/ELA2 DuoSet ELISA	DY9167-05	Bio-Techne/R&D
Human S100A8/S100A9 Heterodimer DuoSet ELISA	DY8226-05	Bio-Techne/R&D
Human Myeloperoxidase DuoSet ELISA	DY3174	Bio-Techne/R&D
Human Lipocalin-2/NGAL DuoSet ELISA	DY1757	Bio-Techne/R&D

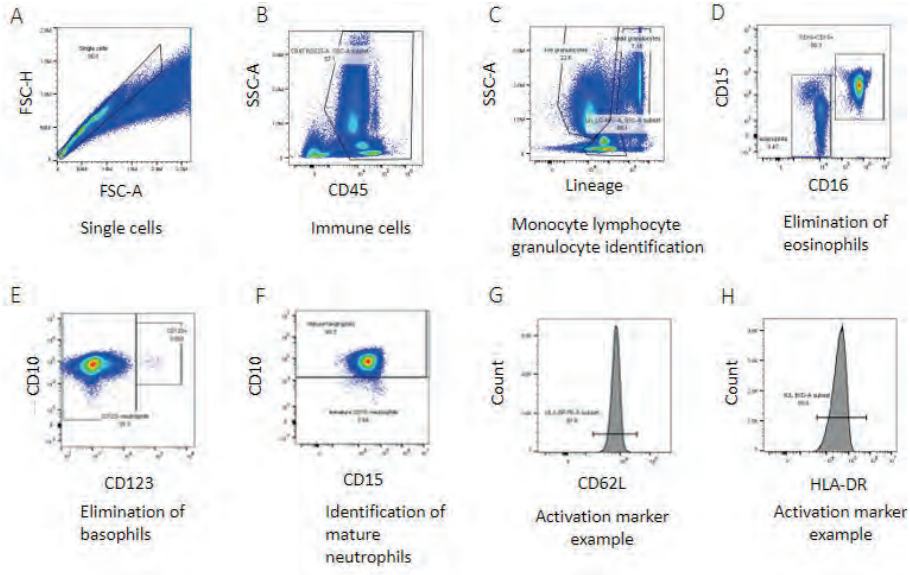


Figure S1: Gating strategy for defining neutrophil population using flow cytometry. Manual gating strategy for neutrophil sub-analysis: gating for **(A)** single cells and **(B)** CD45+. Then granulocytes were selected based FSC/SSC **(C)**. Eosinophils and basophils were eliminated based on low CD16 expression, and high CD123 expression respectively **(D-E)**. Mature neutrophils were determined based on CD10 expression. **(F)** Neutrophils were further analyzed for their median fluorescent intensity of activation markers HLA-DR **(G)** and CD62L **(H)**.

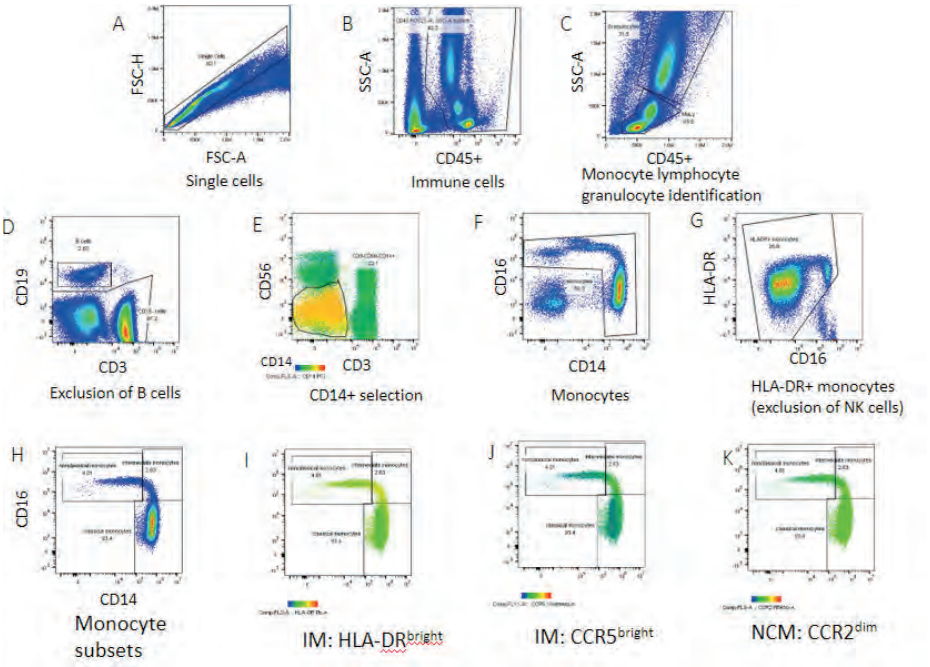


Figure S2: Gating strategy defining monocyte population using flow cytometry. Manual gating strategy for monocyte subsets: single cells **(A)** and CD45+ immune cells **(B)**. Gating for monocytes, lymphocytes and granulocytes based on forward and side scatter **(C)**. We excluded B-cells based on high expression of CD19 **(D)**. Thereafter, monocytes were identified based on the expression of CD14 and CD16 **(E-F)** HLA-DR+ monocytes were selected to eliminate NK cells **(G)**. Monocyte subsets (classical, intermediate and nonclassical) were determined based on CD14 and CD16 expression and the exact gates were put based on HLA-DR and CCR5 expression (highest on intermediate monocytes), and CCR2 expression (highest on nonclassical monocytes) **(H-K)**.

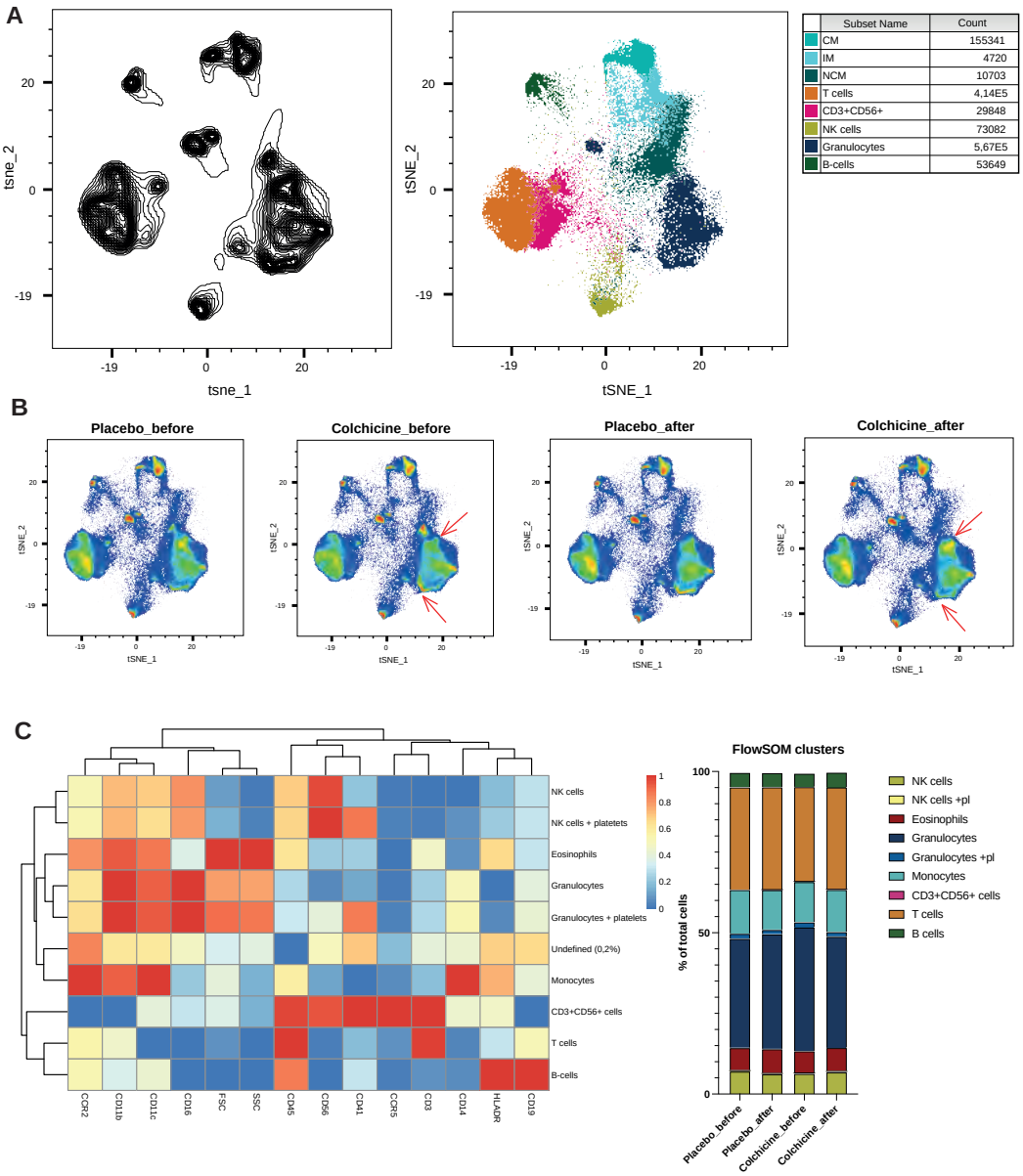
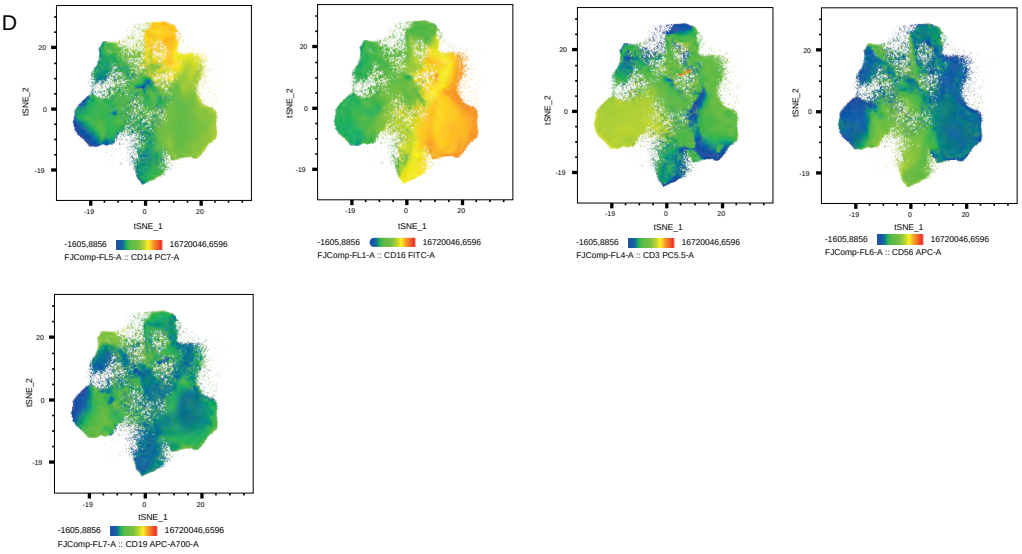
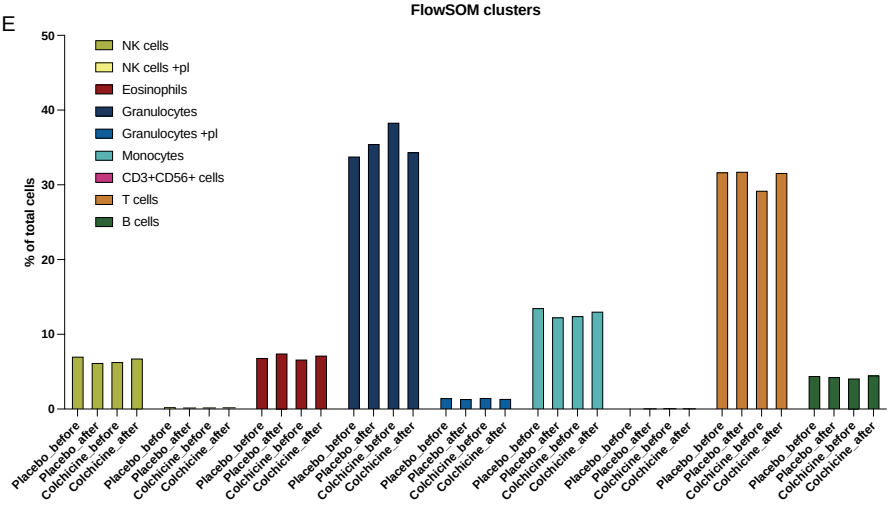


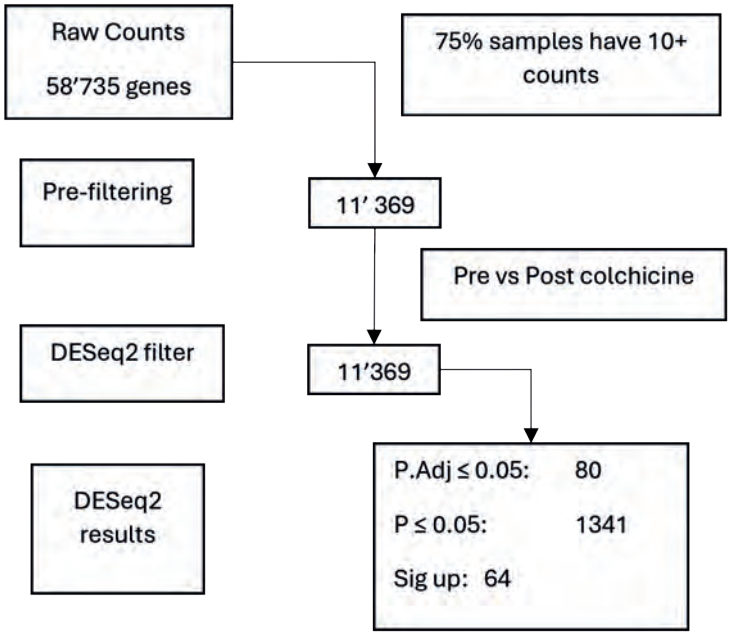
Figure S3: Unbiased analysis of the monocyte flow cytometry panel. **(A)** tSNE of all events, colored according to known cell types. **(B)** Marker expression of the most important cell-type identifying markers are shown (CD14, CD16, CD3, CD56 and CD19), confirming good separation of cell types in the tSNE plot. **(C)** tSNE separation of the 4 groups analyzed: before/after placebo and before/after colchicine. Arrows indicate changes induced by colchicine that need further confirmation using manual gating. **(D)** FlowSOM clustering of cells based on marker expression. All major cell populations can be identified. **(E)** Analysis of FlowSOM clusters for each group: before/after placebo and before/after colchicine. Changes in granulocyte% can be observed upon colchicine treatment.

D


tSNE_1
tSNE_2
-1605,8856
16720046,6596
FJComp-FL5-A :: CD14 PC7-A
FJComp-FL1-A :: CD16 FITC-A
FJComp-FL4-A :: CD3 PC5.5-A
FJComp-FL6-A :: CD56 APC-A
FJComp-FL7-A :: CD19 APC-A700-A

E


FlowSOM clusters
% of total cells
NK cells
NK cells +pl
Eosinophils
Granulocytes
Granulocytes +pl
Monocytes
CD3+CD56+ cells
T cells
B cells
Placebo_before
Placebo_after
Colchicine_before
Colchicine_after



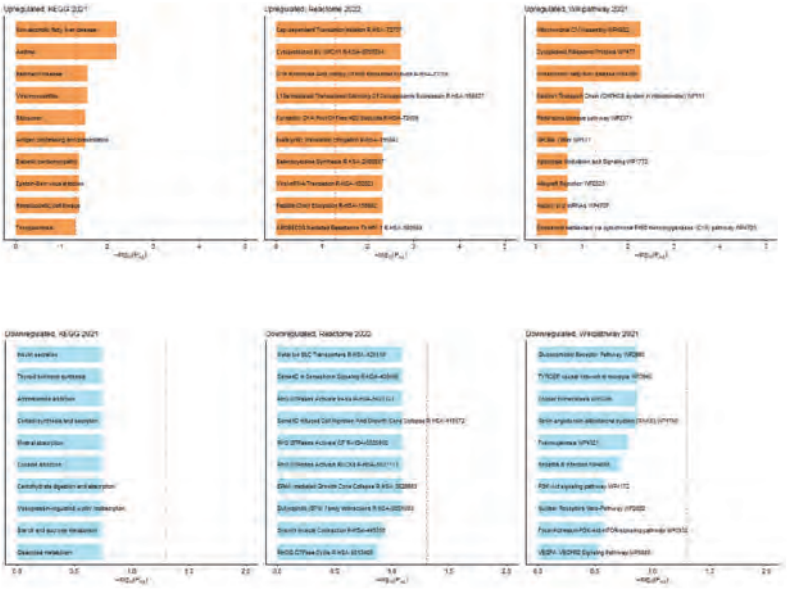
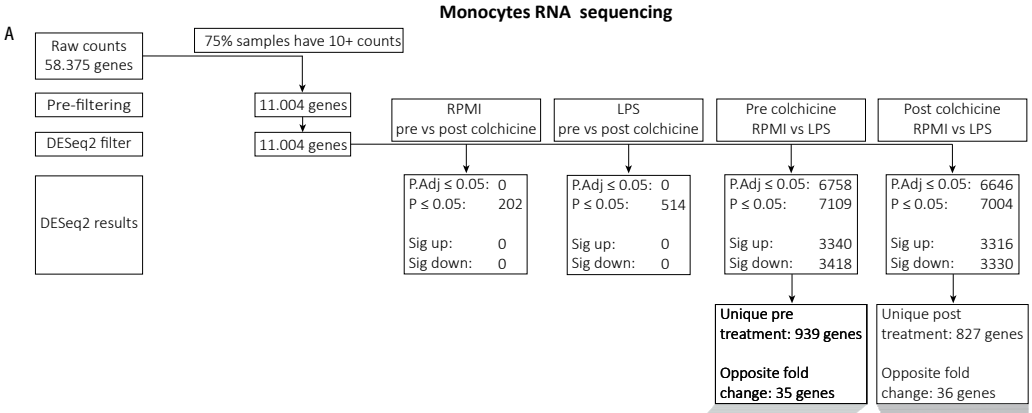


Figure S4: Neutrophil RNA expression upon colchicine treatment.



B

	Pre colchicine RPMI vs LPS		Post colchicine RPMI vs LPS	
gene_name	log2Fold Change	padj	log2Fold Change	padj
BTBD6	-1.44	0.003	0.02	0.980
BTBD3	-1.42	0.014	0.02	0.976
BICRAL	-1.26	0.002	0.02	0.976
ZNF623	-1.25	0.009	0.15	0.780
MOGS	-1.25	0.007	0.28	0.586
PHLPP2	-1.23	0.010	0.56	0.261
WDR47	-1.13	0.004	0.14	0.758
PPFIBP2	-1.07	0.004	0.03	0.954
SIPA1L3	-0.90	0.034	0.09	0.857
ANKS1A	-0.85	0.049	0.40	0.375
PGRMC2	-0.78	0.004	0.07	0.835
ZDHHC18	-0.66	0.046	0.28	0.429
AC073869.1	-0.62	0.005	0.33	0.150
SUGP2	-0.60	0.007	0.05	0.848
SPECC1	-0.56	0.028	0.08	0.777
GALNT1	-0.55	0.025	0.05	0.850
RNF111	-0.52	0.006	0.03	0.908
AAK1	-0.48	0.039	0.10	0.692
C5orf51	-0.36	0.006	0.20	0.153
HDLBP	-0.32	0.045	0.05	0.798
ATP6V1C1	0.34	0.028	-0.04	0.809
BECN1	0.42	0.017	-0.01	0.946
CAPZA2	0.42	0.008	-0.16	0.363
OS9	0.47	0.024	-0.20	0.380
CAP1	0.51	0.013	-0.07	0.772
GCA	0.56	0.042	-0.04	0.890
ACO2	0.62	0.041	-0.10	0.772
AC016727.1	0.66	0.016	-0.25	0.404
CNDP2	0.68	0.035	-0.27	0.429
TTC38	0.83	0.028	-0.47	0.242
OAT	0.85	0.034	-0.06	0.893
TMED2	0.87	0.019	0.00	0.998
PRMT5	0.90	0.000	-0.01	0.970
PEAR1	0.99	0.030	-0.05	0.925
RASL11A	1.53	0.002	-0.92	0.088

	Pre colchicine RPMI vs LPS		Post colchicine RPMI vs LPS	
gene_name	log2Fold Change	padj	log2Fold Change	padj
TCTE3	0.22	0.719	-1.20	0.036
FP671120.4	0.19	0.759	-1.18	0.037
ZNF486	0.18	0.743	-1.16	0.019
C5orf30	0.24	0.635	-1.12	0.017
PEX3	0.02	0.966	-1.07	0.008
SERPING1	0.49	0.360	-1.04	0.042
AC010969.2	0.11	0.829	-0.97	0.041
PPP3CB-AS1	0.02	0.962	-0.94	0.003
TK1	0.31	0.526	-0.92	0.048
MICALL2	0.12	0.700	-0.90	0.002
OXCT1	0.08	0.851	-0.87	0.025
CLYBL	0.24	0.618	-0.87	0.047
ZNF84	0.01	0.978	-0.86	0.044
AL035071.1	0.20	0.548	-0.78	0.012
ATP6AP2	0.03	0.916	-0.67	0.010
MTA2	0.17	0.630	-0.64	0.049
COASY	0.16	0.434	-0.57	0.002
PADI4	0.03	0.929	-0.55	0.045
MICU2	0.02	0.919	-0.47	0.021
TBP	0.19	0.352	-0.44	0.030
PGS1	0.03	0.884	-0.36	0.032
GDI2	0.05	0.755	-0.30	0.043
NIPBL	-0.03	0.810	0.24	0.035
PPP4R1	-0.01	0.959	0.46	0.035
AP3B1	-0.01	0.963	0.54	0.003
TRIM28	-0.05	0.842	0.62	0.005
MMP24OS	-0.18	0.556	0.65	0.020
CLIP2	0.00	0.993	0.74	0.032
TET2-AS1	-0.22	0.579	0.78	0.029
ZNF83	-0.18	0.674	0.81	0.036
AC009951.1	-0.03	0.921	0.82	0.004
PILRB	-0.09	0.826	0.86	0.019
ZNF787	-0.09	0.845	0.91	0.021
PABPC1L	-0.01	0.987	1.08	0.005
TPRG1	-0.02	0.970	1.19	0.011
DCDC2B	-0.43	0.513	1.58	0.007

Figure S5: Transcriptional response to colchicine treatment on monocytes before and after 4 hours RPMI or LPS stimulation. **(A)** Overview of gene filtering and significant genes per group. **(B)** Uniquely expressed genes pre- and post colchicine treatment. Shown are significant genes with opposite fold change (blue indicates negative fold change, yellow indicates positive foldchange) before and after colchicine treatment. P-values are adjusted for multiple testing (FDR 0.05). P-values of less than 0.05 (2-tailed) were considered statistically significant.

Supplementary Methods

Flow cytometry

For flow cytometric analysis, whole blood from EDTA tubes was used. Red blood cells were lysed by BD Pharm Lyse buffer treatment for 15 minutes at room temperature (RT) in the dark. After washing with FACS buffer (1% BSA in PBS with 2mM EDTA) the cells were resuspended in 100 ul of FACS Buffer and incubated with 10 ul Human TruStain FcX (Biolegend, San Diego, CA, USA) Fc block for 10 minutes. Cells were stained with the following two panels. For monocyte analysis, cells were stained using the following anti-human fluorochrome-conjugated antibodies: CD45, CD3, CD19, CD56, CD14, CD16, HLA-DR, CD11b, CD11c, CD41, CCR2, CCR5. For neutrophil analysis, the following anti-human fluorochrome-conjugated antibodies were used: CD45, a lineage cocktail containing CD3, CD56, CD19, CD20 and CD14, CD123, CD15, CD16, CD35, HLA-DR, CD62L, CD49d, CD10 and CD11b. 50ul of cells were incubated with 50ul of antibody mix for 30 min at RT in the dark. Subsequently, the cells were washed and resuspended in FACS buffer. Helix NiR viability dye was added to the neutrophil panel for 15 min at RT in the dark. Cell populations and expression of markers were measured using a CytoFlex cytometer (Beckman Coulter, Brea, USA) which underwent daily quality control to correct for variation in laser settings. Flow Minus One controls were assessed for all markers for which MFI was assessed to set the correct markers. For a full overview of used antibodies see **Table S10**.

Manual gating of the neutrophil panel was performed by removing doublets and debris and selecting for live granulocytes among CD45+ cells. Then, neutrophils were identified based on CD15 and CD16 surface expression. After excluding eosinophils and basophils, we characterized the maturation and activation level of neutrophils by CD49d, CD10, CD66b, HLA-DR, CD16 and CD62L **(Figure S1)**.

Manual gating of the monocyte panel was performed by selecting for single cells and CD45+ immune cells, B cells, T cells and NK cells were removed based on the expression of CD19, CD3 and CD56. CD19-CD3-CD56- cells were used for monocyte gating; monocytes were identified based on the expression of CD14 and CD16 and HLA-DR. Monocyte subsets (classical, intermediate and nonclassical) were determined based on CD14 and CD16 expression and the exact gates were put based on HLA-DR and CCR5 expression (highest

on intermediate monocytes), and CCR2 expression (highest on nonclassical monocytes) (**Figure S2**).

For unsupervised analysis of the monocyte panel, all debris and doublets were removed, after which the CD45+ cells were selected in all FCS files. Subsequently, all the preprocessed files were grouped with keywords for their timepoint and treatment, down sampled to 20,000 cells per file and concatenated into one file for unsupervised analysis. This resulted in a file with a total of 1,400,000 events. Subsequently, FlowSOM and tSNE were conducted on the concatenated file with all compensated values, using the FlowJo FlowSOM and EmbedSOM tsne-plugins. FlowSOM was performed with 12 meta clusters predefined. tSNE was performed following the manufacturer's instructions and the recommended settings. All immune cell types were separated successfully (confirmed by manually gating known immune cell types as described above and layering on top of the tSNE plot) and the density plots corresponding to markers are shown in **Figure S3.**

Immune cell isolation

PBMC isolation

Blood was diluted in PBS (phosphate buffered saline, Gibco) and subsequently peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque PLUS (Cytiva) density gradient centrifugation for 30 minutes at 615g (no brakes, RT). The PBMCs with plasma were collected and washed with PBS containing 0.1% human pooled serum and 1 mM UltraPure EDTA (0.5 M, pH 8, Life Technologies) at 190g for 15min, RT, to separate the platelet rich plasma (PRP) from the PBMCs . The PBMCs were then washed two times with cold PBS and resuspended in RPMI 1640 Dutch-modified culture medium supplemented with 1 mM pyruvate (Invitrogen), 2 mM glutamine (Invitrogen), 50 µg/mL gentamicin (Centrafarm).

The PBMC composition was assessed by the Sysmex- XN 450 hematology analyzer.

Platelet rich plasma (PRP) collection and isolation of platelets

The PRP was collected from the plasma supernatant after Ficoll gradient. PRP was diluted in PBS containing 0.1% human pooled serum and 1 mM UltraPure EDTA (0.5 M, pH 8, Life Technologies) and centrifuged at 190g for 15min. The supernatant containing PRP was collected and centrifuged at 2500g, 5 minutes, 4°C and gently resuspended in previously described RPMI

1640 Dutch-modified culture medium. Platelet count was assessed by the Sysmex – XN 450 hematology analyzer.

Monocyte isolation

Monocytes were isolated from PBMC fraction by MACS pan-monocyte isolation kit (Miltenyi Biotec) according to manufacturer's protocol. The purity and count were assessed by the Sysmex- XN 450 hematology analyzer.

Polymorphonuclear cell isolation

After collecting the PBMC fraction, the neutrophil isolation was performed by hypotonic lysis. In short, leftover cells (peripheral polymorphonuclear neutrophils (PMNs) and erythrocytes) were incubated with hypotonic lysis buffer (155 mM NH4Cl, 10 mM KHCO3) twice for 15 and 10 minutes on ice. Afterwards, PMNs were washed twice in PBS and resuspended in RPMI 1640 medium without phenol red (Gibco, 32404014) supplemented with 50µg/mL gentamicin (Centrafarm), 2mM glutamax (Gibco), and 1mM pyruvate (Gibco). Neutrophils were counted with Sysmex Hematoanalyzer and brought to 5×10^6cells/ml concentration for downstream applications.

Stimulation assays

Whole blood stimulation

Whole blood was stimulated for 24 hours in flat bottom 48-well plates (Corning) with RPMI (Roswell Park Memorial Institute, Life Technologies/ Invitrogen) 1640 Dutch-modified culture medium. *Escherichia coli*-derived lipopolysaccharide (LPS) 10ng/mL purified as previously described[1] or phytohemagglutinin (PHA) 10ug/ml. After 24 hours, the plates were centrifuged for 8 minutes at 350g, and the supernatants were collected and stored at –80°C until measurement.

PBMC stimulation

500.000 PBMCs per well were stimulated for 24 hours in duplicate in round-bottom 96-well plates (Corning, NY, USA) with RPMI, *Escherichia coli*-derived lipopolysaccharide (LPS) 10 ng/mL purified as previously described[15], Monosodium urate (MSU) crystals (300ug/ml), Pam3CYSK4 (Pam3Cys) (L2000, EMC Microcollections) 10 µg/mL, Ethanol (dissolving agent for Nigericin), Nigericin (tlrl-nig, Bioconnect, 10 mg). After 24 hours, the plates were centrifuged for 8 minutes at 350g, and the supernatants were stored at –80°C until measurement.

Neutrophil stimulation

500.000 neutrophils per well were stimulated in duplicate in flat bottom 96-well plates (Corning, NY, USA) for 4 hours with culture medium only (as control), LPS (1 µg/ml), MSU (300 µg/ml), LPS and MSU (1 µg/ml and 300 µg/ml respectively), Pam3Cys (10 µg/ml), Nigericin (1 µM), Ethanol (dissolving agent for Nigericin) and Phorbol-12-myristate-13-acetate (PMA, Sigma) (50 nM) at 37°C with 5% CO_2. After 4 hours, the plates were centrifuged for 8 minutes at 350g, and the supernatants were stored at -80°C until measurement.

Monocyte stimulation

500.000 monocytes were stimulated in round bottom tubes (corning 352063, Fisher Scientific) with RPMI or LPS (10ng/ml) for 4 hours at 37°C with 5% CO_2. After 4 hours, the cells were pelleted at 3420g, 4°C. The cells were lysed in 500µl TRIzol reagent (ThermoFisher) and stored at -80°C until RNA isolation.

Circulating plasma protein and cytokine concentration measurement

Circulating plasma protein concentration (hCRP, S100a8/9, NE, NGAL, MPO) or secreted cytokines (IL-1Ra, IL-1β, IL-6, IL-8, IL-10, TNF, S100a8/9, NE, NGAL, MPO) were measured using DuoSet ELISA kits (Bio-Techne/ R&D Systems) following the manufacturer's instructions. For identifiers of ELISA kits, see **Table S2**.

ROS assay

Neutrophil-derived ROS production was determined by a luminol (5-amino-2,3, dihydro-1,4-phtalazinedione)-enhanced luminescence assay. 2×10^5 neutrophils per well were added to opaque flat-bottom 96-well plates (Corning, NY, USA). Neutrophils were stimulated in quadruplicate with serum-opsonized zymosan, PMA, and culture medium as control. Chemiluminescence was measured at 142s intervals for 1 hour at 37°C in a BioTek Synergy HTreader. The integral of relative luminescence units per second (RLU/sec) was measured.

NETosis assays

NOX-dependent NET formation

200.000 neutrophils per well were added to flat-bottom 96-well plates (Corning, NY, USA) at 37°C for 20 min. After attachment of neutrophils, the supernatants were removed. The cells were stimulated in quadruplicate with Nigericin (1 µM), Ethanol, PMA (50 µM) or culture medium as control for 3 hours at 37°C, 5% CO_2.

NOX-independent NET formation

$1x10^8$ platelets were either kept unstimulated or activated with 156uM Thrombin Receptor Activator Peptide 6 (TRAP6, Sigma) for 30 min at 37°C, 5% CO_2 in round bottom non-stick tubes (corning 352063, Fisher Scientific). In parallel, 200.000 neutrophils per well were attached to flat-bottom 96-well plates (Corning, NY, USA) at 37°C for 20 min. The neutrophils were stimulated in quadruplicate with unstimulated platelets, TRAP6-stimulated platelets or culture medium as control for 1 hour at 37°C, 5% CO_2.

After both assays, the neutrophils were washed twice with warm PBS and NETs were isolated by partial digestion in culture medium supplemented with 5 U/ml micrococcal nuclease (MNase, Worthington biochemical corporation) 20 min at 37°C, 5% CO2. MNase was inactivated by brief vortexing, and cells were pelleted by centrifugation. The supernatant containing partially digested NETs were kept at -80°C until measurement.

Measurement of DNA concentration in NETs with Sytox Orange

The DNA concentrations in the NET formation assay MNase-treated supernatants (NOX-dependent and NOX-independent formation assay) were quantified by adding 5 mM Sytox Orange Nucleic Acid Stain (Life Technologies) solution to undiluted sample. Fluorescence was measured with excitation and emission of 530/560nm using the BioTek Synergy HT multi-reader. All the measurements were done in duplicate.

RNA isolation and bulk RNA sequencing

Bulk RNA sequencing was performed on monocytes and neutrophils in a subset of 8 randomly selected patients before and after colchicine treatment period. RNA samples were isolated from 500.000 monocytes that were either stimulated with RPMI or LPS as described and from 500.000 unstimulated neutrophils.

RNA was extracted by hybrid TRIzol/RNeasy protocol. Per 1 mL of TRIzol 200 µL chloroform was added, mixed thoroughly, and incubated for 5 min at room temperature. After centrifugation for 15 min at 12000 g at 4°C, the upper aqueous phase was transferred to a RNAse free Eppendorf tube and mixed with an equal volume of 70% ethanol. The sample was loaded onto RNeasy mini(monocyte) or micro(neutrophil) columns (Qiagen, Hilden, Germany) and further processed according to the manufacturer's instructions. 30 µL of RNase free water was added to the columns to elute the RNA, incubated for 1 min

and spun down at max speed. RNA concentration and purity were measured with Nanodrop.

Isolated RNA from monocytes and neutrophils was sequenced by the Beijing Genomics Institute. Low-quality filtering and adapter trimming were performed using Trim Galore! v0.4.5 (Babraham Bioinformatics). Reads were mapped to the human reference genome (GRCh38.95, Ensembl) with Star v2.7.5a,[15] resulting in BAM files. BAM files were counted with HTSeq [HTSeq-count tool v0.11.0][16] with default parameters using the complementary gtf file, containing annotation for GRCh38.95 (Ensembl). Prefiltering of the data was performed, requiring ≥10 counts in 75% of samples, followed by data normalization using DESeq2's median of ratios.[17] Differential expression (DE) analysis was then performed using DESeq2. The threshold of FDR adjusted p-value ≤ 0.05 was applied in declaring significant DE genes (DEGs).

References

1. Hirschfeld, M., et al., *Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2.* J Immunol, 2000. **165**(2): p. 618-22.

Chapter 7:

Summary

Cardiovascular disease is the leading cause of death worldwide and is anticipated to hold its position in the coming decade. Atherosclerotic cardiovascular disease (ASCVD) denotes diseases caused by atherosclerosis, such as coronary artery disease, stroke, and peripheral artery disease. Atherosclerosis has formerly been classified solely as a lipid storage disease. However, this lipid centric view has been challenged by substantial evidence that proves inflammation as an additional causal mediator in all stages of disease pathophysiology.

The classical risk factors for atherosclerosis include dyslipidemia, hypertension, diabetes, obesity, and smoking. These risk factors shape the innate immune cells towards a proinflammatory phenotype. In recent years two immunological mechanisms that also mediate the proinflammatory rewiring of the innate immune cells have been described, namely clonal hematopoiesis and trained immunity. In this thesis, I studied these two novel mechanisms in the context of atherosclerosis associated inflammation, and how they can interact. Additionally, I studied modulation of inflammation by an anti-inflammatory therapy in a randomized double-blinded trial with a crossover design.

Monocytes and neutrophils are the innate immune cells that play major roles in the induction of inflammation during atherosclerosis pathophysiology. In this thesis I focused on these two cell types and investigated the mechanisms that shape their phenotype and function. Monocyte derived macrophages are the most common cell type found in the atherosclerotic plaque, playing key roles during various phases of the disease. In the early phases of the atherosclerotic plaque formation endothelial lining becomes more amenable to immune cell infiltration by increased adhesion molecule expression. Macrophages take up oxidized lipoproteins and become foam cells. In addition, plaque macrophages can produce a plethora of proinflammatory cytokines and chemokines such as IL-1β, IL-6, TNF, IL-8 and MCP-1.

Neutrophils have long thought to be bystanders to atherosclerosis and neglected due to their limited life span. However, we now know that they are heavily involved in vascular inflammation and tissue damage. These phagocytes are characterized bygranules packed with cytotoxic and antimicrobial proteins. And crucially, neutrophils can undergo a specific form of cell death, called NETosis, during which they expel their DNA content together with histones and proteases. These properties of neutrophils recruit even more immune cells

and induce tissue damage which could lead to rupture or superficial erosion of the atherosclerotic plaque.

Circulating immune cells, including monocytes and neutrophils are produced in the bone marrow from their corresponding progenitors. During this proliferative process, it is possible that a somatic mutation arises. Clonal hematopoiesis refers to accumulation of somatic mutations in the hematopoietic stem and progenitor cells (HSPCs) that lead to clonal expansion of the selected mutant cell. This can then be traced in the progeny of the HSPCs, the immune cells. Clonal hematopoiesis driver mutations (CHDMs) are predominantly identified in a known set of genes, including DNMT3A, TET2, ASXL1 and JAK2. Variant allele frequency (VAF) is measure of the size of the clone. Clonal hematopoiesis of indeterminate potential (CHIP) is defined as presence of clones with VAF>2%, without a hematological malignancy. The presence of CHIP has been associated with increased risk for major adverse cardiovascular events (MACE), however, the exact mechanism of this association is not fully understood. Preclinical studies demonstrated that plaque macrophages with TET2 mutations are producers of IL-1β, causally linking clonal hematopoiesis to atherosclerosis. However, only a few studies in humans have investigated how presence of CHDMs affect leukocyte phenotype.

Firstly, in **Chapter 3**, we investigated the association between the presence of clonal hematopoiesis and a first MACE in a cohort of patients with stable coronary artery disease. We identified CHDMs using a targeted ultrasensitive smMIP sequencing approach which enabled us to study clones of small sizes (VAF<2%) as well as larger clones (VAF≥2%). We showed that the presence of CHDMs is associated with a first ischemic event and cardiovascular mortality. In fact, this association is valid for CHDMs with VAF >1.07%, below the threshold of 2% CHIP criteria. We did not observe changes in monocyte subsets or cytokine production capacity in individuals with CHDMs. But we identified higher levels of circulating tissue factor, matrilysin, and proteinase-activated receptor-1.

In **Chapter 4**, we investigated how clonal hematopoiesis affects immune cell function, systemic inflammation and vascular and metabolic parameters in a cohort of individuals with obesity, the "300 OB" cohort. In this cohort we show that the presence of CHDMs was associated with higher circulating leukocytes, neutrophils, and circulating IL-6, but with a lower *ex vivo* cytokine production capacity of PBMCs. While CHDM presence was not associated with metabolic

syndrome or insulin resistance, remarkably it was associated with lower presence of carotid atherosclerotic plaques. This initially counterintuitive observation led us to hypothesize that clonal hematopoiesis is not associated with formation of atherosclerotic plaque *per se*, but rather with destabilization of the plaque.

Trained immunity has recently been proposed as a new immunological mechanism that shapes leukocyte phenotypes, particularly that of the innate immune cells. Immunological memory has been traditionally attributed to the adaptive arm of the immune system. This dogma has been challenged by the evidence showing increased responsiveness of innate immune cells upon restimulation with a secondary stimulus that is unrelated to the initial stimulus. This hyperresponsive phenotype has been termed as trained immunity. In Chapter 2, I provide an extensive state-of-the art overview of this immunogical mechanism. IL-1β is a central cytokine involved in the induction of trained immunity. Therefore, one of our hypotheses is that clonal hematopoiesis and trained immunity could be interrelated mechanisms.

To investigate whether clonal hematopoiesis affects the susceptibility to build trained immunity, in **Chapter 5** we selected individuals with DNMT3A mutations and individuals without CHDMs to characterize the innate immune function and phenotype. Recapitulating our findings from the larger 300 OB cohort, we show lower *ex vivo* cytokine production capacity of PBMCs from individuals with DNMT3A mutations. And importantly, an increase in capacity to build trained immunity.

In addition to understanding the mechanisms involved in the induction of inflammation, we also studied regulation of inflammation by low dose colchicine treatment. This ancient drug, extracted from the *Colchicum Autumnale*, has long been used in the treatment of gout. Recent landmark trials (COLCOT and LoDoCo2) showed effectiveness of low dose colchicine (0.5 mg/day) in reducing MACE in patients with recent acute coronary syndrome, and stable coronary artery disease. A targeted proteomic approach performed in a subgroup of LoDoCo2 trial demonstrated that after 4 weeks of low dose colchicine treatment neutrophil degranulation related markers were decreased.

Therefore, lastly in **Chapter 6**, we focused on how treatment with low dose colchicine (0.5 mg/day), alters the phenotype of monocytes and neutrophils in a double-blind randomized placebo-controlled trial with a crossover design.

We showed that in individuals with a history of myocardial infarction, treatment with colchicine mostly affected the neutrophil phenotype and function, demonstrated by decreased surface CD62L expression and NGAL release upon MSU stimulation.

Overall, this thesis's contents expand the knowledge on the role of inflammation, and in particular innate immunity, in atherosclerosis. This can help to improve patient stratification for specific anti-inflammatory therapies. For example, knowing whether an individual carries a CHDM could play a role in deciding which personalized treatment to receive, the gene and the size of the CHDM could also factor in this decision.

Chapter 8:

General Discussion

Despite the availability of various therapy regimens, cardiovascular diseases (CVDs) remain the leading cause of death worldwide[1]. The main underlying cause of CVDs is atherosclerosis. Atherosclerosis has long been thought to be driven only by the accumulation of lipids in the arterial wall. It is now well established that the pathophysiology of atherosclerosis also involves chronic low-grade inflammation of the arterial wall[2]. As most treatment efforts for atherosclerotic cardiovascular disease (ASCVD) are aimed at reducing plasma lipids, there is still a need for adequately addressing this residual inflammatory risk[3]. It is therefore crucial to unveil the players in the inflammatory landscape of atherosclerosis.

This thesis investigates how two recently established immunological mechanisms -clonal hematopoiesis and trained immunity- shape the innate immune phenotype and function and how this relates to development of atherosclerosis and ASCVD. These studies were done in two cohorts that differ in terms of the developmental stages of atherosclerosis. The first cohort, the "300 Obese (OB)" consists of 297 individuals with an increased risk of developing atherosclerosis due to overweight and obesity. Approximately 50% of this cohort had a carotid plaque[4]. And the second cohort "200 Cluj" consisted of 218 patients with established coronary artery disease. The first cohort was recruited in Nijmegen and the second in Cluj-Napoca, Romania in the context of a collaborative EU project[5].

And lastly, I studied how a specific anti-inflammatory therapy (daily low-dose colchicine) affects the neutrophil and monocyte function. In this chapter, I will first scrutinize key findings of this thesis and place them in context with the existing literature. Thereafter, I will discuss the limitations of my studies. And lastly, I will reflect on future research perspectives.

CHDMs with VAF>1.07% is associated with major adverse cardiovascular events (MACE): time to revisit the CHIP cutoff?

The presence of Clonal Hematopoiesis of Indeterminate Potential (CHIP) is associated with an almost twofold increase in the risk of coronary heart disease[6]. In **Chapter 3**, we show for the first time that the presence of CHDMs is associated with a first ischemic event and cardiovascular mortality in a well-characterized cohort of patients with stable coronary artery disease (CAD). In fact, this association is valid for CHDMs with VAF >1.07%, below the threshold of 2% that is traditionally used to define CHIP[5]. Recent work by various groups corroborated our findings on the importance of smaller clones for various CVDs, even suggesting gene specific cutoffs[7–9].

The current consensus of 2% CHIP cutoff is largely based on the sequencing methodology. Most epidemiological studies on clonal hematopoiesis (CH) up to date used whole genome or exome sequencing (WGS, WES). While this is certainly a robust approach in terms of coverage, still most CHDMs identified fall into a list of approximately the same 30 genes. Thus, one could argue that a targeted gene panel might be sufficient for identification of CHDMs. A major limitation of WGS and WES technologies is that they are relatively insensitive to clones of a small size (and hence, most studies using this technique only report clones with a VAF of >2% or sometimes even 10%). Therefore, the intrinsic properties of the sequencing methodology determine the CHIP cutoff.

Single molecule molecular inversion probe (smMIP) is a cost-effective targeted sequencing technology which can be used to study a known set of genes with high coverage depth and minimal DNA quantity. The single-molecule probes increase the specificity by correcting for PCR artifacts[10].

With a targeted smMIP approach, we were able to identify small clones well below the CHIP criteria and demonstrated their clinical relevance, in terms of predicting MACE at an optimal cut-off value of 1.07%[5]. Consequently, we argue that the CHIP cutoff could be revised or applied in a gene specific manner. Specifically, we encourage future clinical studies on CH and ASCVD to use more sensitive assays and also include CHDMs with a VAF <2 % in the analyses.

Sources of inflammation in CHIP: a counterintuitive observation

Data from large clinical studies characterize individuals with CHIP with increased inflammation in circulation. In line with this, in **Chapter 4**, in a cohort of individuals with overweight or obesity we show presence of CHDMs is associated with higher circulating IL-6 and IL-1β concentrations. In contrast, the *ex vivo* cytokine production capacity of PBMCs is lower. This paradoxical observation can be explained by dissecting the cellular sources of inflammation. Immune cells are not only patrolling the circulation but also are residents of virtually all tissues. Tissue resident immune cells and even some non-immune cells can contribute to systemic inflammation. Furthermore, sources of circulating and tissue resident immune cells are distinct which could play a role in determining their function. Long-lived tissue resident macrophages are derived from yolk sac erythroid myeloid progenitors whereas HSCs can give rise to short and long-lived macrophages[11]. This “upbringing” of macrophages determines their phenotype and function.

Adipose tissue is also known to be a source of inflammation in obesity. In murine models of obesity, adipose tissue macrophages are shown to produce TLR4 and NLRP3 inflammasome dependent IL-1β, which in turn engages with the IL-1 receptor on bone marrow myeloid progenitors, further stimulating myelopoiesis[12]. We identified higher expression of TNF in the adipose tissue biopsies of individuals with CHDMs with VAF<2%, a hallmark of adipose tissue inflammation.[4] Therefore, it is plausible that the excessive adiposity in the individuals of the 300 OB cohort contributes to systemic inflammation, and the circulating immune cells are tolerized due to the exposure to chronic low-grade inflammation. Additionally, in a study by Abplanalp *et al.*, unstimulated monocytes from patients with heart failure and DNMT3A mutations showed a significantly increased expression of inflammatory genes compared to monocytes from patients with heart failure without DNMT3A mutations[13]. Thus, it is another possibility that the transcriptome of the unstimulated and stimulated monocytes differs.

Mosaicism of the immune cells: does one rotten apple spoil the barrel?

CH affects a relatively small proportion of immune cells. However, even in individuals with CHDMs with VAF<2% there is elevated systemic inflammation (**Chapter 4**), and small clones at a VAF>1.07% are associated with a first MACE (**Chapter 3**). This raises two possibilities, the first one being that the HSCs with CHDMs and their progeny can influence the rest of the bone marrow niche to produce proinflammatory cytokines. And secondly, even a small proportion of immune cells with CHDMs are enough increase systemic inflammation. In a recent conference abstract describing a single cell DNA/RNA parallel sequencing study on individuals with DNMT3A and TET2 mutations, intra-individual comparison of the mutant and non-mutant monocytes revealed identical transcriptome profiles. However, in the hematopoietic stem and progenitor cell (HSPC) compartment in the bone marrow, specifically the cells carrying the mutations showed upregulation of inflammatory cytokines such as *IL-1B* and *HMGB1* in contrast to the non-mutant counterparts. This finding highlights the potential of few mutant HSPCs being sufficient to influence the rest of the niche, leading to an overall inflammatory transcriptomic profile in the circulating monocytes[14]. In the study by Abplanalp *et al.*, as mentioned in the previous section, in patients with heart failure, transcriptional changes of monocyte corresponded to a larger percentage of cells than the VAF. Thus, it is possible that the wild type cells are activated by the mutant cells[13]. As noted in the section above ("Sources of inflammation in CHIP"), the source of the

immune cells could also contribute to the heterogeneous responses. Lastly, there could be innate differences between the phenotypes of monocytes and macrophages that might explain our contrasting findings of increased systemic inflammation vs *ex vivo* cytokine production. It is therefore highly relevant to perform single cell RNA sequencing studies to scrutinize the sources of this heterogeneity. In addition to performing such studies in circulating monocytes, it would be very informative to also include monocytes and macrophages from various tissues, for example the atherosclerotic plaque.

Chicken or the egg: did atherosclerosis come first?

While it is unequivocally proven that presence of CH is associated with ASCVD, the causal relationship between the two predominantly comes from mouse studies. Moreover, the directionality of this interaction remains a major question. In 2021, Heyde *et al.*, investigated whether atherosclerosis could be the driver of CH. The authors illustrated with a mathematical modelling that the atherosclerosis trait complex (interaction between hyperlipidemia, chronic low-grade inflammation and atherosclerotic plaque formation) could cause CH. This reverse causality is explained by the higher average HSC proliferation rate in atherosclerotic populations. Ultimately, the authors argue that this causes a vicious cycle during which atherosclerosis causes CH and CH in turn exacerbates further atherosclerosis development[15]. While this is an intriguing theory, it warrants further investigation in the form of longitudinal sampling to follow atherosclerosis and clone growth dynamics.

Obesity and metabolic syndrome are risk factors for developing atherosclerosis[16]. Our group and others have shown that obesity is associated with clonal hematopoiesis. By using the same targeted smMIP sequencing approach Andersson-Assarsson and van Deuren *et al.*, showed that clone size increased with age in individuals with obesity, but not in individuals who underwent bariatric surgery[17]. This finding could corroborate the reverse causality of atherosclerosis (or its risk factors) being the driving force for CH.

It is imperative to make a distinction between the effects of CH on the development of atherosclerosis and on established ASCVD (i.e. the incidence of MI and stroke). In the 300 OB cohort (**Chapter 4**) we show that CH was associated with lower presence of asymptomatic carotid atherosclerotic plaques, detected by ultrasound. Whereas in the 200 Cluj cohort (**Chapter 3**) with established CAD, CH was associated with increased MACE. Thus, we argue that CH does not drive atherosclerosis development *per se*, as this starts decades earlier

than the emergence of clones, but it mainly influences the stability of the atherosclerotic plaques, i.e. the tendency to erode or rupture, leading to an acute cardiovascular event.

However, in the 300 OB cohort we did not measure the presence of coronary plaques. Therefore, we cannot eliminate the possibility that presence of coronary plaques might be elevated in individuals with CHDMs.

Crossroads of trained immunity and clonal hematopoiesis

In addition to CH, trained immunity has also been described as a novel mechanism that drives persistent innate immune cell activation. Three recent studies show that trained immunity induced by intermittent high fat diet[18], hyperglycemia[19] and MI[20] can accelerate atherosclerosis. Given joint involvement of shared pathways such as IL-1β in trained immunity and CH, it is an attractive possibility to hypothesize that CH can also have an impact on trained immunity.

DNMT3A and TET2, the most frequent mutated genes in CH, are both epigenetic modifier enzymes with opposing roles. While TET2 is responsible for the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, the first step of DNA demethylation, DNMT3A regulates *de novo* methylation of cytosine residues[21]. As epigenetic modifications, along with metabolic reprogramming, are the orchestrators of trained immunity, it is intriguing to speculate that these enzymes could be intermediaries in the induction of trained immunity. KDM5 activity, an epigenetic modifier involved in trained immunity[22], is attenuated via a-ketoglutarate which is a substrate of TET2[23]. As TCA cycle metabolites are central to maintenance of trained immunity, the interaction of TET2 with α-ketoglutarate might point to its role in this process. It is noteworthy to emphasize that the central trained immunity is maintained at the level of bone marrow. Another shared mechanism of CH and trained immunity is the key role of IL-1β.

The role of IL-1β in trained immunity has been the topic of pivotal studies. Firstly, b-glucan administration in mice was associated with myelopoiesis in the bone marrow and this is mediated via enhanced IL-1β signaling[24]. Additionally, mice fed with a western diet for a short period of time, developed trained immunity in a NLRP3 inflammasome dependent manner. Particularly, training induced by oxLDL was shown to be influenced by several genetic polymorphisms in the putative regulatory region of *PYCARD*, the gene encoding

for the inflammasome adaptor protein ASC, and in the gene for the endogenous IL-1 inhibitory molecule IL-1RAP[25]. In mouse models of TET2 driven CH, IL-1β concentration was also shown to be elevated in white adipose tissue and causal to the age and obesity-related insulin resistance[26].

In **Chapter 5**, we show that while the *ex vivo* cytokine production capacity of PBMCs from individuals with DNMT3A mutations was lower, the capacity to build trained immunity was higher. Future studies are needed to investigate in detail the underlying molecular mechanisms responsible for this observation.

Diving deeper into the world of neutrophils

Plethora of evidence points to the predictive role of neutrophil counts in prognosis of CVDs[27]. As such, we are moving beyond the "bystander neutrophils" to "active player neutrophils" era. It has been shown that neutrophils are also amenable to trained immunity upon BCG vaccination, given the short lifespan of neutrophils this is hypothesized to happen at the level of bone marrow progenitors (i.e. Granulocyte Monocyte Progenitor)[28]. While this hyperresponsiveness can strengthen neutrophil-mediated host defense, it can also lead to chronic inflammation and tissue damage. In particular, mice fed a high-fat diet showed exacerbated atherosclerosis phenotype via an IL-1β-dependent neutrophil progenitor reprogramming. Thus, it is crucial to identify and target this maladaptive responsiveness in the case of cardiometabolic diseases.

Colchicine is one of the anti-inflammatory drugs that selectively accumulate in leukocytes, and bind to tubulin filaments, which is crucial to neutrophil function[29]. The findings from LoDoCo2 proteomic substudy supported this effect of colchicine on neutrophils by the reduced quantity of neutrophil granule associated proteins[30].

The changes we identified in neutrophil phenotype and function upon colchicine treatment pointed to less recruitment of neutrophils to the areas of inflammation (by reduced CD62L expression) and decreased inflammatory granule protein release (marked by lower NGAL). Remarkably, bulk RNAseq revealed reduced expression of KDM5 in neutrophils upon colchicine treatment. KDM5 was identified as a mediator of trained immunity in neutrophils. Therefore, colchicine treatment might have reversed the training of neutrophils induced by chronic low-grade inflammation due to CAD. It would be particularly interesting to investigate which group of individuals with a specific CHDM would benefit from colchicine treatment, potentially bringing the chapters of this thesis together.

Limitations of the studies in this thesis

Sequencing methodology used

The studies presented in this thesis have several limitations. The smMIP sequencing approach in the CH studies uses probes against well-known mutational hotspots in 23 driver genes, and the DNMT3A gene was entirely covered. This relatively cheap targeted approach allowed us to identify CHDMs in known hotspots with increased sensitivity. However, we cannot exclude the possibility that we missed a portion of mutations that fall outside of the sequenced regions. To estimate this, we compared our coverage of TET2 and ASXL1 genes to that of a recent and large UK Biobank study[31]. We filtered for mutational spots that have been observed ≥5 times in the UK Biobank data. Our probes covered 7 out of 108 (6.5%) mutational spots in TET2 and 11 out of 30 (37%) for ASXL1. This strong limitation stems from the fact that the initial smMIP design was based on the hotspots known at that time. Thus, our data on non-DNMT3A genes should be interpreted with caution. As mutations in DNMT3A are the most commonly identified in almost all cohorts and causally linked to atherosclerosis in a mouse model[32], we covered this gene entirely. This could have potentially led to a bias in identifying relatively higher portions of mutations in DNMT3A compared to other genes which were not entirely covered. It is therefore of utmost importance to update the probe design to cover more (if not all) mutational spots in the other well characterized CH-related genes for future studies.

Sample size and design of the cohorts

Cohorts in **Chapters 3** and **4**, 200 Cluj and 300 OB consist of 218 and 297 individuals respectively. While these are relatively large cohorts, when compared to the size of the large epidemiological studies on CHIP, the statistical power is limited. This is especially important when one wants to investigate each clonal hematopoiesis driver gene individually. We assessed the associations for the DNMT3A gene in both cohorts, but we did not have enough data points for other genes.

The participants in **Chapters 5** and **6** consisted of 32 and 19 individuals respectively. Due to the exploratory nature of both studies, we did not perform a formal sample size calculation. This prevented us from drawing robust conclusions with strong statistical power. However, we performed an in-depth phenotypical and functional characterization of the innate immune system. This could not have been possible in a short time frame for cohorts with larger sizes.

Sex distribution

In **Chapters 3**, **4**, and **5**, we strived for balanced distribution of men and women. However, in **Chapter 6**, Crystalo study this was not achieved, only 11% of the cohort was women. Hence, it would be of great importance to replicate our findings in a larger cohort with equal distribution of men and women.

Conclusions and future perspectives

The studies in this thesis provide several novel insights into understanding the role of inflammation in atherosclerosis. Importantly, in **Chapter 4**, we show that in a cohort of individuals with overweight and obesity presence of CHDMs was associated with lower carotid plaque presence. On the other hand, in patients with established CAD (**Chapter 3**), CHDMs with a VAF>1.07 were associated with a first MACE. Taken together, we hypothesize that CH could affect plaque destabilization rather than formation.

And while there is an increase in the concentration of inflammatory cytokines in the circulation, the *ex vivo* cytokine production capacity of PBMCs is decreased in individuals with CHDMs (**Chapter 4**). In a subgroup of DNMT3A mutation carriers the capacity of trained immunity of monocytes is increased (**Chapter 5**). And lastly in **Chapter 6**, we illustrate that treatment with low dose colchicine alters neutrophil phenotype.

Our findings pave the way for future research on CH with particular emphasis on the relevance of small clones. It would be of great importance to perform larger studies to be able to investigate CHDMs in each individual gene in more detail. Prospective studies with a longitudinal design to follow the trajectory of each clone would also provide essential insights. Presence of CH and clonal growth dynamics can be used in cardiovascular risk prediction models which could eventually lead to better patient stratification for specific anti-inflammatory therapies.

TET2 mutations are shown to grow exponentially with age, whereas DNMT3A mutations are relatively stable. Thus, one could propose differential cutoffs per gene with the age of the individual in mind. The fact that individuals with TET2 mutations benefit from canakinumab[33] treatment at a higher rate than individuals with mutations in other genes could stimulate other large clinical trials to perform a post hoc analysis to identify if the corresponding treatment

benefit a certain group of individuals. Therefore, the CH sub analysis of LoDoCo2 study is highly awaited.

Another point that can tie the concepts of trained immunity and inflammation in atherosclerosis would be to further elucidate the capacity of neutrophils to develop trained immunity. Our finding in **Chapter 6**, that KDM5 expression was decreased after low dose colchicine treatment hints to loss of training in the neutrophils. In **Chapter 5**, in the parameters we measured, we did not identify an enhanced responsiveness of neutrophils from individuals with DNMT3A mutations. In contrast, monocytes from those individuals were more amenable to be trained. Therefore, single cell RNA sequencing studies are critical in delineating the transcriptomic profile of monocytes with or without CHDMs. This would be relevant for monocytes in circulation but also in the atherosclerotic plaque[34]. And lastly, exact the molecular mechanisms of the induction of trained immunity in individuals with DNMT3A mutations need to be investigated. These open questions should be the topic of future studies.

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8

Chapter 9:

Nederlandse samenvatting

Hart- en vaatziekten zijn wereldwijd de meest voorkomende doodsoorzaak en men verwacht dat ze deze positie in het komende decennium zullen behouden. Onder de afkorting ASCVD (*Atherosclerotic Cardiovascular Diseases*, Atherosclerotische hart- en vaatziekten) vallen ziekten veroorzaakt door atherosclerose, zoals kransslagaderaandoeningen, beroertes en perifere vaatlijden. Atherosclerose werd vroeger uitsluitend geclassificeerd als een lipidenopslagziekte. Deze visie waarbij lipiden centraal staan, blijkt echter achterhaald door substantieel bewijs dat aantoont dat ontsteking ook een belangrijke rol speelt in alle stadia van de pathofysiologie van de ziekte.

De klassieke risicofactoren voor atherosclerose zijn dyslipidemie, hypertensie, diabetes, obesitas en roken. Deze factoren kunnen, naast een direct effect op de bloedvatwand ook de aangeboren immuuncellen prikkelen zodat ze een 'agressiever' en pro-inflammatoir fenotype krijgen. In de afgelopen jaren zijn twee immunologische mechanismen beschreven die kunnen bijdragen aan deze activatie van aangeboren immuuncellen, namelijk klonale hematopoëse en getrainde immuniteit. In dit proefschrift bestudeerde ik deze twee nieuwe mechanismen in de context van atherosclerose-geassocieerde ontsteking, en hoe ze elkaar beïnvloeden. Daarnaast bestudeerde ik het effect van een ontstekingsremmende therapie in een gerandomiseerde dubbelblinde crossover studie.

Monocyten en neutrofielen zijn de aangeboren immuuncellen die een belangrijke rol spelen bij het induceren van een ontsteking tijdens de pathofysiologie van atherosclerose. In dit proefschrift richtte ik me op deze twee celtypen en onderzocht ik de mechanismen die hun fenotype en functie bepalen. Macrofagen zijn het meest voorkomende celtype in de atherosclerotische plaque en deze komen grotendeels voort uit monocyten uit het bloed. Zij spelen een sleutelrol tijdens verschillende fasen van de ziekte. In de vroege fasen van de vorming van de atherosclerotische plaque wordt de endotheelbekleding vatbaarder voor infiltratie van immuuncellen door een verhoogde expressie van adhesiemoleculen. Macrofagen nemen geoxideerde lipoproteïnen op en worden schuimcellen. Daarnaast kunnen plaque-macrofagen een overvloed aan ontstekingsbevorderende cytokinen en chemokinen produceren, zoals IL-1β, IL-6, TNF, IL-8 en MCP-1.

Lange tijd werd gedacht dat neutrofielen geen belangrijke rol speelden in het ontstaan van atherosclerose, onder andere vanwege hun beperkte levensduur. We weten nu echter dat ze sterk betrokken zijn bij vasculaire ontsteking

en weefselschade. Deze fagocyten worden gekenmerkt door granules die vol zitten met cytotoxische en antimicrobiële eiwitten. En wat cruciaal is, neutrofielen kunnen een specifieke vorm van celdood ondergaan, NETosis genaamd, waarbij ze hun DNA samen met histonen en proteasen uitscheiden. Deze eigenschappen van neutrofielen rekruteren nog meer immuuncellen en veroorzaken weefselschade die kan leiden tot het scheuren van de vaatwand of oppervlakkige erosie van de atherosclerotische plaque.

Circulerende immuuncellen, waaronder monocyten en neutrofielen, worden in het beenmerg geproduceerd uit hun overeenkomstige progenitorcellen. Tijdens dit proliferatieve proces is het mogelijk dat een somatische mutatie ontstaat in deze hematopoietische stam- en progenitorcellen (HSPCs). Als deze mutatie een voordeel geeft in de proliferatie of overleving, ontstaat en een kloon van witte bloedcellen die voortkomen uit deze gemuteerde cel. Dit wordt klonale hematopoëse genoemd. Mutaties die leiden tot klonale hematopoëse (in het Engels *Clonal hematopoiesis driver mutations* (CHDM's) genoemd) worden voornamelijk geïdentificeerd in een bekende set genen, waaronder DNMT3A, TET2, ASXL1 en JAK2. De variante allelfrequentie (VAF) is een maat voor de grootte van de kloon. Klonale hematopoëse van onduidelijk potentieel (*Clonal hematopoiesis of indeterminate potential*; CHIP) wordt gedefinieerd als de aanwezigheid van klonen met VAF>2%, zonder hematologische maligniteit. De aanwezigheid van CHIP is in verband gebracht met een verhoogd risico op cardiovasculaire gebeurtenissen (*Major advserse cardiovascular events*; MACE), maar het exacte mechanisme van deze associatie wordt niet volledig begrepen. Preklinische studies toonden aan dat plaque-macrofagen met TET2-mutaties meer IL-1β produceren, waardoor er een oorzakelijk verband wordt gelegd tussen klonale hematopoëse en atherosclerose. Er zijn echter maar een paar studies die hebben onderzocht hoe de aanwezigheid van CHDM's het leukocytenfenotype beïnvloedt in mensen.

Ten eerste onderzochten we in **Hoofdstuk 3** de associatie tussen de aanwezigheid van klonale hematopoëse en een eerste MACE in een cohort van patiënten met stabiele coronaire hartziekte. We identificeerden CHDM's met behulp van een gerichte ultrasensitieve smMIP-sequencing aanpak waarmee we zowel kleine klonen (VAF<2%) als grotere klonen (VAF≥2%) konden bestuderen. We toonden aan dat de aanwezigheid van CHDM's geassocieerd is met een eerste cardiovasculaire gebeurtenis en cardiovasculaire mortaliteit. In feite geldt deze associatie voor CHDM's met VAF >1,07%, onder de drempel van 2% CHIP-criteria. We zagen geen veranderingen in monocytensubsets of

cytokineproductiecapaciteit van monocyten bij mensen met CHDM. Maar we identificeerden wel hogere niveaus van bepaalde eiwitten in het bloed, zoals circulerende weefselfactor, matrilysine en proteinase-activated receptor-1.

In **Hoofdstuk 4** onderzochten we hoe klonale hematopoëse de immuuncelfunctie, systemische ontsteking en vasculaire en metabole parameters beïnvloedt in een cohort van personen met obesitas, het "300 OB" cohort. In dit cohort laten we zien dat de aanwezigheid van CHDM's geassocieerd is met een hoger circulerend leukocyten-, en neutrofielen aantal en hogere concentraties van de ontstekingsstof IL-6, maar met een lagere *ex vivo* cytokineproductiecapaciteit van PBMC's. Terwijl de aanwezigheid van CHDM's niet geassocieerd was met het metabool syndroom of insulineresistentie, was het opmerkelijk genoeg wel geassocieerd met een verminderde aanwezigheid van atherosclerotische plaques in de halsslagader. Deze aanvankelijk contra-intuïtieve observatie leidde ons tot de hypothese dat klonale hematopoëse niet geassocieerd is met de vorming van atherosclerotische plaque op zich, maar eerder met destabilisatie van de plaque en dus met cardiovasculaire gebeurtenissen, zoals hartinfarct of herseninfarct.

Getrainde immuniteit (*trained immunity*) is onlangs voorgesteld als een ander nieuw immunologisch mechanisme dat het leukocytenfenotype kan beïnvloeden, in het bijzonder die van de aangeboren immuuncellen. Immunologisch geheugen werd traditioneel toegeschreven aan de adaptieve tak van het immuunsysteem. Ongeveer tien jaar geleden werd echter aangetoond dat ook aangeboren immuuncellen een soort geheugen kunnen opbouwen: deze cellen reageren namelijk sterker als ze een tweede keer worden gestimuleerd, in vergelijking met de eerste keer. Dit hyperresponsieve fenotype wordt getrainde immuniteit genoemd. In **Hoofdstuk 2** geef ik een uitgebreid overzicht van dit immunologische mechanisme. IL-1β is een centrale cytokine die betrokken is bij de inductie van getrainde immuniteit. Daarom is één van onze hypotheses dat klonale hematopoëse en getrainde immuniteit onderling gerelateerde mechanismen zouden kunnen zijn.

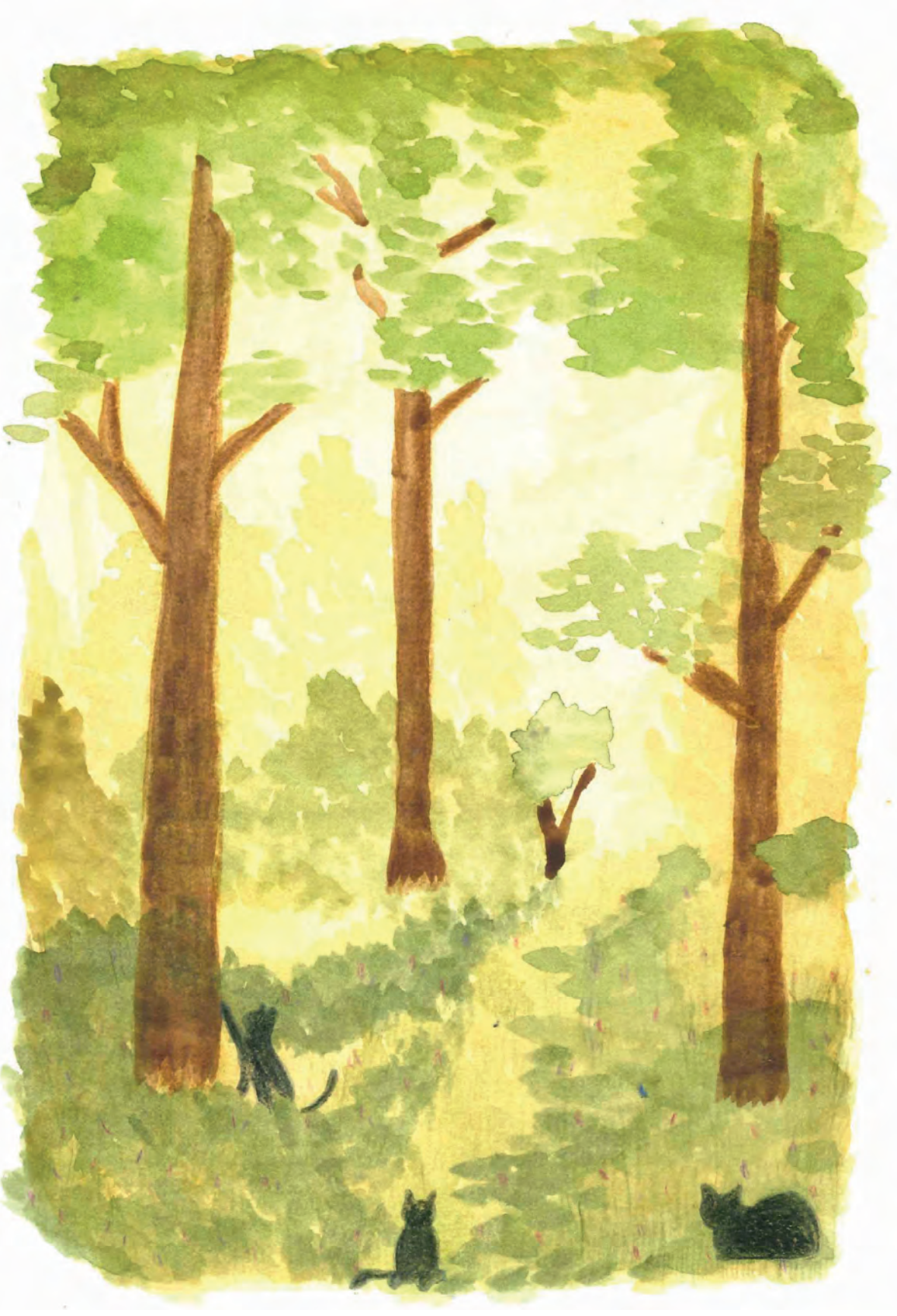
Om te onderzoeken of klonale hematopoëse de gevoeligheid voor het opbouwen van getrainde immuniteit beïnvloedt, selecteerden we in **Hoofdstuk 5** individuen met DNMT3A mutaties en individuen zonder CHDMs om de aangeboren immuunfunctie en het fenotype te karakteriseren. Net als zoals we zagen in het grotere 300 OB cohort, lieten we hier een lagere ex vivo cytokine productiecapaciteit zien van PBMC's van individuen met DNMT3A mutaties. Wat

daarnaast nog belangrijker is, is dat cellen met een DNMT3A mutatie beter in staat waren om getrainde immuniteit op te bouwen.

Naast het begrijpen van de mechanismen die betrokken zijn bij de inductie van ontsteking, bestudeerden we ook de regulatie van ontsteking door behandeling met een lage dosis colchicine. Dit oeroude geneesmiddel, gewonnen uit de Colchicum Autumnale, wordt al lange tijd gebruikt bij de behandeling van jicht. Recente baanbrekende onderzoeken (de COLCOT en LoDoCo2 studies) toonden effectiviteit van lage dosis colchicine (0,5 mg/dag) in het verminderen van MACE bij patiënten met recent acuut coronair syndroom en stabiele coronaire hartziekte. In een recent onderzoek werden allerlei ontstekingseiwitten gemeten in een subgroep van de LoDoCo2 studie en dit toonde aan dat na 4 weken behandeling met een lage dosis colchicine vooral eiwitten die gerelateerd zijn aan neutrofiele degranulatie afnamen.

Vervolgens hebben we ons in **Hoofdstuk 6** gericht op de vraag hoe behandeling met een lage dosis colchicine (0,5 mg/dag) het fenotype van monocyten en neutrofielen precies verandert. Dit deden we in een dubbelblind, gerandomiseerd placebogecontroleerd onderzoek met een cross-overontwerp. We toonden aan dat bij personen met een voorgeschiedenis van myocardinfarct, de behandeling met colchicine vooral het fenotype en de functie van neutrofielen beïnvloedde, aangetoond door verminderde CD62L-expressie aan het oppervlak en verminderde NGAL-afgifte bij MSU-stimulatie.

Over het geheel genomen vergroot de inhoud van dit proefschrift de kennis over de rol van ontstekingen, en in het bijzonder aangeboren immuniteit, in atherosclerose. Dit kan helpen bij het verbeteren van de stratificatie van patiënten voor specifieke ontstekingsremmende therapieën. Bijvoorbeeld, weten of een individu drager is van een CHDM zou een rol kunnen spelen in de beslissing welke gepersonaliseerde behandeling zou kunnen helpen. Daarnaast zouden het gen en de grootte van de CHDM ook mogelijk een rol kunnen spelen in deze beslissing.



Appendices

Research Data Management
PhD portfolio
List of publications
Acknowledgements
Curriculum vitae

Research Data Management

Ethics and privacy

All human studies described in this thesis were conducted according to the principles of the declaration of Helsinki and were approved by the national and international ethical committees. The study in Chapter 3 was approved by the Ethics Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy (approval number 385/2017). Studies in Chapters 4, 5, and 6 were approved the Medical Ethics Committee of the Radboud University Medical Centre (approval numbers: nr. 46846.091.13, NL72552.091.20 and NL73042.091.20 respectively). Written informed consent was obtained from all participants prior to studies.

Data collection and storage

This thesis is based on human data generated in Nijmegen, the Netherlands and Cluj-Napoca, Romania. Data for Chapters 3, 4, 5 and 6 was obtained through laboratory experiments involving pseudonymized human materials.

Raw and processed data for Chapters 3, 4, 5 and 6 are stored on the private server of the department of Experimental Internal Medicine at the Radboudumc, Nijmegen, which is backed-up daily.

Data sharing according to the FAIR principles

The targeted genomic data underlying Chapter 3 is fully published in the supplementary data of the open access publication and can be accessed via doi: 10.1016/j.isci.2024.109472. The proteomic data of Chapter 3 is available on peptideatlas.org and can be accessed via accession no: PASS01721.

300 OB cohort in Chapter 4 is generated as part of the Human Functional Genomics Project (HFGP), and the majority of the data are publicly available on http://www.humanfunctionalgenomics.org/site/. This manuscript used previously developed CHMIP-RsCh-PIPELINE pipeline to identify CHDMs. The code used in the pipeline is publicly available on GitHub (https://github.com/RosanneVanDeuren/CHMIP-RsCh-PIPELINE/tree/main).

The data underlying Chapter 5 will become available after publication.

The data underlying Chapter 6 will be deposited in the Radboud repository with restricted access, only after an embargo period of 12 months to enable publication of new results based on the data.

PhD portfolio

Department: **Internal Medicine**
PhD period: **01/11/2019 – 30/01/2025**
PhD Supervisor(s): **Prof. N.P. Riksen**, **Prof. L.A.B. Joosten**
PhD Co-supervisor(s): **Dr. S. Bekkering**

Training activities	Hours
Courses	
• RIMLS - Introduction course "In the lead of my PhD" (2020)	15.00
• Radboudumc - Scientific integrity (2023)	20.00
• The next step in my career (2023)	24.00
• Radboudumc - eBROK course (2023)	42.00
Seminars	
• ImmunoMetNet (2022)	10.00
• Young@heart Fall Event (2022)	10.00
Conferences	
• 4th International Conference on Innate Immune Memory (2019) *	20.00
• 88th European Atherosclerosis Society Congress (2020)	40.00
• PhD retreat (2020) *	7.00
• PhD retreat (2021) *	7.00
• NVVI Winterschool (2021) *	20.00
• 28th Annual Scandinavian Atherosclerosis Society Conference (2022) ^	32.00
• 90th European Atherosclerosis Society Congress (2022) ^	40.00
• 6th DCVA Translational Cardiology Meeting (2022) ^	20.00
• PhD retreat (2022) ^	42.00
• Summer Conference on Innate Immune Memory (2022) *	40.00
• 29th Annual Scandinavian Atherosclerosis Society Conference (2023) ^	32.00
• IN CONTROL II Consortium meetings (2023) ^	40.00
• 91st European Atherosclerosis Society Congress (2023) ^	40.00
Other	
• Atheromeeting (2019-2023, weekly)	150.00
• Cytokine meeting (20190-2023, weekly) ^	150.00
Teaching activities	
Lecturing	
• VECTOR Student association (2023)	4.00
Supervision of internships / other	
• Supervision Master student (2022)	60.00
• Supervision Master student (2022)	15.00
Total	880.00

^: oral presentation

*: poster presentation

List of Publications

1. **Tercan H**, Riksen NP, Joosten LAB, Netea MG, Bekkering S. Trained Immunity: Long-Term Adaptation in Innate Immune Responses. Arterioscler Thromb Vasc Biol. 2021 Jan;41(1):55-61. doi: 10.1161/ATVBAHA.120.314212. Epub 2020 Oct 22. PMID: 33086868.
2. Domínguez-Andrés J, Arts RJW, Bekkering S, Bahrar H, Blok BA, de Bree LCJ, Bruno M, Bulut Ö, Debisarun PA, Dijkstra H, Cristina Dos Santos J, Ferreira AV, Flores-Gomez D, Groh LA, Grondman I, Helder L, Jacobs C, Jacobs L, Jansen T, Kilic G, Klück V, Koeken VACM, Lemmers H, Moorlag SJCFM, Mourits VP, van Puffelen JH, Rabold K, Röring RJ, Rosati D, **Tercan H**, van Tuijl J, Quintin J, van Crevel R, Riksen NP, Joosten LAB, Netea MG. In vitro induction of trained immunity in adherent human monocytes. STAR Protoc. 2021 Feb 24;2(1):100365. doi: 10.1016/j.xpro.2021.100365. PMID: 33718890; PMCID: PMC7921712.
3. **Tercan H**, Cossins BC, van Deuren RC, Rutten JHW, Joosten LAB, Netea MG, Hoischen A, Bekkering S, Riksen NP. Association Between Clonal Hematopoiesis Driver Mutations, Immune Cell Function, and the Vasculometabolic Complications of Obesity. J Am Heart Assoc. 2024 Jan 16;13(2):e031665. doi: 10.1161/JAHA.123.031665. Epub 2024 Jan 12. PMID: 38214284; PMCID: PMC10926828.
4. Dregoesc MI, **Tercan H**, Țigu AB, Bekkering S, Joosten LA, Netea MG, van Deuren RC, Hoischen A, Riksen NP, Iancu AC. Clonal hematopoiesis is associated with cardiovascular events in patients with stable coronary artery disease. iScience. 2024 Mar 11;27(4):109472. doi: 10.1016/j.isci.2024.109472. PMID: 38558938; PMCID: PMC10981089.
5. **Tercan H**, van Broekhoven A, Bahrar H, Opstal T, Cossins BC, Rother N, Rodwell L, Bekkering S, El Messaoudi S, Riksen NP, Cornel JH. The Effect of Low-Dose Colchicine on the Phenotype and Function of Neutrophils and Monocytes in Patients with Chronic Coronary Artery Disease: A Double-Blind Randomized Placebo-Controlled Cross-Over Study. Clin Pharmacol Ther. 2024 Aug 8. doi: 10.1002/cpt.3394. Epub ahead of print. PMID: 39115262.
6. Liu R, Klück V, Kischkel B, **Tercan H**, Netea MG, Crișan TO, Joosten LAB. Soluble urate-induced effects on cytokine production in vitro - Assessment of methodologies and cell types. Cytokine. 2024 Mar;175:156502. doi: 10.1016/j.cyto.2024.156502. Epub 2024 Jan 17. PMID: 38237388.

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Sevgili ailem, hayatımın her döneminde olduğu gibi doktoramda da hep yanımda oldunuz. Her zaman sizin koşulsuz sevginizi ve desteğinizi yanımda hissediyorum, sizi çok seviyorum. **Anneciğim**, her an bir telefon kadar uzağımda olduğun, her derdimi ve sevincimi dinlediğin için çok teşekkür ederim. Sen gerçek bir Melek'sin. **Babacığım**, her zaman şiirlerinle yanımda olduğun ve servetinin yarısını bana yedirdiğin için teşekkür ediyorum. **Özgürcüğüm**, en sevdiğim kardeşim, iyi ki varsın.

Curriculum Vitae

Helin Tercan was born on 20th of May 1995 in Antalya, Turkey. After completing her primary and secondary education in Antalya, in 2013 she moved to Ankara where she studied Molecular Biology and Genetics at Middle East Technical University. During her undergraduate studies she took several courses on Immunology offered by Prof. Mayda Gürsel. As she quickly and deeply became fascinated by the field of immunology, she joined the laboratory of Mayda Gürsel for her bachelor's thesis project. Under the supervision of Dr. Bilgi Güngör and Dr. Ihsan Cihan Ayanoğlu she worked on vaccine adjuvant applications of CpG ODN nanorings and development of Leishmania extracellular vesicle based cutaneous Leishmaniasis vaccine. After graduating *cum laude* in 2017, she started the Molecular Mechanisms of Disease Master program in Nijmegen. Her first master's internship was in the Department of Rheumatology at Radboudumc. During this 6 months internship, she worked in the group of Dr. Fons van de Loo where she characterized the inflammatory signature of extracellular vesicles from psoriatic arthritis patients. She performed her second master's internship at Boston Children's Hospital, in the group of Prof. Talal Chatila. During her 10 months stay in Boston she worked on DOCK8 primary immunodeficiency.

She started her PhD trajectory in November 2019, at the department of Internal Medicine at Radboudumc under the supervision of Prof. Niels Riksen, Prof. Leo Joosten and Dr. Siroon Bekkering. During her PhD she worked on various collaborative projects focusing on how clonal hematopoiesis and trained immunity shape the phenotype of monocytes and neutrophils in atherosclerosis. The results of these works are part of this thesis and have been presented at various international conferences.

During her PhD she developed a particular interest in the role of neutrophils in inflammation. As neutrophils quickly became her favorite immune cell, she decided to dive deeper into the world of this cell. Therefore, since August 2023 she works as a postdoctoral researcher at Sanquin in the group of Prof. Taco Kuijpers. Her project there is aimed at understanding molecular mechanisms of neutrophil extracellular trap formation.

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