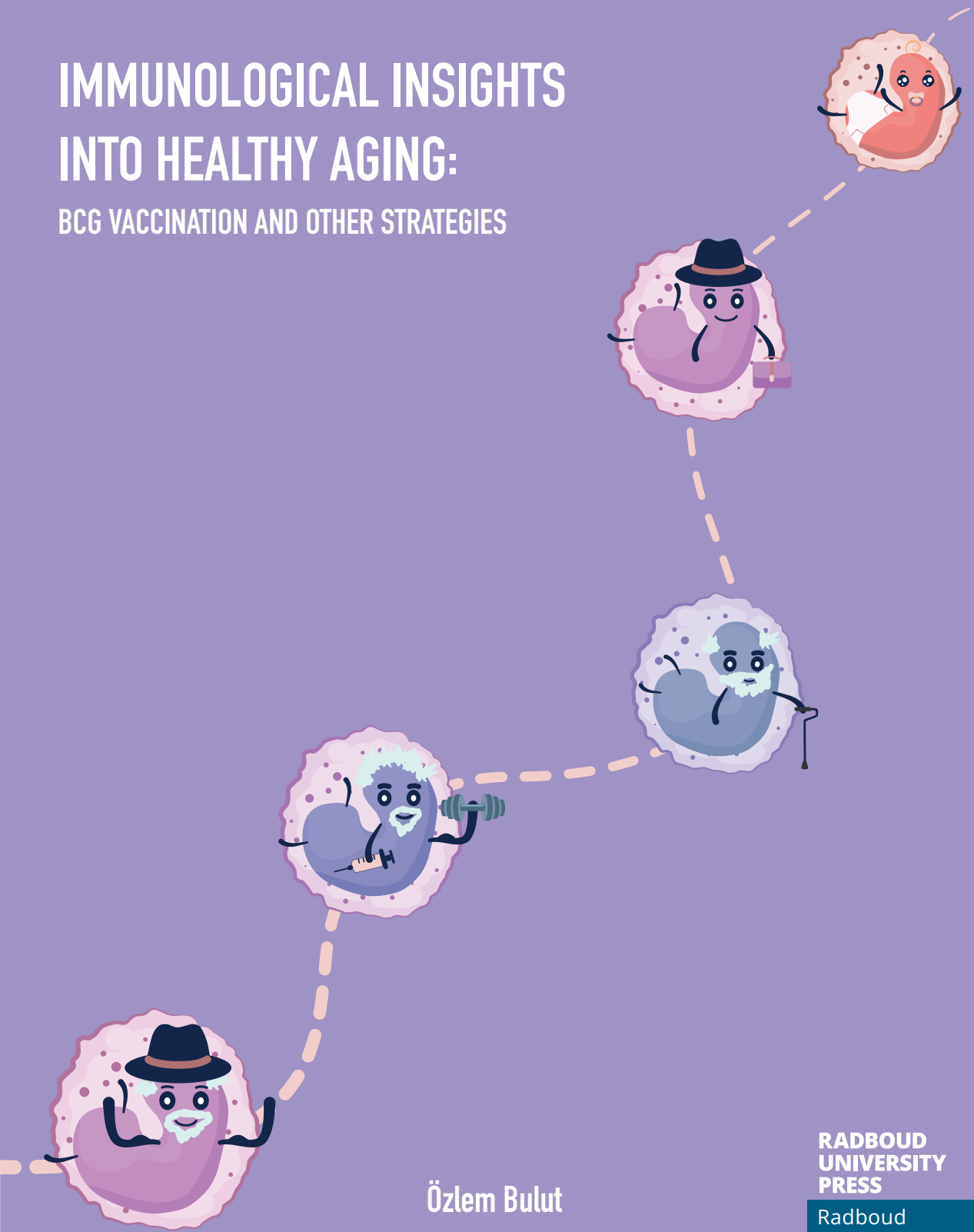
# IMMUNOLOGICAL INSIGHTS INTO HEALTHY AGING:

## BCG VACCINATION AND OTHER STRATEGIES



Özlem Bulut

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# IMMUNOLOGICAL INSIGHTS INTO HEALTHY AGING:

## BCG VACCINATION AND OTHER STRATEGIES

**Özlem Bulut**

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# IMMUNOLOGICAL INSIGHTS INTO HEALTHY AGING:

## BCG VACCINATION AND OTHER STRATEGIES

Proefschrift ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.M. Sanders,
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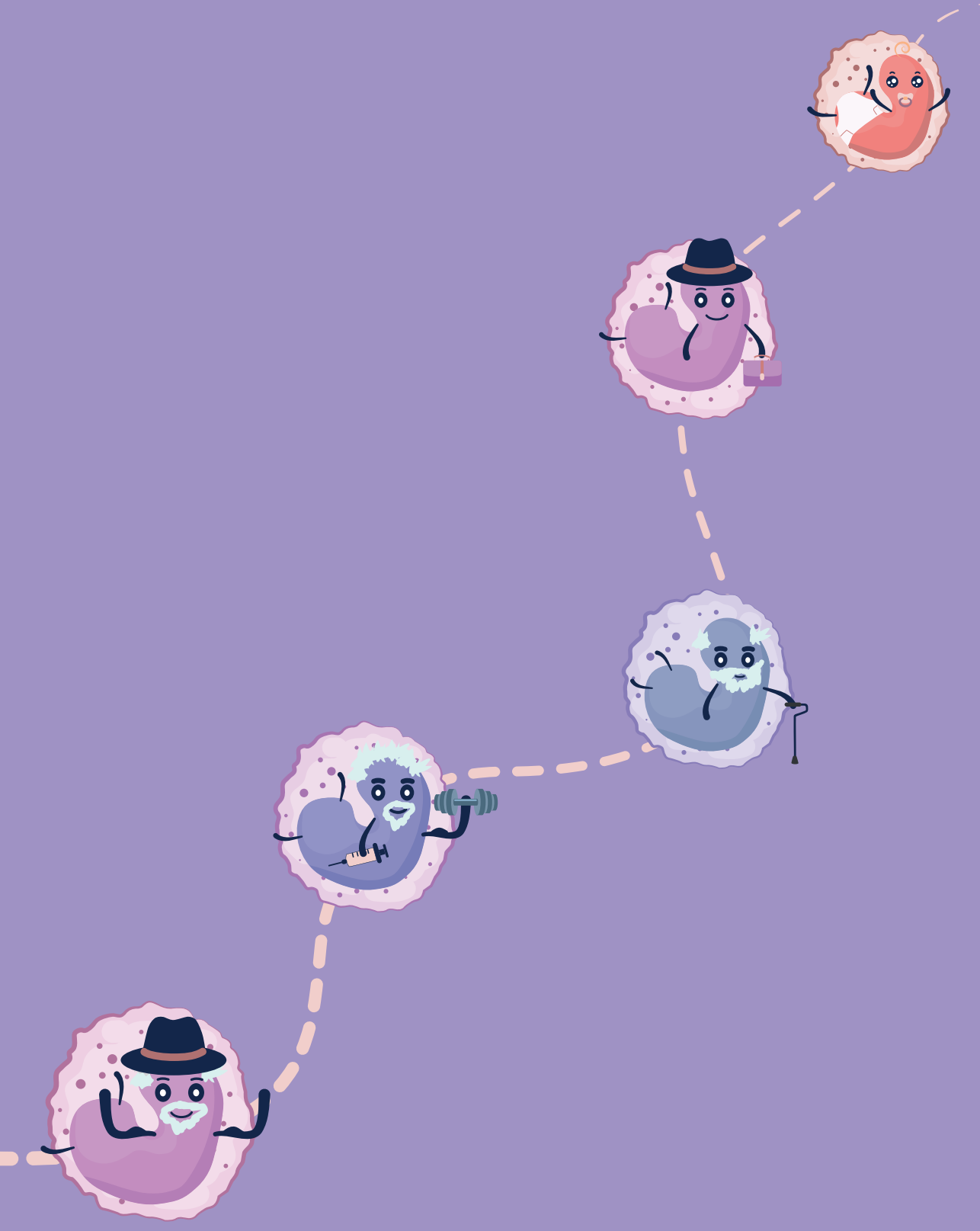
*Canım anneme,*

*To my dear mother,*

## CONTENTS



# CHAPTER 1

# INTRODUCTION, AIMS, AND OUTLINE OF THE THESIS

Age is one of the biggest risk factors for many human diseases, including cancer, diabetes, and cardiovascular diseases, partly due to changes in the immune system associated with accelerating biological aging [1, 2]. As the human body ages, all cells and tissues undergo changes and adapt to cope with them, and the immune system is no exception. The numbers of certain (especially lymphoid) immune cells and their progenitors decline, and cellular responses are inefficient, which is collectively called immunosenescence [3, 4]. Meanwhile, an age-related increase in sterile systemic inflammation occurs (so-called inflammaging). A suboptimal immune system leads to decreased immunosurveillance against cancer, higher susceptibility to infections, and diseases associated with elevated systemic inflammation, such as atherosclerosis and neurodegenerative diseases.

The number of people over the age of 65 is expected to more than double and reach 1.6 billion by 2050 [5]. A rapidly growing older population brings about a decline in the quality of life and immense strain on the medical systems and economies due to age-related diseases and high risk of infections. More than half of the individuals over 65 have multiple chronic age-related disorders [6]. Furthermore, disruptions in the adaptive immune system caused by aging lead to low vaccine efficiency in old age, further emphasizing the importance and urgency of finding ways to promote a robust immune response [7].

Infections are one of the prominent evolutionary forces. Before antibiotics and vaccines, humans had to adapt to the constant presence of a high infectious burden, developing strategies to combat different pathogens. Immune receptors and reactions started evolving early in the evolutionary timeline and exist in different forms in invertebrates, plants, and microorganisms [8-10]. Another critical selection force alongside infections was nutrient availability. This led our cells to develop a complex network of genes and proteins that regulate nutrient sensing and metabolism. Nutrient availability and metabolic processes vastly impact the function of immune cells, which is one of the many threads connecting immunity to metabolic diseases [11]. Although inflammaging can also be observed in healthy aging without comorbidities, it can contribute to and accelerate age-related pathologies in relation to other intrinsic and extrinsic risk factors [12]. This thesis primarily focuses on how the function and metabolism of immune cells are linked to aging-associated processes and identifies different ways of promoting immuno-metabolic homeostasis.

# MECHANISMS OF AGING

Fundamental molecular, cellular, and systemic events that manifest with aging have been clustered into several "aging hallmarks". The current understanding of this complex biological event points to 12 hallmarks: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis [13]. These can be further grouped as primary hallmarks that are the initial causes of damage; antagonistic or compensatory hallmarks that develop to mitigate damage but become deleterious when chronic; and integrative hallmarks that result from the others and ultimately cause functional decline.

## Primary Hallmarks

Damage accumulates at the genome level throughout our lives [14]. Somatic mutations and endogenous DNA replication errors aggregate in nuclear and mitochondrial DNA. External factors such as exposure to radiation and chemical mutagens can also challenge DNA stability. Cells have DNA damage responses (DDR) to repair and prevent further damage [15]. However, consistent DDR, resulting from mutations in DDR genes or too much chronic DNA damage, can cause cellular senescence.

Another form of genomic damage occurs at the level of the telomeres: repeats of TTAGGG nucleotides at the ends of each chromosome. The end of telomeres forms a knot called the T-loop, and together with a group of 6 proteins called the shelterin complex, protect the ends of chromosomes from being recognized as DNA damage [16]. During each cell replication, this DNA-protein complex unravels to allow new DNA synthesis. Each time, telomeric repeats shorten by 30-200 base pairs. Gradual shortening, when it reaches a critical threshold, can lead to aberrant DDR signaling and cause senescence [17]. Telomere damage can occur in other ways in non-proliferating cells, such as due to inflammation, oxidative stress, or environmental exposures.

Epigenetic changes in DNA methylation and histone modifications, such as increased H3K4me3 and H3K27me3 marks, also occur throughout life and regulate gene expression [18]. With aging, DNA shows global hypomethylation [19]. However, particular loci, including important tumor-suppressor genes, are hypermethylated [20]. Using the characteristic age-associated epigenetic changes, a biological age estimate of the organism can be calculated, which is a better indicator of health and mortality than chronological age [21, 22].

Moving from the level of genes to proteins, aging is associated with the loss of protein homeostasis, also termed proteostasis [23]. This involves the correct folding, stabilization, and the timely and proper degradation of proteins. Accumulation of misfolded or damaged proteins contributes to the pathology of some age-related diseases, including Alzheimer's and Parkinson's [24].

Lastly, macroautophagy is compromised during aging. This process is essential for digesting cytoplasmic waste and recycling materials, including proteins, whole organelles, or invading microorganisms. Genetic inhibition of macroautophagy accelerates aging in model organisms while promoting it extends longevity [25], and loss of function mutations in related genes in humans are linked to a wide range of age-related diseases [26].

## Antagonistic or Compensatory Hallmarks

Aging impacts many crucial metabolic processes for nutrient sensing [27] that also heavily regulate immune responses [28]: insulin and insulin-like growth factor 1 (IGF-1) signaling, the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin(mTOR) axis, AMP-activated protein kinase (AMPK), and sirtuins. Overactive nutrient signaling and anabolic processes governed by insulin/IGF-1 and PI3K/AKT/mTOR pathways accelerate the aging phenotype. In contrast, reduced nutrient signaling, similar to ancestral conditions with lower nutrient availability, restores compromised functions and extends longevity. Insulin/IGF-1 signaling is the most conserved aging-related pathway across species, highlighting its significance [29]. Attenuating this pathway at various steps leads to lifespan extension in model organisms, and related mutations were linked to longevity in humans. Similarly, the downregulation of mTOR activity promotes longevity [30]. However, mTOR signaling is critical for immune function. Therefore, there is a delicate balance to achieve for homeostasis and healthy longevity. In contrast, the upregulation of AMPK and sirtuin functions, forming a positive feedback loop among each other, leads to healthier aging [31, 32].

As the metabolic load on cells increases and the efficiency of metabolic pathways is compromised, mitochondria dysfunctions also arise. These include increased production of reactive oxygen species (ROS) causing oxidative stress, reduced mitochondrial biogenesis, ineffective removal of damaged mitochondria, compromised mitochondrial membrane integrity, and leakage of mitochondrial DNA triggering inflammatory responses [33].

The alterations in mitochondrial function and nutrient sensing are thought to be adaptations meant initially to restrict and prevent damage, and improve cellular fitness [34]. However, they end up accelerating the aging process and are thus considered among the antagonistic or compensatory hallmarks of aging. Similarly, cell cycle arrest and the resulting senescence characteristics have protective and compensatory functions, but they can become deleterious. Senescent cells have a characteristic phenotype involving metabolic changes, high oxidative stress causing more pressure on the genome and metabolism, increased resistance to apoptosis, and the senescence-associated secretory phenotype (SASP) [35]. SASP is the collection of secreted cytokines, chemokines, and other molecules with overall pro-inflammatory and tissue-disrupting functions [36].

### Integrative Hallmarks

The aforementioned aging-associated cellular changes occur not only in differentiated cell types but also in stem and progenitor cells [37]. The proliferative and regenerative capacities of tissue-resident stem cells and hematopoietic stem cells (HSCs) are reduced, along with skewed differentiation to more specialized cell types. Eventually, aged and exhausted stem cells contribute to mechanistically and functionally compromised cells and tissues.

Cellular disruptions in the immune and neuroendocrine systems, such as altered receptor expressions, secretion profiles, and overall cellular fitness, lead to progressive changes in intercellular communication throughout the body. This contributes to chronic inflammation, decline of immunosurveillance against pathogens, and disrupted bidirectional interaction with the microbiome resulting in dysbiosis.

Dysbiosis points to a loss of balance in the gut microbiota characterized by reduced microbial diversity, disappearance of core species, and expansion of opportunistic pathogens and species with more pro-inflammatory profiles [38]. These can lead to altered tolerance of the immune system against the microbiota, compromised integrity of the epithelial barrier, and leaking of toxins or inflammatory microbial products into the bloodstream, contributing to tissue damage and inflammaging.

All these hallmarks are intricately linked and contribute to each other's progression. Therefore, it is essential to have a comprehensive view of the fundamental mechanisms and system-level changes in order to design effective anti-aging strategies. Targeting the immune system is an attractive approach with wide-ranging impacts on aging hallmarks.

## IMMUNE MEMORY AND THE AGING IMMUNE SYSTEM

The immune system is classically divided into two arms: the more ancient and non-specific innate immunity, which coordinates the quick response in the first hours and days after infection, and adaptive immunity, which is antigen-specific and can harbor the memory of previous encounters with a pathogen to fight it efficiently next time. An ever-increasing body of research has challenged the concept that only adaptive immune cells can build immunological memory, revealing that innate immune cells also carry memories of past exposures [8]. Innate immune memory is also known as trained immunity, and it is mediated by metabolic and epigenetic reprogramming of innate immune cells and their progenitors upon exposure to certain pathogens or microbial ligands, resulting in a more robust subsequent response to unrelated pathogens [39]. The century-old Bacillus Calmette–Guérin (BCG) vaccine is a safe inducer of trained immunity in humans. Initially developed for tuberculosis prevention, BCG can protect against unrelated infections and significantly reduce all-cause mortality in settings with high infectious burden [40]. A more detailed explanation of the molecular mechanisms of BCG-induced trained immunity and beneficial non-specific effects against other infections is provided in subsequent chapters.

T and B cells of lymphoid origin are the cell types that develop and maintain adaptive immunological memory and, thus, are the primary targets for vaccine development. However, with aging, HSCs are increasingly skewed to differentiate into myeloid cells that constitute the innate arm of the immune system [37]. Additionally, the production of T cells in the thymus critically diminishes throughout life due to thymic involution [41]. Apart from reduced production of new cells resulting in a shrunken naïve lymphocyte pool, existing lymphocytes may become senescent and unable to respond effectively to a challenge. Reduced expression of the co-stimulatory receptor CD28, lower cytotoxic capacity and receptor diversity are among the detrimental age-related changes in T cells [42]. B cells also experience reduced receptor diversity, lower antibody production and weaker antibody affinity [43]. These changes in lymphocytes, detailed further in Chapters 2 and 5, lead to the suboptimal adaptive immune response and vaccine efficacy observed in later stages of life.

Although immunosenescence is mainly defined by the loss of naïve lymphocyte pools, accumulation of terminally-differentiated senescent lymphocytes, and accompanying inflammaging, innate immune cells also undergo age-related changes. Cytokine production by many cell types, antigen presentation capacity of monocytes and dendritic cells (DCs), phagocytic capacities of neutrophils and

macrophages, cytotoxicity of NK cells, and chemotaxis are reduced in old age [4]. Despite the lower cellular response, circulating concentrations of cytokines and chemokines tend to increase with age, taking part in inflammaging [1]. Contributors to inflammaging include the SASP, also secreted by non-immune senescent cells such as epithelial and endothelial, accumulating cell and protein debris, adipose tissue, which is a significant source of interleukin 6 (IL-6), and factors produced by the gut microbiota that increasingly assumes a pro-inflammatory profile with age [44].

Despite the suboptimal functioning of innate immune cells, immunosenescence weighs much heavier on adaptive immunity. With shrunken and dysregulated lymphocyte pools, innate cells become more critical in defense against pathogens or response to vaccinations. Therefore, improving the functions of innate immune cells by stimulating trained immunity is an exciting approach to improving health in old age.

## AIMS AND OUTLINE OF THE THESIS

Life expectancy continues to rise in every part of the world, with vaccines and antibiotics having a vital contribution to the decline of infection-related mortality. With it comes the struggle to maintain the quality of life into old age, and an effective immune system is an essential component of biological resilience to aging. Aging of the organism brings about a complex chain of events, with the immune system at the center. A comprehensive understanding of the mechanisms at play is essential to identify targets for anti-aging interventions. The two main goals of this thesis are to understand better the complex process of biological aging, particularly immune aging, and to investigate strategies to maintain immune homeostasis and overcome aging-associated changes.

**Chapter 2** provides a detailed look into how innate and adaptive immune memory are affected by aging. This review considers the relationships between immune aging, the gut microbiota, and the central nervous system and delves into the epigenetic and metabolic mechanisms that underlie age-related pathologies. This chapter also discusses various efforts to slow, halt, or even reverse age-associated phenotypes that have the opportunity to tackle the complex phenomenon of aging from multiple angles. Caloric restriction, physical exercise, metformin, and resveratrol are highlighted as promising anti-aging interventions.

Genetic and non-genetic factors, including climate, diet, physical activity, smoking, exposure to hazardous environmental contaminants, and infection history, heavily impact immune function [45, 46]. **Chapter 3** emphasizes the importance of researching geographically distinct populations with different genetic backgrounds and lifestyles to tease out the differences and the commonalities in their immune profiles, how they change with age, and the influential factors mediating these changes. We explored two healthy adult cohorts from the Netherlands and Tanzania by combining metabolomics, proteomics, and functional immune assays. This work identified several metabolites with pro- and anti-aging associations common in both cohorts and the metabolites that might play a role in the distinct aged immune profiles of the two populations.

The COVID-19 pandemic made it ever more apparent that older individuals are at a higher risk of severe infection and death. Along with age, male sex is a risk factor for severe COVID-19. The work presented in **Chapter 4** investigates how the circulating proteome and immune cell composition of healthy men and older individuals differ from women and younger individuals, and if these differences could explain the predisposition to severe COVID-19. In addition to age and sex, we also explored the effect of seasons on the immune response against SARS-CoV-2, using cells of healthy individuals sampled four times over a year prior to the pandemic.

**Chapter 5** highlights the promise of trained immunity in overcoming immune aging, providing protection against non-specific infections, and improving the vaccine responses of older individuals. This review first provides an overview of the age-related changes in innate and adaptive immune cell types. It discusses past and current strategies to develop more effective vaccines for older age groups. Then, the concept of trained immunity is explained, and the existing body of research is presented to emphasize the potential of trained immunity induction, such as by BCG vaccination, as a tool to counteract immune aging.

**Chapter 6** focuses on the long-term immunological impact of combining alendronate (alendronic acid) treatment with BCG vaccination. Earlier studies have demonstrated that cholesterol metabolism is critical for the induction of trained immunity [47]. Bisphosphonates, such as alendronate, are known inhibitors of the cholesterol synthesis pathway [48, 49] and are used chronically later in life to treat osteoporosis. We investigated alendronate's effects on BCG-induced trained immunity, which is clinically important considering the wide use of this drug in elderly individuals.

One of the anti-aging interventions highlighted in Chapter 2 is resveratrol, a natural phenolic compound with antioxidant properties. The primary known target of resveratrol, deacetylase Sirtuin 1 (Sirt1), was implicated in trained immunity induction by BCG in humans [50]. Modulating Sirt1 activity also impacts many aging-related cellular processes, and elevated Sirt1 activity in model organisms can revert aged phenotypes and extend lifespan [51, 52]. Apart from Sirt1 activation, resveratrol has many more anti-aging implications [53]. **Chapter 7** explores resveratrol's acute and long-term impacts on human monocyte metabolism and function, alone and in combination with the BCG vaccine. Its effects on β-glucan-, *C. albicans*- and oxLDL-induced trained immunity are also investigated.

By improving innate immune response to pathogens and reducing systemic inflammation, BCG vaccination could help maintain immune homeostasis during aging [54, 55]. However, it is unclear if BCG impacts other hallmarks of aging, such as telomere attrition. Inflammation is intricately linked with genomic damage and cellular senescence. In **Chapter 8**, we investigated the long-term impact of BCG vaccination on telomere length and maintenance in two BCG-vaccinated healthy cohorts and an *in vitro* trained immunity model.

**Chapter 9** concludes this thesis with a summary and discussion of all the work presented, highlighting the most critical messages and their implications in the quest to extend the healthspan of individuals by modulating the immune system with pharmacological or lifestyle interventions.

This body of work provides new insights into how diet, sex, medication use, and vaccination can impact processes related to immune aging or the efficiency of anti-aging interventions. Further research built upon this knowledge should target aging by focusing on fundamental common mechanisms that govern many facets of aging and combine it with a nuanced understanding of the differences between sexes and individuals with varying genetic makeups and lifestyles.

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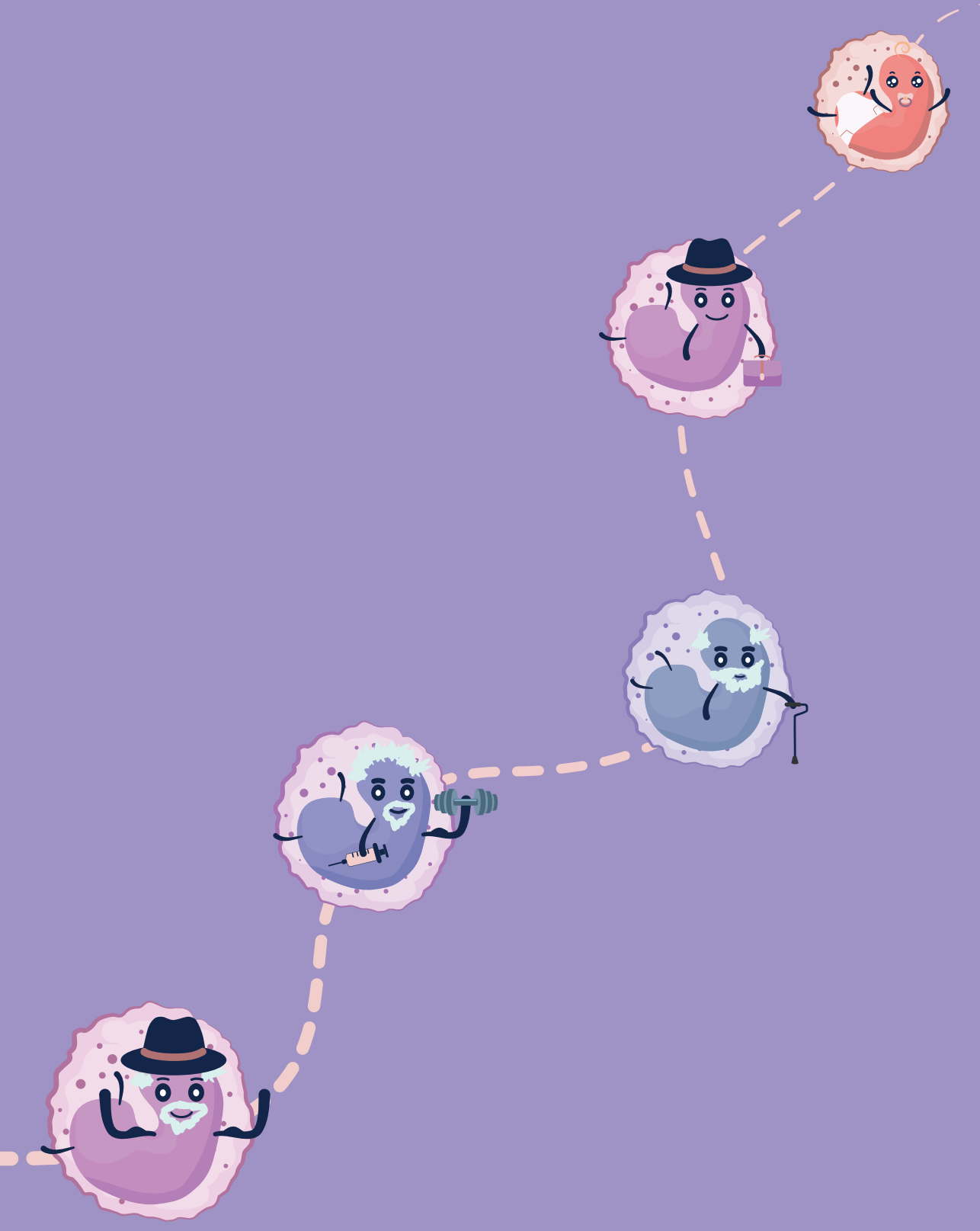

# CHAPTER 2

# IMMUNE MEMORY IN AGING: A WIDE PERSPECTIVE COVERING MICROBIOTA, BRAIN, METABOLISM, AND EPIGENETICS

Ozlem Bulut · Gizem Kilic · Jorge Domínguez-Andrés

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## ABSTRACT

Non-specific innate and antigen-specific adaptive immunological memories are vital evolutionary adaptations that confer long-lasting protection against a wide range of pathogens. Adaptive memory is established by memory T and B lymphocytes following the recognition of an antigen. On the other hand, innate immune memory, also called trained immunity, is imprinted in innate cells such as macrophages and natural killer cells through epigenetic and metabolic reprogramming. However, these mechanisms of memory generation and maintenance are compromised as organisms age. Almost all immune cell types, both mature cells and their progenitors, go through age-related changes concerning numbers and functions. The aging immune system renders the elderly highly susceptible to infections and incapable of mounting a proper immune response upon vaccinations. Besides the increased infectious burden, older individuals also have heightened risks of metabolic and neurodegenerative diseases, which have an immunological component. This review discusses how immune function, particularly the establishment and maintenance of innate and adaptive immunological memory, regulates and is regulated by epigenetics, metabolic processes, gut microbiota, and the central nervous system throughout life, with a focus on old age. We explain in-depth how epigenetics and cellular metabolism impact immune cell function and contribute to or resist the aging process. Microbiota is intimately linked with the immune system of the human host, and therefore, plays an important role in immunological memory during both homeostasis and aging. The brain, which is not an immune-isolated organ despite former opinion, interacts with the peripheral immune cells, and the aging of both systems influences the health of each other. With all these in mind, we aimed to present a comprehensive view of the aging immune system and its consequences, especially in terms of immunological memory. The review also details the mechanisms of promising anti-aging interventions and highlights a few, namely caloric restriction, physical exercise, metformin, and resveratrol, that impact multiple facets of the aging process, including the regulation of innate and adaptive immune memory. We propose that understanding aging as a complex phenomenon, with the immune system at the center role interacting with all the other tissues and systems, would allow for more effective anti-aging strategies.

## INTRODUCTION

Human beings, like all organisms, inevitably age and die. Even if science eventually cracked the code for immortality, that would not end the need to understand the mechanisms of aging and the efforts to slow or revert it. If anything, it will be even more critical to maintain the health of all cells and organs throughout a long life. Tackling aging is always a worthwhile effort to improve the quality of live for the middle-aged and elderly populations, especially since the human population over 60 years of age is expected to reach two billion by 2050 [1].

Infectious diseases of the elderly, especially in low-income countries, represent a significant social and economic burden. The immune system undergoes numerous changes as humans age, leaving older individuals more prone to disease [2]. The age-related dysregulations in the immune system are collectively referred to as "immunosenescence" and include accumulating tissue damage, a low-grade chronic systemic inflammation termed "inflammaging," impaired immune cell function, inadequate response to vaccination, and increased vulnerability to infections [3].

The importance of immune memory has perhaps never been more evident than during the ongoing coronavirus disease 2019 (COVID-19) pandemic, which disproportionately affected the elderly population due to the altered functionality of their immune system [4]. Thanks to the outstanding collaborative effort of governments and scientists, 7 vaccines generating effective immune response and protection against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have been authorized for emergency use by World Health Organization (WHO)–recognized authorities as of June 2021, and many more are in use with authorizations by national regulatory agencies [5]. Due to the increased vulnerability of the elderly, they are the priority group in COVID-19 vaccination rollouts.

Besides the morbidities caused by infection, the elderly also present an increased incidence of metabolic diseases such as type 2 diabetes and obesity [6], and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [7]. However, the development of these age-related conditions is not separated from their aging immunity. All systems and organs exchange signals with and are influenced by the immune system. Combining all the accumulating insights from different lines of research is critical to drawing up a comprehensive view of aging.

In this review focusing on immune memory, we first outline how memory is developed and maintained. Next, we delve into metabolic and epigenetic

mechanisms, their roles in immune memory, how they change with age, and the implications for age-related pathologies. As two examples of the far-reaching impacts of an aging immune system, we highlight the interplay of immune memory with the gut microbiota and the brain. We end the review by presenting the current preventative and therapeutic strategies against aging, approaching from the alternative points of view of epigenetic modulation, metabolic intervention, microbiota reconstitution, and neuroprotection.

## ADAPTIVE IMMUNE MEMORY

Infections have been one of the primary selective forces throughout evolution, so immunological memory has evolved to ensure survival when an organism is exposed to a pathogen that it encountered before [8]. Until the discovery of non-specific innate immune memory in the last decade, the antigen-specific memory established by T and B lymphocytes has been getting all the credit for long-term protection against pathogens.

### T Cells: Thymus-Derived Troops of Immunity

Immunological memory against infections and tumors requires the intervention of T cells. T cells can recognize both self and non-self antigens through their T cell receptors (TCRs) and mount self-tolerance or immunological memory. Different subsets of T cells include naïve T cells that recognize new antigens and memory T cells that are formed upon former exposure to antigens and assure long-lasting immunity.

### T Cell Development

T cells derive from the hematopoietic stem cells (HSCs) in the bone marrow but mature in the thymus. Most mature T cells reside in lymphoid tissues, but they are ubiquitously present throughout the body. After lymphoid progenitors migrate from bone marrow to the thymus, TCR gene rearrangement occurs, and CD4+ CD8+ double-positive cells expressing both co-receptors are generated. Then, these cells undergo positive selection based on TCR-antigen interactions and differentiate into naïve single-positive CD4+ helper or CD8+ cytotoxic T cells, which are released into the periphery [9].

Most of our knowledge on T cell development originates from mouse studies. However, there are substantial differences between mice and humans. For instance, although the peripheral naïve T cell pool is almost exclusively provided by the thymus in mice, humans primarily sustain it by peripheral cell division [10].

When a naïve cell recognizes an antigen presented by antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages, they proliferate and develop into effector cells that can clear the source of the antigen, likely a pathogen. A small portion of these effector cells later become memory cells to establish long-term immunity that can last multiple decades, while the rest die by apoptosis [11]. Early in life, before exposure to many antigens, naïve T cells constitute most of the T cell pool [12]. Meanwhile, regulatory T cells (Treg) are critical for the development of tolerance for innocuous antigens in the environment [13].

Around 5% of all adult CD4+ T cells are Tregs that are able to suppress the immune response [12]. Tregs are produced in the thymus but can also derive from peripheral naïve T cells by acquiring Forkhead Box P3 (FOXP3) expression in response to environmental cues [13]. Recently, Tregs were shown to acquire memory characteristics, mostly against self-antigens, to prevent unwanted inflammation [14].

Memory T cells are divided into three subtypes which are central memory (TCM), effector memory (TEM), and stem cell memory (TSCM). Compared to TEMs, TCMs have more proliferation capacity and are closer to naïve T cells in gene expression profiles [15]. TEMs can perform effector functions such as cytokine production. TSCMs are a stem cell-like, less differentiated cell type with high self-renewal capacity and the ability to differentiate into effector T cells, TEMs, or TCMs [16]. Following TCR stimulation, they are able to secrete interferon-gamma (IFN-γ) and interleukin 2 (IL-2). The long-lasting, multipotent TSCMs might help protect the organism against infections later in life when thymic output is low.

Although 90–95% of the effector T cells die after an infection resolves, a population of terminally differentiated effector cells regaining the naïve T cell marker CD45RA, termed TEMRA cells, remain in circulation. These senescent-like cells have defects in telomerase expression and proliferation; however, they are capable of cytokine production and cytotoxicity, unlike exhausted cells [17].

In many tissues such as lungs, intestines, and spleen, TEMs are the predominant T cell type [18, 19]. Moreover, discrete tissue-resident memory T cell populations (TRM) are identified with enhanced expression of adhesion markers and homing receptors, lower proliferative capacity, and higher production ability of pro-inflammatory and antiinflammatory cytokines [20]. They can quickly react upon tissue injury or infection while also restricting the inflammatory damage. Establishing TRMs is a promising approach to consider in vaccine design, boosting and prolonging vaccine-mediated protection [21–24].

## Effects of Aging on T Cells

Lineage differentiation dynamics of HSCs in the bone marrow are altered with age. They skew towards myeloid differentiation, leading to lower numbers of lymphoid cells in the elderly [25]. HSCs also accumulate DNA damage throughout life and differentiate into leukocytes with chronic DNA damage response [26]. This triggers cellular senescence, which contributes to chronic inflammation by inducing a senescence-associated secretory phenotype (SASP), impacting neighboring immune and non-immune cell types. Another way that DNA damage can contribute to inflammation is the activation of DNA-dependent protein kinase catalytic subunits (DNA-PKcs) that can promote NFκB and inflammasome activity [27, 28].

Involution of the thymus is one of the critical age-dependent changes in the immune system [29]. It is an evolutionarily conserved phenomenon in all vertebrates, starting before puberty, where the total mass, volume, and cellular content of the thymus shrink [30]. Thymic activity does not entirely cease, at least until the sixth decade of life, but thymopoiesis strikingly decreases with age [31, 32]. Thymic epithelial cells gradually lose the ability to produce IL-7, which is crucial to support thymopoiesis [33, 34]. Low thymic output in the elderly is associated with increased vulnerability to infections [35]. In a young adult, the thymus provides around 16% of the naïve T cell pool, the rest of which derives from peripheral proliferation [36]. In the elderly, this number falls below 1%, causing them to entirely rely on the proliferation of existing naïve T cells.

The decline in the number of naïve T cells and the accumulation of terminally differentiated cells are two of the hallmarks of T cell aging [36]. CD4+ and CD8+ naïve cell pools, although more markedly for CD8+ T cells, contract in the elderly. Maintenance of naïve T cells through peripheral proliferation is more successful for CD4+ T cells, but CD8+ T cells are largely lost. Interestingly, while this is mostly the case in cytomegalovirus (CMV) + individuals in women, it is observed in men irrespective of the CMV status [37]. Also, CMV + individuals of both sexes have a higher proportion of late-differentiated senescent T cells than CMV individuals. Chronic CMV infection affects most adults, with an 83% global seroprevalence rate [38]. Even though it usually does not cause active symptoms and is mainly unrecognized, CMV presence significantly shapes the T cell compartments and accelerates immunosenescence. Accumulation of terminally differentiated T cell types such as TEMs and TEMRAs occurs faster in CMV + individuals throughout their lifespan [39]. Expansion of CD8+ TEMRA cells is related to impaired antibody production upon influenza vaccination in the elderly [40]. Latent CMV infection is also associated with inadequate CD4+ T cell response against influenza antigens [41]. Moreover, CMV

positivity is associated with a higher risk of all-cause mortality [42]. Of note, CMV + young adults displayed higher antibody responses to influenza vaccination, compared to CMV – young individuals [43]. In the early stages of the infection, CMV might be potentiating immune responses before the accumulation of CMV-induced senescent cells pass a certain threshold and causes functional impairments.

Not just the numbers but also the receptor diversity of naïve T cells are compromised in aged organisms. Naïve T cells of a young adult carry around 100 million different TCR sequences; however, this repertoire diversity is reduced up to tenfold in the elderly [44]. Moreover, memory T cells experience a narrowing of TCR repertoires [45], and the proliferative capacity of senescent T cells following TCR engagement is defective [46]. Activated CD8+ cells of elderly individuals also produce lower levels of cytotoxins such as granzyme B and perforin [47]. On the other hand, CD4+ naïve T cells of the elderly seem to maintain their differentiation and subsequent cytokine production capacities [48].

Lastly, differentiation of non-Treg cells into Tregs and proliferation of existing Tregs can maintain the Treg pools throughout life, despite reduced thymic output with aging. However, the balance between T cell subsets is altered: as in other T cell types, the naïve subset declines with age while memory Tregs increase [49].

## B Cells: Bone Marrow-Born Battlers

B cells are a vital part of the adaptive immune memory. They have several immunological functions, including antibody and cytokine production, antigen presentation, and regulation of T cell responses [50]. Most vaccines mainly target and rely on B cell activation by inducing long-lived plasma and memory B cell proliferation [51]. However, aging affects the functional capacity of existing B cell subsets drastically, which is evident from the susceptibility to diseases and poor vaccine responses [52].

## B Cell Development

B cells continuously arise from the hematopoietic stem cells (HSCs) and develop in the bone marrow (BM) [53]. HSCs generate multipotent progenitors that eventually diverge to common lymphoid progenitors (CLPs). Certain environmental cues, transcription factors (TFs), cytokines, and chemokines lead CLPs to differentiate into B-cell lineage. Following differentiation, cells undergo a rearrangement in the variable regions of the immunoglobulin (Ig) genes and start to express B-cell receptors (BCRs) and IL-7 receptor (IL-7R) [54]. Each B cell has a unique BCR with a different specificity to antigens.

B cells that finish their developmental process in the bone marrow are called transitional (TR) B cells. They make 4% of all B lymphocytes in healthy individuals [55] and are found in several places, including the bone marrow, peripheral blood, and secondary lymphoid tissues. Transitional B cells become either marginal zone (MZ) or mature follicular (FO) cells partly based on the strength of their BCR signaling. Cells with more robust signaling tend to develop into follicular type, while weaker signaling drives them to be MZ cells [56]. FO B cells have a broad immunoglobulin repertoire and are located in the follicles close to T cell zones [57]. Therefore, they are suited for getting T-cell help and becoming short-lived plasma cells. On the other hand, MZ B cells can get activated easier than FO B cells, which quickly allow them to produce immunoglobulin M (IgM) or induce class switching without T-cell help [58].

The third naïve B cell subset is B-1 cells, which are considered part of the innate immune system [59, 60]. Apart from the other B cell subsets developed in the bone marrow, B-1 B cells originate from a distinct progenitor in the fetal bone marrow [61]. They are mainly found in peritoneal and pleural cavities; however, low numbers can also be located in secondary lymphoid organs. During an infection, they act by producing non-specific antibodies that are crucial for early defense [62, 63].

Advancing age alters the entire course of B cell development, the abundance of distinct B cell subsets, and their function. Furthermore, a B cell subset emerging with increasing age influences immune responses in the elderly.

## Effects of Aging on B Cell Development

B cell development and the influence of old age in this process are extensively studied in mice. First of all, the differentiation capacity of long-term HSCs (LT-HSCs) reduces with advanced age [64]. The genes driving lymphoid cell differentiation and function are downregulated in LT-HSCs, while the genes mediating myeloid cell development are upregulated. Numbers and percentages of early B-cell lineage progenitors decrease as C57BL/6 mice age [65]. Furthermore, these populations exhibit declined IL-7 responsiveness, indicating an impaired B lymphopoiesis.

Following progenitor differentiation, the development of B cells in the bone marrow is also influenced by aging. In different groups of old mice, a severe decrease with more than 80% loss of pre-B cells and 50% loss of pro-B cells, or a moderate decrease with 20–80% loss of pre-B cells were observed [66]. TFs regulating B cell development are altered by age, influencing the abundance of developing B cells [66–68]. Among them, the E2A gene encodes for two proteins, E47 and E12. Transcription and DNA-binding capacity of E47 were shown to decline in aged mice [66]. As E47 is a vital TF

in B cell development during the proto pre-B cell stage [69], lower numbers of pre- and pro-B cells in old mice could partly be explained by the decreased function and expression of E47. PAX5 is another TF regulating early B-cell development that is lower in the elderly [70]. Lastly, BCR expression and diversity are altered upon aging [71, 72], although a study suggested that the changes were not evident until 70 years of age [73].

## The Emergence of Age-Associated B Cells

In 2011, a new subset of B cells was described in aged mice [74, 75]. This mature B cell population is named age-associated B cells (ABCs) since it progressively accumulates with increasing age. The origins of ABCs are not exactly known; however, differentiated FO, MZ, and B-1 cells are thought to contribute to the heterogeneous ABC pool [76]. Although studies define ABCs using different markers, they agree that ABCs are mature B cells with memory characteristics. Unlike the other B cell subtypes, ABCs express the transcription factor T-bet and a unique surface marker combination [77]. Therefore, their activation requirements, functions, and survival conditions are remarkably different. BCR engagement induces FO and MZ B cell proliferation, while Toll-like receptor 9 (TLR9) or TLR7 signaling with or without BCR ligation drives proliferation in ABCs [76]. *In vitro* studies showed that TLR stimulation leads to IL-10 and IFNγ production from ABCs, and an *in vivo* study reported that they also produce tumor necrosis factor-alpha (TNFα) [78].

ABCs are engaged in both protective and autoreactive immune responses, although their protective role seems scarce. Furthermore, they are linked with autoinflammatory and autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis [75, 79, 80], making ABCs a potential underlying reason for the increased incidence of autoimmune diseases in the elderly.

ABCs contribute to immune dysfunctions observed during the aging process. For instance, TNFα produced by ABCs has direct and indirect effects on pro-B cell numbers: ABCs directly induce pro-B cell apoptosis and lead to their loss by altering the bone marrow microenvironment [78]. Besides, increased abundance of ABCs was significantly correlated with the loss of B cell precursors in the bone marrow of aged mice.

ABCs express considerably high major histocompatibility complex II (MHC-II), CD80, and CD86 compared to FO B cells; therefore, they are better inducers of T cell activation and antigen presentation [81]. However, the same study associated

these properties of ABCs with autoimmune diseases in an autoimmune-prone mice strain. Besides, considering that they make the bone marrow environment more inflammatory via the production of TNFα and robustly produce IL-6 and IFNγ upon TLR7 and TLR9 engagement [74, 78], it is plausible to propose that ABCs contribute to inflammaging.

Lastly, a study reported that humoral response depends more on TLR signaling and less on CD4+ T cell help due to decreased FO B cells and increased ABCs in aged mice [82]. This eventually resulted in impaired production of IgG and long-lived plasma cells.

## Abundance and Functions of B Cells in the Elderly

Several studies reported a decrease of mature B cell subsets in humans with aging, although the extent of these changes varies depending on the subsets, experimental approaches, and cohorts of people [53, 83, 84]. For instance, Muggen et al. reported that numbers and relative abundance of several B cell subsets including transitional B cells, memory cells, and plasmablasts reduced with aging, particularly in individuals older than 70 years old [73]. Plasma and memory B cell percentages in the circulation and bone marrow decline, while naïve and immature B cells remain relatively stable in older people [85]. The abundance of B-1 cells, along with their ability to produce IgM, decreases with age [63]. A study found significantly low switched memory B cells, but high naïve and double-negative memory B cells in people over 65 years of age compared to younger adults [86]. The authors concluded that double-negative or so-called late-exhausted memory B cells express senescence markers and are associated with poor immune responses against influenza vaccine. Of note, switched memory B cells play a role in antibody production upon re-infection, generating a rapid response compared to naïve B cells [84]; therefore, a lower abundance of switched memory B cells is another evidence of impaired humoral immune response in the elderly.

Not only the numbers but also functions of B cells are diminished with aging. Poor antibody responses in the elderly after influenza vaccination are due to low binding and neutralization capacity of antibodies, decreased class switch recombination, hypermutations of the antibody variable regions, and higher abundance of inflammatory B cells [87, 88]. Besides, antigen-specific antibody production decreases with age, while self-reactive antibodies become more abundant, rendering old individuals more susceptible to develop autoimmune diseases [89]. All these defects in the humoral immune response lead to increased susceptibility to diseases and reduced efficiency of vaccines [90].

## TRAINED IMMUNITY: A DE FACTO INNATE IMMUNE MEMORY

Although immune memory had been attributed only to the adaptive immune system for a long time, growing evidence consistently shows the existence of memory-like characteristics in innate immune cells [91–94]. Certain infections, vaccinations, or molecules can reprogram innate immune cell types to exhibit increased responsiveness against a secondary insult. This phenomenon is termed trained immunity and mediated through extensive epigenetic and metabolic changes.

Over the last couple of years, innate immune cells, including monocytes [95], natural killer (NK) cells [96], innate lymphoid cells (ILCs) [97], DCs [98], and neutrophils [99], have been reported to exhibit trained immunity response. As innate immune cells can only recognize microbial patterns via their pattern recognition receptors (PRRs), their memory-like response is not specific to pathogens but can work against a wide range of antigens. Thus far, vaccines, such as the tuberculosis vaccine Bacillus-Calmette Guérin (BCG) [100], measles [101], and oral polio vaccine [102]; microbes/microbial patterns, e.g., β-glucan [91], Candida albicans; oxidized low-density lipoprotein (oxLDL) [103]; and metabolites such as fumarate [104] have been reported to induce heterologous protection through trained immunity. Epidemiological studies reporting decreased all-cause mortality after certain vaccinations suggested the existence of an innate immune memory [105]. The existence of trained immunity was first depicted in monocytes with an *In vitro* model and *in vivo* in mice, where C. albicans and β-glucan induced enhanced cytokine productions after the second microbial stimulation [91]. In parallel, BCG vaccination was reported to induce higher TNFα and IL-1β production against unrelated pathogens, even 3 months after the vaccination [100]. Further research demonstrated that trained immunity could persist up to 1 year and possibly even longer [106]. Considering that monocytes have a half-life around 1–2 days in the circulation [107], the programming of progenitor cells could be involved in sustaining the memory-like phenotype. Indeed, β-glucan administration leads to the expansion of myeloid lineage progenitors in the bone marrow of mice [108]. Increased myelopoiesis is associated with upregulated IL-1β and granulocyte macrophage colony-stimulating factor (GM-CSF) signaling, besides alterations in glucose and cholesterol metabolism. Another mouse study demonstrated increased myelopoiesis following BCG vaccination, which is associated with enhanced protection against M. tuberculosis infection [109]. These findings align with a recent study on humans, showing that BCG vaccination leads to the upregulation of myeloid and granulocyte-lineage genes in HSCs [110].

## Trained Immunity in the Elderly

Low-grade chronic inflammation occurring in the elderly is associated with poor innate and adaptive immune responses [111]. Koeken et al. recently reported that BCG vaccination reduces systemic inflammation, and a lower abundance of circulating inflammatory proteins at baseline is correlated with trained immunity response 3 months after vaccination in males [112]. Therefore, BCG vaccination could alleviate inflammaging while providing non-specific protection via trained immunity induction in the elderly. On the other hand, since the cell differentiation capacity of HSCs in the bone marrow changes and is skewed toward myelopoiesis with aging, inducing trained immunity could lead to unfavorable outcomes by further expanding the myeloid cell production in older people.

Nevertheless, a double-blinded placebo-controlled clinical trial demonstrated that trained immunity could be safely induced in the elderly by BCG vaccination, evident from the increased cytokine production compared to the participants who received placebo [113]. Remarkably, the trial showed that BCG prolongs the time until an infection and reduces the risk of all new infections and respiratory infections by 45% and 79% compared to the placebo group, respectively. In line with this, other trials reported a decrease in acute upper respiratory tract infections and pneumonia in older people vaccinated with BCG [114, 115]. However, more research is needed to explore the strength and longevity of trained immunity responses in older individuals compared to adults.

BCG's ability to confer protection against heterologous infections has attracted a lot of attention during the COVID-19 pandemic, which disproportionally affects the elderly. BCG is being tested in more than 20 randomized control trials to investigate if it has a protective effect against SARS-CoV-2 infection [116]. Promisingly, a recently published study from Greece reported 68% risk reduction for COVID-19 6 months after BCG vaccination [117]. Another study revealed that even an early history of BCG vaccination is associated with decreased incidence and symptoms of COVID-19 among healthcare workers [118]. Therefore, induction of trained immunity by BCG vaccination may be utilized as a preventive measure against COVID-19, especially in the vulnerable elderly group.

## AGING AS A MULTISYSTEM MALADY

Aging leaves no part of the body unscathed. Besides tissue-specific damage occurring with advanced age, the aging immune system impacts many other systems and processes. Even the organs that were once thought to be devoid of immune cells, such as the brain, are now known to harbor tissue-resident immune cells and interact extensively with the peripheral immune system. The last few decades have also witnessed a boom in research on the microbiota, the collection of up to 100 trillion microorganisms residing in human bodies, mainly in the gut [119]. The microbiota has close interactions with the host immune system and is also prone to age-related disruptions.

In the following chapters, we discuss the interplay of microbiota and the brain with the aging immune system, mainly focusing on immune memory. We especially approach this body of research from a metabolic perspective, describing various cellular metabolic programs and their impact on immune memory in aging and age-related diseases. Additionally, we point out the role of epigenetic regulation underlying all the topics discussed. By providing such a comprehensive view, visualized in Figure 1, we aim to strengthen the notion of aging as a multisystem problem and accordingly inform counteractive efforts.

### Interplay of Metabolism and Immune Memory

Metabolism and metabolic inflammation are key processes that both influence and get influenced by aging. Metabolic diseases such as type 2 diabetes mellitus, cardiovascular diseases, and obesity are also considered age-related diseases. These conditions are accompanied by chronic inflammation, termed metaflammation, which is driven by nutrient excess. Although the triggers might vary, the mechanisms underlying metaflammation and inflammaging are quite similar.

Mitochondrial dysfunction, accumulation of senescent cells and cellular debris, and hyperactivation of innate immune responses, such as inflammasome, contribute to both processes [120]. Therefore, it is crucial to understand the interplay between cellular aging, metabolism, and inflammation in chronological aging and age-related metabolic diseases to revert them.

### T Cell Metabolism

Quiescent T cells mainly use catabolic processes, while activated cells rely on anabolic processes to support protein production and proliferation. Cells need to activate a critical serine/threonine kinase, mammalian target of rapamycin

(mTOR), to induce anabolic pathways [121]. While driving growth and proliferation, mTOR also upregulates glucose transport and glycolysis. Glycolysis is one of the main pathways to generate energy. Although it is not energetically efficient — only 2 adenosine triphosphate (ATP) molecules can be generated from one glucose molecule — it generates energy very rapidly, which is of use for active and proliferating T cells [122]. Processing of glucose yields ATP, NADH, and pyruvate. Pyruvate is then converted to lactate and exported as lactic acid in the case of glycolysis or otherwise transported to mitochondria for oxidative phosphorylation (OXPHOS).

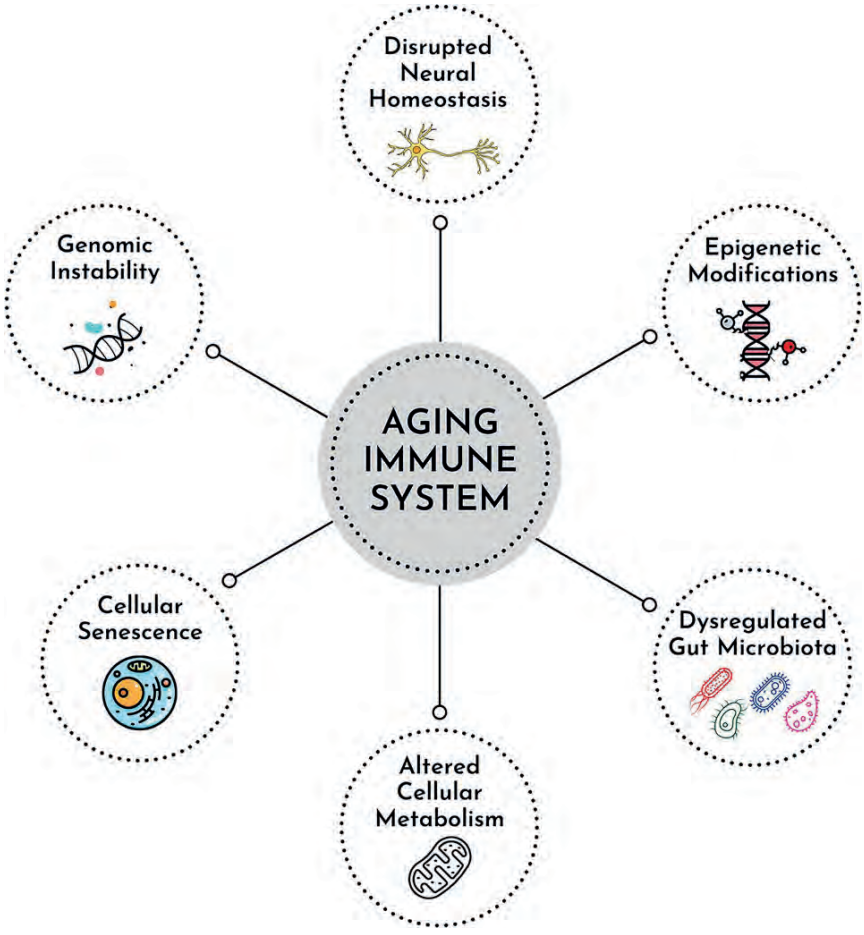


**Figure 1. The far-reaching effects of the aging immune system.** Age-related changes in immune cells include genomic instability, epigenetic modifications, altered cellular metabolism, and cellular senescence. An aged and impaired immune system has broad consequences, affecting many tissues and systems of the body. Gut microbiota and the central nervous system are profoundly impacted by and, in turn, regulate the immune system.

OXPHOS is a much more efficient bioenergetic pathway, producing 36 ATP molecules from every glucose molecule [123]. In this case, pyruvate is converted to acetyl-CoA and enters the tricarboxylic acid cycle (TCA cycle), which is coupled to the electron transport chain (TCA) through electron donors NADH and FADH2. TCA cycle can be replenished by amino acids and oxidation of fatty acids. Fatty acid oxidation (FAO) is mainly used by cells with low energy demands and plays a critical role in CD8+ memory and CD4+ Treg development [124]. Activated T cells upregulate their glutamine uptake and perform glutaminolysis to yield α-ketoglutarate, which enters the TCA cycle.

Additionally, TCA cycle metabolites can regulate immune functions in ways other than energy production. For instance, acetyl-CoA acts as the key cofactor for histone acetylation [125]. In activated T cells, acetyl-CoA is required for IFNγ production through histone acetylation [126]. Acetyl-CoA also contributes to the acetylation of mitochondrial proteins [127], which has vast functional consequences for both innate and adaptive immune cells [128]. Quiescent naïve T cells meet their energy needs with OXPHOS [129]. IL-7 and TCR signaling are essential for their metabolic regulation and survival [130, 131]. When T cells are activated, an immediate need for energy occurs for effector functions and biomass generation. The cells upregulate transporters like glucose transporter 1 (GLUT1) and engage in aerobic glycolysis, promoting cytokine production through pathways, such as the phosphoinositide 3-kinase (PI3K)-AKTmTOR axis and mitogen-activated protein kinase (MAPK) signaling [132]. The glycolytic switch is required for the effector functions, e.g., IFNγ production but not essential for proliferation [133]. OXPHOS can also be utilized for proliferation and survival purposes. Although activated T cells functionally rely on glycolysis, OXPHOS is certainly not dispensable: when OXPHOS is inhibited with oligomycin, T cell activation and proliferation are blocked [133].

Although they rely on OXPHOS and FAO in the resting state, memory T cells need to respond quickly and efficiently upon antigen encounter. Therefore, they can shift to glycolysis quicker than naïve T cells [134]. Greater mitochondrial mass and a strong mitochondrial spare respiratory capacity have been linked to this bioenergetic advantage [135, 136]. Additionally, mitochondrial fusion is essential for the development and function of memory T cells [137].

## Impact of Aging on T Cell Metabolism

Increased p38 MAPK activity is one of the characteristics of senescent T cells. Inhibiting p38 improves telomerase activity, proliferation, autophagy, and

mitochondrial fitness, in an mTOR-independent way [17]. MAPK inhibition also enhances T cell and antibody responses in influenza-vaccinated old mice [138].

Patients with gain-of-function mutations in PI3K have depleted naïve T cells but an accumulation of senescent effector cells, just like in the elderly [139]. Inhibiting mTOR activity with rapamycin treatment partially restores the senescent phenotype in these patients. Therefore, overactive PI3K/AKT/mTOR signaling is suggested as one of the drivers of T cell senescence.

Aged naïve T cells have higher mitochondrial mass, but interestingly, less mitochondrial respiratory capacity, possibly due to transcriptional downregulation of respiratory chain genes [140]. Furthermore, enzymes of one-carbon metabolism are deficient in aged naïve T cells, and supplementation with formate and glycine, one-carbon metabolism metabolites, improves cell survival and activation [141].

Autophagy is important for the generation of T cell memory, and induction of autophagy by spermidine improves CD8+ T cell responses against influenza vaccination in aged mice [142]. CD4+ memory T cells of the elderly display upregulated oxidative phosphorylation, reactive oxygen species (ROS) production, and fatty acid oxidation [143]. They also have a higher expression of Sirtuin 1 (SIRT1), a NAD-dependent deacetylase, compared to younger cells. SIRT1 and AMPK, two important nutrient-sensing molecules and negative regulators of mTOR, positively influence each other [144]. In contrast to CD4+ memory cells, aging-associated terminally differentiated memory CD8+CD28– T cells have a high glycolytic capacity, which is linked to their downregulated SIRT1 expression [145].

CD8+ TEMRA cells have a higher expression of glycolysis and glutaminolysis-related genes and a larger ATP pool compared to naïve and EM cells [146]. Despite upregulated glycolytic transcription in TEMRA cells, basal glycolysis levels are similar to naïve and EM cells. Like EM cells, TEMRA cells can quickly increase glycolysis and OXPHOS upon activation [146]. In terms of function, TEMRA cells are capable of cytotoxicity and cytokine production, despite their senescent state and impaired mitochondrial function [17, 36].

Long-term CMV infection, known to promote immunosenescence, also alters the cellular metabolism of T cells, increasing glucose uptake, promoting glycolysis, restructuring lipid rafts, and disturbing cholesterol metabolism [147, 148]. In addition, chronic inflammation due to lifelong CMV infection disrupts pancreatic β-cells and increases the risk for type 2 diabetes in the elderly [149].

## B Cell Metabolism

The metabolic pathways that regulate T cells are also essential for B cell function, although there has not been much research on B cell metabolism. When a B cell is activated upon antigen recognition by the BCR and T cell help, it activates PI3K/AKT/mTOR signaling [150]. Just like activated T cells, activated B cells need rapid energy production to increase biomass and proliferate. As a result, glucose and glutamine uptake increase, along with oxygen consumption, OXPHOS, and mitochondrial remodeling [151]. OXPHOS and glutamine-fueling of the TCA cycle have been suggested as the critical bioenergetic pathways for B cell growth and function, while glucose was dispensable [152].

A study showed that activated B cells have more mitochondria but similar amounts of mitochondrial DNA, indicating that fission of naïve B cell mitochondria with multiple nucleoids, rather than mitochondrial replication, occurs upon activation [152]. Another study suggested that mitochondrial remodeling and ROS levels determine the fate of activated B cells. Cells with increased mitochondrial mass and higher ROS levels upon activation are destined for class switch recombination, whereas cells with decreased mitochondrial mass undergo plasma cell differentiation [153].

The energy needs of activated B cells in GCs frequently shift [154]. In the hypoxic light zone, cells consume less oxygen and are more glycolytic. mTORC1 is not necessary for the regulation of glycolysis here, but it is critical, together with c-Myc, for the positive selection of the cells and migration to the dark zone for proliferation and somatic hypermutation [155, 156].

Upon GC maturation, when a cell differentiates into memory B cell, the metabolic state becomes more quiescent with dominant OXPHOS. However, rapid re-activation of mTORC1 and glycolysis is possible for later differentiation into antibody-producing plasmablasts [157]. Furthermore, memory B cells have high basal autophagy, which is essential for their survival until antigen encounter [158, 159].

GCs also output long-lasting plasma cells, which can produce thousands of antibodies per second. This, naturally, is highly energy-demanding. mTORC1 is essential for plasma cell generation and antibody synthesis [160]. Plasma cells have high levels of glucose uptake, but most of the glucose is used for protein glycosylation [161]. Still, survival and antibody production of plasma cells were impaired when the glucose transporter Glut1 was deleted [162]. Also, mitochondrial import of pyruvate, provided by glycolysis, is critical for the long-term maintenance of plasma cells [161].

Finally, tissue-resident B1 B cells are more active in glycolysis and OXPHOS than other B cells, the classical antibody-producing and memory B cells. In addition, autophagy is critical for the mitochondrial function and self-renewal of B1 cells [163].

## Impact of Aging on B Cell Metabolism

There is less literature on how B cell metabolism is regulated and impacts function as organisms age. A study showed that antibody-secreting B cells of aged individuals had lower SIRT1 expression, and higher SIRT1 levels were associated with better antibody response to multiple influenza virus strains [164]. Also, naïve and activated B cells of the elderly had slightly less glycolytic capacity and a more striking reduction in OXPHOS. In mice, aged B cells had similar glycolysis and OXPHOS rates as young counterparts but could not further enhance OXPHOS upon stimulation [165]. However, the cells were able to upregulate glycolysis to meet their energy need.

Leptin, a pro-inflammatory hormone secreted by adipocytes, is higher in the circulation of obese individuals [166]. Among non-obese people, leptin concentrations are strikingly more elevated in the elderly [167]. Leptin abundance in the serum is also positively associated with frailty [168]. After exposure to leptin, B cells from young lean individuals exhibit a similar profile as B cells of older lean and young obese individuals regarding the transcriptional profile and antibody secretion [167]. Leptin also decreases influenza-specific antibody production from B cells *In vitro*. Obesity is known to impair B cell responses to vaccination, and studies suggest that leptin might be partially responsible for this [169].

Additionally, post-transcriptional glycosylation of antibodies modulates their function, and altered glycosylation patterns have been linked to aging [170, 171]. β4-Galactosyltransferase activity increases with age [172], which would have functional consequences, although yet unexplored.

## Metabolism in Trained Immunity

Metabolic reprogramming is one of the key mechanisms underlying trained immunity (also known as innate immune memory), along with chromatin remodeling. In fact, metabolic changes can drive epigenetic changes since certain metabolites, e.g., acetyl-CoA, can regulate epigenetic enzymes [173]. Fumarate is one example of TCA metabolites driving epigenetic changes. It can induce trained immunity on its own, and its accumulation during this process induces trimethylation of histone 3 lysine 4 at the promoters of IL-6 and TNFα [104]. This is due to fumarate inhibiting the activity of lysine-specific histone demethylase KDM5.

The AKT/mTOR/HIF1α pathway is the most critical pathway for inducing aerobic glycolysis in β-glucan-trained monocytes [174]. Contrary to β-glucan-induced trained immunity, BCG upregulates not just glycolysis but also OXPHOS [175]. Glutaminolysis and cholesterol synthesis are other crucial metabolic pathways for β-glucan-induced trained immunity [104]. Interrupting these pathways blocks these processes *In vitro* and *in vivo*. BCG also induces glutaminolysis, and glutamine availability is important for the trained response [175].

Synthesis of cholesterol itself is not essential for trained immunity but rather the accumulation of the intermediate mevalonate is. Blocking mevalonate generation inhibits trained immunity, while mevalonate alone can induce trained immunity in monocytes through the activation of insulin-like growth factor 1 (IGF1) receptor and mTOR [176]. Furthermore, the changes in glycolysis and mevalonate pathways are observed not only in monocytes but also in HSPCs [108]. oxLDL, a non-microbial inducer of innate immune memory, upregulates both glycolysis and oxygen consumption, and high glucose availability further enhances the trained immunity response [103]. Similarly, catecholamine-induced trained immunity is accompanied by increased glycolysis and oxygen consumption. Of note, the particular metabolic rewiring might differ for different inducers of innate immune memory. For instance, stimulation with aldosterone is not associated with elevated glycolysis or OXPHOS but is dependent on fatty acid synthesis [177].

As of yet, trained immunity responses and associated metabolic states have not been characterized in the context of aging. However, several ongoing large-scale studies of BCG vaccination in the elderly would soon shed light on the effects of BCG-induced trained immunity on the metabolism of aged immune cells (NCT04537663, NCT04417335).

## Role of Epigenetic Alterations in Immune Memory

Epigenetic changes include histone modifications and DNA methylation that regulate the way a gene works. These modifications are dynamic and affect all cells and tissues throughout life. Environment and lifestyle, as well as aging, can lead to dramatic epigenetic alterations. For the purpose of this review, we will focus on how age-dependent epigenetic modifications alter innate and adaptive immune memory.

## DNA Methylation In Adaptive Immunity

DNA methylation is the most abundant epigenetic modification that occurs by transferring a methyl group to the 5th carbon of the cytosine [178]. DNA methylation

does not always indicate a lower gene expression; however, methylation in gene promoters is generally associated with poor TF binding and reduced transcription [179]. Biological sex, genetic background, environmental factors, and age affect the DNA methylation profile [180]. Among these factors, age-dependent methylation is very well-characterized. Remarkably, different mathematical models are developed to predict the biological age based on the methylation levels of certain CpG sites from various tissues or cells [180–182].

Advancing age is associated with a progressive loss of methylation marks on DNA [183], although abnormal hypermethylation patterns are also observed in some gene promoters [184]. Changes in the methylation landscape are linked to various age-related diseases as well as dysfunctions in the immune system. For instance, age-related macular degeneration, a disease resulting in irreversible blindness in the elderly, has been correlated with the loss of methylation in the promoter region of the IL17RC gene, leading to increased IL17RC protein levels in the blood [185].

A growing number of studies indicate that DNA methylation plays a significant role in the adaptive immune system's functioning. Age-related functional changes in immune cells, such as decreased self-renewal capacity, defects in cell differentiation, and skewed differentiation towards myeloid cell production in the elderly, are strongly correlated with epigenetic modifications occurring in HSCs during aging [186]. Murine studies show that gene expression of HSCs is regulated via hyper and hypomethylation of certain DNA regions, affecting the capacity of those cells to differentiate [187]. Expression of DNA methyltransferases in HSCs are lower in aged mice [186]. Also, HSCs of mice with decreased DNA methyltransferase activity fail to efficiently differentiate into lymphoid progeny [188]. These studies reveal that DNA methylation is essential to finetuning the differentiation capacity of HSCs and therefore the proper activity of the innate and adaptive immune system. Epigenetic modifications also modulate the function of HSCs during aging, which will be elaborated in "Histone Modifications."

Several studies report age-dependent methylation changes in T cells. A study analyzing CD4+ and CD8+ T cell methylome profiles in young individuals and the elderly found that 48,876 and 12,275 CpG sites were differentially methylated in CD8+ T and CD4+ T cells, respectively [189]. Moreover, the methylation profile of CD8+ T cells was strongly associated with aging and inversely correlated with genes linked to T cell differentiation and immune response, suggesting a possible link between weakened T cell responses and age-related alterations in DNA methylation.

The age-associated methylation profile of CD4+ T cells is characterized by hypermethylation of CpG sites enriched in the polycomb repressive complex 2 (PRC2) genes and hypomethylation of CpG sites enriched in enhancer regions [190, 191]. Of note, the PRC2 proteins regulate histone methylation, cell differentiation, and proliferation [192]. These methylation patterns identified by Dozmorov and colleagues were highly similar to the methylation and transcriptomic profile of T cells from lupus patients. Lupus erythematosus, an autoimmune disease leading to autoreactive T cells, is characterized by defects in the MAPK signaling pathway and increased mTOR activity resulting from altered methylation patterns [193]. Therefore, the authors suggested that the age-dependent methylation profile of naïve CD4+ T cells might render the elderly susceptible to autoimmune diseases, such as lupus, though this remains to be formally demonstrated.

Loss of CD28 co-stimulatory protein in CD4+ T cells is one of the well-characterized aging marks, leading to impaired T cell activation and differentiation. Comparison of methylation profiles of CD28+ and CD28null T cells revealed 296 differentially methylated genes associated with poor TCR signaling and cytotoxic response [194]. Furthermore, the expression of the genes involved in inflammasome activation was higher in CD28null T cells, suggesting that these cells have a higher pre-activation state. Another study reported that increased methylation at the BACH2 locus of the CD4+ T cells in the middle and old age groups results in lower BACH2 expression [195]. BACH2 has a regulatory role in immune responses, modulating CD4+ T cell differentiation and controlling inflammation [196]. Overall, alterations in the DNA methylation patterns contribute to CD4+ T cells becoming more inflammatory in the elderly.

A few studies shed light on the DNA methylation profile of B cells during activation and diseases [197–200]; however, whether B cells are affected by age-dependent methylation changes is yet to be known.

## Histone Modifications in Adaptive Immunity

N-terminal histone tails are targets for post-translational enzymatic modifications including acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation [201]; however, this review will focus on methylation and acetylation, which are the most well-characterized alterations regulating histone structure. Methyl groups are added to the histone by histone methyltransferases and removed by histone demethylases [202]. The trimethylation of histone 3 lysine 4 (H3K4me3), histone 3 lysine 36 (H3K36), and histone 3 lysine 79 (H3K79) are linked to open and actively transcribed regions [203]. On the other hand, mono-methylation of histone 3

lysine 9 (H3K9me), histone 3 lysine 27 (H3K27me), and histone 4 lysine 20 (H4K20me) is associated with closed and inactive chromatin regions. Furthermore, histone acetylation is associated with loosened chromatin structure and increased gene transcription [204]. Histone acetyltransferases catalyze lysine acetylation, whereas histone deacetylases (HDACs) reverse the modification [205]. Post-translational modifications of histones do not only influence the accessibility and transcription of genes but also modulate alternative splicing, DNA replication, and repair [206].

Histones and epigenetic marks on histones undergo transitions with aging. HSCs from old mice have more H3K4me3 and H3K27me3 peaks compared to young HSCs [186]. In addition, expression of FLT3, one of the regulators of CLPs, was decreased due to H3K27me3 in the old HSCs, suggesting a link between poor lymphoid differentiation potential of HSCs in the elderly. An extensive study performed in young and old monozygotic twins showed that chromatin modifications during aging are non-heritable [207]. Moreover, histone modification profiles are, to some extent, homogenous in young individuals and heterogeneous among elderly subjects. Heterogeneity in histone modifications was observed between individuals and also cell types in the elderly.

Epigenetic changes are one of the underlying causes of the major defects seen in CD8+ T cells of the elderly. More closed chromatin regions are observed in the enhancer and promoter regions of the genes related to T cells signaling in the elderly compared to the young [208]. Furthermore, IL-7R, in the memory CD8+ T cells, is one of the top genes related to multiple closed chromatin peaks in the elderly. As IL-7 ensures homeostasis and maintenance of T and B cells, poor IL-7 signaling in the elderly might be an underlying reason of impaired adaptive immune response [209]. Furthermore, naïve CD8+ cells in the elderly have lower chromatin accessibility at the gene promoters associated with poor nuclear respiratory factor 1 (NRF1) binding [140]. Considering the role of NRF1 in oxidative phosphorylation, decreased chromatin activity might partially explain the impaired CD8+ T cell metabolism in the elderly [210]. Other significant findings of the study are that open chromatin regions are associated with a memory cell profile, and accessibility of the promoters is diminished in aged individuals.

As mentioned in the DNA methylation section, an age-associated decrease in BACH2 expression is observed in CD4+ T cells. Another mechanism leading to lower BACH2 gene transcription is due to Menin deficiency observed in immune senescence [211]. Menin induces BACH2 expression by binding to its locus and maintaining histone acetylation. Decreased binding of Menin to BACH2 locus and

subsequently reduced BACH2 expression contributes to immunosenescence in CD4+ T cells.

A study investigating the epigenetic changes in B cell precursors in old and young mice associated these alterations with gene expressions [212]. It revealed that aged pre-B cells exhibit a loss of H3K4me3 at the promoter site of insulin receptor substrate 1 (IRS1), which is associated with lower transcription. As insulin signaling is necessary for the development of B cells in the bone marrow [213], decreased insulin growth factor (IGF) signaling might lead to defects in B cell development.

## Epigenetic Reprogramming as a Hallmark of Trained Immunity

A distinct epigenetic profile regulates trained immunity responses following the first insult. As a result of certain infections or stimulations, primed cells undergo an epigenetic reprogramming that allows them to respond stronger upon a heterologous infection by facilitating the transcription of genes related to inflammation and metabolism [106].

H3K4me3 is the first characterized epigenetic mark in monocytes after β-glucan treatment [91]. Further analysis revealed that H3K4me3 peaks are enriched at the promoter sites of TNF, IL6, IL18, DECTIN1, and MYD88 genes, indicating that gene transcriptions are more active in these regions. In addition, increased H3K27ac is a well-characterized histone mark in trained cells, promoting glycolysis and PI3K/AKT pathway activation [174, 214]. Besides the enrichment in H3K4me3 and H3K27ac, decreased H3K9me3 was found in the promoters of genes related to cytokine production and glycolysis [175]. Since H3K9me3 is a repressive mark, reduced trimethylation suggests the presence of open chromatin regions. These studies show that trained immunity responses are modulated by epigenetic modifications that facilitate enhanced cytokine responses and specific metabolic changes. Trained cells share a common epigenetic profile; however, different stimuli could lead to minor unique epigenetic alterations.

Infections and certain stimulations leave marks on the DNA methylation profile, as well as histones, of innate immune cells [215]. Studies demonstrate the role of DNA methylation in anti-mycobacterium response following BCG vaccination, discriminating responders from non-responders [216, 217]. Responders to BCG vaccination were characterized by reduced DNA methylation at the promoters of inflammatory genes [216]. However, whether DNA (de) methylation plays a direct role in the development of nonspecific protective responses is still being investigated.

As in adults, trained immunity is modulated by histone modifications in the elderly. Giamarellos-Bourboulis and colleagues recently showed that increased cytokine production upon BCG vaccination in the elderly was accompanied by acetylation of H3K27 at the promoter regions of TNF and IL6 genes [113]. However, further studies are warranted to compare the epigenetic differences following innate immune memory development between adults and older individuals and explore how aging influences epigenetic marks in the context of trained immunity.

## Gut Microbiota Modulating Immune Memory

Aging causes changes throughout the whole body of humans, and trillions of microbes living there are no exemption. The composition and diversity of gut microbiota dynamically shift in infancy, remain relatively stable during adulthood, and start to decline with old age [218].

## Interactions of Microbiota and the Adaptive Immune System

The gut microbiota has essential roles in educating the adaptive immune system by inducing a certain level of immune response and fine-tuning the inflammation. For instance, Bacteroides fragilis, a commensal in the gut, enhances and regulates CD4+ T cell differentiation into T helper 1 (Th1) and Th2 [219]. In the presence of gut bacteria and TGFβ, naïve CD4+ T cells become Tregs, producing IL-10 to maintain immune homeostasis. On the other hand, Tregs and Th17 cells in the lymphoid follicles of the gut induce B cell class switching, resulting in IgA secretion [220, 221]. Microbiota-associated IgA, IgM, and IgG secretion from B cells also occurs via TLR signaling activation without T cell help [222].

The adaptive immune system can limit the inflammatory response against commensal gut microbes mediated by the innate immune system. IgA produced by B cells is explained as a part of sustainable host-microbe interaction, controlling the inflammatory response against beneficial microorganisms [223]. Besides, intestinal Treg cells express TCRs for intestinal antigens, such as metabolic products and commensals, while other Tregs in the body express TCRs for self-antigens [224]. In this way, intestinal Tregs suppress immune responses against intestinal antigens and play an immunoregulatory role in the guts.

How microbiota strikingly shapes the adaptive immune system development was also demonstrated in germ-free mice: the lack of microbial species in the gut is characterized by defects in secondary lymphoid tissue development [225], low IgA production [226], and reduced Th17 cells and Tregs [227]. It should be noted that

short-chain fatty acids (SCFAs) produced by microbial species in the gut greatly contribute to the immune system development and responses [228].

A healthy gut microbiota composition is important in protecting individuals from diseases. As an example, IL-10 secreting IgA+ plasma cells and plasmablasts originating in the gut confer resistance to experimental autoimmune encephalomyelitis induced in mice [229]. Another study reported that gut microbiota protects against respiratory infections induced by *S. pneumoniae* and *K. pneumoniae* by inducing GM-CSF and IL-17A secretion [230].

## The Role of Dysbiosis in Aging

The incidence of gut dysbiosis, the imbalance of microbial species, increases with age and is associated with numerous health problems [231]. However, it is unclear whether cellular and molecular alterations of the immune cells during aging affect the composition and functioning of the gut microbiota, or if age-related dysbiosis triggers defective immune responses. It is likely that both are concurrently true, but a better understanding of the gut microbiota-immune system interactions is necessary to resolve this question.

As individuals age, a decline in certain beneficial bacterial species, such as *Bifidobacterium*, is replaced by the growth of pathogenic species, i.e., *Enterobacteriaceae* [232]. A decrease in *Firmicutes* and increase in *Proteobacteria* are also reported in older people [233]. Besides, gut dysbiosis is associated with several age-related diseases, including obesity [234], type 2 diabetes [235], Alzheimer's disease [236], and increased incidence of infections [237–239]. The risk of developing cancer is also higher in the elderly due to dysbiosis-associated chronic inflammation, debilitated phagocytosis of senescent and dormant tumor cells, and impaired activation of tumor-specific CD8+ T cells [240].

Dysbiosis was also proposed to be a major reason for various age-associated pathologies and premature death in older individuals by triggering excess inflammation and several complications, including leaky gut and diminished functions of the gastrointestinal tract [228]. In line with this, a particular composition and diversity of microbial species is correlated with health, fitness, and increased survival in the elderly [241, 242]. A recent study revealed that healthy elderly experience a particular drift in their microbiota composition, while this drift is missing in the frail elderly [242]. Furthermore, having high *Bacteroides* abundance during aging correlates with decreased survival rate over the 4-year follow-up. Another recent work with 15 years of follow-up reported that *Enterobacteriaceae* abundance

was significantly linked with deaths related to gastrointestinal and respiratory causes in the elderly [243].

Dysbiosis can lead to defects in intestinal barrier integrity, which results in the translocation of bacterial species to the host tissues. Those bacteria create inflammation through the recruitment of neutrophils and differentiated Th17 cells [244]. For example, translocation of a gram-positive pathobiont *E. gallinarum* that results from defects in the gut barrier induces Th17 response and autoantibody production [245].

*Akkermansia* is a beneficial commensal shown to protect the gut barrier integrity [228] and enhance antibody and T cell responses [246]. Loss of *Akkermansia* is associated with insulin resistance in aged non-human primates and mice [247]. Decreased butyrate and *Akkermansia* abundance increase gut leakage, which in turn increases pro-inflammatory responses.

A human study, on the other hand, reported that *Akkermansia* is more abundant in the elderly [248]. Furthermore, *Akkermansia* was significantly correlated with serum IgA and CD8+ T cells and negatively correlated with CD4+ T cells in older people. *Bacteroidetes*, which are less abundant in the elderly, were positively correlated with serum IgG levels and CD4+ T cell abundance in the middle age group. In conclusion, this study highlights the relationship between the adaptive immune system and gut microbiota composition, although the direct link between them is missing.

Microbiota also affects disease course and vaccine responses in the elderly. Even though the antiviral therapy for human immunodeficiency virus (HIV) is successful and increases the life expectancy of patients, older HIV + people suffer more from comorbidities compared to HIV – elderly. HIV + elderly have less CD4+ T cells and more CD8+ T cells than HIV individuals older than 55 [249]. In addition, the abundance of *Prevotella* in the gut is significantly higher in the individuals with low CD4+ T cell counts. *Prevotella* was previously associated with cardiovascular diseases [250], but how it interacts with the immune system is not yet clear.

Age-dependent alterations in gut microbiota are likely to contribute to poor immune responses after vaccinations [251]. Some studies reported that probiotics supplements increase the antibody titers after influenza vaccine in the elderly [252–255], whereas a few studies showed limited or no effect [87, 256, 257]. Variations in the results could be due to multiple factors, including the sample size,

type of probiotics, and delivery route. Nevertheless, studies strongly suggest that imbalances in microbiota cause impaired immune responses, and restoring the healthy composition might be beneficial for a better vaccine response in the elderly.



## Innate Immune Memory Induction by Gut Microbiota

As the adaptive immune cells, members of the innate immune system closely interact with the gut microbiota. A few studies suggest that microbiota could regulate immune memory development by priming or tolerizing the cells with microbial antigens and SCFAs. For instance, β-glucan, a fungal cell wall component, and BCG act through Dectin-1 and NOD2 signaling pathways, respectively [91, 100]. Since Dectin-1 and Nod-like receptors (NLRs) are found on various cell types in the intestines, including non-immune cells, it is plausible to propose that these cells develop immune memory due to their exposure to the gut microbiome. Supporting this argument, peptidoglycan fragments derived from gut microbiota were shown to prime the innate immune system, promoting the killing capacity of neutrophils [258]. Furthermore, gut microbiota was shown to induce myelopoiesis to protect mice against infection [259], similar to the increase in the number of myeloid progenitors in the bone marrow of mice following trained immunity induction by β-glucan administration [108]. Other microbiota-derived components, such as lipopolysaccharide (LPS), flagellin, and β-glucan, might also be able to induce trained immunity in the guts, although the dose of the stimuli is critical for immune memory or tolerance response [260].

As mentioned before, trained immunity is mediated by extensive metabolic and epigenetic programming. Molecules and metabolites produced by commensal gut microbes and microbes themselves are able to induce such changes in both innate and adaptive immune cells [261]. For example, despite causing an increase in the anti-microbial activity, butyrate produced by gut microbes have effects opposite to trained immunity in macrophages, possibly stemming from decreased mTOR activity and inhibition of HDAC3 [262].

It is important to note that non-immune cells, e.g., fibroblasts [263], epithelial cells [264], and intestinal stromal cells (ISCs) [265] are also capable of forming immune memory, showing increased responsiveness after secondary infection. It was shown that ISCs could clear infection more rapidly during a secondary related or unrelated infection, indicating the presence of immune memory [266]. Therefore, non-immune cells also contribute to the homeostasis between gut microbes and the immune system.

Considering the strong links between gut microbiota and induction of innate immune memory, it would be conceivable to hypothesize that trained immunity response could be dysregulated by the dysbiosis in the elderly. Poor trained immunity response could render the elderly more susceptible to infections, while exuberant response might contribute to disease pathogenesis. However, more research is needed to understand how age-related changes in microbiota affect innate immune memory.

## Cross-talk Between the Immune System and the Brain

Aging causes a great deal of deterioration in the central nervous system (CNS) through DNA damage, accumulation of waste products, oxidative stress, disturbed energy homeostasis, and impaired function [267]. The brain and the rest of the CNS are not immunologically isolated, as once thought: there is extensive cross-talk between the immune system and the CNS. Brain homeostasis and regeneration depend on a robust immune system [268]. Therefore, deterioration of the immune system with old age contributes to and escalates brain aging and neurodegenerative diseases.

In the CNS parenchyma, the resident immune cell type is the microglia, which originates from primitive macrophage progenitors in the yolk sac early in development [269]. Microglia are extremely important for the maintenance of a healthy brain. They perform immunosurveillance, respond to infections, orchestrate the communication with the circulating immune system, regulate neurons, and other cell types in the brain, phagocytose cellular debris, misfolded proteins, toxic products, and even synapses [270]. Microglia are altered by aging and contribute to age-related neurodegenerative diseases [271]. Their phagocytic capacity is reduced with advancing age, and they contribute to a state of chronic low-grade inflammation. Due to this review's focus on immune memory, we will not go into detail on microglia and instead focus on the role of adaptive immunity and trained immunity in the context of brain aging.

The blood–brain barrier (BBB) largely prevents the infiltration of immune cells into the brain. However, certain immune cell types are present in the cerebrospinal fluid (CSF) and the blood-CSF barrier at the choroid plexus (CP) [272]. CP, located in the brain's ventricles, is a CSF-producing epithelial cell network with embedded capillaries. T cells are present in CP, and they regulate immune cell trafficking into CSF by IFNγ-dependent activation of CP epithelium [273].

Immune cells contribute to neuronal survival and neurogenesis during homeostasis, upon injury, or under neurodegenerative conditions [272]. Damage to the CNS induces a protective T cell response that prevents neuronal loss [274]. CD4+ lymphocytes play the most prominent role in this "neuroprotective immunity."

## Neuroprotective T Cell Immunity

CP harbors CD4+ T cells with an effector-memory phenotype that recognize CNS-specific self-antigens [275]. These cells can receive signals from circulation through the epithelium and the CNS through the CSF and orchestrate an integrated response to maintain brain homeostasis [276]. Astrocytes, a cell type that helps maintain synapses and the BBB, among various other functions, assume a neuroprotective phenotype and reduce neuronal apoptosis when co-cultured with T cells [277]. During spinal cord injury, CNS-specific autoreactive T cells migrate to the injury site, inhibit cyst formation, and contribute to the preservation of axons [278]. In T cell-deficient mice, the proliferation of progenitor cells is reduced, leading to lower numbers of new neurons, while neurogenesis is boosted in transgenic mice with excess CNS-specific autoreactive T cells [268]. Supplementation of the T-cell-derived cytokine IFNγ can enhance neurogenesis in old mice with Alzheimer's disease [279]. CNS-specific T cells are also critical for spatial learning and memory. In immunodeficient mice, spatial memory is impaired but can be restored with reconstitution of immune cells even in aged mice [280]. In models of the motor neuron disease amyotrophic lateral sclerosis (ALS), T cell deficiency accelerates the disease, while reconstitution promotes neuroprotection and delays disease progression [281–283]. However, of note, T cells contribute to the death of dopaminergic neurons in mouse models of Parkinson's disease [284].

One mechanism through which T cells improve brain maintenance is the regulation of brain-derived neurotrophic factor (BDNF). BDNF signaling via tropomyosin receptor kinase B (TrkB) plays wide-ranging roles, for example, in adult neurogenesis [285], memory formation and retrieval [286, 287], and is regulated by anti-depressant treatments [288]. BDNF levels are lower in T cell-deficient mice [268]. BDNF is associated with depressive behavior and immunization of mice with a myelin-derived peptide, generating CNS-specific immunity, restores BDNF levels, improves neurogenesis, and reduces depressive behavior [289]. Furthermore, healthy stress response in mice is associated with T cell trafficking in the brain and BDNF levels. Anxious behavior caused by stress is also reduced by immunization with a myelin-derived peptide [290]. Apart from neurons and microglia, T cells themselves are shown to secrete BDNF [291].

Tregs are also shown to be protective and delay disease progression in ALS by reducing microglial activation [292]. In models of Alzheimer's disease, Treg transplantation enhances cognitive abilities and reduces amyloid plaques [293]. Moreover, a lower Treg/Th17 ratio is correlated with more severe disease in patients with multiple sclerosis, a debilitating autoimmune disease affecting neurons [294].

Although an overexuberant immune response would impair brain function, a fine-tuned T cell immunity is clearly vital for healthy brain homeostasis and recovery from injury. Any intervention targeting this phenomenon must be carefully controlled to avoid inflammatory damage; however, the insights into adaptive immunity's role in brain health open up new avenues to counter brain injury or age-related neurodegenerative diseases.

## Trained Immunity in Microglia

Recent studies suggest that innate immune memory can be induced in microglial cells. One study found epigenetic reprogramming in microglia present for at least 6 months upon systemic LPS administration [295]. Interestingly, while a single LPS injection induced a trained phenotype in microglia, repeated LPS injection led to the induction of tolerance. Similarly, low-dose TNFα administration was also found to induce microglia training. In a mouse model of Alzheimer's disease, trained immunity exacerbated the disease while tolerance alleviated it. A recent study confirmed the finding of LPS-induced training and demonstrated that systemic β-glucan administration could also induce trained immunity in microglia [296]. However, the trained phenotype of microglia was only observed two days after the priming and was no longer present at day 7, possibly indicating a lack of sustained epigenetic reprogramming. Therefore, it is worthwhile to investigate the strength and persistence of training with different doses and different injection regimens.

## The Aging Brain

Many brain functions deteriorate with aging, with some even starting to decline after the third decade of life [297]. The impaired functions include processing speed, problem-solving, fluid reasoning, perceptual abilities, verbal fluency, and working memory. However, the impairments do not necessarily correlate with chronological age. It is rather an outcome of increased maintenance demand through the accumulation of damage and the inability of the immune system to monitor the brain to meet these demands. Of course, aging contributes to both the demand and the incapacity of the immune system through the mechanisms discussed earlier. Aged microglia develop a pro-inflammatory phenotype [298]. Following a head injury or infection, they produce an excessive amount of pro-inflammatory cytokines

for a longer time compared to a healthy young brain [299]. This inflammatory state leads to inhibited neurogenesis [300, 301]. A pro-inflammatory environment also inhibits modulators of long-term memory such as BDNF and activity-dependent cytoskeletal-associated protein and causes memory dysfunction [299]. Circulating BDNF levels decrease with age in humans, and brain levels are shown to decline in rodent models [302], which might reflect the age-associated drop in T cell numbers and function.

Aging is also associated with increased recruitment of effector memory CD8+ T cells to the CP and the meninges — the membranes covering the brain [303]. These cells were shown to impair microglial function during homeostasis but enhance pro-inflammatory cytokine production upon injury. Moreover, Treg numbers are elevated in elderly individuals; however, their migratory capacity and function are likely impaired since they are not able to control neurodegeneration. For instance, Tregs of multiple sclerosis patients have less immunosuppressive capacity and are unable to survive in sclerotic lesions in the brain [304].

In the case of chronic inflammation, while innate immune cells typically display tolerance leading to lower cytokine production, microglia acquire a primed to exhibit a more inflammatory phenotype, accelerating cognitive decline [305]. In addition, high levels of circulating TNFα observed in aged organisms might also cause damage by inducing trained immunity in microglia, as discussed above. Therefore, a well-balanced innate immunity is as essential for the healthy maintenance of the brain as adaptive immunity.

## TACKLING IMMUNE AGING FROM ALL ANGLES

Efforts to slow or revert aging are far from scarce. However, the outcome measures assessed by most studies are restricted in the sense that they do not offer mechanistic insights or focus on specific processes. Yet, some exciting interventions, including caloric restriction, metformin, and physical exercise, interfere with aging on multiple levels encompassing immunity, metabolism, epigenetics, microbiota, and the nervous system (Figure 2). The following chapters discuss different ways to tackle the aging problem and detail the mechanisms of the most promising anti-aging treatments.

## Metabolic Interventions

For most of the human evolution, nutrients were scarce, and a great deal of physical activity was required to obtain them. Thus, humans evolved to adapt to those conditions. Our current sedentary lifestyle with an overabundance of nutrients is proposed to cause the high prevalence of metabolic diseases, such as obesity, diabetes, and cardiovascular disease [306]. Furthermore, age is a risk factor for these conditions, as mentioned before, and immunosenescence has a lot in common with metabolic disease profiles. Therefore, focusing on metabolic interventions is a sensible approach to tackle aging and metabolic disorders simultaneously. Caloric restriction (CR) and exercise, bringing us closer to the ancestral conditions, take the lead in this line of research. CR refers to a reduction of total calory intake by 20–40%.

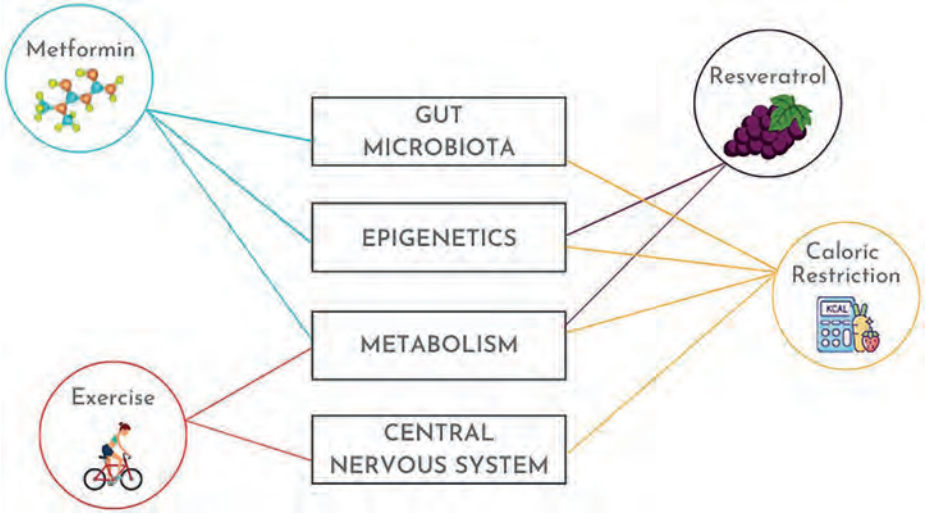


**Figure 2. Promising anti-aging interventions that target multiple facets of the aging process.** Metformin delays stem cell aging, improves mitochondrial function, prevents telomere shortening, reverses age-related epigenetic modifications, and reduces gut leakiness and dysbiosis. Physical exercise, even if initiated late in life, improves immune cell numbers and functions, restores mitochondrial metabolism, prevents cellular senescence, counteracts cognitive decline, and reduces risks for neurodegenerative diseases. Resveratrol, available in grapes and red wine, acts as an antioxidant, extends lifespan in various model organisms, attenuates systemic inflammation, and slows epigenetic aging. Caloric restriction by 20–40% enhances lifespan and reduces all-cause mortality in non-human primates, delays epigenetic aging, restores gut microbiota, and slows cognitive decline. Cellular mechanisms shared by these treatments include limitation of the mTOR/AKT axis and activation of AMPK and SIRT1.

From yeasts to non-primates, CR has repeatedly been shown to enhance lifespan [307]. In rhesus monkeys, CR starting from young adulthood reduced the risk of mortality related to age-related causes by threefold and all-cause mortality by 1.8-fold [308]. In another study, CR decreased the incidence of diabetes, cancer, and cardiovascular disease while also delaying disease onset [309]. A contrasting study reported no improvement in survival, although the incidence of cancer and diabetes was reduced [310].

In a randomized controlled trial of 218 non-obese people, a 2-year CR diet reduced circulating TNFα levels and strikingly decreased cardiometabolic risk markers, such as cholesterol and triglycerides, without any intervention-related adverse effects [311]. So far, there is no human study reporting a significant effect of CR on longevity. Large and extensive studies with genetically diverse populations are needed to solidify the promise of CR in humans.

Various metabolic impacts of CR include downregulation of mTOR and insulin signaling and activation of SIRT1, which all have broad implications on immune cell function [312]. CR is shown to delay T cell senescence in rhesus monkeys [313]. Furthermore, CD4+ and CD8+ naïve T cell pools were expanded, and thymic output and T cell proliferation were increased, but IFNγ production by CD8 + cells was reduced after CR. Although reducing the amount of calories taken seems to reverse age-induced metabolic changes and improve health and longevity, it is important to note that a few studies in rodents reported an impaired adaptive response and increased mortality against influenza A and West Nile viruses in elderly animals after CR [314, 315]. However, a recent mouse study revealed protective effects of CR against M. tuberculosis infection. This effect was related to metabolic shift characterized by mTOR inhibition but enhanced glycolysis and reduced FAO, along with increased autophagy [316]. mTOR inhibitor rapamycin acted synergistically with CR and further enhanced autophagy, leading to more efficient inhibition of M. tuberculosis.

Similar to CR, exercise is promising to interfere with immunosenescence. Regularly exercising older women had better NK and T cell functions compared to age-matched sedentary women [317]. Naïve T cell numbers and thymic output were higher in physically active elderly, similar to young adults, compared to sedentary ones [318]. They also had lower circulating IL-6 and higher IL-7, which is essential for T cell development. However, senescent CD8+ T cell numbers did not differ between groups. After an 8-week training program, immune cells of elderly adults displayed enhanced autophagy and downregulated NLRP3 inflammasome [319].

Exercise also improved mitophagy and mitochondrial biogenesis in skeletal muscle cells and immune cells alike, restoring the cellular metabolic status impaired by aging [320].

Apart from lifestyle interventions, chemical metabolic regulators are also investigated for their anti-aging potential. Metformin, safely used in humans for more than 60 years for its glucose-lowering effect, attenuates age-associated hallmarks through a plethora of mechanisms. These include activation of AMPK, inhibition of mTORC1, improved mitochondrial biogenesis, downregulation of insulin/IGF1 signaling, and activation of SIRT1 [321]. Furthermore, metformin delays stem cell aging and reduces telomere shortening. Overall, it seems to act on all hallmarks of aging. A large clinical trial of more than 3000 individuals aged 65–79 is currently being planned to assess the anti-aging potential of metformin (https://www.afar.org/tame-trial).

Everolimus, another mTOR inhibitor, attenuated immunosenescence and improved antibody responses to influenza vaccination in the elderly [322]. Even though most immune cell subsets were not altered in this study, T cells positive for programmed cell death protein 1 (PD-1), a marker of exhaustion, were markedly reduced. A follow-up study with 264 elderly subjects reported upregulated antiviral expression, improved response to influenza vaccination, and overall fewer infections [323].

SIRT1 activation is another approach to tackle immunosenescence. It is known to improve B cell proliferation and function, and therefore could help improve antibody responses declining with age [324]. SIRT1 can modulate metabolic pathways through protein and histone deacetylation [325]. Targets of SIRT1 include NF-κB, hypoxia-inducible factor 1-alpha (HIF1α), and FOXO transcription factors. Moreover, SIRT1 activation potentiates BCG-induced trained immunity response [326]. Despite mouse studies with SIRT1-activators showing delayed age-related phenotypes and increased lifespan [327, 328], there is no evidence suggesting that SIRT1 is associated with longevity in humans [329].

Resveratrol, a polyphenol compound found in red wine, is a potent activator of SIRT1 [330]. It is also shown to activate AMPK, therefore repressing mTOR signaling [331]. Apart from *In vitro* studies and inflammatory disease models displaying resveratrol's antioxidant and anti-inflammatory activity [332], several mice studies reveal its antiviral capacity [333, 334]. In terms of longevity, studies failed to report a significant lifespan extension by resveratrol in healthy mice [327, 335]. However, in mice fed with a high-calorie diet, resveratrol shifted

the transcriptional profile towards that of standard-fed mice [336]. It also improved insulin sensitivity and increased survival. Similar results were observed in rhesus monkeys on a high-fat, high-sugar diet [337]. Thirty-day supplementation of obese men with resveratrol induced metabolic changes through the AMPK-SIRT1 axis and reduced systemic inflammation, glucose, and triglyceride levels [338]. However, a similar study did not report any beneficial effects of resveratrol [339].

Overall, there are highly promising therapeutic approaches targeting metabolic pathways underlying immunosenescence and age-associated metabolic diseases. However, large-scale randomized control trials in humans are needed to see whether these exciting observations in nonhuman primates and smaller model organisms are translatable for human use.

## Strategies Modulating Epigenetics

Epigenetic interventions have been employed for several age-related diseases, e.g., cancer, diabetes, and Alzheimer's disease; however, only a few studies specifically target aged-dependent changes in the epigenetic structure [340]. Instead, metabolic interventions employed to halt immunoaging also work by altering the age-associated epigenetic landscape. Resveratrol, CR, and metformin are three promising therapy options for reconfiguring age-related DNA methylation and histone modifications in the elderly.

An intriguing study revealed that regenerating the thymus resulted in a 2.5-year younger epigenetic age [341]. Participants between 51 and 65 years of age received a 1-year treatment with recombinant human growth hormone, dehydroepiandrosterone (DHEA), which is a steroid hormone precursor, and metformin. The treatment led to restored functional thymic mass, changes in the immune cell subsets, and cytokine production, as well as altered epigenetic profile, which was associated with younger age.

Rhesus monkeys, who were exposed to 40% caloric restriction, were late to display the methylation changes found in the older monkeys [342]. Although this study does not provide direct evidence of a longer lifespan associated with delayed methylation drift, it suggests that CR could be used to slow down the aging process. In line with this, improving the lifespan of mice with resveratrol or CR resulted in slower epigenetic aging [343]. Life-long CR has also shown to prevent age-related DNA methylation changes in the brain, providing neuroprotection [344].

A few studies explain how CR could affect epigenetics. These mechanisms include decreased histone acetylation mediated by increased SIRT1 expression, higher DNA methyltransferase (DNMT) activity, and hypermethylation of specific regulatory genes, such as Ras [340]. Similarly, metformin acts on epigenetic marks via activating SIRT1 and inhibiting HDACs [345]. To our knowledge, there is no research investigating the effects of CR on aging-related epigenetic alterations, possibly due to the limitations of implementing such long-term interventions on humans.

## Potential Treatments Targeting Microbiota

Since gut microbiota regulates host metabolism, anti-aging interventions targeting metabolism inevitably affect the gut microbiota. As an example, besides acting on metabolic pathways, metformin modulates the gut microbiota. A study investigating the effects of metformin in obese and aged mice found a decrease in IL-1β and IL-6 in the epididymal fat, which was associated with changes in the gut microbes [346]. Furthermore, type 2 diabetes patients who take metformin had a higher abundance of *Akkermansia* in their guts [347], which was correlated with lower bacterial translocation and risk of dysbiosis [348]. In line with these, metformin reduced age-related leaky gut and inflammation in mice [349].

Another treatment strategy to halt immunoaging by targeting the microbiota is the use of pro and prebiotics. Probiotics are supplements containing live microorganisms, while prebiotics is substrates that microorganisms can utilize for a living [350]. Although there is conflicting evidence, studies suggest that regular probiotics use can modulate the diversity and abundance of the gut microbes, decreasing the incidence of dysbiosis [351, 352]. Probiotics are associated with improved immune responses evident from increased B and T cell counts, enhanced NK cell activity [353] and higher IgA production against influenza virus in older individuals [354]. Furthermore, supplementation with probiotics helped reduce the growth of opportunistic bacteria *Clostridium difficile* among the elderly [355]. Contrary to these findings, a meta-analysis of 10 randomized controlled studies showed no beneficial effect of probiotics on decreasing inflammatory cytokine production [356].

The combination of probiotics with prebiotics, i.e., synbiotics, also has beneficial effects, like probiotics supplementation. Two months of treatment in elderly individuals with a synbiotic formula significantly improved the metabolic syndrome parameters in circulation and decreased inflammatory proteins, such as TNFα and C-reactive protein [357]. A double-blind 4-week symbiotic treatment study reported an increase in *Bifidobacteria, Actinobacteria, Firmicutes*, and the metabolite

butyrate in the treatment group compared to placebo, while *Proteobacteria* and pro-inflammatory cytokines were lower [358].



Caloric restriction could be another treatment strategy to improve cognitive functions, metabolic parameters, and gut microbiota in the elderly. CR slowed the cognitive decline in a mouse model of Alzheimer's disease, associated with increased Bacteroides in the guts. Aged mice receiving 30% fewer calories for 2 months displayed significant shifts in their microbiota towards a more balanced composition similar to that of young mice [359]. Lifelong CR induced more extensive changes in the microbiota, reduced the concentration of inflammatory peptides, and increased the lifespan of mice [360]. However, a recent study revealed that severe CR, more than 50%, disrupts the diversity of microbiota and leads to the growth of pathogenic bacteria *C. difficile* [361]. Thus, it is critical to carefully determine the extent and duration of CR.

## Interventions for Brain Aging

Physical exercise is an excellent way of promoting brain health. Exercise counteracts cognitive impairment, reduces dementia risk, improves spatial memory, and enhances neuroplasticity [362]. Physical activity can attenuate the effects of risk alleles for memory impairment [363] and protect against the development of Alzheimer's disease [364, 365]. A systematic review of 16 studies with a total of 163,797 participants reported that regular exercise led to 28% and 45% risk reduction in dementia and Alzheimer's, respectively [366]. Of note, exercise-associated risk reduction was observed in most of the individual studies irrespective of the frequency and intensity of the exercise.

Studies suggest the antioxidant and anti-inflammatory effects of exercise as potential mechanisms behind neuroprotection [367, 368]. Anti-inflammatory consequences of exercise include reduced circulating IL-6 but increased IL-10 and IL1RA, lower numbers of Treg, and higher numbers of inflammatory monocytes in circulation, and inhibited monocyte function [369]. Besides these, physical exercise is associated with reduced senescent T cells, increased NK cell cytotoxicity and neutrophil phagocytosis, and longer telomeres in leukocytes [370]. Additionally, moderate cardiovascular exercise improved seroprotection after influenza vaccination in the elderly [371]. Slowing down immunosenescence would limit brain aging and cognitive decline through improved immunosurveillance and repair of the CNS.

Moreover, even a single exercise session increases BDNF levels which is further enhanced with regular exercise [372]. Interestingly, the exercise-related increase in BDNF is more pronounced in males compared to females. Ketone bodies are also shown to induce BDNF expression [373,374], possibly contributing to the neuroprotective effect of ketogenic diets in neurological diseases [375].

CR is another intervention shown to prevent neuronal damage. It leads to increased BDNF expression and enhanced neurogenesis [376], causes an energetic shift from glycolysis to the use of ketone bodies, protects white matter integrity, and improves long-term memory in mice [377]. In rats, an alternate-day CR regimen promotes neuronal resistance to chemically induced damage [378]. One mechanism of CR-induced neuroprotection is likely due to the suppression of oxidative stress in the brain [379, 380]. However, severe CR with 50% reduction of calorie intake was reported to cause depressive behavior in rats [381]. In mouse models of Alzheimer's disease, CR is able to limit amyloid plaque deposition [382, 383], possibly through a mechanism involving SIRT1 activation [384].

Despite all the positive results in rodents, neuroprotective effects of CR are not very clear in non-human primates, while large human studies are lacking [385]. Nevertheless, a small randomized controlled trial with humans resulted in no significant improvement in cognitive function [386]. Another clinical study on older adults showed improved memory scores upon 3 months of CR [387]. Improved memory, along with higher functional connectivity in the hippocampus, was reported in obese women that underwent a 3-month CR diet [388]. More extensive human studies with CR are necessary to understand the extent of the neuroprotective effects.

Interestingly, BCG vaccination was recently shown to reduce the risk of Alzheimer's and Parkinson's diseases in bladder cancer patients treated with BCG immunotherapy, compared to non-treated patients [389, 390]. In bladder cancer treatment, BCG is applied directly into the bladder, rather than the usual intradermal route of administration. Exciting future research projects would be assessing the effects of intradermal BCG on neurodegenerative diseases and investigating the underlying mechanisms to find out if trained immunity plays a role in the neuroprotective effects. Currently, a clinical trial is underway using intradermal BCG injections in late-onset Alzheimer's patients (NCT04449926).

## CONCLUDING REMARKS

Biological aging is a complex process involving all systems of the organism. The immune system is at the very center of it, interacting with all the others. The aging immune system is a culprit for the high susceptibility of the elderly to infections and age-related metabolic and neurodegenerative diseases, among others. Therefore, improving innate and adaptive immunological responses is immensely important to reduce infection-related morbidity and mortality and enhance vaccine responsiveness in older individuals. Here, we also presented a large body of research hinting towards new roles of immune memory in metabolic regulation and maintaining a healthy central nervous system. Approaching aging from all angles, with immunity as a central node, and designing anti-aging interventions targeting the common mechanisms ubiquitously affected by aging is a sensible way to further research. Behavioral interventions such as caloric restriction and physical exercise as well as pharmacological agents such as metformin and resveratrol are able to regulate many facets of aging and have yielded promising results in animal models and humans. A comprehensive strategy is essential for human beings striving to lead long lives with healthy guts, functional brains, and free of severe infections.

### Conflict of Interest

The authors declare no competing interests.

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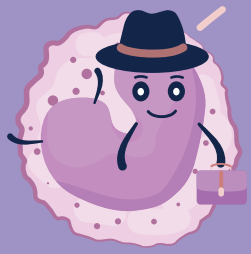

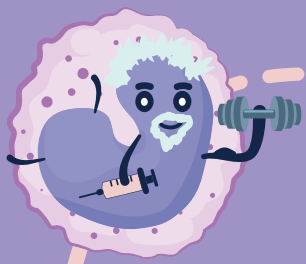

# CHAPTER 3

# COMMON AND DISTINCT METABOLOMIC MARKERS RELATED TO IMMUNE AGING IN WESTERN EUROPEAN AND EAST AFRICAN POPULATIONS

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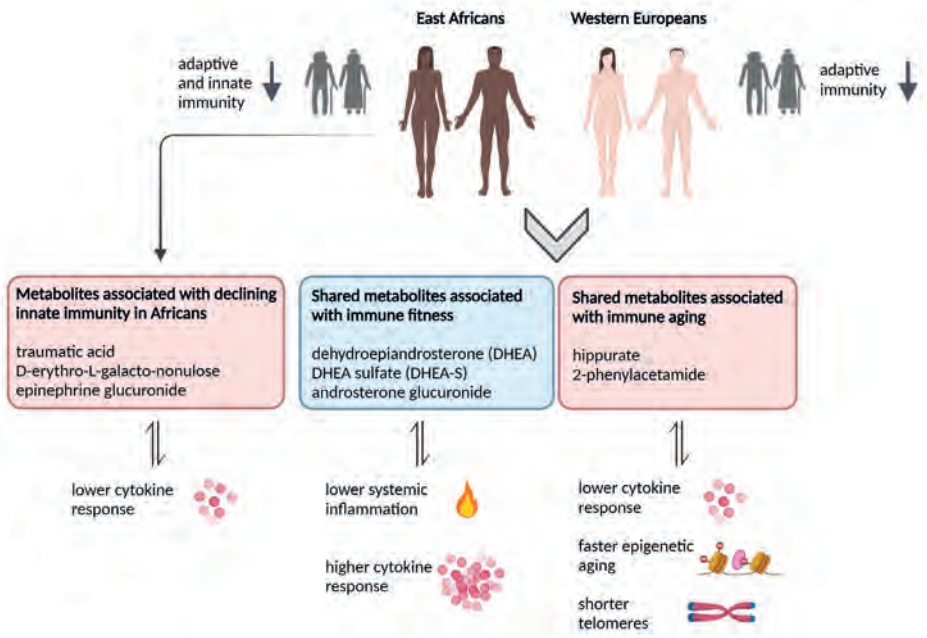
## ABSTRACT

In old age, impaired immunity causes a high susceptibility to infections and cancer, higher morbidity and mortality, and poorer vaccination efficiency. Many factors, such as genetics, diet, and lifestyle, impact aging. This study aimed to investigate how immune responses change with age in healthy Dutch and Tanzanian individuals and identify common metabolites associated with an aged immune profile. We performed untargeted metabolomics from plasma to identify age-associated metabolites, and we correlated their concentrations with ex-vivo cytokine production by immune cells, DNA methylation-based epigenetic aging, and telomere length. Innate immune responses were impacted differently by age in Dutch and Tanzanian cohorts. Age-related decline in steroid hormone precursors common in both populations was associated with higher systemic inflammation and lower cytokine responses. Hippurate and 2-phenylacetamide, commonly more abundant in older individuals, were negatively correlated with cytokine responses and telomere length and positively correlated with epigenetic aging. Lastly, we identified several metabolites that might contribute to the stronger decline in innate immunity with age in Tanzanians. The shared metabolomic signatures of the two cohorts suggest common mechanisms of immune aging, revealing metabolites with potential contributions. These findings also reflect genetic or environmental effects on circulating metabolites that modulate immune responses.



# INTRODUCTION

Aging is a physiological process of decline in the function of different organs and systems of an organism, often associated with increased morbidity. Population aging will become an increasing health and economic issue in the coming decades, with individuals over 65 estimated to reach 1.5 billion by 2050 (Nations U, 2020). The aging process affects virtually all tissues and systems, including the immune system. An aged immune system is generally characterized by chronic subclinical systemic inflammation that can lead to tissue damage, a cellular senescence-associated pro-inflammatory secretory profile, impaired immune function causing vulnerability to infections, and suboptimal response to vaccination (Bulut et al., 2020; Pawelec, 2018).

Finding biomarkers of an aged immune system is essential to monitor the immune health of individuals and to identify potential targets to delay the aging process. Many biomarkers have been suggested, particularly since the increased availability of omics-based approaches, but only a few markers that represent age-related systemic inflammation have been identified, such as interleukin 6 (IL-6) and C-reactive protein (CRP) (Pawelec, 2020). Many genetic and non-genetic factors, such as diet and climate, affect the inflammatory response (Ter Horst et al., 2016). A deeper understanding of the factors influencing immune aging in various populations with different genetic backgrounds and lifestyles is needed.

Metabolic processes modulate immune function (Loftus and Finlay, 2016), are influenced by age, and influence the process of aging itself (Bulut et al., 2021). In addition, gut microbiota alters the body's metabolic status (Fan and Pedersen, 2021) and is also implicated in aging (Badal et al., 2020). The metabolites derived from the gut microbiota might help accelerate or slow down the aging of the immune system. This study hypothesizes that changes in the immune system in old age might be partly induced by changes in metabolism. The potential identification of metabolic pathways associated with immune aging would open the door for specific lifestyle and pharmacological interventions to slow the aging of the immune system.

Combining untargeted metabolomics with *ex vivo* cytokine responses against various pathogenic stimuli, the present study aimed to identify the circulating metabolites associated with age and their impact on immune responses mainly in two similar-sized healthy cohorts: one from East Africa (Tanzania) and one from Western Europe (Netherlands). Another healthy Dutch cohort was used for the validation of the findings. Understanding the similarities and differences of

metabolic-immune interaction between geographically distinct populations may lead to better population-specific approaches.

## MATERIALS AND METHODS

### Study cohorts

The cohorts were recruited as part of the Human Functional Genomics Project (see www.humanfunctionalgenomics.org) and consisted of healthy individuals without chronic illnesses or medication use, with the exception of oral contraceptives. The East African cohort consists of 323 healthy Tanzanian individuals between the ages of 18–65. Enrollment took place at the Kilimanjaro Christian Medical Center and Lucy Lameck Research Center between March and December 2017. The demographics of the cohort were previously described in detail (Temba et al., 2021). The Western European cohort includes 324 healthy individuals between the ages of 18 and 71 enrolled at Radboud University Medical Center (Radboudumc) between April 2017 and June 2018. The second Western European cohort used for validation consisted of 534 healthy participants between 18 and 75 years old, also recruited at Radboudumc, between August 2013 and December 2014. General characteristics of the European cohorts were previously published (Ter Horst et al., 2016; Mourits et al., 2020). Characteristics of the cohorts investigated in the current analyses are provided in Supplementary Table 1.

The European cohort studies were approved by the Arnhem-Nijmegen Medical Ethical Committee (NL42561.091.12 and NL58553.091.16). The cohort study in Tanzania was approved by the ethical committees of Kilimanjaro Christian Medical University College (2443) and the National Institute for Medical Research in Tanzania (NIMR/HQ/R.8a/Vol.IX/2290 and NIMR/HQ/R.8a/Vol.IX/3318). All participants signed a written informed consent prior to sample collection, and the study procedures were conducted in accordance with the Declaration of Helsinki.

### Plasma metabolome measurement and analysis

Untargeted metabolomics measurements in plasma were performed by high-throughput flow injection-time-of-flight mass spectrometry (Fuhrer et al., 2011). The platform employed an Agilent (CA, USA) 6520 Series Quadrupole Time-of-flight mass spectrometer and Agilent Series 1100 LC pump coupled to a Gerstel MPS2 autosampler. The metabolites were matched and annotated with HMDB (www.hmdb.ca), KEGG (www.genome.jp/kegg/) and ChEBI (www.ebi.ac.uk/chebi/) identifiers.

The MetaboAnalyst v.4.0 platform (www.metaboanalyst.ca) was employed to perform comprehensive data analysis using the peak intensity table of annotated metabolites (Chong et al., 2019). Log transformation and Pareto scaling were applied before data analysis. Significant differences between the metabolome of different age groups were calculated with t-tests and visualized in volcano plots. Pathway enrichment analysis of identified metabolite lists was performed using the pathway analysis function of MetaboAnalyst, which combines network topology analysis and functional enrichment analysis. KEGG library was selected as the reference pathway library.

### Measurement of circulating protein concentrations

Cytokine and chemokine concentrations in plasma were measured with targeted proteomics using the Olink Inflammation Panel consisting of 92 markers (Olink Biosciences, Sweden) as previously described (Koeken et al., 2020). This method employs proximity extension assay and provides relative protein quantification expressed as normalized protein expression (NPX) values (Assarsson et al., 2014).

### Whole blood (WB) or peripheral blood mononuclear cell (PBMC) stimulations

Depending on the cohort, WB and/or PBMC stimulations were performed to assess cytokine production. Details of the stimuli used for each cohort are provided in Supplementary Table 2.

For WB stimulations of the African and European validation cohorts, 100 µl of heparin blood was diluted 1/5 with culture medium containing stimuli in 48-well plates and incubated for 48 hours with 100 ng/mL *Escherichia coli*-derived LPS, 50 µg/mL Poly(I:C), $10^6$/mL *Candida albicans*, $10^6$/mL *Staphylococcus aureus*, 5 µg/mL *Mycobacterium tuberculosis* (MTB), $10^6$/mL E. coli, $10^7$/mL *Coxiella burnetii*, $10^7$/mL *Streptococcus pneumoniae*, $10^6$/mL *Salmonella typhimurium* or $10^6$/mL *Salmonella enteritidis*.

For PBMC stimulations of the two European cohorts, $5\times10^5$ PBMCs per well were stimulated in round-bottom 96-well plates with 5 µg/mL *M. tuberculosis* (MTB), $10^6$/mL *S. aureus*, 10 µg/mL LPS, $10^6$/mL *E. coli*, $10^6$ CFU/mL *Candida albicans*, or 50 µg/mL Poly(I:C) for 24 hours or 7 days. RPMI 1640 Medium Dutch modification (Gibco, MA, USA) supplemented with 1 mM sodium pyruvate (Gibco), 2 mM GlutaMAX (Gibco), and 5 µg/mL gentamicin (Centrafarm, the Netherlands) was used in all cell culture experiments. Supernatants were collected and stored at -20 ˚C until cytokine quantification by ELISA. Cytokines were quantified using

DuoSet ELISA Development Systems or Luminex assay (R&D, MN, USA) according to the manufacturer's instructions.

## Telomere length analysis

DNA was isolated from whole blood samples of the European cohort, and average telomere length was determined with the Absolute Human Telomere Length Quantification qPCR Assay Kit (ScienCell, CA, USA) following the supplier's instructions. This qPCR-based assay includes primers for the telomere sequence and a single copy reference gene for normalizing genome numbers. A reference genomic DNA sample with a known telomere length is used to calculate the telomere length of the samples.

## Epigenetic aging analysis

The DNA methylation profile of the European cohort was measured using the Illumina MethylationEPIC array. DNA methylation data were pre-processed in R with the Bioconductor package Minfi (Aryee et al., 2014) using the original IDAT files extracted from the HiScanSQ scanner. Quality control was performed to filter bad quality probes with a detection P-value > 0.01, cross-reactive probes, polymorphic probes, and probes in the sex chromosome. We subsequently implemented stratified quantile normalization. Epigenetic age acceleration (EAA) was generated from methylation profiles using Horvath's online DNA Methylation Age Calculator (https://dnamage.genetics.ucla.edu/). The EAA corresponded to the residuals (difference between the actual epigenetic age and the predicted value) resulting from the linear regression of each epigenetic age estimator and chronological age. EAA was correlated with candidate metabolites using Spearman's correlation.

## Statistical analyses

Apart from the ones conducted on the MetaboAnalyst v.4.0 platform, statistical analyses were performed using R 3.6.1 (www.R-project.org) and GraphPad Prism 8 (GraphPad Software Inc.). Correlation heatmaps were built by the R packages 'corrplot' or 'gplots' upon Spearman's rank correlation and Benjamini-Hochberg correction using the 'corr.test' function. False discovery rate (FDR) values smaller than 0.05 after multiple testing correction were considered statistically significant. 'hclust' function was used for hierarchical clustering in dendrograms. R function 'PCA' and package 'ggpubr' were utilized for the principle component analyses after scaling the data. To compare two groups for a single variable in box plots, the Mann-Whitney test or Wilcoxon matched-pairs signed-rank test was used for unpaired and paired conditions, respectively. P values below 0.05 were considered statistically significant.

# RESULTS

## Aging leads to distinct profiles of cytokine production capacity in East Africans and Western Europeans

In order to determine the shared and distinct immune characteristics in the African and European cohorts, we investigated their *ex-vivo* cytokine production. We correlated the age of participants with their corresponding cytokine production capacity in response to various microbial stimuli (Supplementary Table 2) in both cohorts. Data was corrected for sex to detect generalizable patterns. In the African cohort, IFNγ production upon stimulation with *E. coli, S. aureus, S. pneumoniae*, and *C. albicans* was negatively correlated with age (Figure 1A). Similarly, IL-6 and TNFα production also decreased with age in Africans. In contrast, IL-1β production was not significantly affected by age, except for the *C. albicans*-induced response.

In contrast, we could not observe significant correlations of pro-inflammatory cytokine production with age in the European cohort (Figure 1B). Of note, the stimulations in the African cohort were performed with whole blood, whereas PBMCs were used for the European cohort. To test the robustness of the results, we validated them in a larger European cohort of similar demographics (Ter Horst et al., 2016), for which both types of stimulations (whole blood and PBMCs) were performed. While we observed a consistent decline in IFNγ production by PBMCs with age, similar to the African cohort, there was no effect on IL-6 or TNFα responses (Figure 1C). Whole blood stimulations of this cohort also did not reveal a significant effect of age on IL-6 and TNFα responses (Figure 1D).

Overall, these data suggest that a decline in T cell function with age is common in various populations, while the production of pro-inflammatory cytokines, mainly released by innate immune cells, is affected differently in African and European populations.

## Aging influences the metabolomes of Africans and Europeans

To move forward with analyses comparing young and older dividual, 50 was selected as the age cut-off, although the elderly are usually defined as 65 and older. The main reason was the low number of individuals over 65 years of age in all cohorts (Figure 1E): importantly, however, various immunological markers of aging were already significantly different between people below or above 50 years of age (Supplementary Figure 1).

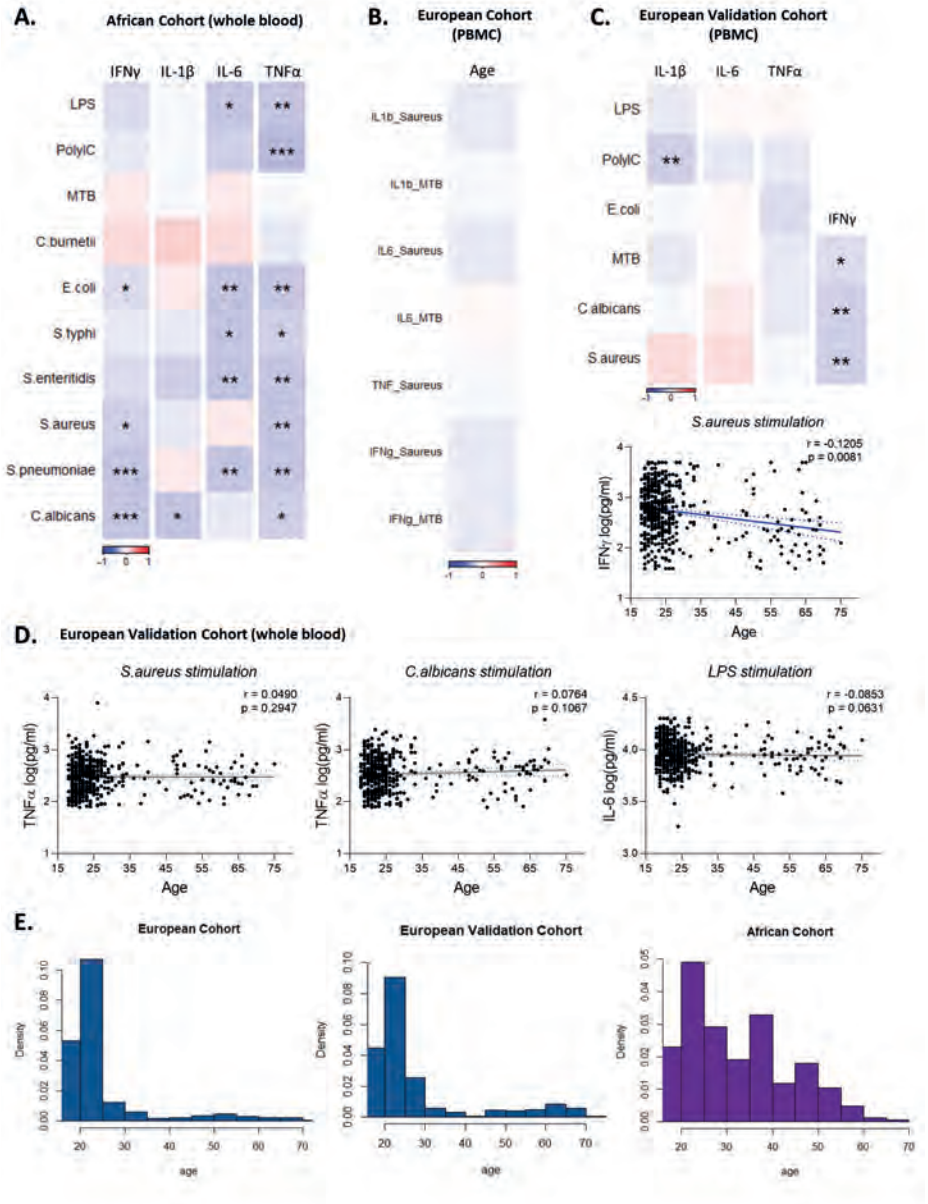



**Figure 1. Aging leads to distinct profiles of cytokine production capacity in Africans and Europeans.** Correlation of age with ex vivo cytokine production upon stimulation in the (A) African and (B-D) European cohorts, and (E) age distribution of each cohort. All data was controlled for sex. Red represents a positive correlation with age in the heatmaps, while blue represents a negative correlation. Multiple testing correction was performed. Solid lines depict the best fit in correlation dot plots, and dashed lines depict the 95% confidence interval. r: Spearman's correlation coefficient. *p<0.05, **p<0.01, ***p<0.001.

We used an untargeted approach to identify the circulating metabolites influencing age-related immunological changes. 1376 metabolites could be detected and annotated. Principal component analyses revealed that geographical location and genetic background exert a more substantial effect than age on the circulating metabolites (Figure 2A- B). In this respect, while the circulating metabolomes of people over 50 and younger people were significantly different in both cohorts (Figure 2A), there were much greater differences between the two geographically different cohorts in both younger and older individuals (Figure 2B).

In the European cohort, 246 metabolites were significantly more abundant, and 265 were less abundant in people over 50 compared to younger individuals (Figure 2C). In the African cohort, the number of metabolites that were more and less abundant in older individuals were 132 and 141, respectively. A complete list of these metabolites and the relevant metrics are provided in Supplementary File 1. 61 of metabolites with higher circulating concentrations in individuals over 50 were shared between the cohorts, while 81 metabolites were commonly less abundant (Supplementary Table 3). The top 5 commonly abundant metabolites with the highest fold changes were quinic acid, 15-Keto-13, 14-dihydroprostaglandin A2, hexonic acid, D-Glycero-D-galacto- heptitol, and Ferulic acid 4-sulfate. The top 5 commonly less abundant metabolites in older individuals were dehydroepiandrosterone sulfate, 2-oxoglutarate (2-), niazicinin A, curcumin, and androsterone sulfate. These data indicate that metabolomes of different demographics differ in young and old individuals, but they also display significant common age-related changes.

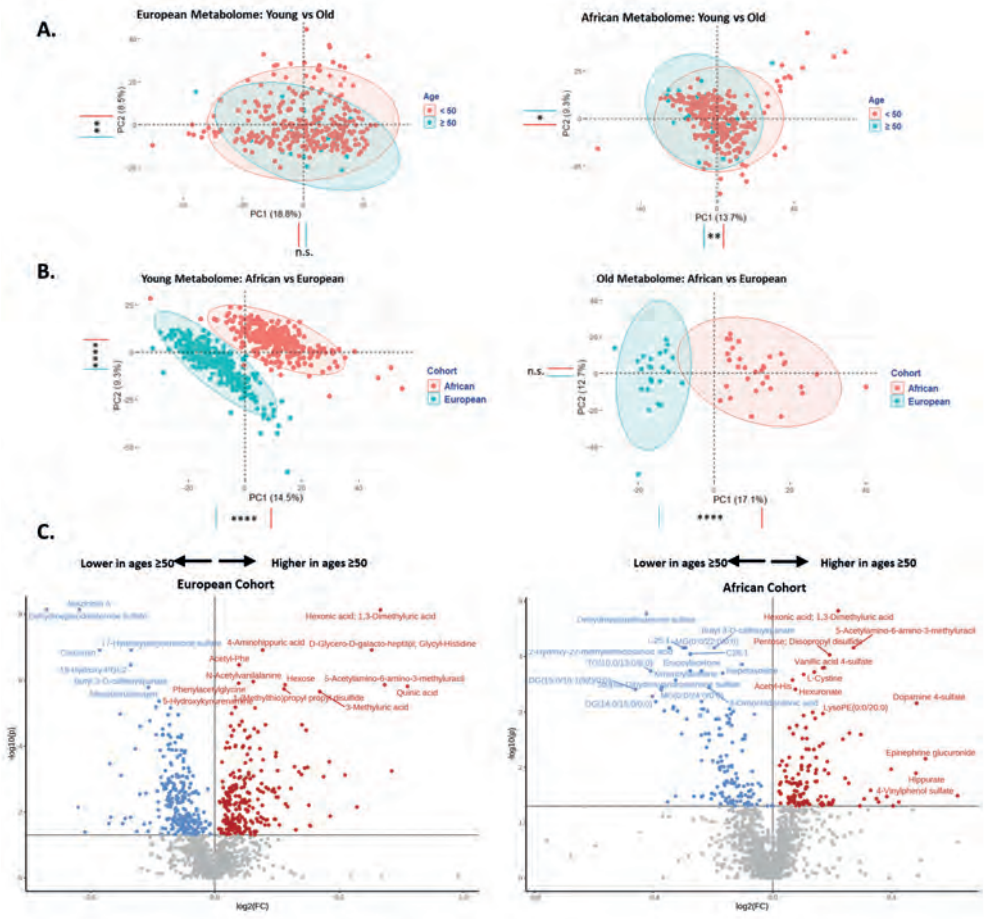



**Figure 2. Aging influences the distinct metabolomes of Africans and Europeans.** (A-B) Principal component analysis of metabolome data and (C) volcano plots depicting the differential abundance of metabolites in different age groups in the African and European cohorts. In the PCA plots, significance stars reflect the difference between the two groups' distribution along each PC axis. In the volcano plots, significantly different metabolites are depicted in pink. PC: principal component, ns: not significant, FC: fold change. *p<0.05, **p<0.01, ****p<0.0001.

## Age-related decline in steroid hormone precursors is associated with increased systemic inflammation and lower cytokine response in both Africans and Europeans

Next, we performed pathway analysis using the 61 more and 81 less abundant metabolites in older individuals shared in both cohorts. Additionally, using the information provided by the Human Metabolome Database, we could identify whether these metabolites are endogenous or exogenous (e.g., food-derived).

While most of the 61 upregulated metabolites in the older dividual were endogenous or both endogenous and food-derived, 16.4% were strictly derived from food or beverages (Figure 3A). The significantly overrepresented pathways in this metabolite list were caffeine metabolism and the pentose phosphate pathway (PPP). PPP is known to shape immune responses (Jansen et al., 2021). On the other hand, the 81 metabolites less abundant in older individuals included 9.9% exclusively food-derived metabolites (Figure 3B). Pathway analysis revealed that the steroid hormone biosynthesis pathway was over-represented among the 81 metabolites with decreased concentrations in older individuals. The declining metabolites belonging to that pathway included dehydroepiandrosterone (DHEA), DHEA sulfate (DHEA-S), and androsterone glucuronide (ADT-G). Their concentrations were much lower in people older than 50 than younger people in both cohorts (Figure 3C). ADT-G was also lower in older individuals of the European validation cohort, while DHEA was not significantly different, and DHEA-S measurements were not available (Supplementary Figure 2).

To investigate whether the age-related decline in the metabolites involved in steroid hormone biosynthesis might have immunological implications, we correlated the concentrations of DHEA and DHEA-S with circulating inflammatory markers and cytokine responses upon microbial stimulation after correcting for the effect of age. DHEA-S, the dominant form of DHEA found in circulation, and ADT-G concentrations were significantly negatively correlated with serum IL-6 concentration in the African cohort (Figure 3D). In contrast, DHEA-S was significantly positively correlated with TNFα and IFNγ production upon stimulation with *S. pneumoniae*. Similarly, ADT-G levels positively correlated with TNFα secretion after *S. pneumoniae* or *S. aureus* stimulation. In the European cohort, no association was found between these metabolites and *ex-vivo* cytokine production. However, DHEA-S significantly negatively correlated to circulating IL-6, IL-8, and IL-18 concentrations (Supplementary Figure 3). ADT-G was also associated with lower IL-6 circulating concentrations. Collectively, these data imply that the decline of steroid hormone precursors might contribute to age-related low-grade systemic inflammation on the one hand and defective cytokine response against pathogens on the other. This seems to be a general feature in populations of both African and European ancestry.

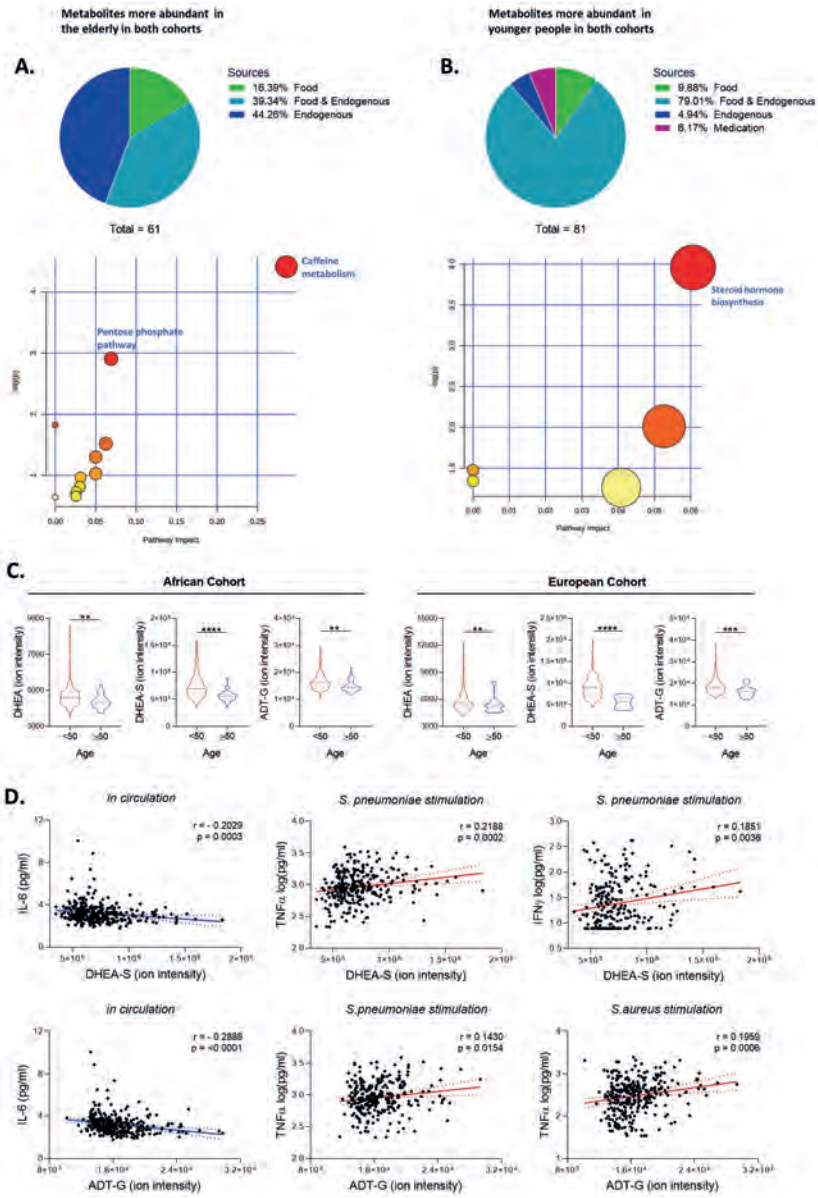



**Figure 3. Age-related decline in steroid hormone precursors is associated with increased systemic inflammation and defective cytokine response in both African and European populations.** Sources and pathway enrichment analyses of metabolites (A) significantly more abundant in people over 50 years of age and (B) more abundant in younger people in both cohorts. (C) Levels of metabolites that belong to the steroid hormone biosynthesis pathway in the two cohorts. (D) Association of DHEA-S and ADT-G with circulating IL-6 and cytokine response against pathogens in the African cohort. In pathway analysis graphs, node colors reflect the p values, and diameters reflect the pathway impact values. Only the statistically significant pathways are labeled. Solid lines depict the best fit in correlation dot plots, and dashed lines depict the 95% confidence interval. DHEA: dehydroepiandrosterone, DHEA-S: dehydroepiandrosterone sulfate, ADT-G: androsterone glucuronide, r: Spearman's correlation coefficient. **p<0.01, ***p<0.001, ****p<0.0001.

## Hippurate and 2-phenylacetamide are aging-related metabolites associated with low cytokine production in both cohorts

We next tested if the 61 metabolites with higher concentration in people over 50 years of age in both populations were associated with the *ex-vivo* cytokine production capacity. An overall negative correlation pattern between these metabolites and IFNγ, IL-6, and especially TNFα production was identified in the African cohort after correcting for the effect of age (Supplementary Figure 4 and Figure 4A). Among those, hippurate and 2-phenylacetamide were also negatively correlated with IL-6 and IL-1β production in the European cohort (Figure 4B). These two metabolites were also more abundant in individuals over 50 in the Euro-pean validation cohort (Supplementary Figure 2).

Although the observations remained broadly similar for innate cytokines when men and women were analyzed separately, some intriguing sex differences were observed for IFNγ production in the African cohort (Supplementary Figure 5 A). Contrary to the general trend, age-related metabolites were positively correlated with IFNγ production against viral stimulus poly(I:C) in women, but did not have a significant association in men. Moreover, a strong negative correlation was observed with the IFNγ response to *C. burnetii* in women, with no association in men. In the European cohort, negative correlations with hippurate and 2-phenylacetamide could only be seen in women when sex-specific assessment was performed (Supplementary Figure 6 C).

The 81 metabolites with lower concentrations in older individuals were positively correlated with cytokine production in the African cohort (Supplementary Figure 7). Among those, ximenoylacetone, KB2, and 14,16-nonacosanedione, all food-derived metabolites, were strongly correlated with ex-vivo IFNγ, IL-6, and TNFα production (Figure 4C). However, such associations were not found in the European cohort (Figure 4D), arguing for population-specific consumption or biological effects. Only ximenoylacetone concentrations among these metabolites negatively correlated with *S. aureus*-induced IFNγ production. Of note, circulating concentrations of all three metabolites were much lower in the European cohort compared with the African counterparts (Figure 4E), which could explain the lack of association with cytokine production capacity in Europeans. 14,16-nonacosanedione was also found in lower levels in the older individuals of the European validation cohort, although KB2 and ximenoylacetone were not among the detected- metabolites (Supplementary Figure 2).

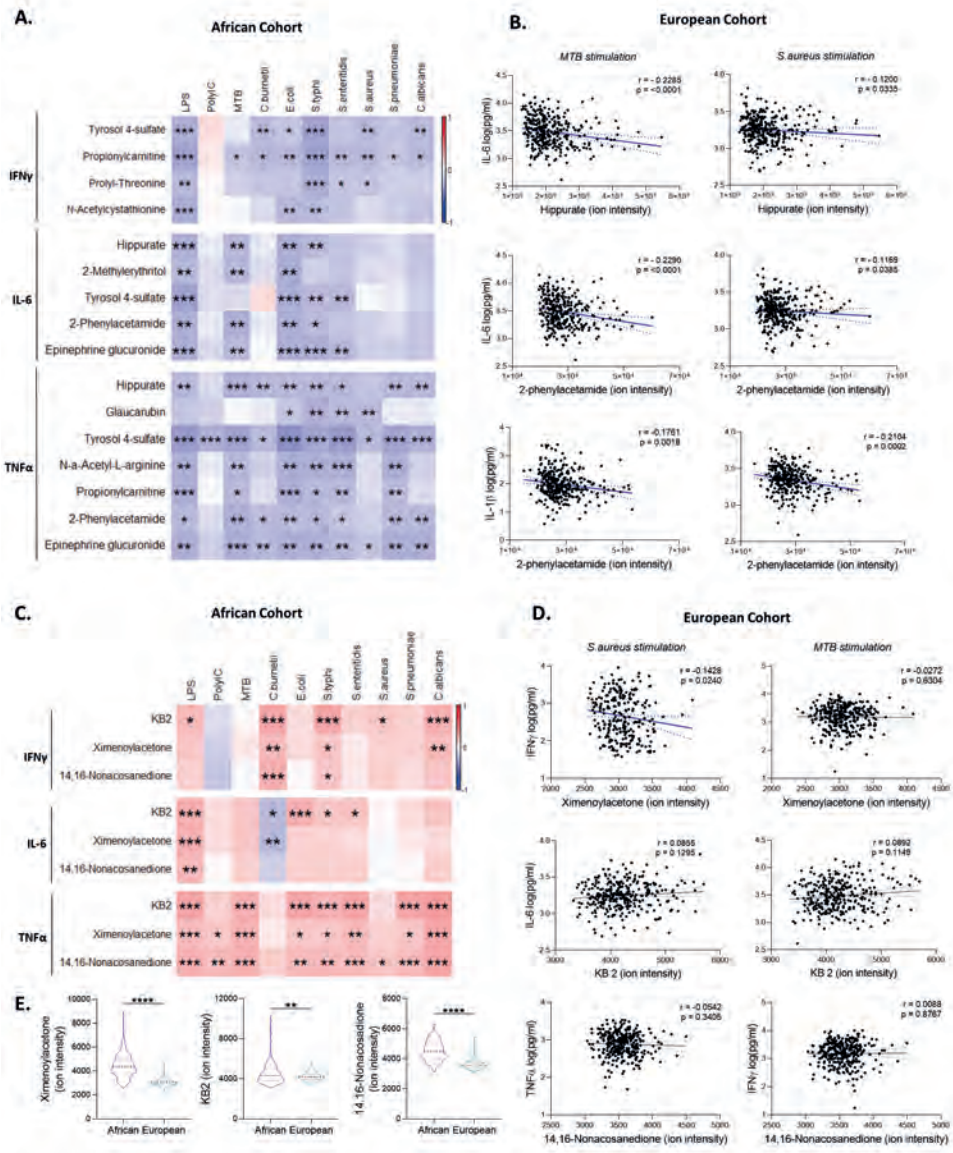



**Figure 4. Hippurate and 2-phenylacetamide are aging-related metabolites associated with low cytokine production in both cohorts.** Correlation of *ex-vivo* cytokine production with levels of selected metabolites commonly (A-B) more abundant or (C-D) less abundant in people over 50 years of age in the African and European cohorts. (E) Levels of ximenoylacetone, KB2, and 14,16-Nonacosanedione in the two cohorts. Only the metabolites with the strongest correlations are shown in the heatmaps. Red depicts a positive correlation, while blue depicts a negative correlation. Multiple testing correction was performed. Solid lines depict the best fit in dot plots, and dotted lines depict the 95% confidence interval. r: Spearman's correlation coefficient. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

These metabolites also differed in their association with the lymphoid response to poly(I:C) in women and men with African ancestry (Supplementary Figure 5B). There was no correlation in men, while the metabolites were negatively correlated with IFNγ production in women. Moreover, a positive association of the metabolites with *C. burnetii*-induced IFNγ production was only observed in women.



Altogether, these findings suggest that the changing metabolic landscape as people age is linked to the modifications in immune function with some sex differences, particularly for antiviral response. Hippurate and 2-phenylacetamide emerge as metabolites possibly contributing to immune dysfunction in old age in both populations. On the other hand, the food-derived metabolites ximenoylacetone, KB2, and 14,16-nonacosanedione might have protective effects against the aging of immune cells, specifically in the African cohort in which they are at higher concentrations.

## Hippurate and 2-phenylacetamide are associated with faster epigenetic aging and shorter telomeres in males

Epigenetic clocks based on DNA methylation, such as Horvath's clock, and telomere length have been strongly associated with immune aging. These measures correlate poorly; however, each has been linked to age-related disorders (Jansen et al., 2021). Epigenetic age acceleration (EAA) analysis based on DNA methylation of whole blood was performed for the European cohort. Also, average telomere length was assessed from whole blood DNA samples for 98 participants, half of whom were female and half male. Because of the consistent associations of hippurate and 2-phenylacetamide with immune function, we assessed their relationship with either of the aging clocks.

In both analyses, another sex difference in the associations of these two metabolites was observed. In males but not females, both hippurate and 2-phenylacetamide concentrations showed a positive correlation with EAA ($p = 0.0321$ and 0.0339, respectively) (Figure 5A). Similarly, the circulating concentrations of these two metabolites were negatively correlated with telomere length in males but not females (Figure 5B). Of note, circulating concentrations of both metabolites were not significantly different between males and females (Figure 5C). These associations support the possibility that hippurate and 2-phenylacetamide might contribute to aging, while the sex-specific findings should be confirmed with larger sample sizes.

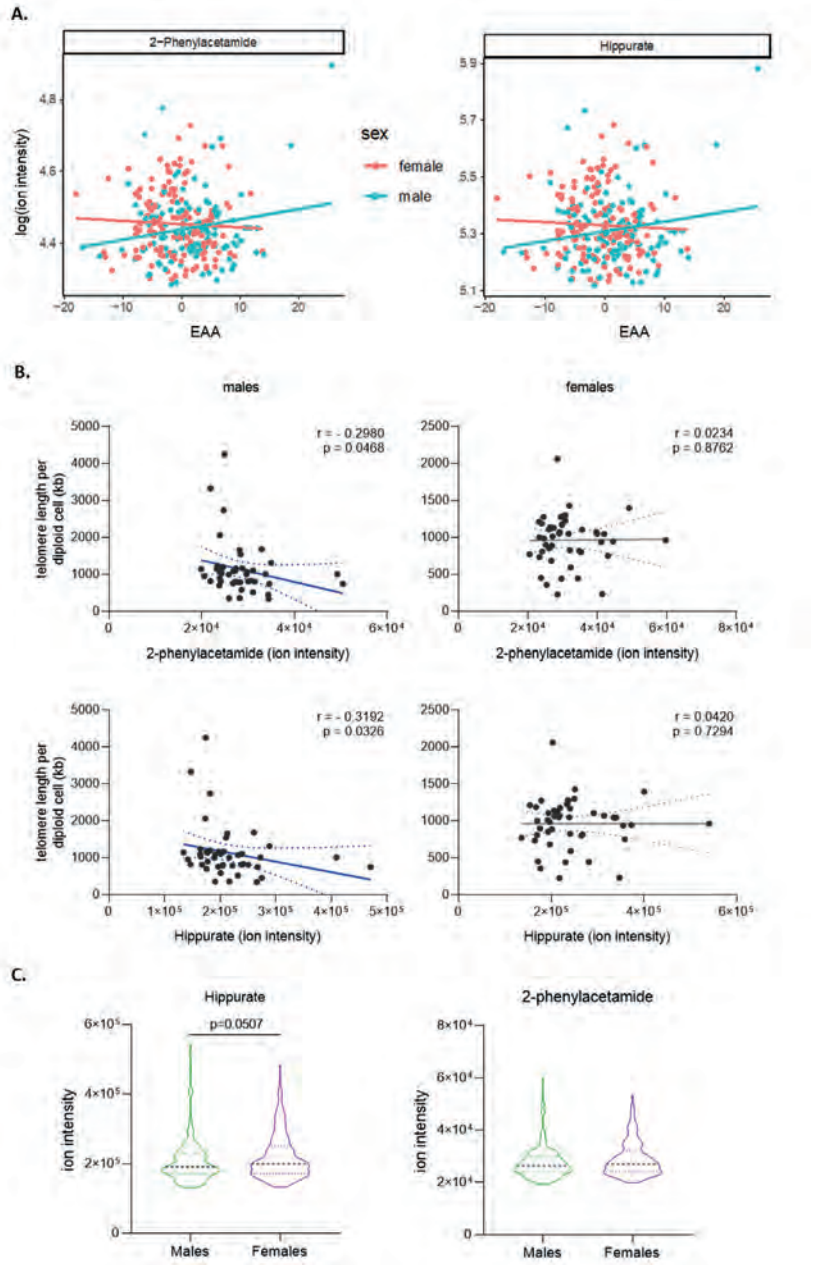



**Figure 5. Hippurate and 2-phenylacetamide are associated with faster epigenetic aging and shorter telomeres in males.** Sex-dependent associations of hippurate and 2- phenylacetamide on (A) epigenetic age acceleration (B) average telomere length of the cells in whole blood in the European cohort. (C) Hippurate and 2-phenylacetamide levels in males and females of the European cohort. In correlation plots, solid lines depict the best fit, and dashed lines depict the 95% confidence interval. In violin plots, dashed lines depict the median and quartiles. EAA: epigenetic aging acceleration, r: Spearman's correlation coefficient.

## Lifestyle differences lead to distinct metabolic profiles in older individuals, possibly contributing to a different immune response

In order to identify the metabolic profile unique to the older individuals of the African cohort and to determine if that can explain their lower innate immune response compared to the young individuals, we compared the metabolomes of the 24 and 32 participants over 50 years of age in the European and African cohorts, respectively. 370 metabolites were significantly higher in the Africans, while 353 were higher in the Europeans (Figure 6A). When we assessed the sources of the top 50 metabolites with the biggest fold change in either direction, 10% and 32% were purely endogenous, while 10 and 23% of metabolites were strictly diet-derived in Africans and Europeans, respectively (Figure 6B). A total of 10% of the metabolites higher in Europeans were related to medication use and environmental contaminants.

Pathway analysis of the 370 metabolites more abundant in older individuals of the African cohort revealed that many pathways related to amino acid metabolism, including glycine, serine, threonine, alanine, aspartate, glutamate, arginine, and histidine, were overrepresented (Figure 6C). Primary bile acid biosynthesis, linoleic acid metabolism, and glutathione metabolism were also overrepresented. Among the 353 metabolites more abundant in older individuals of the European cohort, only phenylalanine, tyrosine, and tryptophan biosynthesis were significantly overrepresented.

When the top 50 differentially found metabolites in Africans were correlated with *ex vivo* cytokine response, traumatic acid, d-erythro-l-galacto-nonulose, and epinephrine glucuronide showed a robust negative correlation with IFNγ, IL-6, and TNFα production (Supplementary Figure 8 and Figure 6D). Collectively, these data imply distinct regulation of amino acid metabolism in the two populations, reveal the impact of diet and environmental factors on the metabolome of different groups of older individuals, and identify metabolites that might contribute to the modulation of innate immune responses in the African cohort.

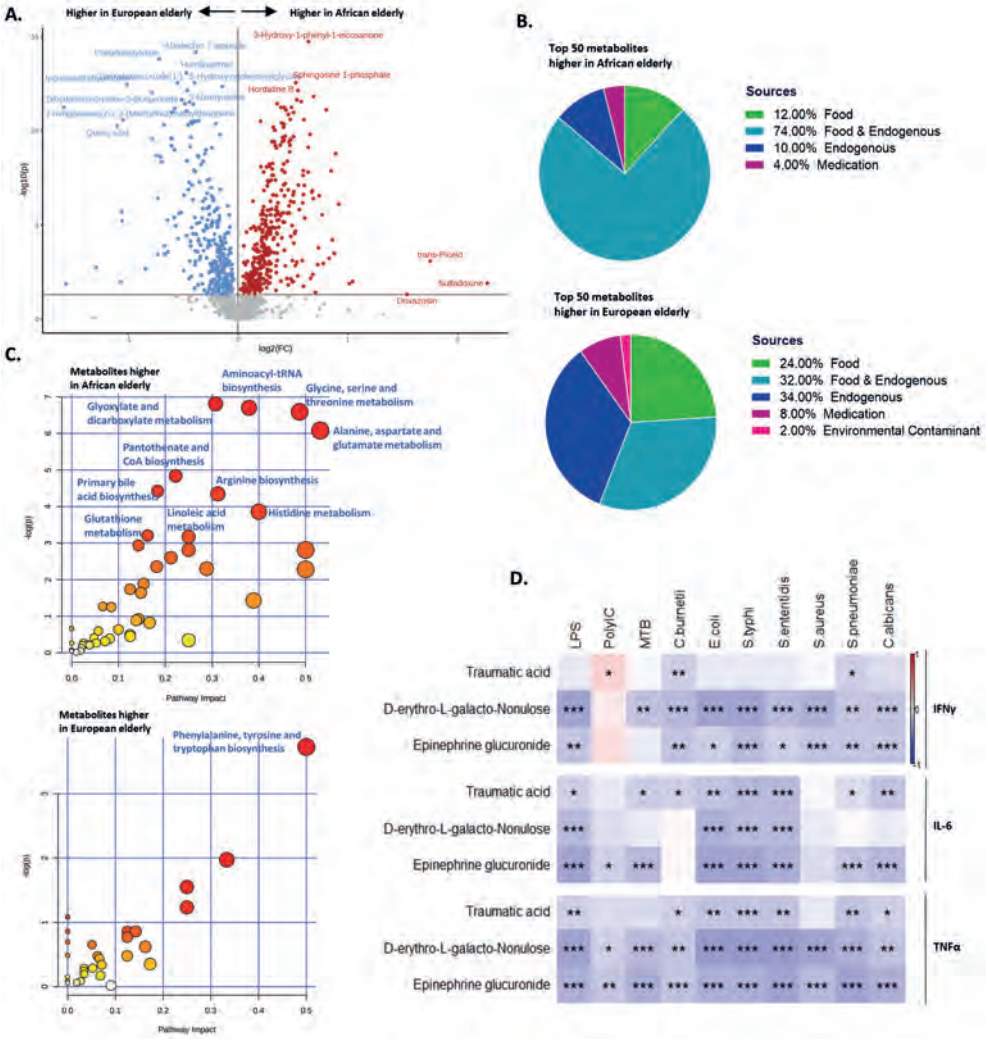



**Figure 6. Lifestyle differences might lead to distinct metabolic profiles in older individuals, possibly contributing to a different immune response.** (A) Volcano plot depicting the differential abundance of metabolites in individuals over 50 years of age in two cohorts, (B) sources of the 50 metabolites with the biggest fold change that are higher in Africans or Europeans, (C) pathway enrichment analyses of metabolites significantly higher in Africans or Europeans, (D) correlation of *ex-vivo* cytokine production in the African cohort with levels of selected metabolites that are overrepresented in African individuals over 50. In pathway analysis graphs, node colors reflect the p values, and diameters reflect the pathway impact values. Only the statistically significant pathways are labeled. In the heatmaps, only the metabolites with the strongest correlations are shown. Red shows a positive correlation, while blue shows a negative correlation. Multiple testing correction was performed for correlations. *p<0.05,**p<0.01, ***p<0.001.

## DISCUSSION

Aging impacts the function of various organs and systems in the body, determines longevity, and strongly influences morbidity and quality of life. Understanding the factors that influence aging can thus have significant implications for improving the quality of life and reducing the burden of disease at the societal level. Proper immune system function is crucial for the homeostasis of the human body, and aging of the immune system influences morbidity and disease complications in humans (Alpert et al., 2019; Sayed et al., 2021). In the present study, we investigated the age-related changes in the function of the immune system in East African and Western European populations.



We identified a general decrease in T cell function with age in both populations, similar to other studies (Goronzy and Weyand, 2017), whereas innate immune responses declined with aging only in the African population but not in Europeans. One main aim of the study was to assess the circulating metabolites associated with decreased immune function in older individuals. Although the circulating metabolome differed between African and European populations, we identified several metabolites that increased with age and were associated with decreased immune function in both populations, as well as metabolites associated with potent immune responses but were deficient in individuals of older age.

Circulating metabolites have an important impact on the immune response (Koeken et al., 2022), and we aimed to identify metabolites that change with age while influencing immune responses. We identified 61 common metabolites with higher plasma concentrations in older individuals in both populations and pathway analysis revealed that pentose phosphate pathway (PPP) metabolites were enriched among them. By providing redox equivalents and nucleotide precursors, PPP plays an essential role in feeding the demands of immune cells (Nagy and Haschemi, 2015). The upregulated pathway might reflect the increased demand against high infectious burden in old age or mirror chronic low-grade inflammation (Franceschi et al., 2018).

Metabolites of the steroid hormone biosynthesis pathway were enriched among the 81 metabolites with reduced circulating concentrations in the older individuals of African and European ancestry. Age-related decrease in steroid hormone biosynthesis is highly likely to have immunological consequences, considering the impact of steroid hormones on immune cell function (Hoffmann et al., 2023). Steroid hormones like estradiol, testosterone, and DHEA regulate both innate and adaptive

immune responses and are responsible for many immunological differences between males and females (Klein and Flanagan, 2016). DHEA and its sulfated form DHEA-S are the most abundant circulating steroid hormones in humans, primarily produced by the adrenal cortex but also synthesized in the brain (Powrie and Smith, 2018; Webb et al., 2006). Prior studies have linked low DHEA levels to aging, various age-related comorbidities, and neurodegenerative disorders (Ravaglia et al., 2002). As a result, DHEA is a popular anti-aging supplement, available over-the-counter and online, although sufficient clinical trial data to show its effectiveness is missing. Experimental data have shown anti-inflammatory effects, such as inhibiting leukocyte migration and reducing neural inflammation (Alexaki et al., 2018; Ziogas et al., 2020). A relatively small study of elderly men receiving 20 days of DHEA supplementation reported higher monocyte numbers and lymphocyte response to mitogens than before treatment (Khorram et al., 1997). However, a 3-week treatment with DHEA in post-menopausal women reduced T helper cell numbers and mitogenic response, but enhanced NK cell cytotoxicity (Casson et al., 1993).

Our study suggests DHEA and DHEA-S have complex regulatory effects: while reducing systemic inflammation in both cohorts, they also seem to support the homeostasis of cytokine production in the Tanzanian cohort, maintaining an effective response against microbial stimuli. These data may thus support an approach in which DHEA supplementation might be beneficial as an anti-aging supplement, maintaining the tonus of the immune system while inhibiting systemic inflammation. However, large randomized controlled trials assessing the immunological effects of DHEA supplementation are needed to validate this assumption.

We also identified several metabolites with higher circulating concentrations in the older individuals in both cohorts, including tyrosol-4-sulfate, propionyl carnitine, hippuric acid (hippurate), and 2-phenylacetamide, which were associated with lower cytokine production in the African cohort. These data suggest that higher concentrations of these metabolites observed in the circulation of older individuals might contribute to their declined innate immune responses. The inverse associations with cytokine production were replicated for hippurate and 2-phenylacetamide in the European cohort, although sex-specific analysis revealed this only in females. Interestingly, the concentrations of these metabolites were higher in the Africans than in the Europeans, which might be one of the reasons why Africans exhibit more decline in innate immunity as they age. Lastly, both metabolites were associated with higher EAA and lower telomere length in European males, supporting the hypothesis that they are involved in aging.

However, separating the sexes diminished the statistical power of the analyses, so the observed sex discrepancies should be confirmed with larger sample sizes.

Hippurate levels were previously reported to increase in healthy aging but not in frailty (De Simone et al., 2021), and it has been associated with metabolic health (Brial et al., 2021). Its concentrations are elevated upon consumption of polyphenol-rich dietary sources such as wine, fruit juices, coffee, and tea. The intestinal microbiota is critical in hippurate synthesis from these sources (Lees et al., 2013). Our results suggest potential anti-inflammatory effects of hippurate. However, this may be associated with defective responsiveness of immune cells to microbial challenges, and it remains to be studied whether this may be disadvantageous for susceptibility to infections. There is not much prior biological research on 2-phenylacetamide, which can also be derived from plant-based food sources, but it was shown to be elevated in older mice (Osada et al., 2003). More research is needed on this metabolite associated with decreased immune function along with genetic and epigenetic aging in our study. The gut-derived source for these metabolites may support the hypothesis that manipulating diet and microbiota to regulate plasma metabolite composition and aging-related processes could be an attractive strategy.

Among the metabolites with lower plasma concentrations in both European and African older individuals, KB-2, ximenoylacetone, and 14,16-nonacosanedione, all food-derived metabolites, were associated with higher adaptive and innate immune responses in the African cohort. Interestingly, this association was not observed in the European cohort, probably due to the lower concentrations of these metabolites in the Europeans, lacking thus the variability necessary to identify the stimulatory effects. The higher concentration of these metabolites in Africans is likely a diet-induced difference. For instance, KB-2 is a flavone derived from tropical breadfruit cultivated in Tanzania (Mgembe and Maerere, 2007). These diet-derived metabolites might exert an immunoprotective effect by maintaining the homeostasis of the immune responses. Temba et al. also previously reported immunomodulatory effects of food-derived metabolites in the African cohort, with rural residents showing a significant abundance of plant-based polyphenols, such as apigenin, known to have anti-inflammatory effects (Temba et al., 2021).

We observed intriguing sex differences in how age-associated metabolites relate to antiviral lymphoid cytokine responses. IFNγ production against the viral ligand poly(I:C) was positively correlated with tyrosol-4-sulfate, propionyl carnitine, and epinephrine glucuronide levels in women, contrary to the negative correlation

with responses against all other pathogens. Hippurate and 2-phenylacetamide also showed a similar trend, but were not statistically significant. Men and women differ in their susceptibility to viral infections and the strength of antiviral responses induced by vaccines (Klein and Flanagan, 2016). Overall, women mount a stronger antiviral response after vaccination. The human X chromosome contains many immune-related genes including TLRs 7 and 8, which are crucial for viral recognition (Fish, 2008), and both genes can escape X chromosome inactivation (Souyris et al., 2018; Youness et al., 2023), likely contributing to the more robust antiviral response in women. The metabolites associated with immune aging might support a stronger antiviral response in women, potentially leading to both lower susceptibility to initial infection, but more severe disease in later stages of infection. Furthermore, ximenoylacetone and 14,16-nonacosanedione were negatively correlated with poly(I:C)-induced IFNγ production in women. These two metabolites with seemingly restorative effects on immune responses to other pathogens might also help dim overactive antiviral processes in women and maintain immune homeostasis.

Lastly, the circulating plasma metabolomes differed significantly in individuals over 50 years of age from Tanzania and the Netherlands. The metabolic differences highlighted the importance of diet and lifestyle. Notably, more metabolites derived from medication use and environmental contaminants were found in older European individuals compared to Africans. Three out of 50 most abundant metabolites in older Africans strongly correlated with lower IFNγ, IL-6, and TNFα production. Traumatic acid is a regenerative plant hormone that is produced upon tissue damage (Farmer, 1994), D-erythro-L-galacto-nonulose is derived from avocado (Sephton and Richtmyer, 1963), and epinephrine glucuronide is a natural derivative of epinephrine converted in the liver (Axelrod, 1959). These metabolites might be involved in the declining innate immune response as people age in Tanzania.

The cohorts analyzed in this study were previously recruited as part of the Human Functional Genomics Project (HFGP). A limitation of the present study is that aging-related analyses were not the initial focus of HFGP, and the age distribution of the cohorts is not ideal for exploring aging processes. Another limitation, due to the design of the cohorts, was that stimulations were performed only in whole blood stimulation assay for the Tanzanian cohort, while cytokine stimulation assays in either whole blood or PBMCs were used in two Dutch cohorts. Nevertheless, the analyses presented here provide consistent and valuable information regarding the age-related differences in metabolome and their impact on the homeostasis of immune responses. An important strength of our study is the investigation and

comparison of metabolic-immune interactions in cohorts of African and European ancestry, which allowed us to identify potential general mechanisms of immune modulation in human populations.

In conclusion, this study identified several common and specific age-related metabolites in people with African and European ancestry that are likely to mediate the changes in the immune system as they age. Although correlations of metabolite concentrations with immunological parameters were statistically robust, future *in vitro* and *in vivo* functional validation studies are necessary to establish whether these metabolites causally influence immune response. Studying diverse populations with advanced omics and immunological methods is necessary to improve our understanding of the shared mechanisms of aging and the particular biological processes in each population linked to genetics, environmental factors, and diet.



## CRediT authorship contribution statement

Bulut Ozlem: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. Temba Godfrey S.: Project administration, Investigation, Data curation. Koeken Valerie A. C.M.: Investigation, Data curation. Moorlag Simone J.C.F.M.: Investigation, Data curation. de Bree Charlotte J.: Investigation, Data curation. Mourits Vera P.: Investigation, Data curation. Kullaya Vesla I.: Project administration, Investigation, Data curation. Jaeger Martin: Investigation, Data curation. Qi Cancan: Visualization, Formal analysis, Data curation. Riksen Niels P.: Writing – review & editing, Supervision, Funding acquisition. Dominguez-Andres Jorge: Writing – review & editing, Supervision, Conceptualization. Xu Cheng-Jian: Supervision, Data curation. Joosten Leo A.B.: Project administration, Funding acquisition, Conceptualization. Li Yang: Supervision, Funding acquisition, Data curation. de Mast Quirijn: Supervision, Data curation. Netea Mihai G.: Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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## Declaration of interests

M.G.N. is a scientific founder and scientific advisory board member of Trained Therapeutix Discovery (TTxD), and is a scientific founder of Lemba and Biotrip. The other authors declare that they have no conflicts of interest.

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# SUPPLEMENTARY TABLES

**Supplementary Table 1.** Basic characteristics of the healthy cohorts used in the study.

| | African Cohort | European Cohort | European Validation Cohort |
|---|---|---|---|
| Total n (with metabolomics measurements) | 323 | 324 | 458 |
| n (≥ 50 years) | 32 | 24 | 43 |
| Age (median, IQR) | 30.3 (23.4 – 40.2) | 22.2 (20.8 – 24.4) | 23 (21 – 26) |
| Sex (female %) | 50.8 | 56.5 | 56.3 |
| BMI (median, IQR) | 23.8 (21.5 – 27.2) | 22.2 (20.8 – 23.6) | 22.3 (20.7 – 22.3) |

**Supplementary Table 2.** Overview of the stimuli used for the ex vivo experiments and the cohorts they were used for.

| Stimulus | Stimulus Type | Cohorts |
|---|---|---|
| Poly(I:C) | Viral molecular pattern, TLR3 ligand | African, European, European Validation |
| *Escherichia coli*-derived LPS | Bacterial molecular pattern, TLR4 ligand | |
| *Escherichia coli* | Bacteria, primarily extracellular | |
| *Streptococcus pneumoniae* | | African |
| *Staphylococcus aureus* | Bacteria, facultative intracellular | African, European, European Validation |
| *Mycobacterium tuberculosis* | | |
| *Salmonella typhimurium* | | African |
| *Salmonella enteritidis* | | |
| *Coxiella burnetii* | Bacteria, obligate intracellular | African, European Validation |
| *Candida albicans (conidia)* | Fungi, facultative intracellular | |

**Supplementary Table 3.** Annotated metabolites that were significantly different in individuals over 50 years old compared to younger individuals in both Europeans and Africans.

| Higher in ≥50 years old | Lower in ≥50 years old |
|---|---|
| Quinic acid | Dehydroepiandrosterone sulfate |
| 5-Acetylamino-6-amino-3-methyluracil | Isopetasoside |
| Hexonic acid-1,3-Dimethyluric acid | Androsterone sulfate |
| Glycyl-Histidine | 3b,16a-Dihydroxyandrostenone sulfate |
| D-Glycero-D-galacto-heptitol | (2S)-2,3-bis(octanoyloxy)propyl 10-methyldodecanoate |
| 3-Methyluric acid | 19-Hydroxy-PGE2 |
| Hippurate | 17-Hydroxypregnenolone sulfate |
| (2E)-3-[3-(sulfooxy)phenyl]prop-2-enoic acid | (2S)-1-hydroxy-3-(octanoyloxy) propan-2-yl pentadecanoate |

**Supplementary Table 3.** Continued

| Higher in ≥50 years old | Lower in ≥50 years old |
|---|---|
| Arabinonic acid | MG(0:0/15:0/0:0) |
| S-Propyl 1-propanesulfinothioate | (2R)-1-(decanoyloxy)-3-(octanoyloxy) propan-2-yl tridecanoate |
| Methylxanthine | Butyl 3-O-caffeoylquinate |
| Pentose | Glycerol triundecanoate |
| Diisopropyl disulfide | 3-Oxohexadecanoic acid |
| Protocatechuic acid 3-O-sulfate | Hydroxyethylpromethazine |
| Glaucarubin | 2-Hydroxy-22-methyltetracosanoic acid |
| Deoxyribose | Androsterone glucuronide |
| (2-hydroxyphenyl)oxidanesulfonic acid | 3-Hexaprenyl-4-hydroxy-5-methoxybenzoic acid |
| 2-Phenylacetamide | MG(0:0/24:0/0:0) |
| Epinephrine glucuronide | Gentiobiosyl 2-methyl-6-oxo-2E,4E-heptadienoate |
| Hexose | 3-Oxooctadecanoic acid |
| 1-(Methylthio)propyl propyl disulfide | DG(14:1(9Z)/15:0/0:0) |
| trans-Ferulic acid | MG(0:0/22:0/0:0) |
| 3-hydroxy-2-{[(2E)-3-(4-hydroxyphenyl) prop-2-enoyl]oxy}propanoic acid | 13'-Carboxy-alpha-tocopherol |
| 2-[(2-phenylacetyl)oxy]-2-propanamidoacetic acid | 4-Deoxyannoreticuin |
| Catechol | (2S)-1-hydroxy-3-(octanoyloxy) propan-2-yl hexadecanoate |
| (2-methoxyphenyl)oxidanesulfonic acid | (2R)-2,3-bis(octanoyloxy)propyl decanoate |
| Salicyluric acid | 3a,7a-Dihydroxy-5b-cholestane |
| 2-Methylerythritol | 3,4,5-trihydroxy-6-{[5-(4-methoxyphenyl)-3-oxopentan-2-yl]oxy}oxane-2-carboxylic acid |
| Vanillylmandelic acid | 16-Acetylpriverogenin A |
| Prolyl-Threonine | 14,15-DiHETrE |
| [(3-methyl-2-oxo-4-phenylbut-3-en-1-yl)oxy]sulfonic acid | DG(18:0e/2:0/0:0) |
| [4-(2-hydroxyethyl)phenyl]oxidanesulfonic acid | (2Z)-4-(octadecyloxy)-4-oxobut-2-enoic acid |
| Salsoline-1-carboxylate | (2S)-2,3-bis(octanoyloxy)propyl 10-methylundecanoate |
| 3-(6-hydroxy-7-methoxy-2H-1,3-benzodioxol-5-yl)prop-2-enal | MG(0:0/22:4(7Z,10Z,13Z,16Z)/0:0) |
| D-4-O-Methyl-myo-inositol | Momordol |
| Caffeine | 6-Deoxohomodolichosterone |
| N-a-Acetyl-L-arginine | 4a-Formyl-5a-cholesta-8,24-dien-3b-ol |
| N-Phenylacetylaspartic acid | 22-Acetylpriverogenin B |

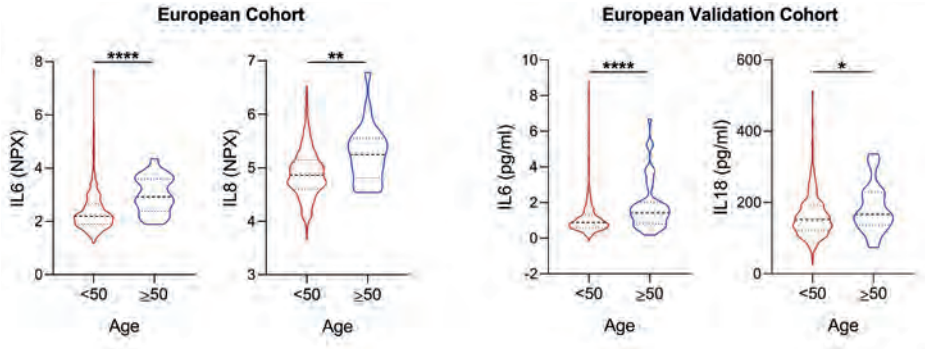
**Supplementary Table 3.** Continued

| Higher in ≥50 years old | Lower in ≥50 years old |
|---|---|
| Mevalonate | 6-({13,14-dimethoxy-9-oxo-8,17-dioxatetracyclo [8.7.0.0$^{2,7}$.0$^{11,16}$] heptadeca-1(10),2(7),3,5,11,13,15-heptaen-5-yl}oxy)-3,4,5-trihydroxyoxane-2-carboxylic acid |
| 3-[3-methoxy-4-(sulfooxy)phenyl]-2-oxopropanoic acid | MG(0:0/18:2(9Z,12Z)/0:0) |
| {[4-(7-methoxy-2-oxo-2H-chromen-6-yl)-2-oxobut-3-en-1-yl]oxy}sulfonic acid | (2S)-1-hydroxy-3-(octanoyloxy) propan-2-yl tridecanoate |
| N-[2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)ethyl]-3-(4-hydroxy-3-methoxyphenyl)prop-2-enimidic acid | 5-Nonadecyl-1,3-benzenediol |
| Phenylacetylglycine | DG(14:0/15:0/0:0) |
| L-Cystine | MG(0:0/22:2(13Z,16Z)/0:0) |
| Propionylcarnitine | DG(14:1(9Z)/14:1(9Z)/0:0) |
| 7-Methoxy-6-methyl-2H-1-benzopyran-2-one | MG(0:0/22:1(13Z)/0:0) |
| Citrulline | beta-Citraurinene |
| L-2-Amino-5-hydroxypentanoic acid | 24-Hydroxycalcitriol |
| Mandelonitrile | 3-Oxo-4,6-choladienoic acid |
| N-Acetylhistidine | MG(0:0/20:3(11Z,14Z,17Z)/0:0) |
| Allitridin | 2,6,6,10,11-pentamethyl-14-(2,6,6-trimethyloxan-2-yl)tetracyclo[8.7.0.0$^{2,7}$.0$^{11,15}$] heptadecane-5,8,16-triol |
| 5-Sulfosalicylic acid | MG(0:0/24:1(15Z)/0:0) |
| Calystegine B2 | (2S)-1-(decanoyloxy)-3-(octanoyloxy) propan-2-yl tetradecanoate |
| (2S)-3-(4-hydroxyphenyl)-2-({[(3S,4S,5R)-2,3,4-trihydroxy-5-(hydroxymethyl)oxolan-2-yl]methyl}amino)propanoic acid | MG(0:0/18:3(6Z,9Z,12Z)/0:0) |
| Oxoadipate | 3-(Acetyloxy)-2-hydroxypropyl icosanoate |
| {[3-(6,7-dimethoxy-2H-1,3-benzodioxol-5-yl)prop-2-en-1-yl]oxy}sulfonic acid | Latanoprost |
| Vanillic acid 4-O-sulfate | Dehydroepiandrosterone |
| Homovanillic acid sulfate | 1-Phenyl-1,3-eicosanedione |
| 2-hydroxy-3-[4-hydroxy-3-(sulfooxy) phenyl]propanoic acid | 5a-Cholestane-3a,7a,12a,25-tetrol |
| N-Acetylcystathionine | 4-[(1-hydroxyoctadecylidene) amino]butanoic acid |

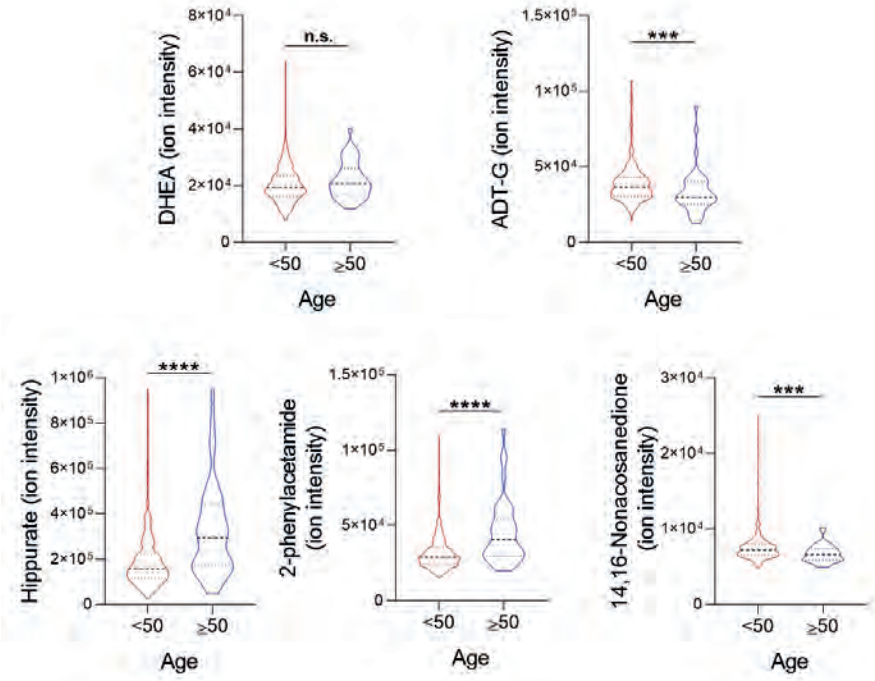
**Supplementary Table 3.** Continued

| Higher in ≥50 years old | Lower in ≥50 years old |
|---|---|
| Gerberinol | 9-Oxoasimicinone |
| | [(2R)-3-[(11Z)-octadec-11-enoyloxy]-2-(pentadecanoyloxy)propoxy]phosphonic acid |
| | 5,7',9',13'-tetramethyl-5'-oxaspiro[oxane-2,6'-pentacyclo[10.8.0.0$^{2,9}$.0$^{4,8}$.0$^{13,18}$]icosane]-16'-one |
| | 13'-Hydroxy-gamma-tocopherol |
| | Tetracosatetraenoic acid (24:4n-6) |
| | Aliskiren |
| | 15-(3,4-dimethyl-5-pentylfuran-2-yl)pentadecanoic acid |
| | Triton X 100 |
| | 5-pentacosylbenzene-1,3-diol |
| | Resveratrol 4'-(6-galloylglucoside) |
| | Lucidone A |
| | 9'-Carboxy-gamma-tocotrienol |
| | DG(14:0/20:5(5Z,8Z,11Z,14Z,17Z)/0:0) |
| | Cilazapril |
| | Prostaglandin F1a |
| | 3-Oxotetradecanoic acid |
| | KB 2 |
| | (9Z,11E,13E,15Z)-4-Oxo-9,11,13,15-octadecatetraenoic acid |
| | Ximenoylacetone |
| | 3-hydroxy-1-phenylicosan-1-one |
| | Delavirdine |
| | 14,16-Nonacosanedione |

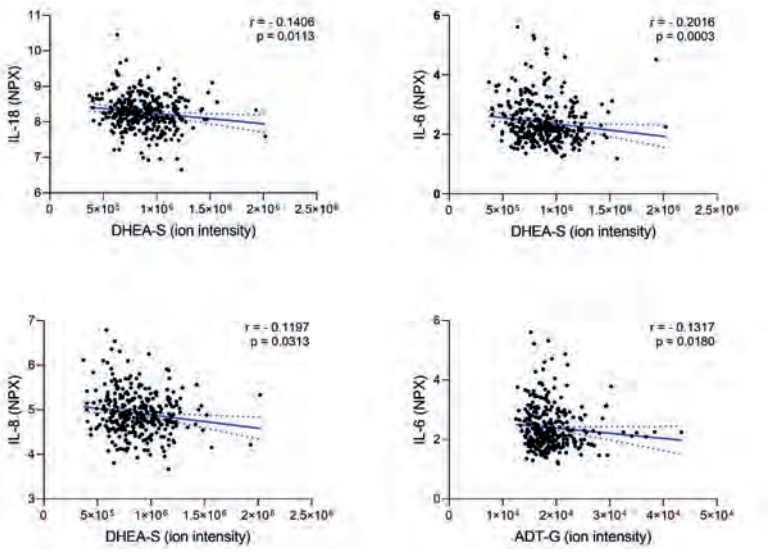
# SUPPLEMENTARY FIGURES



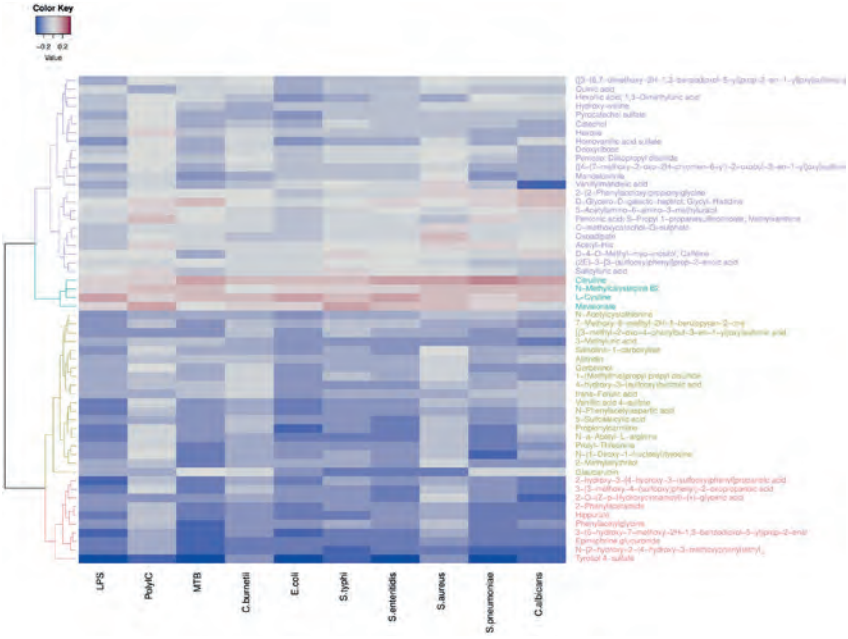

**Supplementary Figure 1. Circulating inflammatory cytokines in different age groups of the European cohorts.** Dashed lines depict the median and the quartiles. NPX: normalized protein expression. *p<0.05, **p<0.01, ****p<0.0001.



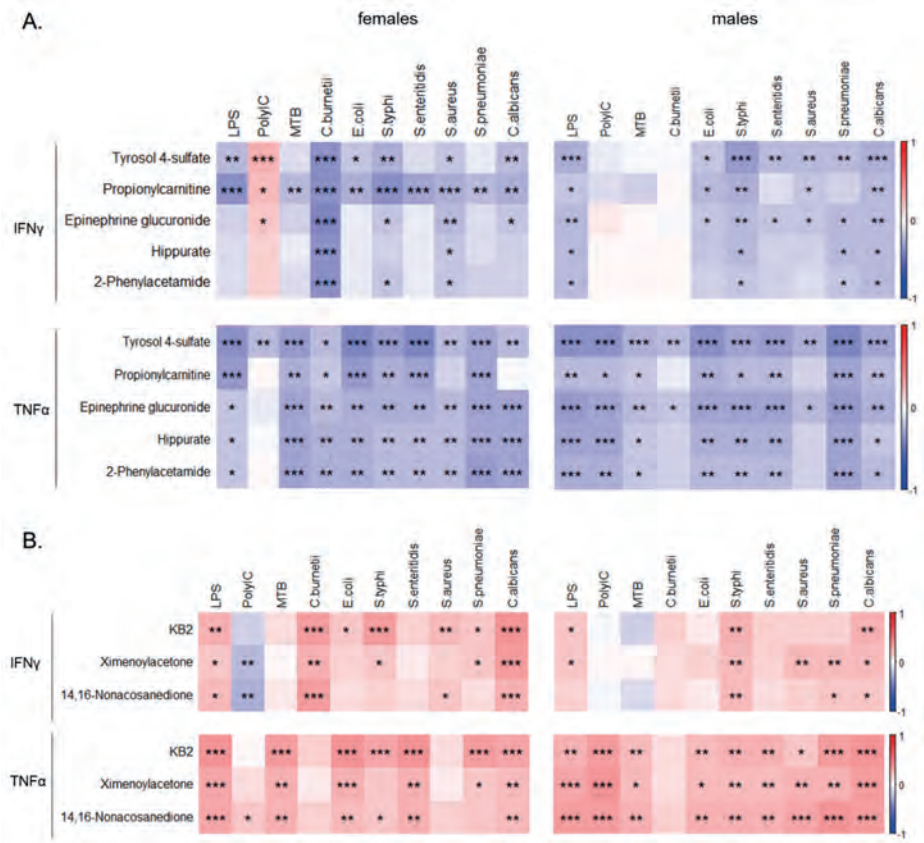

**Supplementary Figure 2. Levels of selected metabolites in individuals below and above 50 years of age in the European Validation Cohort.** Dashed lines depict the median and the quartiles. n.s.: not significant, ***p<0.001, ****p<0.0001.



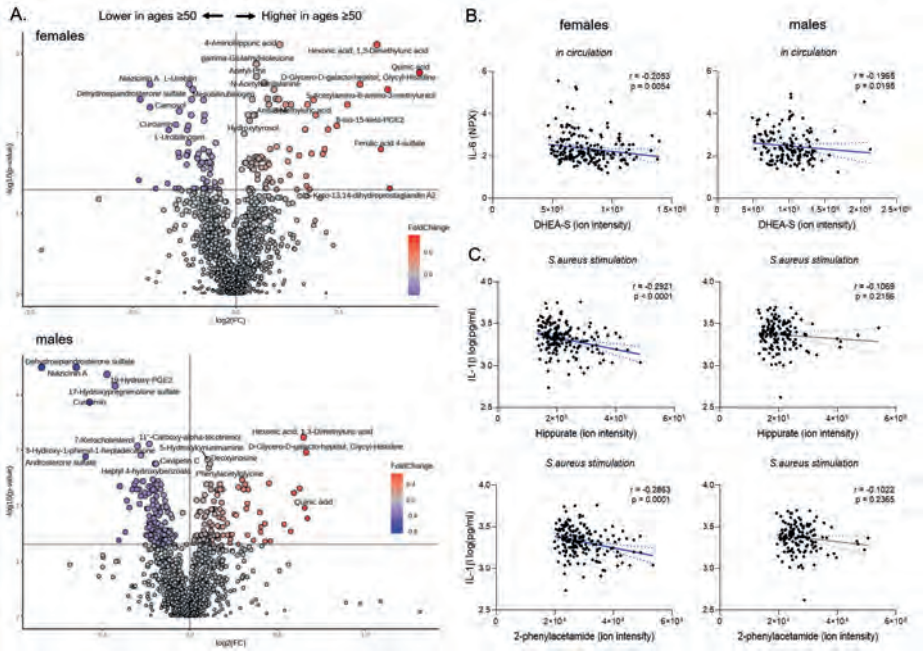

**Supplementary Figure 3. Association of DHEA-S and ADT-G with circulating inflammatory cytokines in the European cohort.** Solid lines depict the best fit and dotted lines depict the 95% confidence interval. DHEA-S: dehydroepiandrosterone sulfate, ADT-G: androsterone glucuronide, r: Spearman's correlation coefficient, NPX: normalized protein expression.



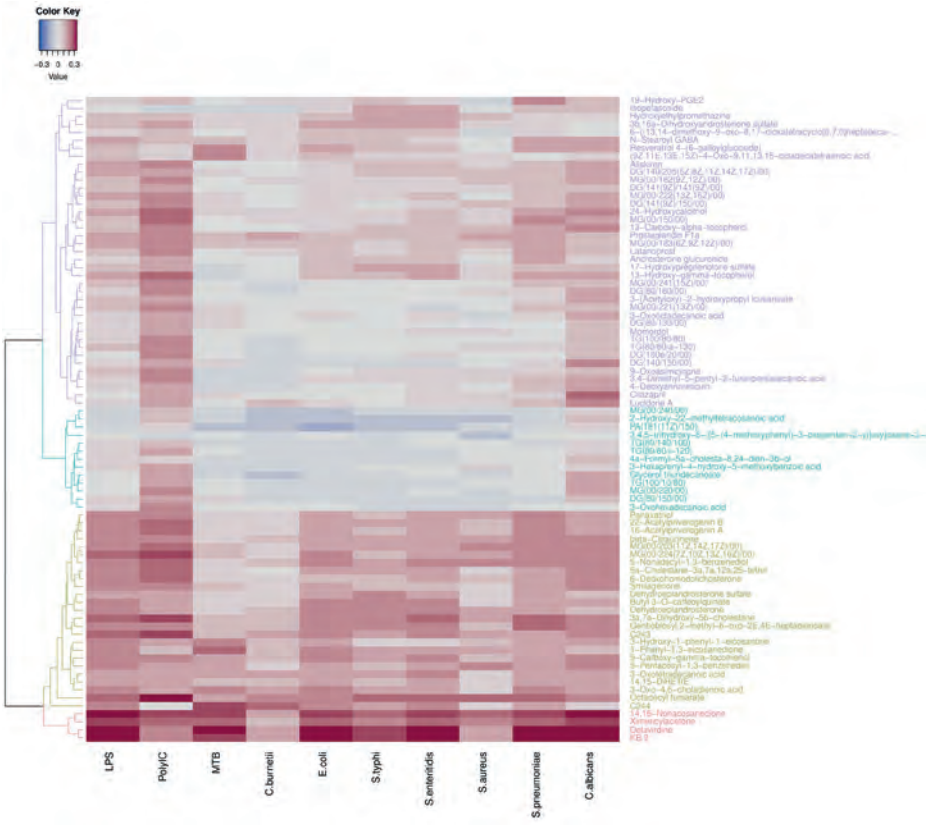

**Supplementary Figure 4. Clustered correlation heatmap of metabolites higher in the elderly in both cohorts and *ex vivo* TNFα production in the African cohort.** Red depicts a positive correlation, while blue depicts a negative correlation. Values of the color key represent Spearman's correlation coefficient. Multiple testing correction was applied.



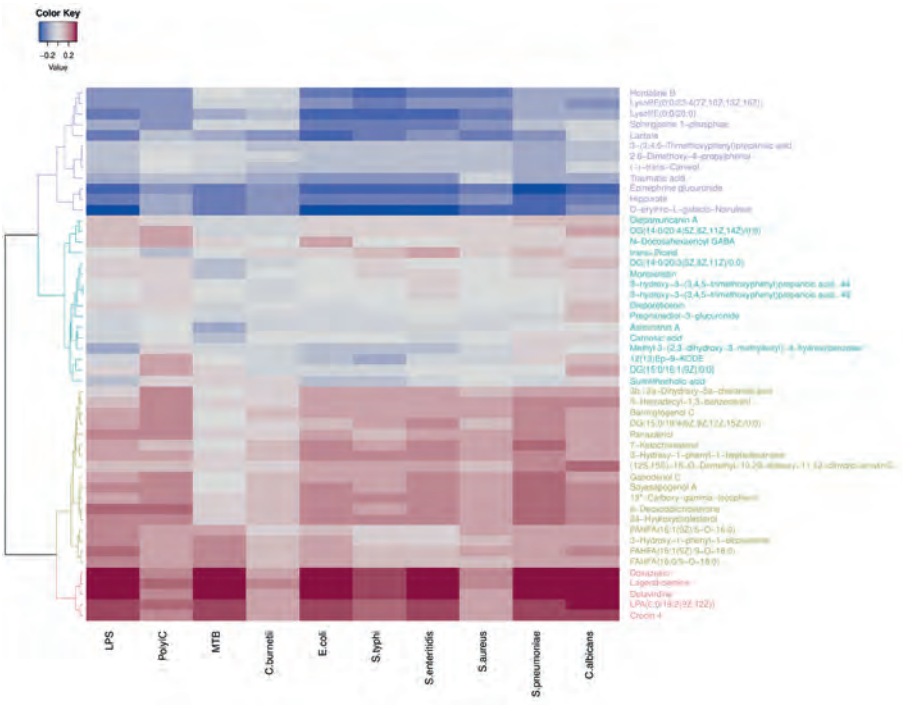

**Supplementary Figure 5. Sex-specific correlations of selected metabolites and *ex vivo* IFNγ and TNFα production in the African Cohort.** Metabolites from Figure 4 that are A) more abundant and B) less abundant in older (≥50) individuals in both cohorts. Red depicts a positive correlation, while blue depicts a negative correlation. Multiple testing correction was performed. *p<0.05, **p<0.01, ***p<0.001.




**Supplementary Figure 6. Highlighted similarities and differences between sexes in the European Cohort.** A) Volcano plots depicting the differential abundance of metabolites in different age groups for females (above) and males (below). B) Association of DHEA-S and circulating IL-6, and C) association of hippurate and 2-phenylacetamide with the IL-1β response against S. aureus stimulation for females (left) and males (right). Solid lines depict the best fit in correlation dot plots, and dashed lines depict the 95% confidence interval. DHEA-S: dehydroepiandrosterone sulfate, r: Spearman's correlation coefficient.




**Supplementary Figure 7. Clustered correlation heatmap of metabolites lower in the elderly in both cohorts and *ex vivo* TNFα production in the African cohort.** Red depicts a positive correlation, while blue depicts a negative correlation. Values of the color key represent Spearman's correlation coefficient. Multiple testing correction was applied.




**Supplementary Figure 8. Clustered correlation heatmap of top 50 metabolites with the biggest fold change that were higher in the African elderly compared to the European elderly and *ex vivo* TNFα production in the African cohort.** Red depicts a positive correlation, while blue depicts a negative correlation. Values of the color key represent Spearman's correlation coefficient. Multiple testing correction was applied.

# CHAPTER 4

# THE IMMUNOLOGICAL FACTORS PREDISPOSING TO SEVERE COVID-19 ARE ALREADY PRESENT IN HEALTHY ELDERLY AND MEN

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* These authors have contributed equally to this work.

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## ABSTRACT

Male sex and old age are risk factors for COVID-19 severity, but the underlying causes are unknown. A possible explanation for this might be the differences in immunological profiles in males and the elderly before the infection. With this in mind, we analyzed the abundance of circulating proteins and immune populations associated with severe COVID-19 in 2 healthy cohorts. Besides, given the seasonal profile of COVID-19, the seasonal response against SARS-CoV-2 could also be different in the elderly and males. Therefore, PBMCs of female, male, young, and old subjects in different seasons of the year were stimulated with heat-inactivated SARS-CoV-2 to investigate the season-dependent anti-SARS-CoV-2 immune response. We found that several T cell subsets, which are known to be depleted in severe COVID-19 patients, were intrinsically less abundant in men and older individuals. Plasma proteins increasing with disease severity, including HGF, IL-8, and MCP-1, were more abundant in the elderly and males. Upon *in vitro* SARS-CoV-2 stimulation, the elderly produced significantly more IL-1RA and had a dysregulated IFNγ response with lower production in the fall compared with young individuals. Our results suggest that the immune characteristics of severe COVID-19, described by a differential abundance of immune cells and circulating inflammatory proteins, are intrinsically present in healthy men and the elderly. This might explain the susceptibility of men and the elderly to SARS-CoV-2 infection.

# INTRODUCTION

Having emerged in China in December 2019, the coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has become a major health crisis. As of early June, 2021, SARS-CoV-2 has led to over 171 million infections and more than 3.5 million deaths worldwide (1).

The most vulnerable groups are people older than 70 years old and adults with underlying health conditions such as chronic respiratory problems and diabetes (2). While age is the strongest predictor of death from COVID-19, the sharp increase in fatality after 50 years of age is more critical in men (3, 4). Association of male sex with higher mortality has been consistently reported in different populations (5–7).



The factors underlying the impact of age and sex on the susceptibility to severe COVID-19 are, however, incompletely understood. Most studies to date have focused on the differences between either young and old or men and women during the disease process. A recent study has shown that male COVID-19 patients had higher circulating concentrations of cytokines such as IL-8 and IL-18, as well as a greater abundance of non-classical monocytes (8). In contrast, a higher degree of T cell activation was observed in females than males during SARS-CoV-2 infection.

We previously analyzed 269 circulating proteins to identify COVID-19 severity markers by comparing patients in the intensive care unit (ICU) to patients who do not require ICU admission (9). Several cytokines and chemokines such as IL-8 and monocyte chemoattractant protein-3 (MCP-3), and growth factors, e.g., hepatocyte growth factor (HGF), were increased in ICU patients, whereas stem cell factor (SCF) and several TNF-family proteins, e.g., TNF-related activation-induced cytokine (TRANCE) were decreased in ICU patients. Besides, frequently reported severe COVID-19 characteristics include elevated TNFa, IL-6, IL-7, MCP- 1, IP-10, G-CSF, and IL-10 concentrations, lower numbers and activity of CD4+, CD8+ cells, Tregs, B cells, and NK cells, increased number of plasmablasts and neutrophils, lower antigen presentation, and downregulated type I interferon signaling (10–15). Changes in cell populations and plasma proteins related to COVID-19 severity are also summarized in Supplementary Table 1. However, it is unknown whether such differences are induced by the disease severity itself, or the potential to respond differently was already present in the healthy steady-state condition.

In this study, we analyzed some of the immune cell populations and circulating proteins linked to COVID-19 severity (Supplementary Table 2) in two Dutch cohorts

of healthy individuals. Our reasoning for choosing these parameters is based on the type of data available from the two cohorts. We investigated if healthy men and individuals over 50 years old already have an immunological profile that predisposes them to severe COVID-19 progression upon SARS-CoV-2 infection. Although the pandemic's seasonal character is not completely clear, several studies reported links to temperature and humidity (16, 17). Therefore, we also hypothesized that immunological differences induced by the seasons could influence the disease outcome. Hence, we investigated SARS-CoV-2-induced immune responses in healthy individuals *in vitro* at different time points of a year using cryo-preserved PBMCs from one of the cohorts. This study provides new insights into how age, sex, and seasons influence COVID-19 response (Figure 1).

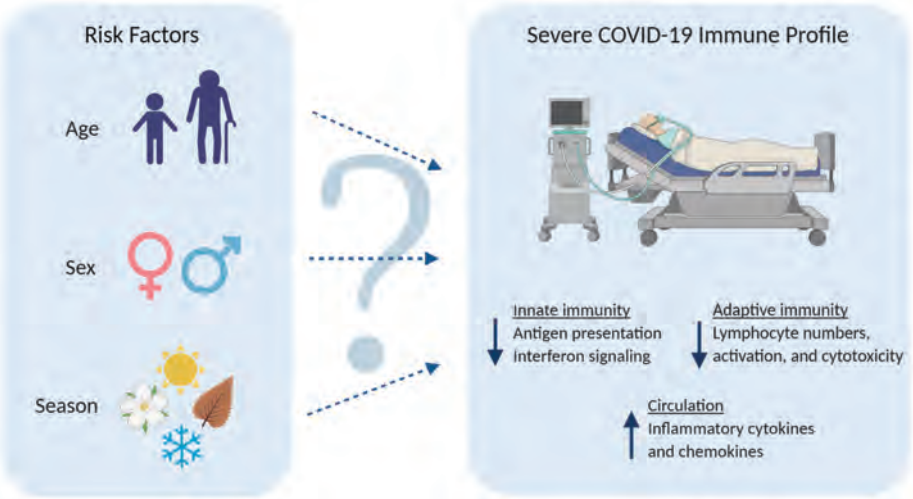



**Figure 1.** (Left) Potential COVID-19 risk factors investigated in this study. (Right) Frequently reported immunological characteristics of severe COVID-19 patients. Downward arrows depict a decline, while upward arrow represents an increase. (Created with BioRender.com).

# MATERIALS AND METHODS

## Study Cohorts

534 healthy individuals of Western European origin were included in Cohort 1 (500 Functional Genomics Project, see www.humanfunctionalgenomics.org) at the Radboud University Medical Center between August 2013 and December 2014. 45 volunteers were initially excluded due to medication use and chronic diseases, while 37 participants were later excluded from the analysis because one or more measurements were unavailable. Data of 452 participants, 229 females and 223 males with age ranges of 18-70 and 18-75, respectively, were used for analysis.

324 healthy individuals of Western European origin, Cohort 2, were included from April 2017 until June 2018 at the Radboud University Medical Center. 183 participants were female, and 141 were male with age ranges of 18-62 and 18-71, respectively. This cohort served as a validation cohort for proteomics.

Both studies were approved by the Arnhem-Nijmegen Medical Ethical Committee (NL42561.091.12 and NL58553.091.16). Inclusion and experimentation procedures were conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all volunteers before sample collection.



### Proteomics

Plasma proteins were measured using the Proximity Extension Assay (PEA) by Olink Proteomics (Uppsala, Sweden). The Olink Inflammation Panel consisting of 92 inflammation-related biomarkers was measured. This assay provides relative protein quantification expressed as normalized protein expression (NPX) values on a log2 scale. The proteins for which the missing data frequency was over 20% were excluded from the analysis. The remaining data under the detection limit was replaced with the lower limit of detection for each protein. Measurements were normalized according to inter-plate controls.

### Flow Cytometry

Immune cell types in Cohort 1 were measured from whole blood by 10-color flow cytometry with Navios flow cytometer (Beckman Coulter, CA, USA). Staining and gating strategies were previously described in detail by Aguirre-Gamboa et al. (18).

### *In Vitro* Stimulations and Cytokine Measurements

From the 452 individuals in Cohort 1, a sub-cohort of 50 people were asked to donate blood at 4 different time points in a year. Peripheral blood mononuclear cells (PBMCs) were collected and cryo-preserved between February 2016 and February 2017 to assess the seasonality of immune responses. From those 50, we selected 20 individuals considering the optimal age and sex matching of young-old and male-female comparisons. Therefore, cells isolated from 5 young males, 5 old males, 5 young females, and 5 old females were included for an *in vitro* study assessing seasonality's impact on the cytokine responses to SARS-CoV-2. Cohort demographics are shown in Supplementary Table 3. Upon thawing, PBMCs were stimulated with heat-inactivated SARS-CoV-2 at a concentration of $3.3\times10^3$ TCID50/mL and heat-inactivated Influenza A H1N1 (California strain) at a concentration of $3.3\times10^5$/mL. To measure the innate immune response, the cytokines TNFα, IL-6, IL-1b, and IL-1RA were measured after 24 hours incubation

with SARS-CoV-2 and influenza A, whereas IFNg response was measured after a 5-day incubation with the viruses. A control condition without a stimulus was included in the experiment. Cytokine concentrations in the supernatants were measured with DuoSet® ELISA kits (R&D Systems, MN, USA) according to the manufacturer's protocols.

### Statistical Analyses

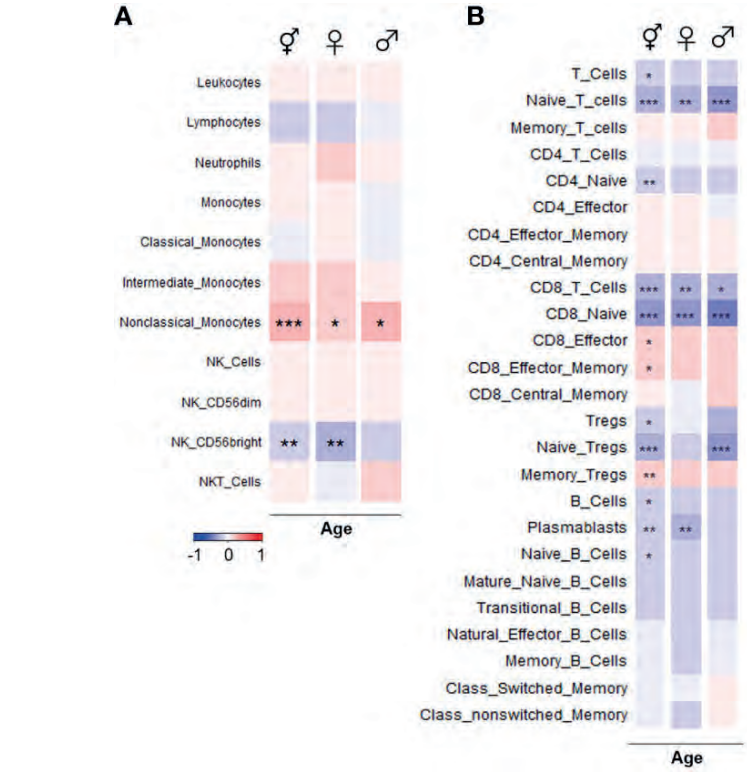
Statistical analyses were performed using R 3.6.1 (www.R-project. org) and GraphPad Prism 8 (GraphPad Software Inc., CA, USA). After adjusting the data for the covariate sex using linear regression, correlation of cell numbers or protein levels with age was done using Spearman's rank-order correlation (Figures 2, 4). Correction for sex was not applied in the heatmaps where two sexes were analyzed separately. After adjusting the data for the covariate age, differential protein expression or cell numbers between males and females was tested using the Mann-Whitney test (Figures 3, 5). For box plots comparing different age groups, the two sexes, or four seasons, the Mann-Whitney test was used between any two groups. The Benjamini-Hochberg procedure was employed to correct multiple testing errors for the heatmaps and volcano plots. False discovery rate (FDR)-adjusted p-values smaller than 0.05 were considered statistically significant.

## RESULTS

### Age-Dependent Changes in Immune Cell Populations Linked to COVID-19 Severity

Various immune cell subtypes were correlated with age in Cohort 1. Non-classical monocytes increased with advancing age in both sexes, but intermediate and classical monocyte numbers were not significantly correlated with age. $CD56^{bright}$ NK population decreased with age in females while other NK cell types remained unchanged (Figure 2A). Previously, these results were partly reported by our group (18).

Total T cell count was negatively correlated with age, indicating the lymphopenia already experienced by the elderly, even in health, is likely to predispose to COVID-19 severity. Naïve T cells, especially CD8+, exhibited the most striking age-dependent decline in both sexes (Figures 2B, C). Naïve regulatory T cells (Tregs) also decreased considerably with age in males, whereas memory Treg numbers were elevated. Total and naïve B cell numbers were negatively correlated with age. Plasmablasts similarly declined significantly in females. No significant age-related changes were observed among the other B cell sub-types.



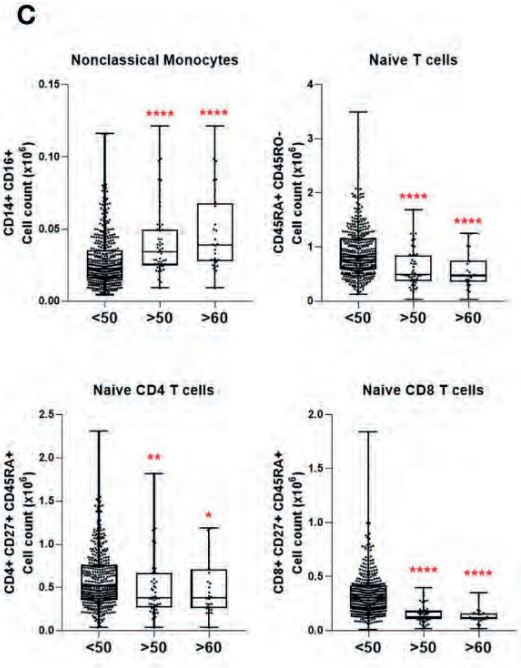




**Figure 2. Heatmaps depicting Spearman correlation of age with cell numbers in (A) Cohort 1 and (B) Cohort 2.** Red depicts a positive correlation, while blue depicts a negative correlation. Data were controlled for the covariate sex when analyzing the whole cohort. (C) Exemplary bar plots of selected cell populations in individuals aged less than 50, more than 50, and more than 60. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. ⚥ whole cohort, ♀ females (n = 229), ♂ males (n = 223).

Overall, these results demonstrate that naïve CD4+, CD8+, Treg, and B cell pools, as well as CD56$^{bright}$ NK cells, which are all depleted in severe COVID-19, also decrease with age. The differences are clear even from the age of 50.

## Sex-Dependent Patterns of Immune Cell Populations Linked to COVID-19 Severity

Next, we investigated the differential abundance of the same immune cell populations influencing COVID-19 severity in females vs. males. Only CD8+ effector memory T cells were significantly more abundant in males (Figure 3A). Almost all cell types, including neutrophils, naïve CD4+ and CD8+ T cells, memory T cells, class-switched memory B cell, and CD56$^{bright}$ NK cell counts, were significantly higher in females (Figures 3A, B). The T cell types and CD56$^{bright}$ NK cells, which are depleted in severe COVID-19 and elderly healthy people, are also apparently less abundant in males.

## Age-Dependent Changes in Immune Mediator Proteins Linked to COVID-19 Severity

We selected 28 proteins whose plasma concentrations have been associated with severe COVID-19 and correlated them with age in healthy cohorts. Circulating IL-6 concentrations increased with age in healthy women, while IL-18 concentrations were higher in healthy men with advancing age in Cohort 1 (Figure 4A). IL-8 concentrations positively correlated with old age in both females and males; however, it was only significant when sexes were combined. Among investigated chemokines, only MCP-1 was positively correlated with age in females in Cohort 2 (Figure 4B). TNF-family proteins in plasma also changed with increasing age: TNF and TNFB concentrations were significantly lower in the circulation of older men in Cohort 1 and 2, respectively (Figures 4A, B). TRANCE sharply declined in older individuals, more strikingly in males, while TWEAK was positively correlated only in females with advancing age. Moreover, aging in males was associated with elevated osteoprotegerin (OPG) concentrations, and HGF concentrations exhibited a considerable age-dependent increase in females in both cohorts.

In summary, several proteins in plasma that are increased in severe COVID-19 patients, such as IL-6, IL-8, IL-18, MCP-1, OPG, and HGF, are more abundant in healthy elderly compared to young individuals (Figures 4A–C). Furthermore, proteins that are lower in severe COVID-19, e.g., TRANCE, decline with age.

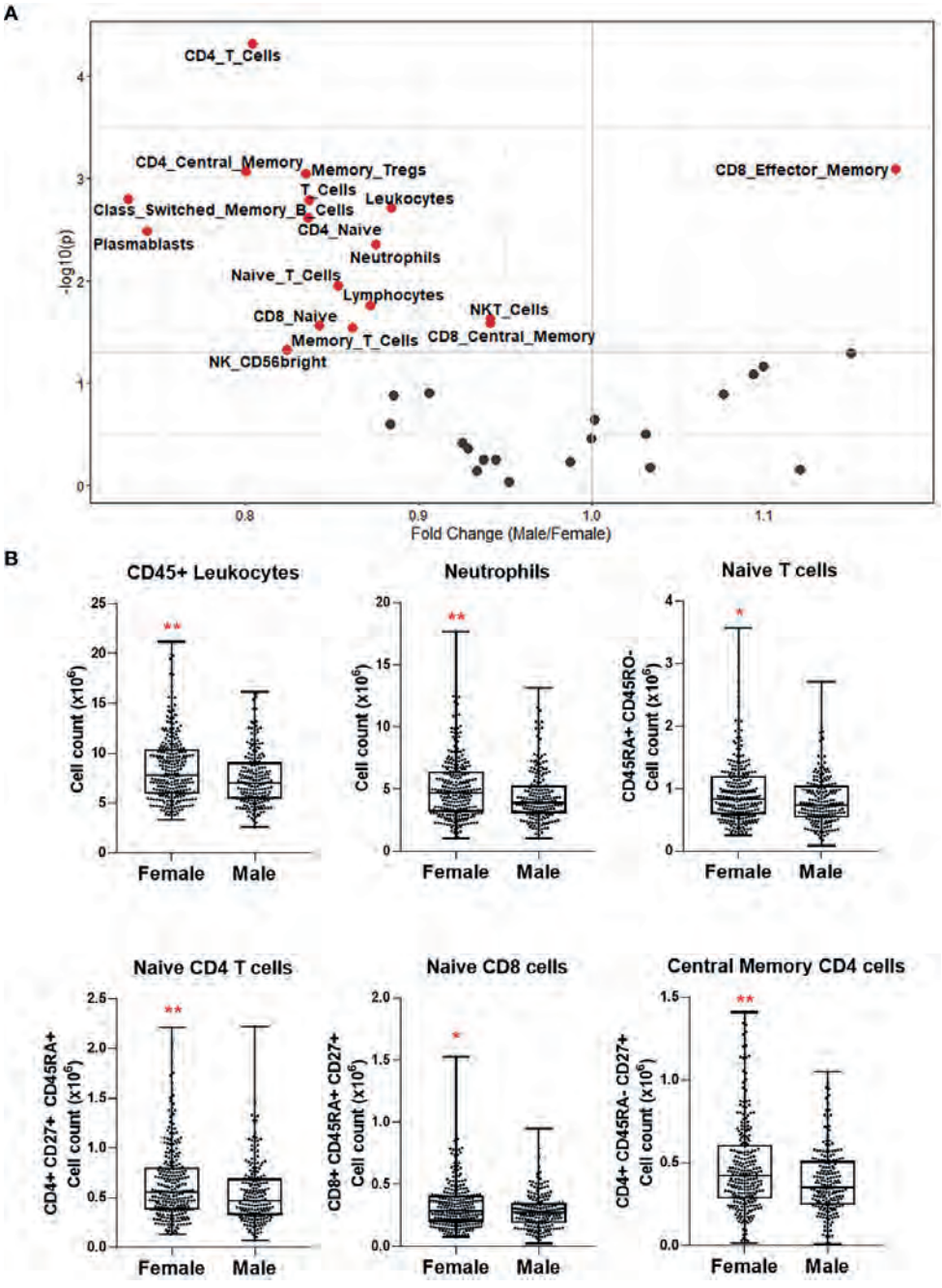



**Figure 3. (A) Volcano plot depicting differential numbers of cell populations depending on sex in Cohort 1.** Significant results are depicted in red. Data were controlled for the covariate age. (B) Exemplary bar plots of selected cell populations in females and males. *p ≤ 0.05, **p ≤ 0.01.

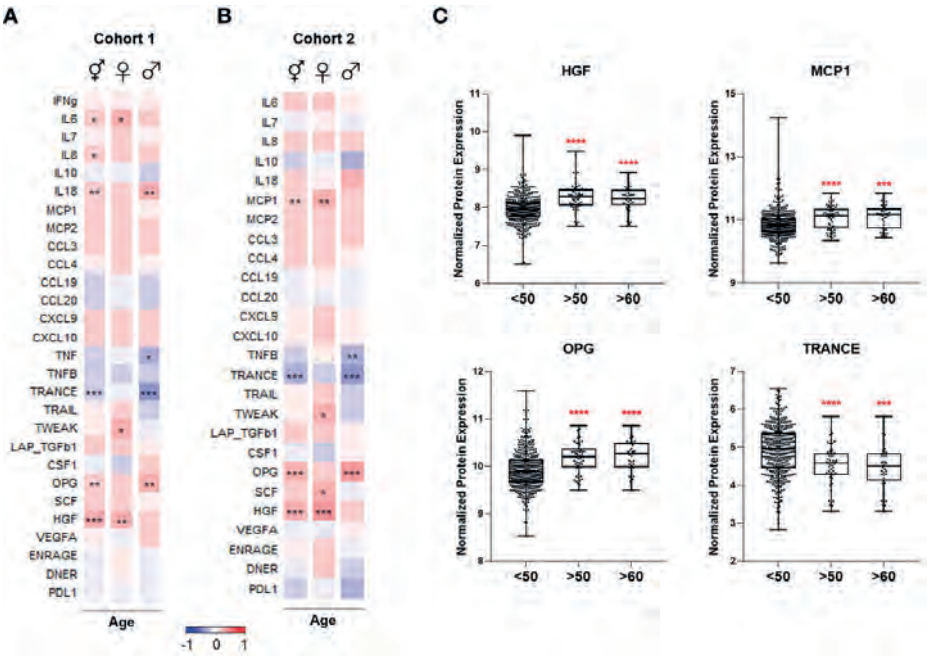



**Figure 4. Heatmaps depicting Spearman correlation of age with plasma levels of proteins linked to COVID-19 severity in (A) Cohort 1 and (B) Cohort 2.** Red depicts a positive correlation, while blue depicts a negative correlation. Data were controlled for the covariate sex when analyzing the whole cohort. (C) Exemplary bar plots of plasma levels of selected proteins in individuals aged less than 50, more than 50, and more than 60. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. ⚥ whole cohort, ♀ females ($n_{Cohort1}$ = 229, $n_{Cohort2}$ = 183), ♂ males ($n_{Cohort1}$ = 223, $n_{Cohort2}$ = 141).

## Sex-Dependent Patterns of Immune Mediator Proteins Linked to COVID-19 Severity

We also compared plasma protein concentrations between sexes. It must be noted that Koeken et al. previously reported that males and females of Cohort 2 exhibit differences in baseline levels of many inflammatory markers (19). Here we provide a more detailed analysis of COVID-19-related proteins among those in Cohorts 1 and 2. We observed a similar sex-dependent trend in both cohorts (Figures 5A, B). Only OPG and colony-stimulating factor-1 (CSF- 1), related to severe COVID-19, were significantly higher in women (Figure 5C). On the other hand, plasma concentrations of other severity markers such as IL-8, IL-18, MCP-1, MCP-2, CCL3, and CCL4 were all higher in men. Furthermore, TRAIL, TWEAK, and TRANCE, which are all lower in COVID-19 patients in ICU, were more abundant in males (9). Males exhibited more anti- inflammatory proteins, e.g., PD-L1 and IL-10. Growth factors HGF and SCF were also more abundant in male plasma. These analyses show that most of the inflammatory mediators playing a role in infection severity are already higher in the circulation of healthy men.

We hypothesized that circulating inflammatory proteins could be related to impaired cytokine response against the virus. Therefore, we correlated SARS-CoV-2-induced *in vitro* cytokine productions with baseline circulating protein concentrations in a sub-cohort of Cohort 1. Indeed, PBMCs of the individuals with higher baseline plasma levels of MCP-2 and IL-8 produced more IL-1RA against SARS-CoV-2 *in vitro* (Supplementary Figure 1). Furthermore, MCP-1 was negatively correlated with IFNg production. The data indicate that higher baseline plasma concentrations of MCP-2, IL-8, and MCP-1 are associated with the inability to produce an optimal defense against SARS-CoV-2 infection.



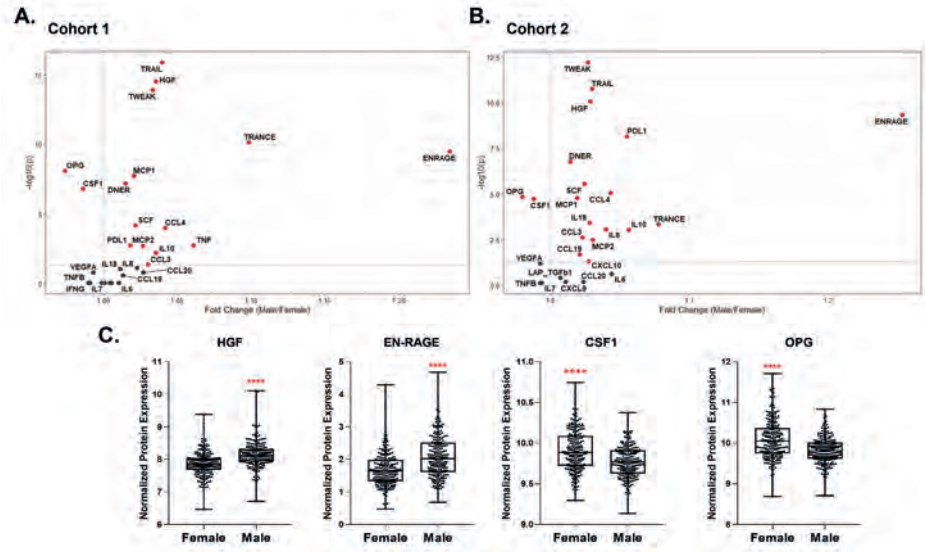



**Figure 5. Volcano plots depicting differential plasma levels of proteins linked to COVID-19 severity depending on sex in (A) Cohort 1 and (B) Cohort 2.** Significant results are depicted in red. Data were controlled for the covariate age. (C) Exemplary bar plots of selected proteins in females and males. ****$p \leq 0.0001$.

## Sex, Age, and Season as Influencing Factors of Immune Response Against SARS-CoV-2

Next, we investigated the impact of seasonality on the SARS-CoV-2-induced immune response and assessed the contribution of age and sex. To this end, we selected 20 individuals from Cohort 1, for which cryo-preserved cells collected at 4 roughly equidistant time points in one year were available. We stimulated their PBMCs with heat-inactivated SARS-CoV-2 and influenza A H1N1.

We found that cytokine production upon SARS-CoV-2 stimulation did not substantially vary during the year, considering all 20 individuals (Supplementary

Figure 2). However, SARS-CoV-2-induced cytokine production did differ for different age groups and sexes throughout the year. Cytokines of the IL-1 biological pathway were higher in the elderly: IL-1β production tended to be greater in the elderly than in young individuals (Figure 6A), while SARS-CoV-2 induced more IL-1RA all-year-round in the old individuals (Figure 6B). Of note, basal IL-1RA production in the absence of any stimulus was also significantly higher in the elderly, but this was not the case for IL-1β (Supplementary Figures 3A, B). Interestingly, IFNγ production upon stimulation with SARS-CoV-2 had a different seasonal profile in the young and elderly: young individuals produced more IFNγ in the summer and fall (Figure 6C). Remarkably, the elderly did not display this seasonal effect, with low IFNg production throughout the year. In contrast to IL-1RA, the basal IFNγ production of the young and the elderly was very low and not different between groups (Supplementary Figure 3C). TNFα and IL-6 productions upon stimulation were similar in the young and the elderly (Supplementary Figure 4A, B). In addition, IL-1β production in response to SARS-CoV-2 in spring and summer was higher in females; however, the average yearly response failed to reach statistical significance (Figure 6D). IL-1RA and IFNγ production in males and females were comparable (Figures 6E, F).

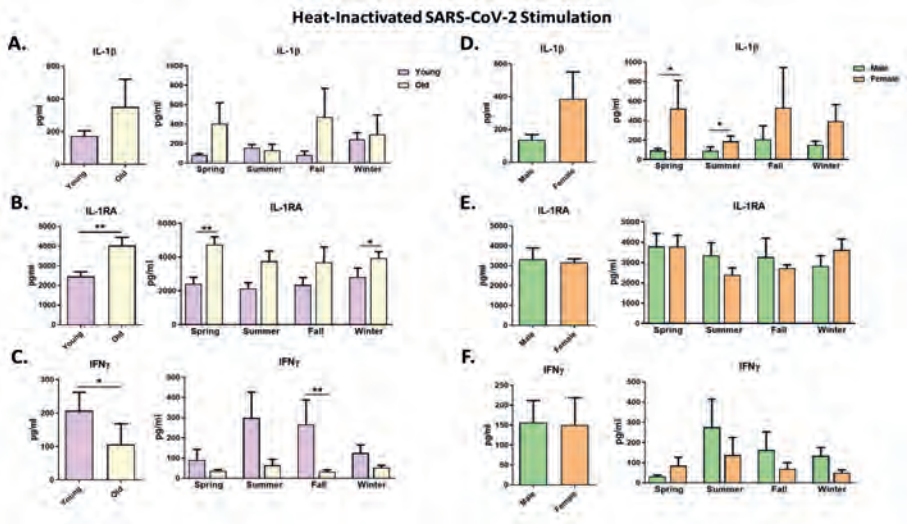



**Figure 6. Cytokine responses against heat-inactivated SARS-CoV-2 in healthy individuals.** Each panel's left graphs show the yearly average production, while the right graphs demonstrate cytokine production in every season. Responses were compared between young and old individuals (A–C) and between males and females (D–F). IL-1β and IL-1RA cytokine levels were measured after 24 hours, while IFNγ was measured after 5 days. *p ≤ 0.05, **p ≤ 0.01. n = 8-10. Error bars depict the standard error of the mean (SEM).

Stimulating PBMCs using another RNA virus, influenza H1N1, resulted in a similar pattern to SARS-CoV-2 stimulation regarding the age and sex effects. The elderly tended to produce more IL-1β and IL-1RA, while young individuals could produce higher IFNγ amounts upon influenza stimulation (Supplementary Figure 5). Similar amounts of IL-1β, IL-1RA, and IFNγ were induced in males and females on average, with few exceptions (Supplementary Figures 5D–F). These data show that individuals of distinct ages and sexes respond differently to SARS-CoV-2 infection depending on the seasons of the year.



## DISCUSSION

Old age and male sex are important risk factors for COVID-19 severity. Several studies have investigated whether immune responses during SARS-CoV-2 infection are influenced by demographic factors such as age and sex (20, 21). Although they identified immune profiles associated with severe COVID-19, they could not assess whether these were secondarily induced by the disease or due to a priori immune differences between different groups. In the present study, we show that the immune characteristics associated with severe COVID-19, such as specific changes in cell populations and circulating inflammatory proteins, are already present in healthy elderly and men. Interestingly, while the season did not impact the immune response to SARS-CoV-2 stimulation in the entire group, there was a clear difference in the responses between the young and old. Young individuals, but not the elderly, improve their IFNγ responses to SARS-CoV-2 during the summer.

COVID-19 progression to a severe clinical picture is related to the depletion of several immune cell types, including naïve CD4+ and CD8+, and $CD56^{high}$ NK cells. Our study demonstrates the age-related decline of these cells in healthy individuals even before the infection, which likely contributes to their incapacity to eliminate the virus. These data are supported by studies suggesting that some of these cell types are scarcer in uninfected elderly and males (22, 23). Naïve B lymphocyte numbers were also reduced with advanced age, which would undermine the development of adaptive immunity and antibody production upon infection (24). The aging process does not only alter cell numbers, but also the functions (Figure 6C) (25). All these might cumulatively disrupt the response against SARS-CoV-2 infection.

Sex differences in immune cell types have been documented before (26). In line with the literature, we showed that one of the striking differences in immune cell types between males and females was the number of CD4+ T cells. Although we

cannot rule out that significant differences in cell populations might not determine the disease severity, SARS-CoV-2-specific CD4+ T cells were strongly linked with milder COVID-19, unlike antibodies and CD8+ T cell numbers (27). Fast induction of CD4+ T cells was related to a milder disease, while defects in inducing SARS-CoV-2-specific CD4+ T cells were associated with severe or fatal COVID-19.

An exaggerated systemic inflammation has been associated with severe COVID-19, mirrored by high circulating concentrations of pro-inflammatory mediators (28). Among those, IL-8, IL-18, and MCP-1 have been frequently reported, and the first two characterize the immune response of men with a severe outcome (8). Notably, their concentrations are already higher in both men and the elderly in our healthy cohorts. Another protein displaying the same pattern is HGF, acting on epithelial and T cells promoting migration (29), which has higher concentrations in the circulation of severe patients (30). The circulating HGF concentrations are higher in men, but also increases with age in women.

Chemokines are critical inflammatory mediators, and MCP-2, CCL3, CCL4, CCL19, and CXCL10 are all more abundant in males. ENRAGE (S100A12), produced by neutrophils and monocytes, is also higher in healthy males than females. Monocytes expressing high S100A12 and IL-8 are linked to COVID-19 severity (31). Additionally, anti-inflammatory proteins IL-10 and PD-L1 are elevated in healthy males and severe COVID-19. Initially considered a negative feedback mechanism for infection-induced inflammation, there are arguments suggesting these proteins as biomarkers of immune exhaustion, which is likely to play a substantial role in the pathophysiology of COVID-19 (32, 33). Notably, the association of early IL-10 production with COVID-19 severity supports this idea (34).

Severity markers increasing with old age, but not affected by sex, include IL-6 and OPG. IL-6 secreted by hyperactive monocytes contributes to low HLA-DR expression and lymphopenia in severe COVID-19 (35). TNF-family cytokine receptor OPG, abundant in ICU patients, increases with old age in our healthy cohorts (9). High OPG concentrations in females are likely due to estrogen's effects promoting OPG expression to inhibit bone resorption (36). Another TNF-family member, TRANCE (RANKL), which is lower in severe COVID-19 cases, also declines with advancing age in healthy individuals. T cells are one of the primary TRANCE sources, which might explain its scarcity in the elderly and severe COVID-19 patients with lymphopenia (37).

Of note, concentrations of circulating IL-7 and IFNg, which are increased in severe COVID-19, are similar in men and women. Therefore, T cell numbers being higher in women is unlikely due to IL-7-induced lymphopoiesis, whereas higher T cell numbers do not necessarily lead to more circulating IFNg. An overview of the age- and sex-dependent immune profiles in healthy individuals potentially predisposing to severe COVID-19 upon infection is provided in Figure 7.

| CELL TYPE | HIGH IN SEVERE COVID-19 | HEALTHY COHORTS: INCREASES WITH AGE | HEALTHY COHORTS: HIGHER IN MALES |
|---|---|---|---|
| Memory Tregs | ✓ | ✓ | |
| **PROTEIN** | | | |
| IL6 | ✓ | ✓ | |
| IL8 | ✓ | ✓ | ✓ |
| IL10 | ✓ | | ✓ |
| IL18 | ✓ | ✓ | ✓ |
| MCP1 | ✓ | ✓ | ✓ |
| MCP2 | ✓ | | ✓ |
| CCL3 | ✓ | | ✓ |
| CCL4 | ✓ | | ✓ |
| CCL19 | ✓ | | ✓ |
| CXCL10 | ✓ | | ✓ |
| PDL1 | ✓ | | ✓ |
| HGF | ✓ | ✓ | ✓ |
| OPG | ✓ | ✓ | |
| ENRAGE | ✓ | | ✓ |

| CELL TYPE | LOW IN SEVERE COVID-19 | HEALTHY COHORTS: DECREASES WITH AGE | HEALTHY COHORTS: HIGHER IN FEMALES |
|---|---|---|---|
| $CD56^{high}$ NK cells | ✓ | ✓ | ✓ |
| T cells | ✓ | ✓ | ✓ |
| Naive CD4 T Cells | ✓ | ✓ | ✓ |
| Naive CD8 T Cells | ✓ | ✓ | ✓ |
| Tregs | ✓ | ✓ | |
| Naive Tregs | ✓ | ✓ | |
| B Cells | ✓ | ✓ | |
| Naive B Cells | ✓ | ✓ | |
| **PROTEIN** | | | |
| TRANCE | ✓ | ✓ | |

**Figure 7. Age- and sex-dependent factors in healthy individuals that are in line with the severe COVID-19 phenotype.**

Aging is already known to alter the immune system in numerous ways. The collective impairments in the aging immune system, termed immunosenescence, and the chronic systemic inflammatory state called inflammaging renders the elderly more susceptible to infections (38, 39). Some of the hallmarks of immune aging observed in both sexes, such as the decline in CD8+ naïve T cells and elevated circulating levels of IL-6 and IL-8, are shared with the pathophysiology of severe COVID-19. Thus, the high COVID-19-related morbidity and mortality observed in the elderly is pathophysiologically plausible. On top of that, in this study, we identified

new factors in the elderly, such as elevated OPG and decreased TRANCE, that are associated with COVID-19 severity but not classically linked to immunosenescence.

Immune responses are more robust and homogenous in young adults, while heterogenous and more variable in the elderly. Along with the age-dependent changes in the immune system, immunobiography of individuals, which is defined by the dose, magnitude, and type of the antigen that each person is exposed to during their lifetime, can explain the large heterogeneity in immune responses, especially in older people (40). Although history of infections affects the immune system of both males and females, our group and others identified a clear distinction between the aged immune systems of men and women (41). As an example, older males have higher pro-inflammatory cytokine levels along with more innate immune activity, but a lower adaptive immune function compared to older females. These variations surely underlie the differences in susceptibility of elderly males and females to infections, including COVID-19.

Environmental factors are also known to affect immune responses. Certain infections such as influenza follow a seasonal pattern (42), and the evolution of the pandemic last year also suggested that COVID-19 incidence might follow a seasonal variation (16). Therefore, we questioned whether this might be due to seasonal changes in the immune response to the virus. In the entire group, we found no clear seasonal response against SARS-CoV-2, although this could be due to the limited sample size. Interestingly, young individuals improve their IFNg response to SARS-CoV-2 during the summer, while the elderly do not. Further research with larger cohorts is required to validate seasonality's full impact on anti-SARS-CoV-2 host defense.

Our results indicate that the immune response upon *in vitro* SARS-CoV-2 stimulation varies depending on age and sex. The response of the elderly is characterized by low IFNγ and elevated IL-1RA production. IFNγ is crucial for an effective response of T and NK cells to viral infections, and its deficiency is associated with severe COVID-19 (43). Depleted NK and T cell pools might explain why the elderly are less capable of producing IFNg upon SARS-CoV-2 stimulation. Poor IFNγ response, especially in the fall, might put the elderly at higher risk for severe COVID-19. Men and women produce comparable amounts of IFNγ, although the T cell numbers are higher in women. Other roles of T cells besides IFNγ production may contribute to the better prognosis of women with COVID-19. One important point is that these defects are present in the whole population, not at the individual level: while the elderly as a group have lower immune responses, there are certainly aged

individuals who have effective immune reaction. This inter-individual variability due to immunobiography of each person could be the reason why some elderly or some men have good responses against SARS- CoV-2 and develop only mild disease.

In COVID-19, an overproduction of pro-inflammatory cytokines contributes to the pathophysiology late in the disease (44). On the other hand, cytokines such as IL-1β might also be crucial for an early anti-viral response. The deficiency of IL-1β or its receptor causes higher viral load and mortality in murine models (45). Furthermore, genetic variants in IL1B contribute to influenza susceptibility in humans (46). We observed higher IL-1β production in women in response to SARS-CoV-2 *in vitro*, arguing that an initial potent anti-viral defense is essential to prevent severe disease.



Moreover, IL-1RA is an antagonist of IL-1 bioactivity, helping prevent excessive inflammation (47). However, early IL-1RA production in patients is associated with COVID-19 severity (34), while our finding of higher IL-1RA production in the elderly suggests that IL-1RA might be hindering their ability to mount an optimal immune response against SARS-CoV-2. Alternatively, high IL-1RA production with increasing age might mirror the general inflammatory profile of elderly individuals, as we have shown that baseline IL-1RA produced by PBMCs is higher in the elderly compared to young individuals. Due to limited statistical power, it was not possible to assess the impact of season depending on both age and sex of the volunteers, e.g. comparing old men, old women, young men, and young women. Assessing how age, sex, and season collectively affect the anti-SARS-CoV-2 response of each subgroup should be assessed in larger future studies. An additional aspect to be assessed in future studies is the potential contribution of innate immune cell reprogramming (also called 'trained immunity') to the hyperinflammation in COVID-19. Trained immunity can be beneficial for the host in terms of protection against heterologous infection, but dysregulated trained immunity response could result in pathological conditions as it was described in autoinflammatory diseases (48, 49). A recent study has described induction of trained immunity by SARS-CoV-2 infection (50), and it has been proposed that this contributes to the acute dysregulation during the disease both an endothelial and immune cell level (51).

In conclusion, our findings shed light on the immunological factors that might explain why men and the elderly have a higher risk of developing severe COVID-19. These results also emphasize the importance of the IL1β/IL-1RA axis and IFNγ in the anti-SARS-CoV-2 response. We propose that intrinsically different immune characteristics, including plasma inflammatory mediators and immune cell

populations, in healthy people would influence their immune response upon SARS-CoV-2 infection and the severity of the disease. The results of this study inform prophylactic and therapeutic efforts.

## Data availability statement

The data is available on request through http://www.humanfunctionalgenomics.org.

## Ethics statement

The studies involving human participants were reviewed and approved by The Arnhem-Nijmegen Medical Ethical Committee: NL42561.091.12 and NL58553.091.16. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

GK, OB, and MGN conceptualized and designed the study. MJ, RH, SJCFMM, VACMK, and CB conducted the cohort studies and provided the data. GK and OB performed the analyses and *ex vivo* experiments. LABJ and MGN provided funding. GK and OB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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# SUPPLEMENTARY TABLES

**Supplementary Table 1.** Plasma proteins and immune cell populations linked with severe COVID-19.

| Reference | Severe COVID-19 Immune Profile |
|---|---|
| **Janssen et al. (2021)** | Higher CRP, D-dimer, TNFα, IL-6, IL-8, IL-18, HGF, CCL3, CCL19, CCL20, ENRAGE, MCP3, VEGFA, and CD40. Lower SCF, DNER, TRAIL, TRANCE, TNFB, VEGFD, and HLA-DR expression. |
| **Qin et al. (2020)** ***Clin Infect Dis*** | Higher TNFα, IL-6, IL-8, IL-10, CRP, ferritin, memory CD4+ T cells, and neutrophils. Lower lymphocytes, T cells, CD4+ T cells, and Tregs. |
| **Huang et al. (2020)** ***Cytometry A*** | Lower lymphocytes, CD4+ T cells, CD8+ T cells, CD56+ NK cells and B cells. |
| **Yang et al. (2020)** ***J Allergy Clin Immunol*** | Higher IP-10, MCP-3, HGF, MIP-1α, IL-1RA, and CSF1. |
| **Bergamaschi et al. (2021)** ***MedRxiv*** | Higher plasmablasts, classical monocytes, and neutrophils. Lower CD4+ naïve, CD4+ central memory, CD4+ effector memory, Tregs, CD4+ follicular helper, CD8+ naïve, CD8+ effector memory, B cells, transitional B cells, memory B cells, pDCs, and non-classical monocytes. |
| **Chen et al. (2020)** | Higher lactate dehydrogenase, D-dimers, CRP, IL-6, IL-10, TNFα, and neutrophils. Lower lymphocytes, CD4+ T cells, CD8+ T cells, and naïve T regs. |
| **Huang et al. (2020)** | Higher IL-2, IL-7, IL-10, IP-10, MCP-1, MIP1α, TNFα, and G-CSF. |

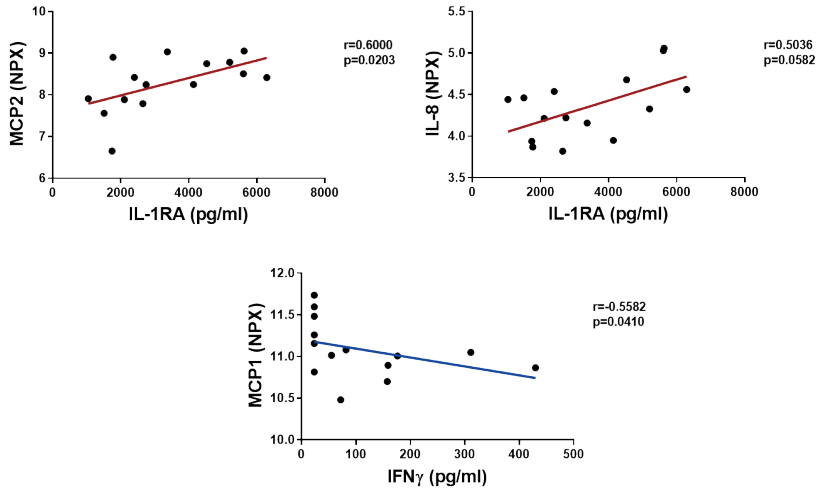
**Supplementary Table 2.** List of immune cell types and circulating proteins investigated in the study. These have been previously linked to COVID-19 severity.

| IMMUNE CELL TYPES | CIRCULATING PROTEINS |
|---|---|
| Leukocytes | Interferon gamma (IFNγ) |
| Lymphocytes | Interleukin 6 (IL-6) |
| Neutrophils | Interleukin 7 (IL-7) |
| Monocytes | Interleukin 8 (IL-8) |
| Classical Monocytes | Interleukin 10 (IL-10) |
| Intermediate Monocytes | Interleukin 18 (IL-18) |
| Non-classical Monocytes | Monocyte chemoattractant protein 1 (MCP-1) |
| Natural Killer (NK) Cells | Monocyte chemoattractant protein 2 (MCP-2) |
| $CD56^{dim}$ NK Cells | Chemokine (C-C motif) ligand 3 (CCL3) |
| $CD56^{bright}$ NK Cells | Chemokine (C-C motif) ligand 4 (CCL4) |
| Natural Killer T Cells | Chemokine (C-C motif) ligand 19 (CCL19) |
| T Cells | Chemokine (C-C motif) ligand 20 (CCL20) |
| Naïve T Cells | Chemokine (C-X-C motif) ligand 9 (CXCL9) |
| Memory T Cells | Chemokine (C-X-C motif) ligand 10 (CXCL10) |
| $CD4^+$ T Cells | Tumor necrosis factor (TNF) |
| Naïve $CD4^+$ T Cells | Tumor necrosis factor beta (TNFB) |
| Effector $CD4^+$ T Cells | TNF-related activation-induced cytokine (TRANCE) |
| Effector Memory $CD4^+$ T Cells | TNF-related apoptosis-inducing ligand (TRAIL) |
| Central Memory $CD4^+$ T Cells | TNF-related weak inducer of apoptosis (TWEAK) |
| $CD8^+$ T Cells | Latency associated peptide - transforming growth factor beta (LAP-TGFB1) |
| Naïve $CD8^+$ T Cells | Osteoprotegerin (OPG) |
| Effector $CD8^+$ T Cells | Colony stimulating factor 1 (CSF1) |
| Effector Memory $CD8^+$ T Cells | Stem cell factor (SCF) |
| Central Memory $CD8^+$ T Cells | Hepatocyte growth factor (HGF) |
| Regulatory T cells (Tregs) | Vascular endothelial growth factor alpha (VEGFA) |
| Naïve Tregs | Extracellular newly identified RAGE-binding protein (EN-RAGE) |
| Memory Tregs | Delta and Notch-like epidermal growth factor-related receptor (DNER) |
| B Cells | Programmed death-ligand 1 (PD-L1) |
| Plasmablasts | Naïve B Cells |
| Mature Naïve B Cells | |
| Transitional B Cells | |
| Natural Effector B Cells | |
| Memory B Cells | |
| Class-switched Memory B Cells | |
| Non-class-switched Memory B Cells | |

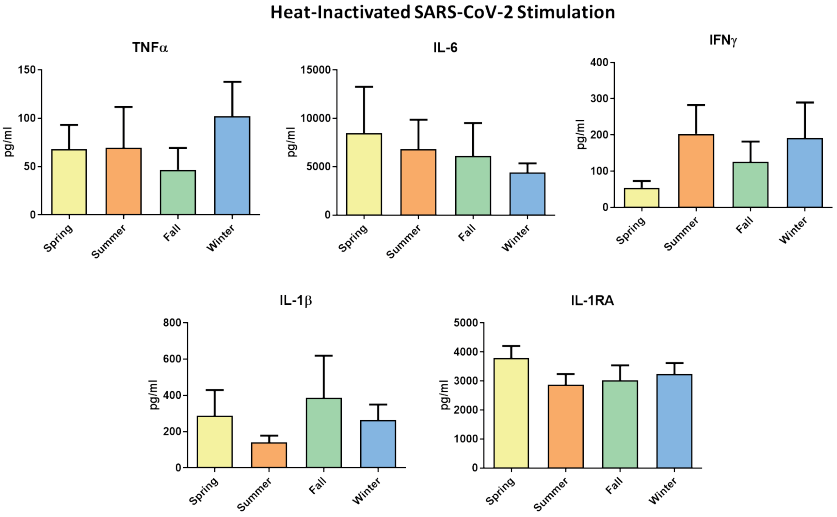
**Supplementary Table 3.** Demographics of the 20 individuals selected from Cohort 1 for seasonality analysis. Age and BMI are depicted as mean ± standard deviation.

| Group (n=5) | Age | Body mass index (BMI) |
|---|---|---|
| Young male | 23.2 ± 3.11 | 24.46 ± 1.47 |
| Old male | 64.6 ± 3.65 | 24.39 ± 2.46 |
| Young female | 23.8 ± 3.11 | 22.40 ± 1.63 |
| Old female | 60.8 ± 6.22 | 24.07 ± 3.70 |

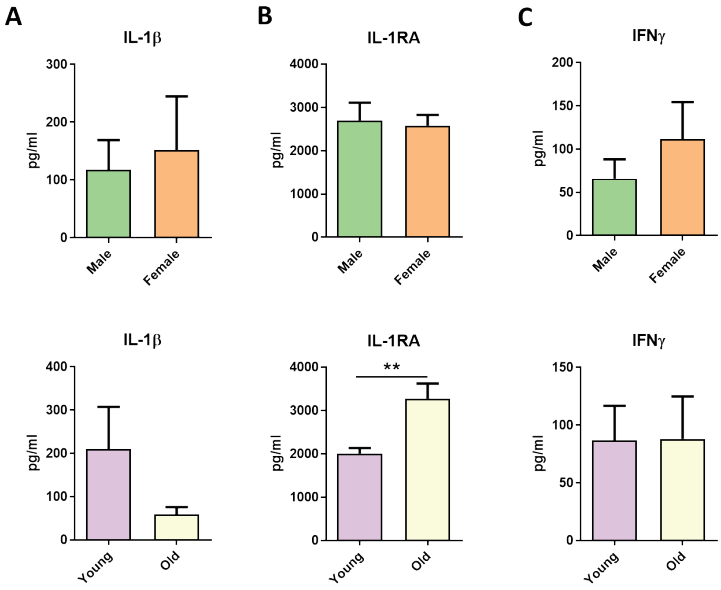
# SUPPLEMENTARY FIGURES



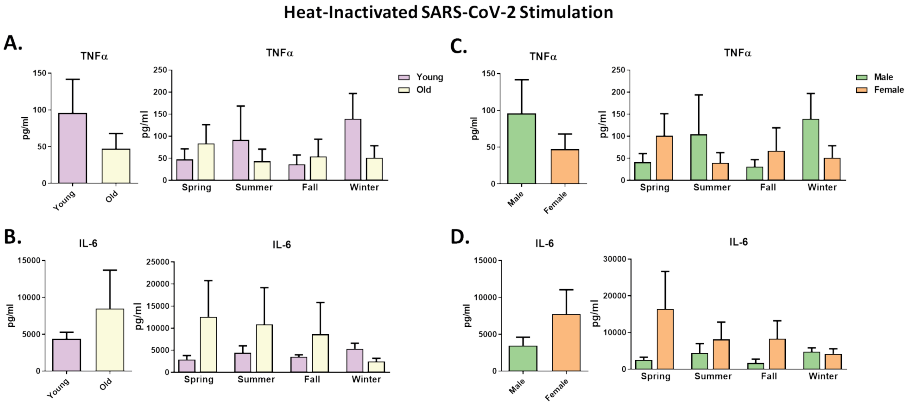

**Supplementary Figure 1. Correlation between baseline plasma protein levels and cytokine productions against *in vitro* SARS-CoV-2 stimulation in healthy individuals.** The x-axis shows the cytokine productions of PBMCs after stimulation with heat-inactivated SARS-CoV-2 while the y-axis demonstrates the baseline plasma protein levels of healthy individuals. Red indicates a positive correlation whereas blue indicates a negative correlation. NPX: normalized protein expression, r = Spearman correlation coefficient, n=14-15.



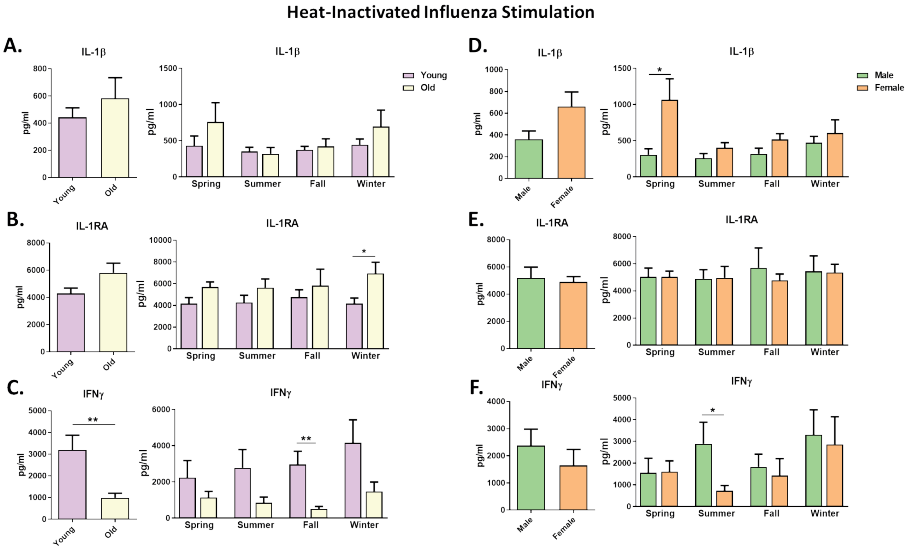

**Supplementary Figure 2. The cytokine productions of PBMCs that were stimulated with heat-inactivated SARS-CoV-2.** The PBMCs from healthy individuals were collected and frozen at different times of the year. n=7-20. Error bars depict the standard error of the mean (SEM).



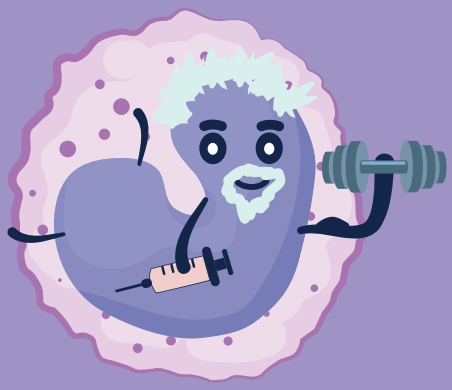

**Supplementary Figure 3. Basal cytokine responses of healthy individuals in the absence of any stimulus.** Responses were compared between males and females (upper row) and between young and old individuals (lower row). The graphs show the yearly average production. (A) IL-1β and (B) IL-1RA cytokine levels were measured after 24 hours, while (C) IFNγ was measured after 5 days. **p ≤ 0.01. n=8-10. Error bars depict the SEM.




**Supplementary Figure 4. Cytokine responses against heat-inactivated SARS-CoV-2 in healthy individuals.** Each panel's left graphs show the yearly average production, while the right graphs demonstrate cytokine production in every season. Responses were compared between young and old individuals (A-B) and between males and females (C-D). Cytokine levels were measured after 24 hours. n=3-8. Error bars depict the SEM.




**Supplementary Figure 5. Immune responses against heat-inactivated influenza A (H1N1) in healthy individuals.** Each panel's left graphs show the yearly average production, while the right graphs demonstrate cytokine production in every season. Responses were compared between young and old individuals (A-C) and between males and females (D-F). IL-1β and IL-1RA cytokine levels were measured after 24 hours, while IFNγ was measured after 5 days. *p ≤ 0.05, **p ≤ 0.01. n=5-10. Error bars depict the SEM.



# CHAPTER 5

# OVERCOMING IMMUNE DYSFUNCTION IN THE ELDERLY: TRAINED IMMUNITY AS A NOVEL APPROACH

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## ABSTRACT

People with advanced age have a higher susceptibility to infections and exhibit increased mortality and morbidity as the ability of the immune system to combat infections decreases with age. While innate immune cells display functional defects such as decreased phagocytosis, chemotaxis and cytokine production, adaptive immune cells exhibit reduced receptor diversity, defective antibody production and a sharp decline in naive cell populations. Successful responses to vaccination in the elderly are critical to prevent common infections such as influenza and pneumonia, but vaccine efficacy decreases in older individuals compared with young adults. Trained immunity is a newly emerging concept that showed that innate immune cells possess non-specific immunological memory established through epigenetic and metabolic reprogramming upon encountering certain pathogenic stimuli. Clinical studies suggest that trained immunity can be utilized to enhance immune responses against infections and improve the efficiency of vaccinations in adults; however, how trained immunity responses are shaped with advanced age is still an open question. In this review, we provide an overview of the age-related changes in the immune system with a focus on innate immunity, discuss current vaccination strategies for the elderly, present the concept of trained immunity, and propose it as a novel approach to enhance responses against infections and vaccinations in the elderly population.

## INTRODUCTION

The rapid aging of the world population is one of the most crucial social shifts taking place in the twenty-first century, with an extensive impact on different fields, including economics and health care. According to the United Nations Population Division, the number of people over 60 years of age in urban areas increased by 68% between 2000 and 2015 (1). This number is predicted to grow by another 56% until 2030, reaching 1.4 billion. By 2050, the population over 60 years will more than double its current size, exceeding 2 billion people.

As humans age, their immune system undergoes age-related changes that are collectively termed immunosenescence (2). Besides other age-related conditions such as Alzheimer's disease and cardiovascular diseases, aging of the immune system leads to increased susceptibility to infections and autoimmune diseases, and poor response to vaccination, followed by high hospitalization and increased mortality rates (3). Morbidity associated with infectious diseases in the elderly population is a significant burden on the healthcare systems and economies of countries all around the globe. Because of these reasons, counteracting immunosenescence and developing new immunization strategies for elderly people are considered priority research areas by the World Health Organization (4). Understanding the mechanisms of immunosenescence and developing counteractive measures are of great importance.



Here, we describe the mechanisms of immunosenescence, with a particular emphasis on the innate immune system. We then review the impact of vaccine responses in the elderly and the current approaches to improve vaccine efficacy. Lastly, we describe the concept of trained immunity, the adaptation of innate host defense that leads to non-specific immunological memory in innate immune cells through epigenetic and metabolic reprogramming (5). We finally detail recent studies utilizing trained immunity to boost vaccine responses and propose trained immunity as a promising approach to increase vaccine efficiency in the elderly population.

## AGING OF THE IMMUNE SYSTEM: A BRIEF OVERVIEW

The most established features of immunosenescence —the dysregulated state of an aged immune system— include short-lived memory responses, defective response to new antigens, higher disposition to autoimmunity and the chronic low-grade

systemic inflammation that is termed inflammaging (6). The main cellular culprits behind these dysregulated responses are a sharp decrease of naive T- and B-cell pools with increasing age, reduced natural killer (NK) cell cytotoxicity, impaired signaling, and decreased function of some innate immune cell subsets (2) (Figure 1).

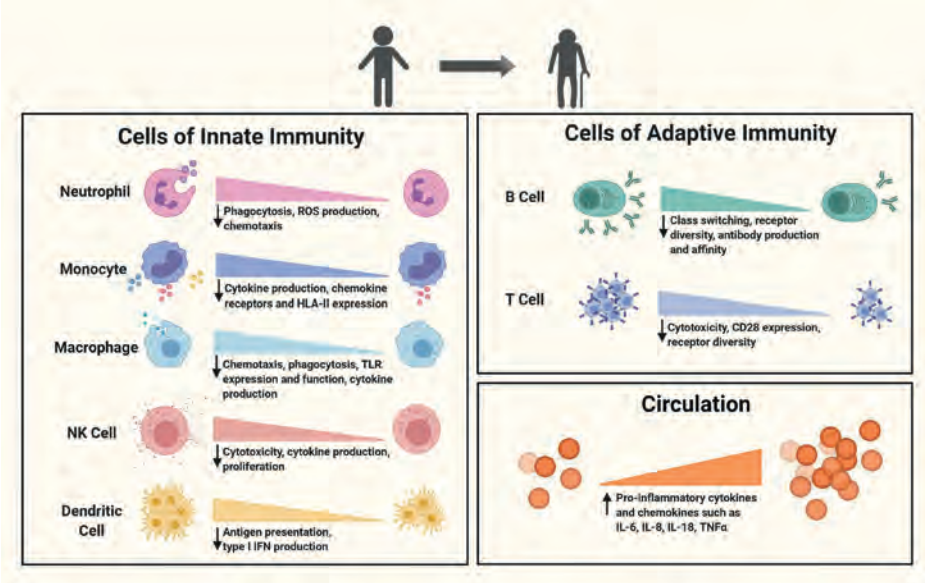


**Figure 1. Age-associated functional changes in the immune system.** Both innate and adaptive immune systems undergo age-related alterations in terms of cell numbers and functions toward the later decades of human life. Multiple human and murine studies revealed that the cells of innate immunity, such as neutrophils, monocytes, macrophages, dendritic cells, and NK cells, display impaired receptor expression, chemotaxis, phagocytosis, antigen presentation, cytotoxicity, ROS, and cytokine production. Adaptive immune cells (B cells and T cells) experience shifts in sub-populations such as the depletion of naive cell pools and accumulation of late-differentiated effector and memory cells. Apart from those, both display reduced receptor diversity. Functionally, expression of the co-stimulatory molecule CD28 is critically diminished in T cells while B cells become weaker in class-switching and affinity maturation. Numbers of plasma cells and production of antibodies also decrease. Despite these functional down-regulations at the cellular level, levels of pro-inflammatory cytokines and chemokines are elevated in circulation with advancing age.

Lingering inflammation causes tissue damage and contributes to the development and progression of age-related diseases. Elevated circulating levels of pro-inflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor α (TNFα) along with C-reactive protein (CRP) are some of the most reliable markers of inflammaging, their circulating concentrations predicting frailty and mortality in the elderly (7, 8). Inflammaging is the result of the accumulated long-term stimulation of the innate immune system with increasing age. As the current life expectancy of humans exceeds the life span that characterized human evolution

for hundreds of thousands of years, beneficial physiological responses may become damaging as humans age (9).

One of the mechanisms proposed to drive inflammaging is the accumulation of damage-associated molecular patterns (DAMPs), which are essential for effective tissue repair and inflammatory response against pathogens, but can also cause maladaptive responses and chronic disease, as disposal of the accumulating material by autophagy or mitophagy declines with age (10). Another likely source is the senescence-associated secretory phenotype (SASP) of senescent epithelial and endothelial cells, which secrete pro-inflammatory cytokines and modify the response of neighboring cells (11, 12). Products of microbiota might also contribute to inflammaging. As the body ages, the gut is less efficient in sequestering microbes and their products (13). Contents of the gut microbiota change with age as well, becoming more inflammatory (14). Age-related expansion of Proteobacteria and a decline in butyrate-producing bacteria, for example, have been correlated to increased IL-6 and IL-8 levels (15).



## AGE-RELATED CHANGES IN INNATE IMMUNITY

Hematopoietic stem cells (HSCs) in the bone marrow increase in number with age and become more likely to commit to the myeloid lineage, which gives rise to the majority of the innate immune cells including dendritic cells (DCs), monocytes, macrophages, mast cells and granulocytes (e.g. neutrophils) (16, 17). However, despite the skewing to the myeloid lineage in the bone marrow, numerous age-related declines in terms of cell number and function have been described for the cells of innate immunity. On the one hand, elderly people tend to develop low-grade systemic inflammation although their immune cells present defective capacities of migration, phagocytosis, and cytokine production (18). Impaired functions of innate immunity can further exacerbate the flaws in adaptive immunity, for instance, by not providing efficient antigen presentation to T cells. Here, we detail the age-related changes in different innate immune cell subsets and their consequences.

### Neutrophils

Neutrophils are the most abundant type of immune cell in cir-culation. They internalize pathogens through phagocytosis and destroy them using reactive oxygen species (ROS) and degradative proteases, while also recruiting and activating DCs, monocytes and lymphocytes (19). Neutrophils also efficiently trap and kill extracellular pathogens by forming neutrophil extracellular traps composed of web-like structures of chromatin and proteases (20).

Many functions of neutrophils, including chemotaxis, phagocytosis, ROS production, signal transduction, and apoptosis, have been reported to be dysfunctional in the elderly (21–25). However, neutrophil numbers are mostly preserved during aging (21). Healthy centenarians —people aged 100 years or older— have well-conserved neutrophil functions (26). Increased activation of constitutive phosphoinositide 3-kinase (PI3K) was associated with impaired chemotaxis in the elderly (22). Expression of CD16, an Fc receptor, is low in neutrophils of people aged over 65, which potentially restricts Fc-mediated phagocytic activity (24). Intracellular killing of the phagocytosed pathogens is also defective in the elderly (27, 28). Defects in ROS production have been linked to the changing composition of cell membranes with age (29). Moreover, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2 have anti-apoptotic effects on neutrophils of young adults but not in adults over 65 years of age (30, 31). Increased neutrophil susceptibility to apoptosis might also contribute to the weakened response of the elderly against pathogens (3).

## Monocytes

Monocytes present a large range of functions, including phagocytosis, cytokine production, and antigen presentation. They circulate in the blood and migrate into tissues in response to infection or tissue damage, where they can differentiate into macrophages or DCs (32). In humans, there are three major monocyte subsets with different functions, identified based on their CD14 and CD16 expression: CD14+ CD16– classical monocytes, CD14+ CD16+ intermediate monocytes and CD14dim CD16+ non-classical monocytes (33).

Circulating monocyte numbers are stable with advancing age (21). However, the ratios of different monocyte subsets are altered. Classical monocytes are reportedly reduced, while intermediate and non-classical monocytes are increased with age (34). Of note, expanding non-classical monocytes present lower expression of the CX3CR1 chemokine receptor and the human leukocyte antigen class II molecule HLA-DR, whereas macrophages derived from monocytes of elderly subjects display intact cytokine production (35, 36). Monocytes from subjects over 60 years old present higher Toll-like receptor 5 (TLR5) expression, produce more IL-8 and show increased phosphorylation of mitogen-activated protein kinases (MAPKs) p38 and extracellular signal-regulated kinase (ERK) upon activation with TLR ligands, but they are defective in activation of nuclear factor κB (NF-κB) (37).

Another study reported lower TLR1 expression, less ERK1/ ERK2 phosphorylation upon TLR1/TLR2 activation, and reduced IL-6 and TNFα production in monocytes of people over 66 years of age (38). A recent study investigating innate immune

responses in a healthy population of individuals of various ages has shown intact cytokine production capacity and normal numbers of innate immune cells in the circulation (39). Moreover, the production of some of the inflammatory cytokines was even higher in the elderly, underscoring the development of inflammaging.

## Macrophages

Macrophages are phagocytic cells present in nearly all tissues, where they contribute to tissue homeostasis, tissue repair, and host defense (40). They exhibit high plasticity and heterogeneity and secrete a wide variety of cytokines and chemokines upon recognition of pathogen-derived or damage-associated signals (41).



Although circulating monocyte numbers are stable through life, numbers of macrophage precursors are reportedly reduced with advancing age (42, 43). Similar to neutrophils, macrophages display age-related defects in chemotaxis, TLR expression and function, signal transduction, phagocytosis, and superoxide production (21). Upon lipopolysaccharide (LPS) stimulation, peritoneal macrophages of aged mice had 70% decreased p38 MAPK and c-Jun N-terminal kinase (c-JNK) activation, which are critical for TLR-mediated responses (44, 45). Decreased expression of inducible nitric oxide synthase (iNOS) and impaired production of nitric oxide were also observed in macrophages from aged mice (46).

Aged mouse macrophages also had less major histocompatibility complex (MHC) class II expression, lower levels of TLRs, and reduced IL-6 and TNFα production upon stimulation with TLR ligands (47, 48). MHC II expression on the surface was 50% less in macrophages of old mice following interferon γ (IFNγ) stimulation (49). LPS-induced IL-1β and IL-12 production was also reduced in splenic macrophages from aged mice (50). Macrophages in aged mice were also less capable of clearing apoptotic debris (51).

Most published studies investigating age-related changes in macrophages are murine studies, because of difficulties in obtaining tissue macrophages from humans. Nevertheless, there are a few studies suggesting decreased macrophage function in the elderly. The numbers of bone marrow macrophages in the later decades of life were found to be comparable to younger adults (52). Monocyte-derived macrophages from the elderly produced less TNFα, IL-6, IL-8 and IL-1β when incubated with Streptococcus pneumoniae, even though their phagocytic ability seemed to be intact (53). This functional defect was linked to impaired PI3K–AKT (Ak-strain thymoma oncogene; also called protein kinase B) signaling. Another study with monocyte/macrophage cultures infected with dengue virus revealed

lower TNFα, IL-6, and IL-1β pro-duction by cells of elderly subjects over 65 years old compared with younger adults (54).

## Dendritic cells

DCs are very potent antigen-presenting cells (APCs) that are usually considered as the bridge between innate and adaptive immunity (55, 56). The two main subsets of DCs are myeloid DCs (mDCs) or conventional DCs (cDCs) of myeloid origin and plasmacytoid DCs (pDCs) of lymphoid origin, which are crucial for anti-viral defense (57).

Total peripheral DC and mDC numbers are lower in people aged over 60 years, although pDC numbers remain stable (58). Thymic DCs are also reduced in the elderly and are less efficient in stimulating T cells (59). Even though pDC numbers do not change with age, they have lower type I IFN-releasing capacity due to impaired interferon regulatory factor 7 (IRF7) phosphorylation, which is associated with a reduced response to the influenza virus (60). Their antigen-presentation capacity is also decreased. mDCs of elderly people have restricted migratory and phagocytic capacities (61).

People over 60 years of age present higher production of TNFα and IL-6 by mDCs upon TLR4 stimulation, despite defective AKT phosphorylation and PI3K signaling (62, 63). Also, when derived from elderly individuals, mDCs that ap-pear to have a more mature phenotype produced less IL-12 upon LPS stimulation (58). In addition, Langerhans cells, which are specialized DCs in epidermis and are critical for skin immunity, are lower in number in elderly people and mi-grate less in response to TNFα (64).

## NK cells

NK cells are cytotoxic cells that are heavily studied in the context of anti-tumor responses, but they also exert cytotoxic activity upon recognition of infected cells, particularly in viral infections, or cytokines such as IL-2, IL-12, IL-15 and IL-18 (65, 66).

Studies reported increased or maintained NK numbers in the elderly, although proliferation rates appear to have decreased (67, 68). This is suggestive of the existence of long-lived NK cells. Recently, memory-like NK cells, defined as NKG2C+ CD57+, were indeed described in people with cytomegalovirus (CMV) infection and were also detected in CMV– individuals later (69, 70).

Despite some studies reporting preserved NK cytotoxicity, it is considered to be impaired on a per-cell basis (71, 72). Low NK cytotoxicity is associated with higher

infection rates and infection-related deaths in the elderly (73). Higher NK cytotoxic activity is also linked to higher antibody titers after influenza vaccination in people over 65 years of age (74). Production of IFNγ and proliferation upon IL-2 stimulation were also reduced in this group (71). The NK cell receptor repertoire was also found to be altered with age (75). Additionally, the $CD56^{bright}$ NK cell subset, which constitutes around 10% of peripheral NK cells, was critically diminished in the elderly (76).

## AGE-RELATED CHANGES IN ADAPTIVE IMMUNITY



Antigen-specific adaptive immunity with memory-generating capabilities is crucial for responding against tumors, allergens, and pathogens. The most profound changes in the immune system related with aging are observed in adaptive immunity. In the following paragraphs, we summarize the age-related defects in T cells and B cells.

### T cells

T cells, through their diverse range of antigen receptors [T-cell receptors (TCRs)], recognize pathogenic or tumor-derived antigens and develop antigen-specific memory or tolerance (77). Upon recognition of antigen and receiving co-stimulatory signals, naive T cells differentiate into effector cells. Most of the effector cells are short-lived; however, a portion persists as memory cells and establish long-term immunity. The two main lineages of T cells are CD4+ helper and CD8+ cytotoxic T cells (78).

Maturation and selection of T cells take place in the thymus. Thymic involution — the gradual atrophy of the thymus with age— starts from the first year of life and progresses until the end of life (79). The thymopoietic space, where T-cell maturation occurs, is shrunk to <10% in volume by the age of 70 (80). Processes underlying this include loss of thymic epi-thelium, reduced IL-7 production by thymic epithelium, which is essential for the maturation of thymocytes, and defective rearrangement of the TCR β-chain (81, 82). People who had undergone thymectomy in early childhood show a premature immunosenescent phenotype (83).

The typical immunosenescent profile includes reduced output of naive T cells and a T-cell pool consisting mostly of differentiated effector cells and memory cells (84). It is important to note that most age-related changes in T-cell profiles are either only seen in, or are more pronounced in, individuals seropositive for CMV, which is

a chronic infection present in almost 70% of people over 60 years of age (85, 86). Among CD8+ T cells, the CD28– effector population is markedly increased in the elderly (87, 88). In contrast, the naive CD8+ T-cell pool is depleted with age (89). Loss of CD28, which plays a critical role in T-cell activation in effector cells, is among the hallmarks of immunosenescence in T cells (90).

Furthermore, the limited number of existing CD28+ cells have a more restricted TCR repertoire and shorter telomeres in people over 65 years of age (91). Clonal expansion of CD28– CD8+ T cells was inversely correlated with antibody production against influenza vaccination (92). Because of the extreme expansion of these cells and the reduced naive T-cell output, the T-cell repertoire diversity is restricted, and susceptibility to novel infections is increased (93).

The naive CD4+ T-cell pool does not undergo such a critical change as CD8+ T cells, although there is a decline in numbers (94, 95). Upon probing with novel antigens, IL-2 production by naive CD4+ T cells of elderly people was also comparable to young individuals (96), even though there is defective TCR-induced ERK signaling (97). In contrast to naive cells, central memory CD4+ cells accumulate in people over 65 years of age (94, 98). Effector memory cells, on the other hand, are found at a lower frequency in the elderly and their numbers were correlated with anti-influenza response upon vaccination (98). The accumulation of effector cells and loss of CD28 seen in CD8+ T cells are not pronounced in CD4+ cells (95).

## B cells

B cells mediate humoral immunity against pathogens and allergens by producing antibodies with high specificity and affinity (99). The B-cell antibody response is one of the crucial outcomes that vaccination strategies strive to achieve. B cells develop and mature in the bone marrow. In contrast to T cells, whose output is severely affected by thymic involution, B-cell lymphopoiesis continues throughout life, but B-cell precursor numbers in the bone marrow and the antibody-producing plasma cells decrease with age (100, 101).

Similar to T cells, accumulation of memory B cells with restricted receptor diversity was reported in the elderly (102). Impaired class-switching and somatic recombination along with lower diversity of antibodies are also observed in this group, leading to weak antibody responses with low affinity (103). Age-related alterations in the number and size of germinal centers, where B cells proliferate and undergo somatic hypermutation, partly contributed by sub-optimal T cells, help underlie these defects (104, 105). The percentage of switched memory

B cells, which have been positively correlated with influenza vaccine responses, also declines significantly with age (106–108). This population has very short telomeres in the elderly compared with younger individuals (109). In contrast, late exhausted memory B cells are expanded in the elderly, filling up the immunological space (109). Another age-related change is the increase of auto-antibodies in the elderly, likely contributing to the prevalence of autoimmune diseases (110).

## VACCINE RESPONSES IN THE ELDERLY AND CURRENT IMPROVEMENT STRATEGIES



In order to prevent and reduce the number of infections in elderly people, vaccines are the most cost-effective and safe approach. However, the overall vaccination efficiency of currently available vaccines remains low in the elderly population, because of the impaired ability of their immune system to respond to immune stimulation (111).

Influenza is one of the major infections worldwide, and it represents a considerable threat to vulnerable populations such as the elderly and young children. There are up to 500,000 deaths reported every year in people aged >65 years because of influenza (112). Along with increased risk of hospitalization and deaths linked with influenza-associated respiratory diseases, vaccine efficiency is also lower at 17–53% in the elderly, compared with 70–90% efficacy in young adults (113). Suggested reasons for the impaired influenza vaccine response included decreased somatic mutations in B cells (114), an increased regulatory T cell (Treg) population (115), impaired expression of the co-stimulatory molecule CD28 in T cells (116), the reduced antigen-presenting capacity of pDCs (60) and low NK cell cytotoxicity (21).

Currently, there are two commonly available influenza vaccines: inactivated vaccines and live-attenuated vaccines. A high-dose inactivated vaccine with 60 μg hemagglutinin (HA) antigen from each strain demonstrated improved anti-body responses with 24.2% more efficiency in people over 65 years of age compared with the 15 μg standard dose (117–119). In 2019, a high-dose influenza vaccine was approved by the Food and Drug Administration (FDA) for use in people older than 65 years, reported as well-tolerated and more effective (120). Nonetheless, vaccination of elderly with the high-dose vaccine still induced lower antibody responses and Th1 T-cell responses in comparison with young adults vaccinated with the standard dose (121). Another study demonstrated that intradermal injection instead of intramuscular injection significantly improved antibody titers

in people over 65 years of age; however, intradermal injection of the high-dose (60 µg) influenza vaccine was not significantly different than that of the normal dose (15 µg) in terms of protection (119).

Adjuvanting the vaccines is another promising strategy to boost immune responses in the elderly. Adjuvants are a crucial part of vaccines, contributing to better vaccine responses by increasing antigen presentation and activating the innate immune system (122). Considering that antigen presentation, responsiveness, and chemotaxis of immune cells are mostly impaired in old individuals, improvements in adjuvant systems would increase the efficacy of vaccinations.

MF59® (Fluad), an emulsion-based adjuvant, was significantly immunogenic, and it reduced influenza-related hospitalizations by 25% in the elderly in comparison with the non-adjuvanted influenza vaccine (123–125). MF59 has been reported to increase viral antigen uptake and antigen presentation, hence enhancing immunization efficacy. Additionally, the MF59-adjuvanted subunit influenza vaccine-induced antibody responses against non-specific seasonal viral strains (126). TLR ligands are also utilized as adjuvants. A phase 2b/3 trial demonstrated that topical application of the syn-thetic TLR7/TLR8 agonist imiquimod prior to intra-dermal trivalent influenza vaccination significantly elevated the immunogenicity of the vaccine in the elderly (127).

Streptococcus pneumoniae is another prevalent cause of severe infections in the elderly that might result in several complications such as upper respiratory disease, bacteremia and meningitis (128). There are two commonly used vaccines: a 23-valent pneumococcal polysaccharide vaccine (PPSV23), which is mostly used for adults and the elderly; and a 13-valent pneumococcal conjugate vaccine (PCV13) for children older than 2 years of age (129). Although PPV23 has been recommended for a long time to vaccinate the elderly, a meta-analysis assessing vac-cine efficiency showed that PPV23 had a moderate effect on invasive pneumococcal disease while it was not potent against pneumococcal pneumonia (130). On the other hand, PCV13 has been reported as partly effective against pneumococcal diseases in old individuals; however, age still influences the potency of PCV13 with efficacy of 65% and 40% in 65-year-old and 75-year-old participants, respectively (131). A study argued that the combination of PCV13 with PPV23 possibly enhances protection in the elderly; however, clinical data demonstrating elevated antibody production and reduced disease incidence are still missing (132).

Varicella zoster virus (VZV) is another important pathogen affecting the elderly. This virus remains latent in the nerve cells of infected individuals after an episode of chickenpox in early life (133). Herpes zoster or shingles is caused by the re-activation of latent VZV, and the risk of developing shingles increases with age because of the reduced activity of cell-based immunity (134); therefore, most of the cases that require hospitalization are people older than 50 years (135). Two vaccines are licensed for usage against shingles: a live-attenuated vaccine (Zostavax™) developed by Merck; and a subunit zoster vaccine (Shingrix™) formulated by GSK. A double-blind, placebo-controlled study with people older than 60 years showed that the live-attenuated vaccine lowered the burden of illness by 61.1% and prevalence of herpes zoster by 51.3% (136).



The novel adjuvant AS01b, consisting of MPL (3-O-desacyl-monophosphoryl lipid A), a TLR4 agonist as a derivative of LPS from *Salmonella minnesota*, and saponin QS-21, has been shown to effectively promote antigen presentation and CD4+ T-cell-mediated immune responses, and demonstrated high efficacy in combination with different vaccines in clinical trials (137). An inactivated vaccine utilizing AS01b as a liposome-based adjuvant exhibited promising results in elderly people, with 97.2% efficacy in people over 50 years of age (138). Of note, the vaccine potency did not decrease with age; the efficiency in people older than 70 years of age is similar to that in people between 50 and 70 years old. Additionally, vaccine-induced antibody production was still higher than the pre-vaccination level even after 9 years (139). A phase II trial comparing the AS01b-adjuvanted vaccine with non-adjuvanted vaccine reported that immunogenicity of the viral subunit vaccine increased with the adjuvant in a dose-dependent manner (140). The very special behavior of this AS01-containing vaccine with high efficacy in the elderly provides a potential tool to investigate the mechanisms needed to induce proper vaccination responses in the elderly and gives hope that similar levels of efficacy may be achieved with other vaccines as well.

## TRAINED IMMUNITY AND VACCINATION IN THE ELDERLY

For a long time, the development of immunological memory was solely attributed to adaptive immunity, which is maintained by antigen-specific long-lasting memory lymphocytes upon recognition of a pathogen. On the other hand, innate immune responses are mediated by non-specific effector molecules and have been considered devoid of memory properties. However, recent studies consistently

reported the capacity of the innate immune system to develop memory-like features (141–144).

Our group and others showed that, following an insult with certain infections or vaccinations, members of the innate immune system, for example, monocytes, DCs, and NK cells, exhibit enhanced responsiveness to a second infection that might be the same or a different pathogen. This phenomenon was later termed as 'trained immunity' or 'innate immune memory' (144). Although the concept of trained immunity was first demonstrated and mostly studied in monocytes, there is evidence that memory-like properties are also present in other innate immune cells. For instance, ex vivo stimulation of human NK cells with heterologous pathogens 3 months after Bacillus Calmette–Guérin (BCG)—a live-attenuated vaccine against tuberculosis (145)—results in enhanced pro-inflammatory cytokine production but not IFNγ production compared with before vaccination (146). Notably, BCG neither induced NK cell expansion nor altered the expression of NK cell markers. A recent study suggested that DCs from immunized mice showed a long-term memory response upon a fungal challenge that was mediated by specific epigenetic modifications (147).

The underlying mechanisms of trained immunity are explained by epigenetic and metabolic reprogramming (Figure 2). Immunological signal pathways, for example pattern-recognition receptors (PRRs) engaged with DAMPs or bacterial products, induce epigenetic changes (i.e. increase in H3K4me3, H3K4me and H3K27Ac and removal of H3K9me3) at the promoter and enhancer sites of genes coding for pro-inflammatory cytokines and metabolic re-wiring such as up-regulation of glycolysis, cholesterol synthesis and glutaminolysis (148–151). Certain metabolites of these pathways, such as α-ketoglutarate and fumarate, subsequently modulate the activities of epigenetic remodeling enzymes, such as histone demethylases or his-tone acetyltransferases (5). As a result, increased chromatin accessibility of pro-inflammatory genes eventually leads to elevated pro-inflammatory cytokine production when a secondary challenge occurs. Another remarkable finding is that memory-like properties can persist for a long time beyond the limited life spans of immune cells, owing to the reprogramming of HSCs and myeloid progenitors in the bone marrow (152, 153).

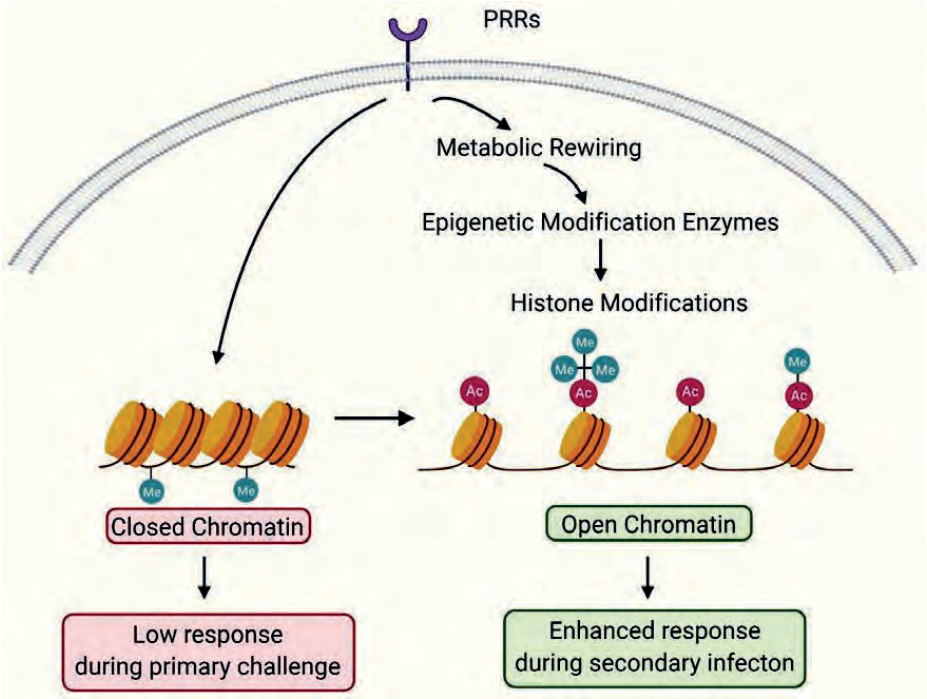


**Figure 2. Overview of fundamental mechanisms in trained immunity.** Certain infections and vaccinations alter metabolic pathways, leading to histone modifications that enable chromatin regions to be more open for transcription. Increased gene expression results in improved responses against pathogens during secondary infection.

Trained immunity can be used as an effective way to boost vaccine responses by conferring wide protection against a diverse range of pathogens (154). For instance, trained immunity induced by β-glucan protected mice against bacterial infections causing peritonitis, enteritis and pneumonia by increasing inflammatory monocyte and granulocyte numbers and IL-1β production (155). Clinical trials and epidemiological studies revealed that certain vaccines such as vaccinia, BCG and measles have non-specific protective effects (156, 157). Among them, BCG is the most extensively studied vaccine for its heterologous protective effects. It has been used for treatment and decreasing the progression of non-muscle invasive bladder cancer for >40 years, although its mode of action has not been fully understood yet (158–160). The wide range of protection conferred by BCG is mainly attributed to increased cytokine production as a result of metabolic and epigenetic reprogramming of innate immune cells. It is also important to point out that in addition to induction of trained immunity, BCG and other live attenuated vaccines can induce heterologous adaptive immune responses, such as Th1-dependent IFNγ production (161, 162). It is conceivable that the complete beneficial effects of

BCG vaccination are due to a combination of trained immunity and heterologous T-cell immunity. In addition, it is important to note that BCG vaccination prior to influenza and childhood vaccines can also act as an adjuvant and enhance antibody responses; however, the mechanisms in play are yet to be established (163, 164).

Evidence from several animal and human studies suggests that BCG vaccination is also effective in protecting against *Leishmania spp.* and *Plasmodium falciparum* infections (165). In a double-blinded, placebo-controlled study, individuals vaccinated with BCG 1 month before experimental viral infection induced by yellow fever vaccine displayed less viremia in their blood compared with people vaccinated with placebo. Protection against the yellow fever virus was reported to be associated with epigenetic modifications of monocytes, and high IL-1β production was inversely related to viremia (166). In a clinical trial investigating protective effects of BCG on malaria infection, BCG-vaccinated subjects 5 weeks prior to controlled malaria infection presented early activation of NK cells and monocytes which were correlated with lower parasitemia (167).

Remarkably, heterologous protection by BCG was not limited to an enhanced innate immune/trained immunity response. It has been shown that BCG vaccination induced heterologous Th1 and Th17 responses even 1 year after immunization (168). Another study from our group demonstrated that BCG vaccine could be used to improve the beneficial effects of diphtheria tetanus pertussis (DTP) and influenza vaccines. BCG vaccination prevented the immuno-suppressive effects of acellular diphtheria tetanus pertussis combined vaccine (DTaP) and induced trained immunity in adults when it was given concurrently with or 3 months after DTaP (169). BCG vaccination 2 weeks before trivalent influenza vaccination significantly boosted HA-inhibiting antibody production in healthy adults. Moreover, BCG-priming induced higher production of pro-inflammatory cytokines after *ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs) with unrelated pathogens such as *Candida albicans* and *Staphylococcus aureus* (164).

Although literature for trained immunity in the elderly is very scarce, a few studies in the elderly suggest that not only children and adults, but also the elderly, might benefit from protection against heterologous infections. It has been recently shown that BCG-vaccinated individuals in Guinea-Bissau who are older than 50 years of age displayed increased pro-inflammatory cytokine production following *ex vivo* stimulation with heterologous stimuli 2 months after vaccination (170). Considering the impaired ability of innate immune cells to respond against infections in the elderly, this study suggests that trained immunity could indeed be induced

in elderly people and might be utilized as a powerful tool to increase vaccine responses and protect this vulnerable population from various infections by counteracting the effects of immunosenescence. Another clinical study, in which participants between 60 and 75 years old received BCG once a month for 3 months, demonstrated that BCG vaccination significantly prevented acute upper respiratory tract infections while increasing IFNγ and IL-10 production (171). Furthermore, the scar diameter at the vaccination site was correlated to the circulating IFNγ levels. Another study performed in Japan with elderly people indicated a lower risk of pneumonia following immunization with BCG (172).

Utilizing the trained immunity response to increase resistance and defense against infections is advantageous in many settings. First of all, since trained immunity confers a broad range of protection, it might be useful in illnesses in which secondary infections or co-infections play a role. As an example, bacterial infections following influenza can worsen the outcome by increasing morbidity and mortality (173, 174). As viruses frequently undergo mutations, conventional vaccines remain ineffective in some cases. Therefore, trained immunity can be employed to protect people from newly emerged bacterial or viral strains. Lastly, clinical conditions such as immunoparalysis could be rescued by inducing trained immunity (175).



Improving innate immune responses to provide protection is crucial for vulnerable populations such as the elderly and people with immune deficiencies. In a recent review by Sánchez-Ramón et al., approaches to employing trained immunity in vaccine formulations were explicitly discussed (154). According to that, it was suggested that trained immunity inducers can be used as immunostimulants and adjuvants, the former promoting innate and adaptive immune responses leading to enhanced protection against bystander pathogens, while the latter delivered with a specific antigen further enhance adaptive immune response against that specific pathogen.

It is important to note that trained immunity might be damaging in situations where people have excessive inflammation as a result of endogenous and exogenous stimuli, and thus vaccines based on trained immunity should be mainly aimed for groups at high risk of infections. Indeed, people with atherosclerosis and hyper-IgD syndrome have been shown to have chronic inflammation due to continuously active trained immunity (176, 177).

The prolonged presence of certain DAMPs induces reprogramming of innate immune cells by providing a basis for sustained low-grade and chronic inflammation. For

instance, pre-incubation of splenocytes with high-mobility group box protein 1 (HMGB1) was shown to increase TNFα production after secondary infection, indicating that HMGB1 might prime the cells to protect against infections (178). Another molecule, oxidized low-density lipoprotein (oxLDL), leads to epigenetic reprogramming of monocytes, eventually causing long-term elevated pro-inflammatory cytokine production (179). Similarly, pre-treatment of healthy PBMCs with soluble uric acid-induced cytokine secretion that was mediated by histone methylation (180). Along with advanced age, accumulation of DAMPs —for example, HMGB1, sodium monourate, and uric acid crystals— results in sterile inflammation, which is one of the underlying causes of several diseases including but not limited to atherosclerosis, cardiovascular diseases, gout and ischemia–reperfusion injury (10, 181, 182). Nevertheless, our group demonstrated that BCG vaccination lowers systemic inflammation by decreasing circulating inflammatory markers in healthy individuals while enhancing cellular responses (L. C. J. de Bree et al., unpublished data); therefore, it would serve to reduce chronic inflammation while overcoming functional impairments at a cellular level.

## CONCLUSIONS

Age-related alterations in the immune system result in high susceptibility to infections, increased risk of hospitalization and mortality. Defects in adaptive immunity underlie the markedly low vaccine efficiency in the elderly. Additionally, many functional defects in chemotaxis, phagocytosis, antigen presentation, ROS production, TLR signaling, and cytokine production are present in aged innate immune cells such as neutrophils, monocytes, macrophages, DCs, and NK cells. Despite reduced cellular functions, a systemic increase in inflammatory markers, so-called inflammaging, is observed in aged individuals.

In addition to numerous efforts underway to develop new vaccines with higher efficacy in the elderly, novel approaches targeting innate immunity to improve host responses are crucial to evade the consequences of the aged immune system. It is an emerging concept that innate immune cells can manifest memory-like properties that are not antigen-specific and exhibit enhanced responsiveness upon later challenges with heterologous stimuli. This concept of 'trained immunity' has been reported to enhance immunization efficiency. However, whether trained immune responses change as people age is yet to be explored. Further investigation is crucial to understand if and how trained immunity can be employed to protect the elderly from a broad range of infections. Besides the possibility that impaired

innate immune cell functions could be reversed by inducing trained immunity, recent data suggest that BCG down-regulates circulating inflammatory markers, which would help alleviate the detrimental effects of inflammaging in the elderly. Therefore, it would be worthwhile to explore the potential of trained immunity for overcoming age-related immune dysregulation and protecting the vulnerable elderly population against infections.

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## Conflicts of interest statement:

M.G.N. is a scientific founder of TTxD. The other authors declare that they have no conflicts of interest.

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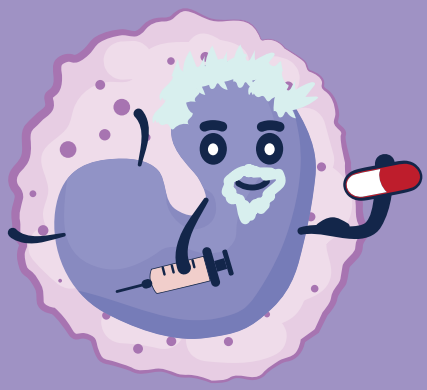

# CHAPTER 6

# ALENDRONATE MODULATES CYTOKINE RESPONSES IN HEALTHY YOUNG INDIVIDUALS AFTER BCG VACCINATION

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## ABSTRACT

Bacillus Calmette-Guérin (BCG) vaccination induces memory characteristics in innate immune cells and their progenitors, a process called trained immunity mediated by epigenetic and metabolic reprogramming. Cholesterol synthesis plays an amplifying role in trained immunity through mevalonate release. Nitrogen-containing bisphosphonates (N-BPs), such as alendronate, can inhibit cholesterol synthesis. We explored their effects on trained immunity induced by BCG in a placebo-controlled clinical study (NL74082.091.20) in young, healthy individuals. Participants receiving single-dose oral alendronate on the day of BCG vaccination had more neutrophils and plasma cells one month after treatment. Alendronate led to reduced proinflammatory cytokine production by PBMCs stimulated with heterologous bacterial and viral stimuli one month later. Furthermore, the addition of alendronate transcriptionally suppressed multiple immune response pathways in PBMCs upon stimulation. Our findings indicate that N-BPs modulate the long-lasting effects of BCG vaccination on the cytokine production capacity of innate immune cells.

# INTRODUCTION

The innate immune system responds early and rapidly after an infection. Although able to distinguish self from non-self, responses generated by innate immunity are antigen-agnostic [1]. Traditionally, only the adaptive immune system was thought capable of developing immunological memory. However, more recent research revealed that the innate immune system also mounts a memory-like response through epigenetic and metabolic programming of innate immune cells, which subsequently exhibit a more robust response to secondary infections. This trait, also termed trained immunity, can be induced by several live-attenuated vaccines, including Bacillus Calmette–Guérin (BCG), measles, and oral polio, that were shown to induce protection against heterologous infections [2].

Initially developed against tuberculosis, BCG also reduces all-cause childhood mortality through protection against a wide range of infections [3]. This non-specific protection is partly mediated by the changes in the epigenetic regulation of the function of innate immune cells and metabolic reprogramming marked by an increase in glycolysis and oxidative phosphorylation [4]. Moreover, BCG vaccination leads to persistent transcriptomic changes in human hematopoietic stem and progenitor cells and a myeloid differentiation bias in the bone marrow [5]. However, the efficacy of inducing trained immunity differs between individuals [6], and strategies to improve trained immune responses are needed.



Bisphosphonates (BPs) are a class of drugs mainly used to treat and prevent bone resorption [7]. They have an affinity for the bone and work as a calcium-chelating agent. So far, there are around ten BPs for human use in different conditions, varying from osteoporosis to Paget's disease to cancer. Some BPs, including alendronate, contain nitrogen in their side chains and have a different mechanism of action compared to simple BPs. Aside from targeting bone-resorbing osteoclasts, nitrogen-containing BPs (N-BPs) are internalized by monocytes and macrophages [8–10], and they inhibit cholesterol synthesis by blocking the activity of farnesyl pyrophosphate (FPP) synthase [11]. This inhibition leads to the accumulation of isopentenyl diphosphate and dimethylallyl diphosphate in peripheral blood mononuclear cells (PBMCs), with immunomodulatory activities such as activation of γδ T cells [8, 12].

Blockade of FPP synthase by alendronate can also lead to the accumulation of metabolites in the mevalonate pathway. Mevalonate accumulation in monocytes was previously linked to a stronger trained immunity phenotype [13]. Furthermore, another N-BP zoledronate drove both peritoneal and tumor-associated macrophages

toward the pro-inflammatory and tumoricidal M1 phenotype in a mouse model of breast cancer [14]. A few observational studies found a lower risk of infections and cancer in people with chronic use of N-BPs [15–17]. Due to its low adverse event profile and potential immune-stimulating properties, we hypothesized that N-BPs could be a good candidate to improve BCG vaccine efficacy.

To test the potential of N-BPs as an adjuvant to improve trained immunity, we designed a clinical study in which the participants received either a placebo vaccination, the BCG vaccine, or the BCG vaccine together with oral alendronate tablets. We collected blood samples from participants before and one month after the intervention and measured cytokine responses following ex vivo stimulation of PBMCs with various stimuli. Furthermore, we performed flow cytometry from the whole blood to identify immune cell subsets and RNA sequencing (RNAseq) from PBMCs to assess the transcriptional responses.

## MATERIALS AND METHODS

### Study design and subjects

Healthy adults were recruited between June and August 2020 at the Radboud University Medical Center. Study subjects did not use any chronic medication except for oral contraceptives, did not have comorbidities, and were BCG-naïve by the time of inclusion. No power calculation was possible due to the absence of information on alendronate's potential effect size; therefore, this clinical trial was designed as an exploratory study. Participants were randomized as 1:1:1 to each group using the Castor electronic data capture platform to receive 9 mg/ ml intradermal sodium chloride (Centrafarm, Netherlands) in 0.1 ml as placebo for BCG vaccination, 0.75 mg/ml intradermal BCG vaccine (AJ Vaccines, Denmark) in 0.1 ml, or intradermal BCG vaccine right after orally ingesting a 70 mg alendronate tablet (Aurobindo Pharma, India). 18, 21 and 18 participants completed the study in each arm, respectively. Age, sex, and BMI distributions in each group are provided in Table 1. Blood was collected from participants before and 1 month after vaccination. Ethical approval for the study (NL74082.091.20) was granted by the local ethics committee CMO region Arnhem-Nijmegen.

### Flow cytometry from whole blood

Before flow cytometry staining, the number of immune cells in the whole blood was determined using a hematology analyzer (Sysmex, Japan). The hematology analyzer relies on flow cytometry principles to identify different cell populations

in the blood: the forward scatter light determines cell volume, the side scatter light gives information about cell nuclei and granules, while the side fluorescence indicates nucleic acids and organelles. As a result, cells having similar properties appear in a cluster.

For flow cytometry staining, erythrocytes were first lysed in isotonic NH4CL buffer and washed twice with PBS. White blood cell counts were determined by Coulter Ac-T Diff® cell counter (Beckman Coulter, CA, USA) and used to calculate the absolute numbers of CD45+ leukocytes identified by flow cytometry. 500.000 total leukocytes were used per staining panel. Cells were transferred to a V-bottom 96-well plate, washed twice with PBS + 0.2% bovine serum albumin (BSA) (Sigma-Aldrich, MO, USA), and stained in the dark for 20 min at room temperature. Afterward, cells were washed twice more with PBS + 0.2 % BSA and measured with the Navios™ flow cytometer (Beckman Coulter). Details of the panels and antibodies are provided in Supplementary Table 1. Kaluza 2.1® software (Beckman Coulter) was used for data analysis.



## PBMC isolation and *ex-vivo* stimulation

Whole blood was diluted with PBS, and PBMCs were isolated using density gradient centrifugation with Ficoll-Paque (GE Healthcare, IL, USA). The middle PBMC fraction was collected and washed three times with cold PBS. The cells were resuspended and stimulated in RPMI 1640 Medium (Dutch modification) (Thermo Fisher Scientific, MA, USA) supplemented with 1 mM sodium pyruvate (Thermo Fisher Scientific), 2 mM GlutaMAX supplement (Thermo Fisher Scientific), and 50 µg/mL gentamicin (Centrafarm, Netherlands). The PBMCs were isolated and used for experiments within 4 h after blood collection.

500.000 PBMCs per well were stimulated with 10 ng/ml *E. coli*-derived LPS, $10^6$ /ml *S. aureus*, 10 µg/ml poly(I:C) (Invivogen, CA, USA), 3 µg/ml R848 (Invivogen), $3.3 \times 10^5$ K/mL TCID50 heat-inactivated influenza A H1N1 and $1.4 \times 10^3$ K/mL TCID50 heat-inactivated SARSCoV-2 Wuhan strain for 24 h or 7 days in the presence of 10% pooled human serum at 37 °C with 5 % CO2. Virus inactivation was performed at 60 °C for 30 min. After stimulation, cytokine levels in supernatants were measured using DuoSet® ELISA kits (R&D Systems, MN, USA) following the manufacturer's protocols. Only for IFNα, Human IFNAlpha ELISA Kit from PBL Assay Science (NJ, USA) was used.

## RNA isolation and sequencing

10 subjects from each intervention group were selected for RNA sequencing. The demographics of the subjects were given in Supplementary Table 2. Cryopreserved PBMCs were thawed and cultured in RPMI 1640 supplemented with 10 % fetal bovine serum (Corning, NY, USA) and 2 mM l-glutamine (Thermo Fisher) for 2 h. After incubation, samples were washed with PBS, filtered, and counted. For samples which were also stimulated with Poly(I:C) (limited to 5 individuals per group with sufficient remaining cells, details in Supplementary Table 3), an additional 1 million cells were plated into one well of a 12-well plate and stimulated with 20 ug/mL poly(I:C) (Invivogen) for 4 h. For each sample, 1 million cells were lysed in RLT buffer (Qiagen, MD, USA) and stored at -80 °C for RNA extraction and sequencing. RNA extractions were performed using the miRNeasy mini or miRNeasy micro kits (Qiagen). RNA quality was evaluated with the 2100 Bioanalyzer (Agilent Technologies).

RNA library preparations were carried out on 100–500 ng of RNA using the Illumina TruSeq Stranded Total RNA Sample preparation kit, according to the manufacturer's instructions. The libraries were size-selected using Ampure XP Beads (Beckman Coulter) and quantified using the KAPA Library Quantification kit – Universal (KAPA Biosystems). The RNA-Seq libraries were sequenced on the Illumina NovaSeq 6000 system using 100-bp single-end sequencing. The quality of raw sequencing reads was assessed using FastQC v0.11.5 [18]. Reads were mapped to GENCODE human genome model (GRCh38 V34) using STAR 2.7.9a [19]. Gene transcripts were assembled and quantified on their corresponding human genome using the count-based method featureCounts [20] available in R from package Subread 2.0.3.

## Differential gene expression and gene set enrichment analyses

Gene expression levels across all samples were first normalized using the calcNormFactors function implemented in the edgeR R package (version 3.34.0), which utilizes the TMM algorithm (weighted trimmed mean of M-values) to compute normalization factors. Then, the voom function implemented in the limma package (version 3.38.3) was used to log-transform the data and to calculate precision weights. A weighted fit using the voom-calculated weights was performed with the lmFit function from limma.

To investigate the impact of vaccination on baseline (before intervention) PBMC gene expression, normalized, log-transformed gene expression levels of unstimulated samples were fit to the linear model Expression ~ 1 + individual +

timepoint:vaccination, which corrects for natural differences in baseline gene expression between individuals and therefore captures the independent effect of each vaccination condition on gene expression after 1 month.

To investigate the impact of vaccination on the PBMC response to poly(I:C) stimulation, we fit normalized, log-transformed gene expression levels to the linear model Expression ~ 1 + individual + timepoint + stimulus:timepoint and used the makeContrasts and contrasts.fit functions implemented in limma to compare the gene expression response to poly(I:C) before vaccination, with the response to poly(I:C) one month post-vaccination.

Gene set enrichment analyses (GSEA) were performed using the fgsea R package (version 1.18.0) with parameters: minSize = 15. To investigate biological pathway enrichments among genes responsive to vaccination (placebo, BCG, or BCG+Alendronate) or with altered responses to poly(I:C) stimulation before compared to after vaccination (placebo, BCG, or BCG+Alendronate), genes were ordered by the rank statistic: -log10(pvalue)*logFC and compared with the Hallmark genesets from the MSigDB collections.



### IL-6 concentration measurements in plasma

Whole blood in EDTA tubes was centrifuged at 3800 RPM for 10 min to obtain plasma. The plasma samples were then stored at -80 °C until testing. IL-6 levels in the plasma samples were measured using the Human IL-6 Quantikine HS ELISA (R&D Systems) according to the manufacturer's instructions.

### Statistical analyses

Statistical analyses apart from RNA sequencing were performed using GraphPad Prism 8 (GraphPad Software Inc., CA, USA) or R 3.6.1 (www.R-project.org). Comparisons between the two time points were performed with the Wilcoxon matched-pairs signed rank test. Mann– Whitney U test was used for comparisons between treatment groups. p values below 0.05 were considered statistically significant.

# RESULTS

## Study design and demographics

Eighteen, twenty-one, and eighteen participants who completed the study were randomized to receive a placebo vaccine, BCG vaccine, and BCG vaccine with an oral alendronate tablet, respectively. Venous blood was collected before the intervention and during the follow-up visit one month later. Flow cytometry was performed to analyze the changes in the immune cell populations in the blood. PBMCs isolated from blood were incubated with different bacterial and viral stimuli for 24 h and 7 days, and cytokine levels were measured. The study design is visually presented in Figure 1.

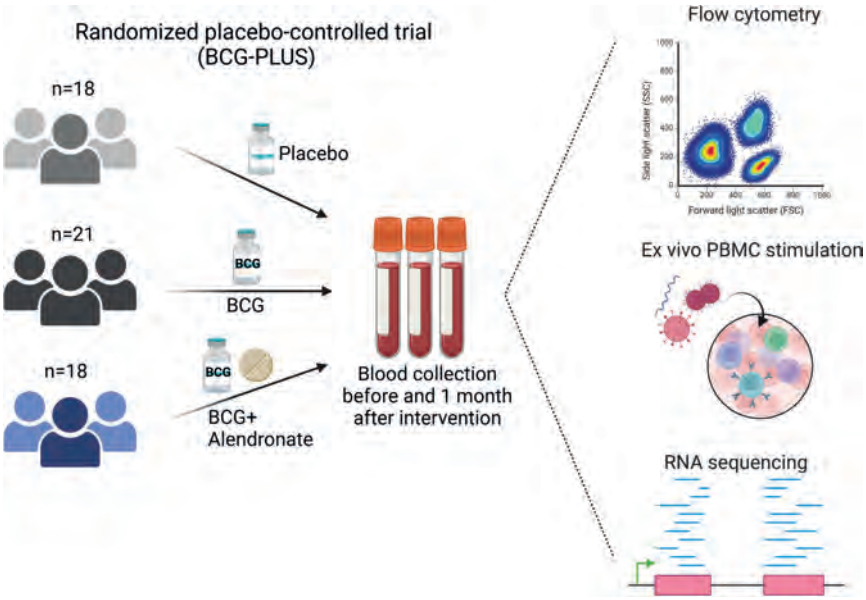


**Figure 1.** Study Design. Blood was collected from the study participants before and 1 month after the intervention. Flow cytometry was performed using whole blood. Following PBMC isolation from the blood, *ex vivo* stimulations were performed to measure cytokine production, and cells were stored for RNA sequencing.

The participant demographics are given in Table 1. There were no significant age and BMI differences between the intervention groups; however, there were more females in the BCG group compared to the other study groups.

**Table 1.** Demographics of the study participants.

| | Placebo ($n$ = 18) | BCG ($n$ = 21) | BCG+Alendronate ($n$ = 18) | p-value |
|---|---|---|---|---|
| Age (years, mean ±sd) | 26.9 ± 7.4 | 26.9 ± 8.1 | 30.5 ± 9.5 | 0.296 |
| Sex (F/M) | 7/11 | 16/5 | 8/10 | 0.039 |
| BMI (kg/m2, mean±sd) | 23.6 ± 1.8 | 22.9 ± 3.1 | 23.8 ± 3.7 | 0.604 |

## Alendronate used together with BCG vaccination led to increased plasma cell and neutrophil numbers in the circulation

Using different cell surface markers, we identified circulating immune cell populations in the blood by flow cytometry. Principal component analysis (PCA) showed that the baseline immune cell counts were similar between the groups (Figure 2A, left). One month after treatment, there was no significant difference in immune cell populations between different treatment groups (Figure 2A, right). Although the abundance of immune cells at the baseline and after treatment remained similar between the groups, the size of plasma and naïve B cell populations exhibited differences after treatment compared to baseline (Figure 2B). The number of naïve B cells increased after the intervention in all groups, including the placebo, although the increase was higher in the BCG and BCG+alendronate groups. Furthermore, the number of plasma cells in the blood became significantly higher in the BCG+alendronate group after the treatment compared to the other groups. We found no additional differences in immune cell numbers after the interventions, except for more intermediate monocytes and regulatory T cells (Tregs) one month after treatment in all groups (Supplementary Figure 1A). The reason for this is unclear, but the fact that it was observed in all groups argues for either a seasonality effect or batch effect between time points.

Since neutrophils were not included in the flow cytometry panel, we also used the whole blood counts obtained with a hematology analyzer. Interestingly, we found that the total numbers of white blood cells and neutrophils were significantly increased only in the BCG+alendronate group one month after treatment compared to baseline (Figure 2C). Monocyte and lymphocyte numbers did not change after the treatments (Supplementary Figure 1B). These data show increased peripheral neutrophil and plasma cell counts one month after receiving the BCG vaccine and oral alendronate treatment.

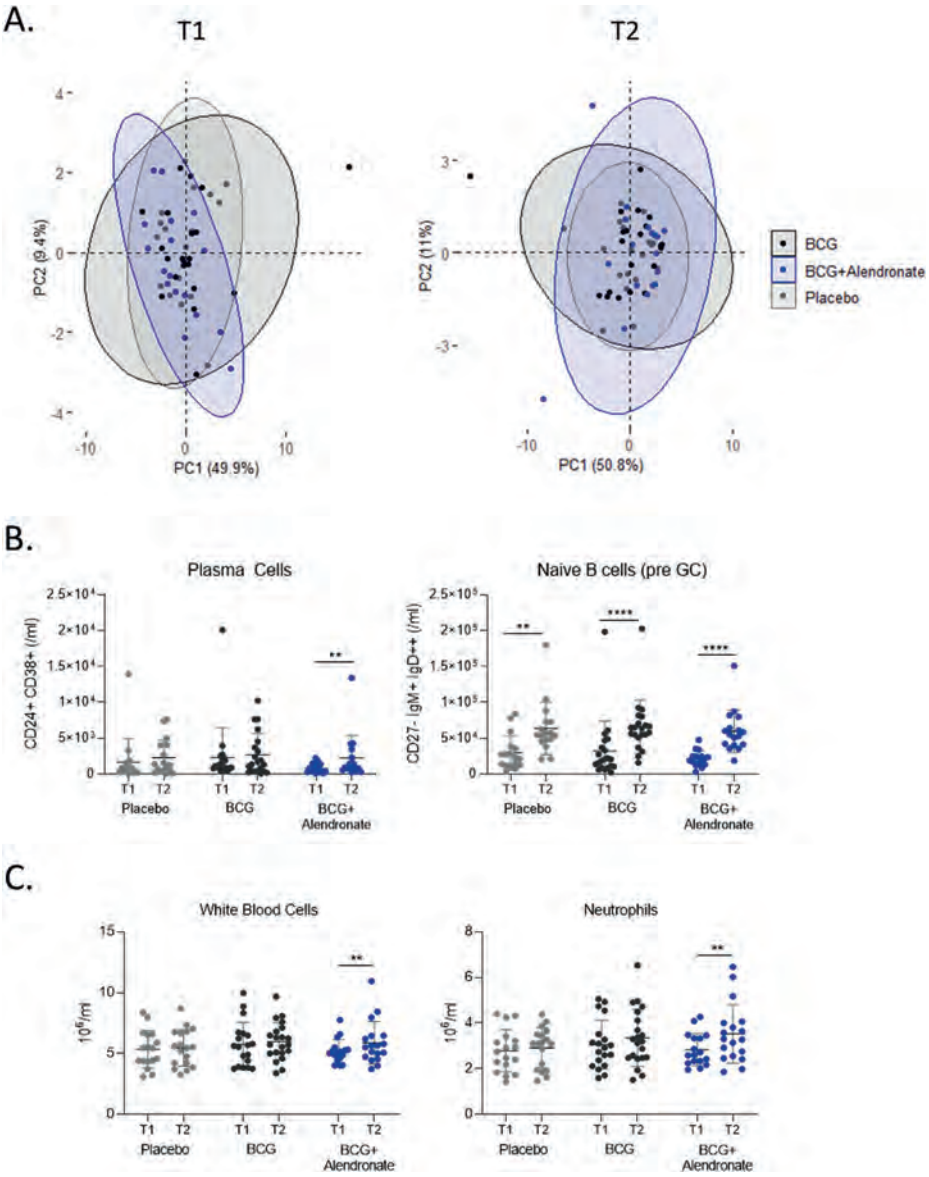


**Figure 2. Immune cell counts measured by flow cytometry and a hematology analyzer before (T1) and after (T2) treatment.** A) Principal component analyses of the immune cell populations before (left) and one month after (right) treatment. B) Plasma and naïve B cell counts measured using flow cytometry. C) Total white blood cell and neutrophil counts measured by a hematology analyzer. Cell numbers before and after treatment were compared using the Wilcoxon matched-pairs signed rank test. **p ≤ 0.01, ****p ≤ 0.0001.

## Combining alendronate with BCG vaccination reduced TNFα production against bacterial and viral stimuli

BCG vaccine can improve cytokine response to unrelated pathogens starting from 2 weeks up to a year after vaccination [21,22]. However, the effects of BCG vaccination in the present study were lower, and IL-6, TNFα, and IL-1RA production did not significantly increase one month after BCG vaccination (Figure 3). Without any secondary stimulation, BCG vaccination (with or without alendronate) led to a trend of higher basal IL-1RA production, but no statistical significance was reached (Supplementary Figure 2). Of note, when sexes were analyzed separately, BCG vaccination's impact was more apparent in female participants, although statistical significance was reached only for the TNFα response against poly(I:C) (Supplementary Figure 3). However, this might simply be due to the low number of BCG-vaccinated male participants in the study.

The simultaneous administration of alendronate and BCG did not significantly impact IL-6 production (Figure 3A). Upon LPS or *S. aureus* stimulation, TNFα production was lower in the group that received alendronate combined with BCG compared to the group receiving BCG alone (Figure 3B). Alendronate also decreased the TNFα response against the Influenza A virus in the combination group compared to the BCGalone group. IL-1RA production was similar between groups in all stimulation conditions (Figure 3C).



Next, we assessed the impact of BCG vaccination and alendronate on the modulation of interferon responses. Generally, BCG vaccination led to a trend of enhanced IFNα and IFNγ production at baseline and after viral stimulation, although statistical significance was not reached (Supplementary Figure 2 and Figure 4). IFNα and IFNγ production was overall not significantly modulated by alendronate (Figure 4). However, sex-specific analyses revealed a significant reduction of IFNα production against poly(I:C) in females of the combination group compared to the placebo (Supplementary Figure 4).

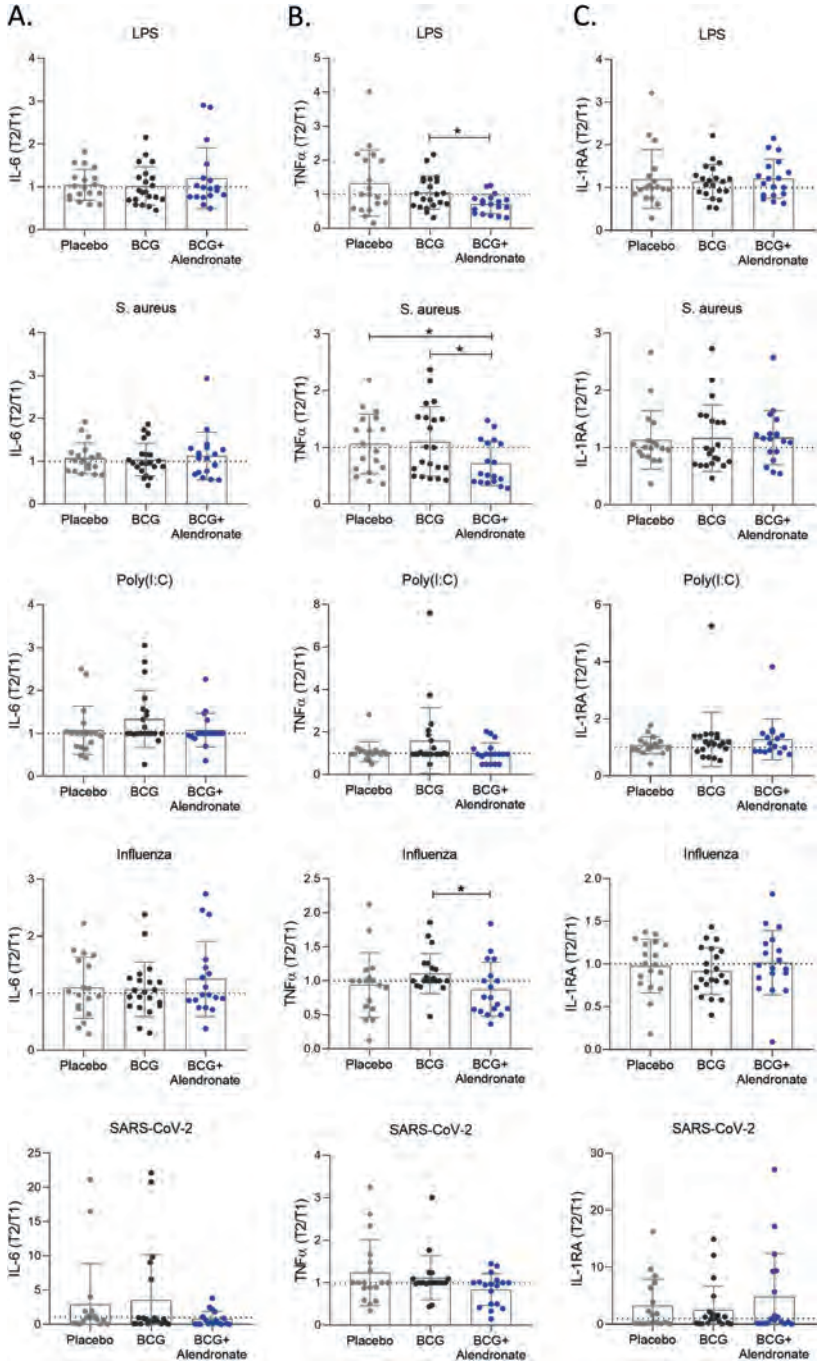


**Figure 3. Fold changes (T2/T1) of A) IL-6, B) TNFα, and C) IL-1RA production by PBMCs upon bacterial and viral stimulation.** Dotted lines depict the fold change of 1. Groups were compared with the Mann-Whitney U test. T1: before the intervention, T2: one month after the intervention. *p ≤ 0.05.

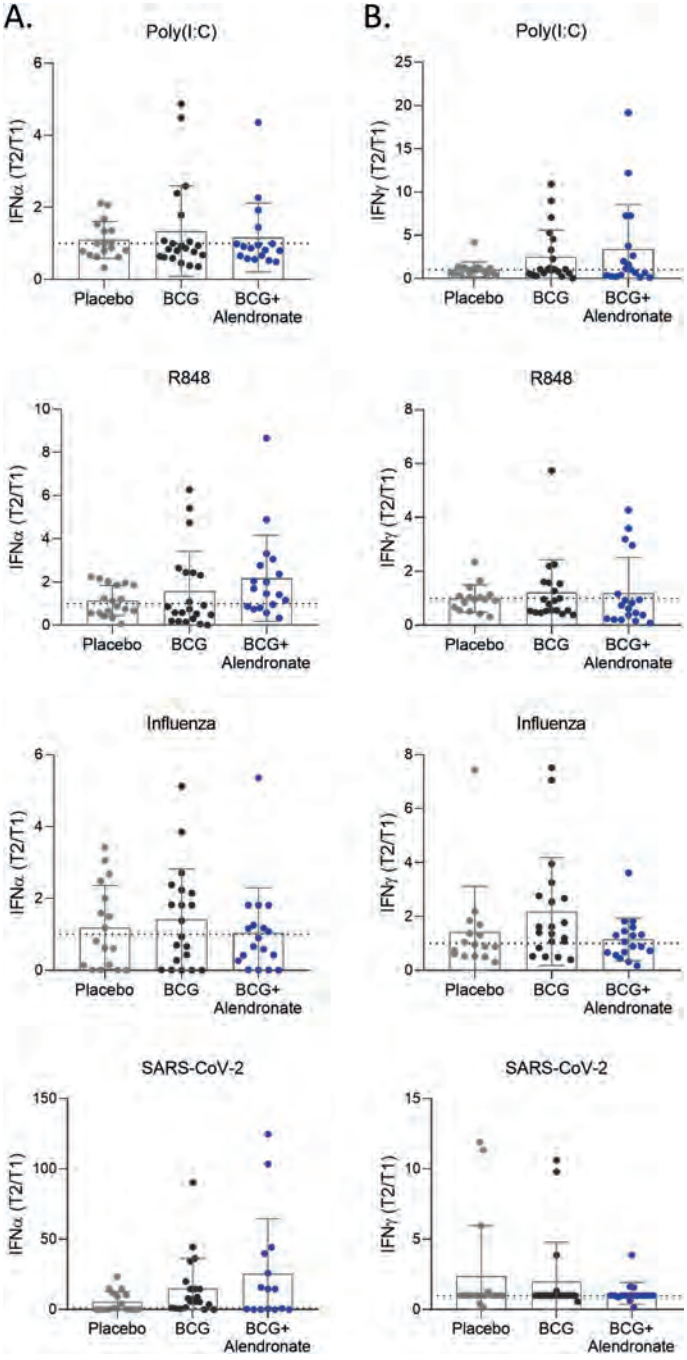


**Figure 4. Fold changes (T2/T1) of A) IFNα and B) IFNγ production by PBMCs upon viral stimulation.** Dotted lines depict the fold change of 1. Groups were compared with the Mann-Whitney U test. T1: before the intervention, T2: one month after the intervention.

Lastly, we compared the cytokine productions at baseline and one month later for each group with a paired analysis to complement the fold change analysis (Supplementary Figures 5 and 6). Although most data were consistent, we have observed a few differences between these two representations: IL-6 production was significantly lower one month later after SARS-CoV-2 stimulation only in the combination group (Supplementary Figure 5A), while the fold changes of the treatment groups were not statistically different (Figure 3A). Figure 3B showed a lower TNFα production capacity in the combination group than in the BCG-only group after LPS stimulation. Supplementary Figure 5B showed no statistical significance on TNFα production against LPS. Finally, the placebo group produced more IFNα after poly (I:C), and less IFNα after influenza and SARS-CoV-2 stimulation 1 month after treatment. However, there was a stronger decline in IFNα after influenza in the BCG+alendronate group (Supplementary Figure 6).

## Single alendronate treatment suppressed transcriptional priming by BCG vaccination

RNA sequencing was performed with PBMCs from 10 individuals per group before and one month after the interventions to investigate if any transcriptional differences were present. When baseline gene expression patterns were compared, BCG and BCG+alendronate treatments led to similar upregulation of pathways, including glycolysis, inflammatory response, and IFNγ response (Figure 5A). However, the addition of alendronate together with BCG vaccination led to higher TNFα signaling and IFNα response in the unstimulated condition.

We also wanted to compare the transcriptomic changes in a stimulated condition. Only 5 participants per group with adequate number of cells for the stimulation were included in this analysis. Upon poly(I:C) stimulation, alendronate treatment led to the striking downregulation of the pathways upregulated by BCG (Figure 5B). These pathways included cholesterol homeostasis, glycolysis, IL-2/STAT5 signaling, IL-6/JAK/ STAT3, inflammatory response, IFNα and IFNγ responses, and reactive oxygen species pathway. When the TNFA and IL1B gene expressions were individually analyzed, priming by BCG compared to placebo and its reversal by alendronate was evident, although not statistically significant due to the low sample size (Figure 5C-D). Overall, the RNA sequencing data support the functional observations suggesting that alendronate suppresses the induction of trained immunity by BCG.

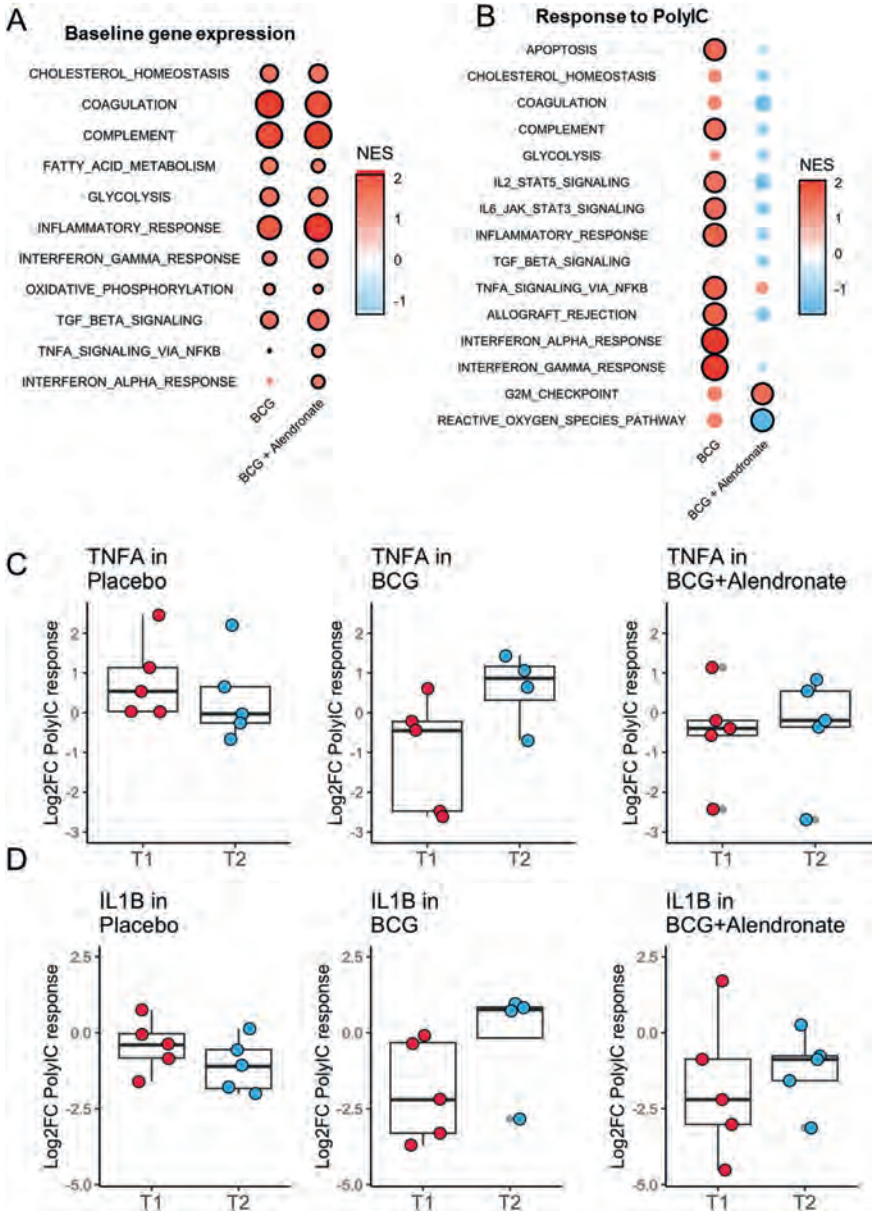


**Figure 5. Gene set enrichment analysis (GSEA) from A) unstimulated and B) poly(I:C)-stimulated PBMCs of treatment groups compared to placebo, and example fold change plots of C) TNFA and D) IL1B expression upon poly(I:C) stimulation.** Genes were ordered by the rank statistic – log10(pval)*logFC for the effect of priming on baseline gene expression and for response to poly(I:C) in the indicated primed condition and compared against Hallmark gene sets. Circle size and shading are scaled to a normalized enrichment score (NES). All circles with black border have padj ≤ 0.05. On the boxplots showing log2FC change in gene expression to poly(I:C), padj > 0.05 (non-significant) for all panels. T1: before the intervention, T2: one month after the intervention.

## DISCUSSION

In this study, we explored the effect of alendronate on the induction of trained immunity responses by BCG vaccination in healthy individuals. We observed that oral administration of alendronate simultaneous with BCG vaccination reduced the cytokine production capacity of PBMCs upon immunological challenge with various stimuli. At the transcriptional level, PBMCs of the individuals who received BCG with alendronate daily for one year, neutrophil counts were elevated at one month and continued to rise throughout the year [23]. Together with our observations, this suggests a stimulatory effect of alendronate on granulopoiesis in the bone marrow. A recent murine study showed that bisphosphonates promote B cell proliferation and antibody production after antigen encounter [24]. Although the study was primarily on clodronate, alendronate was also shown to improve antibody responses. This corroborates the findings that subjects receiving alendronate had significantly higher numbers of plasma cells one month later compared to baseline.

Bisphosphonates are heavily negatively charged molecules that cannot easily permeate the cell membrane. Fluid-phase endocytosis is required for their intracellular uptake [25]. Immune cells such as neutrophils, monocytes, and macrophages are capable of fluid-phase endocytosis and, therefore, could be susceptible to alendronate's immunomodulatory actions. Alendronate's effects on innate immune cells have not been thoroughly investigated, and the existing literature is inconsistent. A few studies have explored alendronate uptake by macrophages and its functional consequences. However, these were mostly performed with macrophage-like J774 or RAW 264 cell lines and not primary human cells [11,26,27]. Simple BPs such as clodronate, etidronate, and nitrogen-containing BP pamidronate suppressed LPS-induced IL-1β, IL-6, and TNFα production from RAW 264 cells [26]. Of note, pamidronate was cytotoxic in high concentrations. In another study with RAW 264 cells, alendronate also proved to be cytotoxic, but it enhanced LPS-induced IL-1β, IL-6, and TNFα production [28]. In J774.1 cells, alendronate increased lipid A-induced IL-1β production and caused cell death dependent on the activation of the Smad3/NLRP3/ ASC axis [29]. A study using PBMCs and monocytes from healthy humans showed that a wide dose range of alendronate inhibited PBMC proliferation in response to lectins or tetanus toxoid (TT) and suppressed IL-1β production from monocytes after LPS or TT stimulation [30].

Our study is the first to explore the effect of alendronate on trained immunity induced *in vivo* by BCG vaccination. Surprisingly, we have not observed a significant

trained immunity response one month after BCG vaccination in this study. A reason for this could be the time of vaccination: the participants were vaccinated with BCG during the summer. It was recently shown that the training capacity of BCG is the highest in the winter, while it is lower in the summer [31]. Despite weak BCG-induced training, the results suggest an overall inhibitory effect of alendronate on proinflammatory cytokine production by innate immune cells, particularly TNFα. The observed effects could be considered relatively small, as they generally amounted to 20–25 % differences in the group that received alendronate and BCG compared to the group receiving BCG alone. However, while this may be a relatively small difference at the individual level, inhibition of BCG effects in this range in patients with bladder cancer treated with BCG immunotherapy [32] could have significant deleterious effects at an epidemiological level in large cohorts.

The transcriptome of PBMCs after poly(I:C) stimulation also corroborates our findings on decreased cytokine production capacity. In the unstimulated condition, we did not observe significant differences between the gene expression in PBMCs of the BCG vs. BCG+alendronate group. However, several pathways related to innate and adaptive immune response and signaling were transcriptionally downregulated in the BCG+alendronate group after incubation of PBMCs with poly(I:C). This suggests that specific genes might be epigenetically modulated by alendronate treatment, but future studies are needed to characterize the context and duration of these effects.



The observed decrease in the cytokine production capacity of immune cells when volunteers were vaccinated with BCG in the presence of alendronate administration is somewhat surprising: inhibition of FPP synthase by alendronate leads to the accumulation of mevalonate, and this was reported to enhance trained immunity responses [13]. Further studies are needed to study the possible molecular mechanisms of these effects of alendronate on BCG-induced immune modulation.

Alendronate is used in both men and women with osteoporosis, although it is more common in women. The tolerability profile and side effects were similar between the two sexes [33], as well as the drug's effectiveness [34]. However, it is not known if bisphosphonates have sex-specific effects on the molecular level. Considering that bisphosphonates, including alendronate, have immunomodulatory effects and considerable differences exist between men's and women's immune responses [35], it would be plausible that alendronate would differentially influence the male and female immune responses. Although this study was not designed to answer how alendronate influences the immune system of young or older men and women, future studies

must address this question. Within the limited sample size of our study, we observed a greater suppressive effect of alendronate on LPS-induced TNFα and poly(I:C)-induced TNFα and IFNα in women. Since a significantly improved cytokine response upon BCG vaccination was also only observed in women, the impact of alendronate might be sex-specific. However, these findings need to be confirmed in larger studies.

An increasing body of research suggests a lower risk of infections and cancer associated with bisphosphonate use. Injection of N-BPs 3 days after infection with influenza resulted in protection against influenza in mice through the expansion of γδ T cells [16]. N-BP use was also linked to a reduced rate of epithelial ovarian cancer compared to no use in women over 50 [17]. An observational study in people with hip fractures reported a lower risk of pneumonia and pneumonia mortality in people using N-BPs than those using non-NB-P anti-osteoporosis medications [15]. On the other hand, a study assessing the incidence of COVID-19 hospitalization and mortality in people using N-BPs found no effect of bisphosphonate use on the risk of getting severe COVID-19 [36]. As these studies are only observational and cannot prove causality, randomized controlled trials are needed to determine whether bisphosphonate use has beneficial or detrimental effects on immunity.

Our results indicate a lower ex-vivo cytokine production against viral and bacterial stimuli in individuals receiving alendronate with BCG, but whether this translates to clinical outcomes such as infection incidence and severity remains to be investigated. Though this study investigated alendronate's immunological effects on healthy young individuals, alendronate is primarily used to prevent and treat osteoporosis, a disease of old age [37]. Immune cell numbers and functions are compromised in advanced age, accompanied by sustained low-grade systemic inflammation, which leads to increased susceptibility to infections, higher morbidity, and mortality [38]. Induction of trained immunity is a promising approach to overcoming immune dysregulation in the elderly and relieving the healthcare burden due to infections. BCG vaccination improves cytokine responses of innate immune cells while decreasing systemic inflammation in healthy elderly [39]. Our observations show that even a single use of oral alendronate tablets together with BCG vaccination can have long-term modulatory effects on cytokine and interferon responses against bacterial, viral, and fungal pathogens. Whether this effect would be beneficial or deleterious remains to be investigated in larger studies. As a systemic inflammation marker, we also measured IL-6 concentrations in the plasma before and after placebo, BCG, or BCG+alendronate treatment. However, no differences existed between time points in any group (Supplementary Figure 7). Whether chronic use of alendronate in older individuals would impact systemic inflammation, prevent the induction of trained immunity, or

further suppress the host response to pathogens are important open questions to be considered.

One limitation of this study was the sample size. Although decreasing cytokine production patterns were observed after the simultaneous use of BCG and alendronate compared to the BCG-only group, these changes sometimes failed to reach statistical significance. A study with a larger sample size is necessary to further validate the effects of alendronate on trained immunity induction.

In conclusion, we show that simultaneous administration of BCG with oral alendronate reduces the cytokine production capacity of PBMCs against heterologous stimuli one month later in young, healthy stimulated PBMCs where BCG+Alendronate treatment results in downregulated inflammatory pathways. These observations could have implications for BCG vaccination in the elderly with chronic use of NBPs, and larger studies investigating the effect of long-term alendronate use on BCG-induced trained immunity are required.



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## CRediT authorship contribution statement

Ozlem Bulut: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. Gizem Kilic: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. Priya A. Debisarun: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Software. Rutger Jan R¨oring: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Software. Sarah Sun: Data curation, Formal analysis, Investigation, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. Manon Kolkman: Data curation, Formal analysis, Investigation, Resources. Esther van Rijssen: Data curation, Formal analysis, Investigation, Resources. Jaap ten Oever: Conceptualization, Project administration, Supervision. Hans Koenen: Resources, Supervision. Luis Barreiro: Resources, Supervision, Writing – review & editing. Jorge Domínguez-Andr´es: Resources, Supervision. Mihai G. Netea: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

## Declaration of competing interest

Mihai G. Netea reports financial support was provided by European Research Council. Mihai G. Netea reports financial support was provided by Dutch Research Council. Jorge Dominguez-Andres reports financial support was provided by Dutch Research Council. Mihai G. Netea reports a relationship with Trained Therapeutix Discovery (TTxD) that includes: board membership and equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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# SUPPLEMENTARY TABLES

**Supplementary Table 1.** Flow cytometry antibodies used in the study

| Antibody | Source | Identifier |
|---|---|---|
| **General panel** | | |
| anti-CD16 FITC (clone 3G8) | Beckman Coulter | Cat#B49215; RRID: AB_2848116 |
| anti-HLA-DR PE (clone immu-357) | Beckman Coulter | Cat#IM1639; RRID: AB_131284 |
| anti-CD14 ECD (clone RM052) | Beckman Coulter | Cat#B92391; RRID: AB_130853 |
| anti-CD4 PE-Cy5.5 (clone 13B8.2) | Beckman Coulter | Cat#B16491; RRID: Unknown |
| anti-CD25 PE-Cy7 (clone M-A251) | BD Biosciences | Cat#557741; RRID: AB_396847 |
| anti-CD56 APC (clone N901) | Beckman Coulter | Cat#IM2474; RRID: AB_130791 |
| anti-CD8 APC-AF700 (clone B9.11) | Beckman Coulter | Cat#B49181; RRID: AB_2750854 |
| anti-CD19 APC-AF750 (clone J3-119) | Beckman Coulter | Cat#A94681; RRID: AB_2833030 |
| anti-CD3 Pacific Blue (clone UCHT1) | Beckman Coulter | Cat#A93687; RRID: AB_2728095 |
| anti-CD45 Krome Orange (clone J33) | Beckman Coulter | Cat#A96416; RRID: AB_2833027 |
| **B cell panel** | | |
| anti-IgD FITC (clone IADB6) | Southern Biotech | Cat#2032-02; RRID: AB_2687521 |
| anti-IgM PE (clone SA-DA4) | Beckman Coulter | Cat#B30657; RRID: unknown |
| anti-CD3 ECD (clone UCHT1) | Beckman Coulter | Cat#A07748; RRID: unknown |
| anti-CD27 PE-Cy5.5 (clone 1A4CD27) | Beckman Coulter | Cat#B21444; RRID: unknown |
| anti-CD38 PE-Cy7 (clone LS198-4-3) | Beckman Coulter | Cat#B49198; RRID: unknown |
| anti-CD24 APC (clone ALB9) | Beckman Coulter | Cat#A87785; RRID: unknown |
| anti-CD5 APC-AF700 (clone BL1a) | Beckman Coulter | Cat#A78835; RRID: unknown |
| anti-CD19 APC-AF750 (clone J3-119) | Beckman Coulter | Cat#A94681; RRID: AB_2833030 |
| anti-CD20 Pacific Blue (clone B9E9) | Beckman Coulter | Cat#B49208; RRID: unknown |
| anti-CD45 Krome Orange (clone J33) | Beckman Coulter | Cat#A96416; RRID: AB_2833027 |

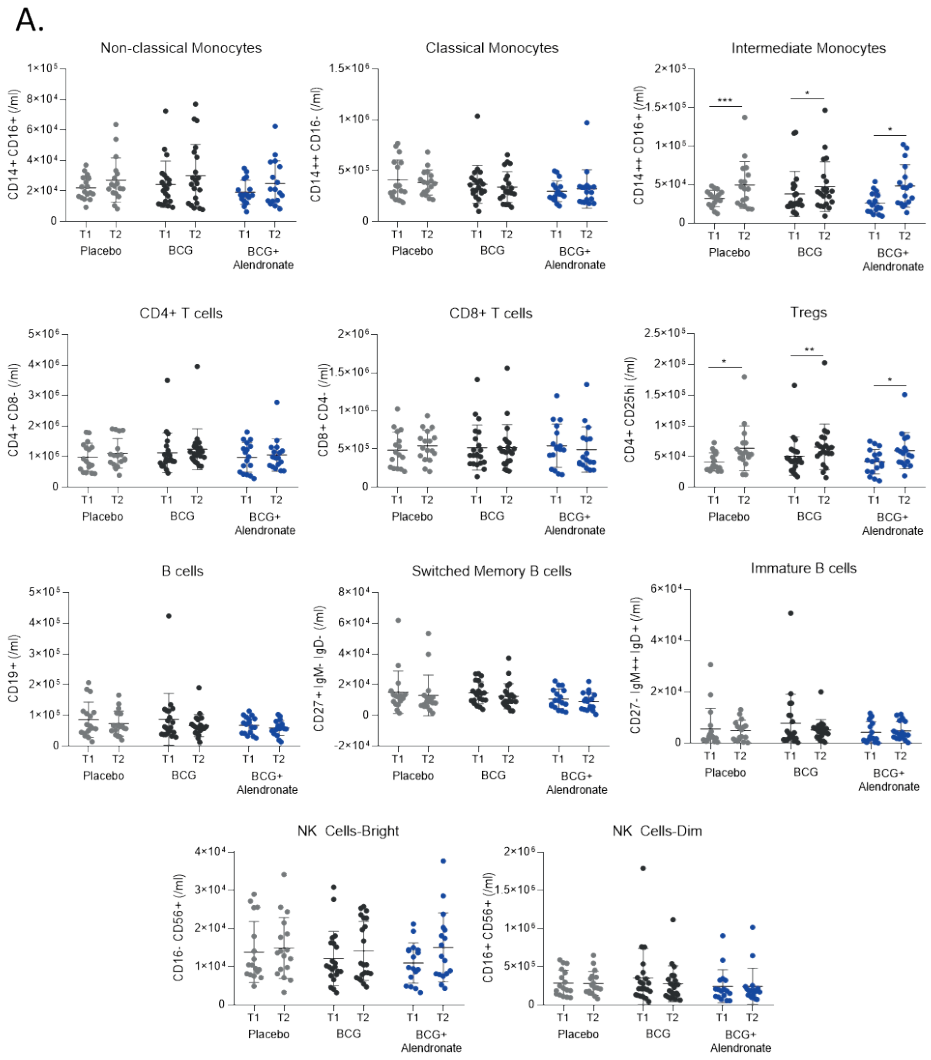
**Supplementary Table 2.** Demographics of the study participants who were selected for RNA sequencing.

| | Placebo (n=10) | BCG (n=10) | BCG+Alendronate (n=10) | p-value |
|---|---|---|---|---|
| Age (years, mean ± sd) | 27.7±7.0 | 25.6±7.7 | 27.6±8.6 | 0.79 |
| Sex (F/M) | 5/5 | 7/3 | 8/2 | 0.35 |
| BMI ($kg/m^2$, mean ± sd) | 23.7±1.7 | 21.3±1.8 | 23.0±3.5 | 0.11 |

**Supplementary Table 3.** Demographics of the study participants for whom RNA sequencing was possible upon poly(I:C) stimulation.

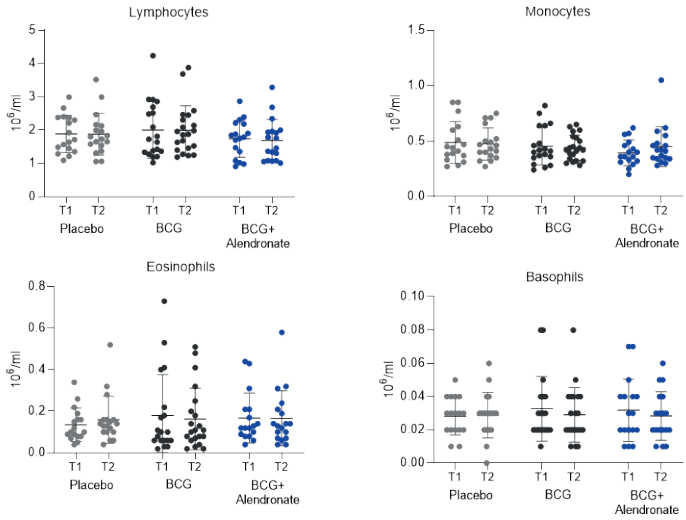
| | **Placebo (n=5)** | **BCG (n=5)** | **BCG+Alendronate (n=5)** | **p-value** |
|---|---|---|---|---|
| Age (years, mean ± sd) | 27.2±6.1 | 28.6±9.8 | 28.4±10.1 | 0.96 |
| Sex (F/M) | 1/4 | 5/0 | 4/1 | 0.02 |
| BMI ($kg/m^2$, mean ± sd) | 24.6±1.8 | 21.4±2.2 | 23.1±3.9 | 0.23 |

# SUPPLEMENTARY FIGURES

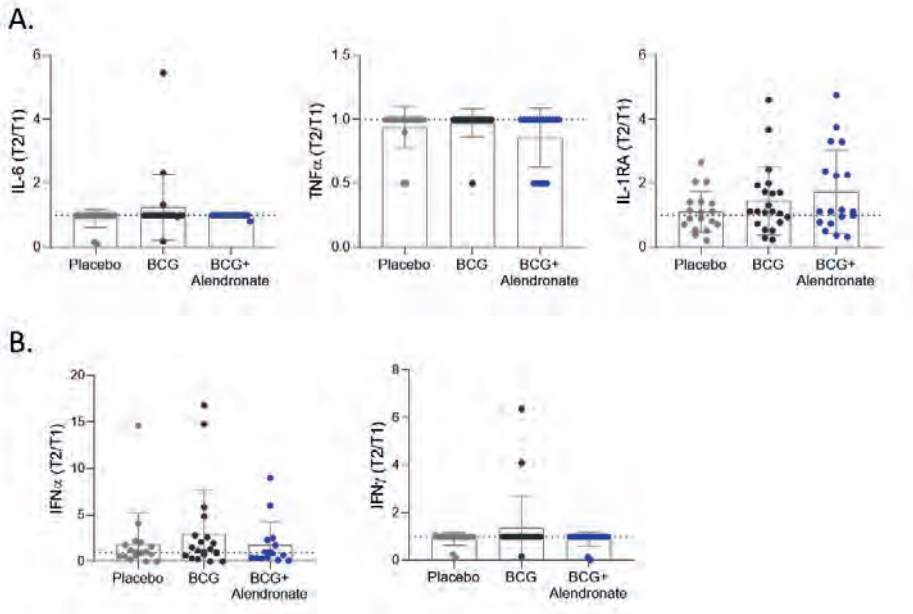



**Supplementary Figure 1. Numbers of the immune cell populations in the blood measured by A) flow cytometry and B) a hematology analyzer before and after treatment.** Cell numbers before (T1) and one month after (T2) treatment were compared with the Wilcoxon matched-pairs signed rank test. *p<0.05, **p<0.01, ***p<0.001.

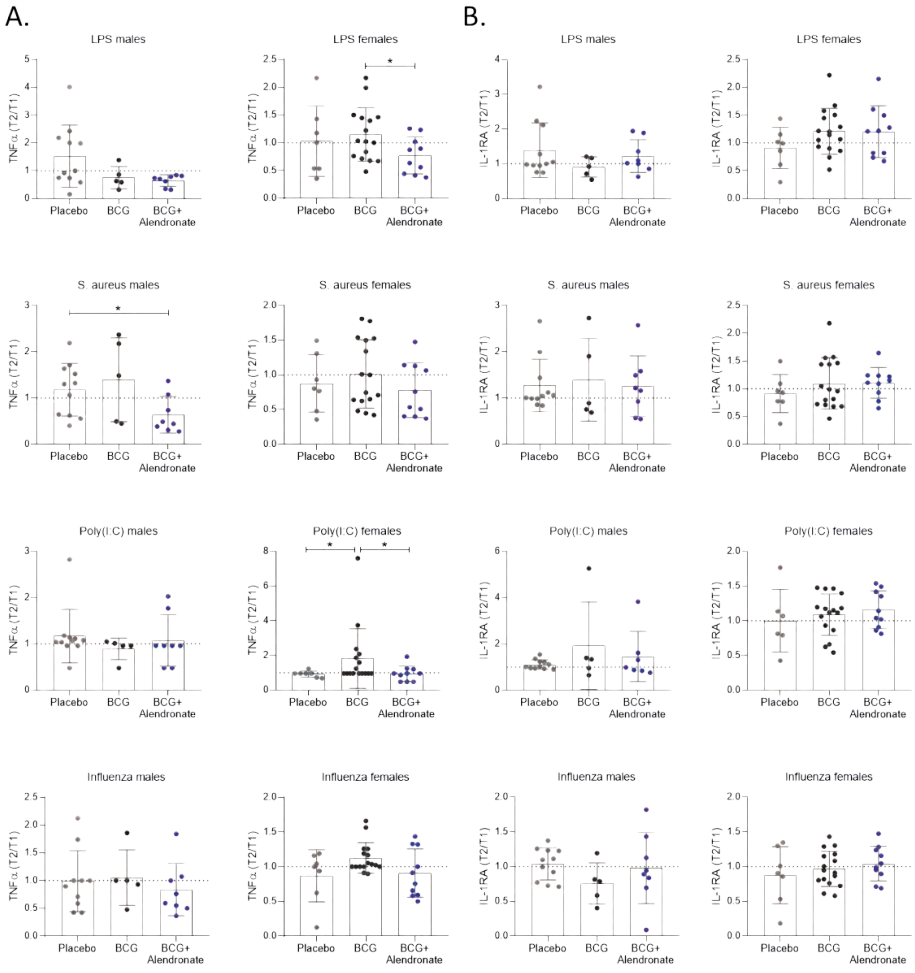
B.



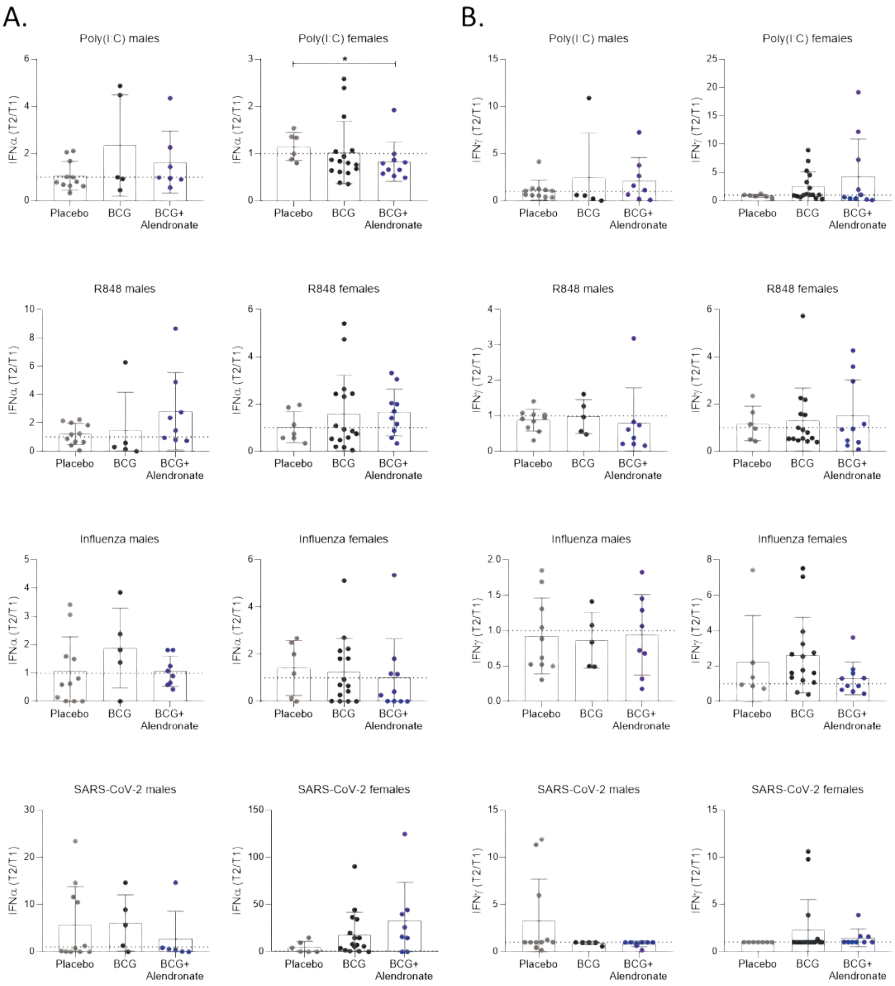

**Supplementary Figure 1. Continued**



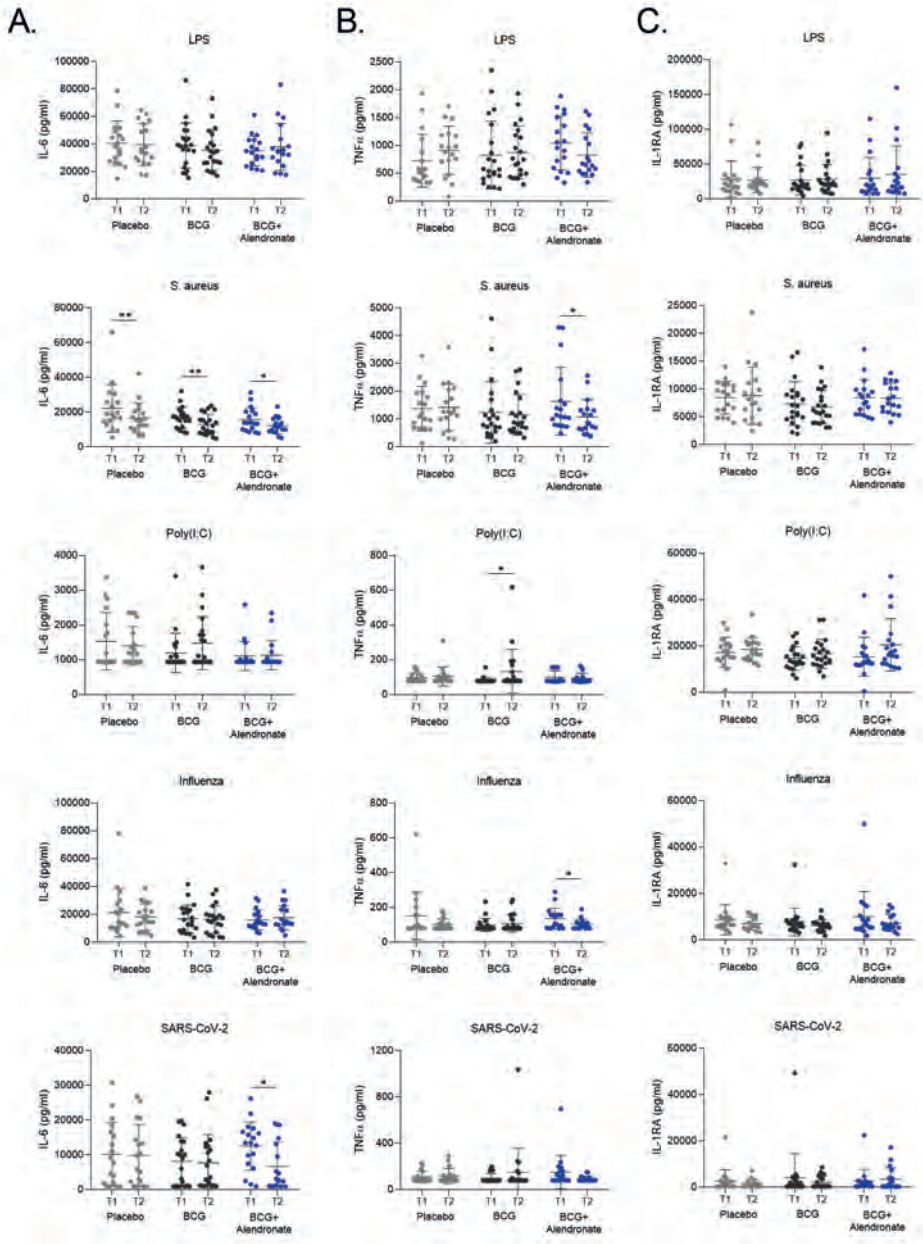

**Supplementary Figure 2. Fold changes of cytokine levels in the absence of stimuli.** Dotted lines depict the fold change of 1. Groups were compared with the Mann-Whitney U test. T1: before the intervention, T2: one month after the intervention. *p<0.05.



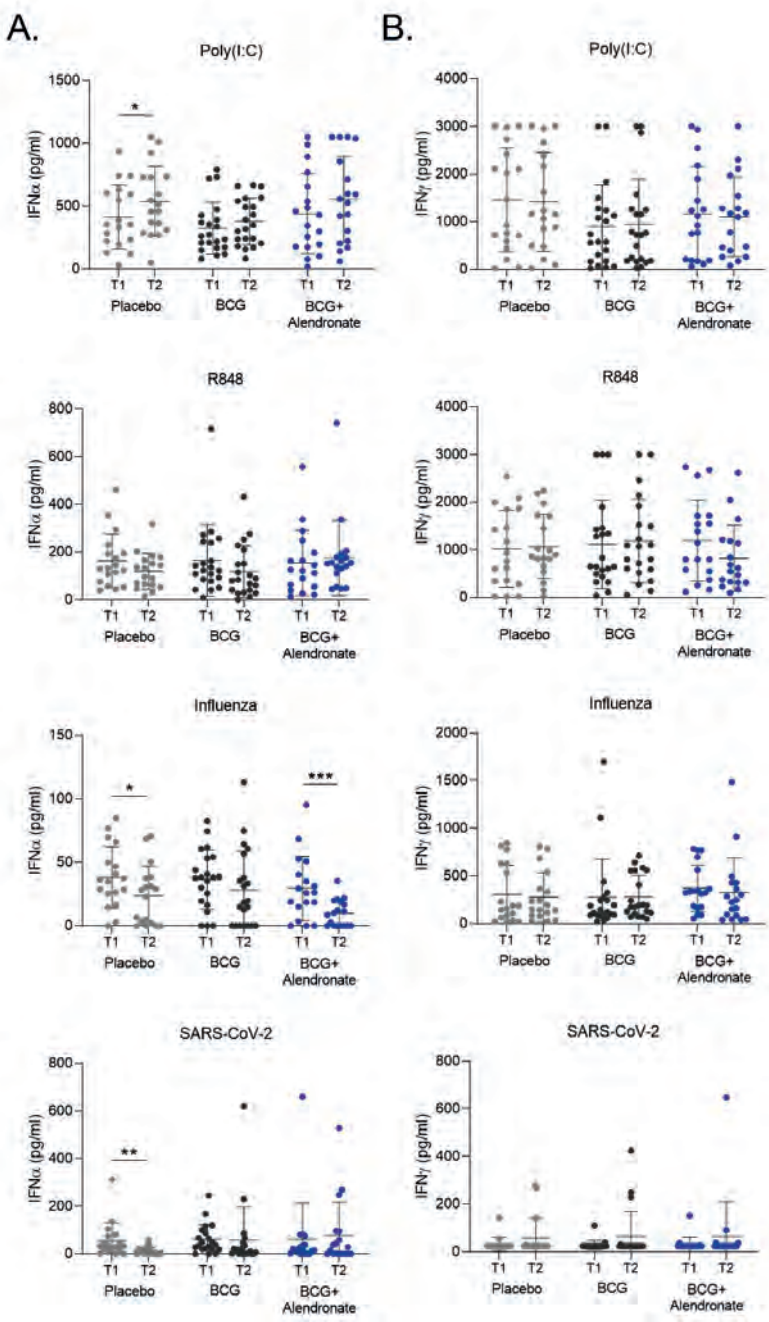

**Supplementary Figure 3. Fold changes (T2/T1) of A) TNFα and B) IL-1RA production by PBMCs upon bacterial and viral stimulation in males and females.** Dotted lines depict the fold change of 1. Groups were compared with the Mann-Whitney U test. T1: before the intervention, T2: one month after the intervention. *p≤0.05.



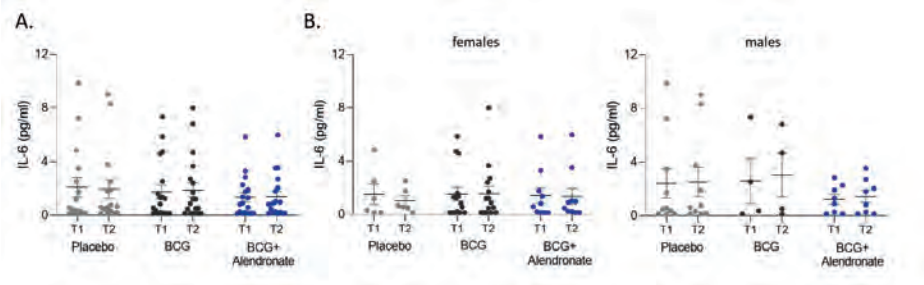

**Supplementary Figure 4. Fold changes (T2/T1) of A) IFNα and B) IFNγ production by PBMCs upon viral stimulation in males and females.** Dotted lines depict the fold change of 1. Groups were compared with the Mann-Whitney U test. T1: before the intervention, T2: one month after the intervention. *p≤0.05.




**Supplementary Figure 5. A) IL-6, B) TNFα, and C) IL-1RA production by PBMCs before and 1 month after intervention upon stimulation.** Time points within the same group were compared with the Wilcoxon matched-pairs signed rank test. T1: before the intervention, T2: one month after the intervention. *p≤0.05.




**Supplementary Figure 6. A) IFNα and B) IFNγ production by PBMCs before and 1 month after intervention upon stimulation.** Time points within the same group were compared with the Wilcoxon matched-pairs signed rank test. T1: before the intervention, T2: one month after the intervention. *p≤0.05, **p<0.01, ***p<0.001.




**Supplementary Figure 7. Plasma IL-6 levels of A) all participants and B) females and males separately before (T1) and one month after (T2) treatment.** T1 and T2 levels were compared with the Wilcoxon matched-pairs signed rank test.

# CHAPTER 7

# RESVERATROL POTENTIATES BCG-INDUCED TRAINED IMMUNITY IN HUMAN MONOCYTES

Ozlem Bulut · Ilayda Baydemir · Gizem Kilic · Jorge Domínguez-Andrés · Mihai G. Netea

Submitted

## ABSTRACT

Resveratrol is a natural polyphenol derived from plants such as grapes and berries. In addition to its role in plants during injury and infection, various cardioprotective, neuroprotective, and longevity-promoting effects were reported in diverse model organisms. The primary target of resveratrol is the deacetylase Sirtuin 1 (SIRT1), which regulates many immunological processes, including BCG-induced trained immunity response in humans. We, therefore, investigated the effect of resveratrol on trained immunity induced by BCG, β-glucan, *C. albicans*, or oxidized low-density lipoprotein (oxLDL).

Using an in-vitro model of trained immunity with monocytes obtained from healthy donors, we demonstrate that resveratrol amplifies BCG-induced trained immunity regarding IL-6 and TNFα production after a secondary challenge. Although resveratrol did not improve and even limited glycolysis, oxidative phosphorylation, and reactive oxygen species production, it enhanced the permissive epigenetic mark H3K27Ac on IL-6 and TNFα promoters. In contrast to BCG-induced trained immunity, resveratrol potently inhibited training induced by β-glucan, *C. albicans*, oxLDL, and muramyl dipeptide, a peptidoglycan component of BCG. Resveratrol's unique boosting effect on BCG training depended on BCG being alive and metabolically active.

These results suggest that resveratrol might amplify the effects of BCG vaccination, which should be mechanistically characterized further. In addition, resveratrol might alleviate oxLDL-induced training of innate immune cells in atherosclerosis, and in-vivo studies of trained immunity combined with resveratrol are warranted to explore these therapeutic possibilities.

# INTRODUCTION

Resveratrol (3,5,4′-trihydroxystilbene) is a natural polyphenol that was first isolated from the plant white hellebore in 1940 [1] and identified in many dietary sources in the following decades. These sources include but are not limited to grapes, blueberries, and peanuts. In plants, its synthesis is amplified in response to infection, injury, or other environmental stressors [2-4]. In various model organisms, from yeasts to mammals, resveratrol has been shown to extend lifespan [5, 6] and slow or prevent age-related disorders such as cardiovascular disease [7, 8], Alzheimer's disease [9, 10], and cancer [11]. In non-human primate and human studies, resveratrol was shown to induce improvements in glucose metabolism, insulin sensitivity, and cardiovascular outcomes [12-15].

The mechanisms through which resveratrol exerts its beneficial effects have not been fully elucidated. However, it is known that molecular targets of resveratrol include Sirtuin-1 (SIRT1) and adenosine monophosphate-activated protein kinase (AMPK), both of which are consistently implicated in longevity and metabolic fitness [14, 16]. Sirtuins (SIRT1-7 in mammals) are nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases [17]. Increased NAD+ concentrations and SIRT activity mimic the effects of caloric restriction; therefore, NAD+ boosters and SIRT activators are under investigation as a way to improve healthspan and lifespan [18]. SIRT1 localizes primarily in the nucleus, regulates p53, nuclear factor κB (NF-κB), sterol regulatory element binding protein (SREBP)-related gene transcription, and impacts cell survival, DNA repair, energy metabolism, inflammation, and neuronal signaling [19-22]. Although synthetic sirtuin-activating compounds with higher specificity and potency are now available [23], resveratrol has been an important research focus on sirtuin modulation.



A SIRT1 polymorphism was reported to impact Bacillus Calmette–Guérin (BCG, attenuated Mycobacterium bovis)-induced trained immunity, and the synthetic SIRT1 activator SRT1720 improved BCG-training in monocytes *in-vitro* [24]. Trained immunity refers to improved long-term responsiveness of innate immune cells upon encounter with certain live-attenuated vaccines such as BCG and pathogen- or damage-associated molecular patterns [25]. Training of innate immune cells involves epigenetic and metabolic rewiring that provides protection against subsequent microbial insults in an antigen-agnostic manner. Besides improving the innate immune response, BCG vaccination can reduce systemic inflammation in young and old individuals [26, 27].

Considering the modulation of trained immunity by SIRT1 and the effects of resveratrol on this epigenetic regulator and other inflammation-related targets, we hypothesized that resveratrol may modulate the induction of trained immunity. We, therefore, assessed the impact of resveratrol on trained immunity induced *in-vitro* [28] by either live attenuated BCG vaccine or non-viable microorganisms and ligands.

# METHODS

## In-vitro trained immunity model

Buffy coats from healthy donors were obtained after written informed consent (Sanquin Blood Bank, Nijmegen, the Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated by differential density centrifugation using Ficoll-Paque (GE Healthcare). Monocytes were enriched by additional density-gradient centrifugation using a hyper-osmotic Percoll (Sigma-Aldrich) solution. Cells were washed with cold phosphate-buffered saline (PBS) and resuspended in RPMI medium Dutch modified (Thermo Fisher Scientific) supplemented with 1 mM pyruvate (Gibco), 2 mM Glutamax (Gibco), and 50 µg/ml gentamicin (Centrafarm).

$10^5$ monocytes per well were seeded on flat-bottom 96-well plates (Sarstedt). After 1 hour, non-attached cells were washed with warm PBS, and stimulated with RPMI (unstimulated control), 1-5 µg/ml BCG (AJ Vaccines, Denmark), 5 µg/ml γ-irradiated BCG, 1 µg/ml β-glucan from *C. albicans*, $10^5$/ml heat-killed *C. albicans*, 10 µg/ml oxidized low-density lipoprotein (oxLDL), or 10 µg/ml MDP in the absence or presence of 25-100 µM of resveratrol (Sigma-Aldrich). In some experiments, BCG was pre-incubated with 1-25 µg/ml rifampicin for 2 hours, or monocytes were pre-incubated for 2 hours with 1-10 µM EX527 (Sigma-Aldrich). After 24 hours, cells were washed with warm PBS and left to differentiate for 5 days in RPMI with 10% pooled human serum. At day 6, cells were restimulated with 10 ng/ml E. coli-derived lipopolysaccharide (LPS) (E. coli 055:B5 LPS, Sigma-Aldrich). After 24 hours, supernatants were collected for cytokine measurements. For Seahorse and chromatin immunoprecipitation (ChIP), $20x10^6$ PBMCs per dish were seeded in 60 mm culture dishes. After 1 hour, cells were washed with warm PBS, and the training protocol as described was followed with adherent PBMCs. Cells were harvested on day 6 by gently scraping in cold PBS.

### Chromatin immunoprecipitation (ChIP) and qRT-PCR

Cells harvested at day 6 were fixed with 1% formaldehyde (Sigma-Aldrich) and sonicated in a lysis buffer with Covaris S220 using 400 bp target length. Sonicated chromatin was immunoprecipitated using H3K27Ac or H3K9me3 antibodies (Diagenode). Chromatin was pulled down using magnetic Dynabeads (Thermo Fisher Scientific) and eluted with The MinElute PCR Purification Kit (Qiagen). RT-qPCR was performed with StepOnePlus Real-Time PCR System (Applied Biosciences) using primers targeting the promoter regions of IL6 and TNFA. (IL6-forward: AGGGAGAGCCAGAACACAGA, IL6-reverse: GAGTTTCCTCTGACTCCATCG, TNFA-forward: GTGCTTGTTCCTCAGCCTCT, TNFA-reverse: ATCACTCCAAAGTGCAGCAG)

### Quantification of reactive oxygen species (ROS) production

ROS levels were evaluated using a luminol-based chemiluminescence assay. 105 monocytes per well were set up for the training protocol in opaque flat-bottom 96 well-plates and treated with RPMI, 25-50 µM resveratrol, 5 µg/ml BCG, or a combination of resveratrol and BCG. After 24 hours and on day 6 of the protocol, cells were treated with 1 mg/ml opsonized zymosan (Sigma-Aldrich) for 1 hour. 0.1 mM Luminol (Sigma-Aldrich) was added to start the chemiluminescence reaction. Measurements were performed with Biotek Synergy HT (Agilent). Chemiluminescence was measured at 37 °C for 1 hour and expressed as the area under the curve (AUC).



### Seahorse metabolic flux assays

On day 6 of the training protocol, cells were harvested by gently scraping and seeded as $10^5$ per well in 96-well Seahorse plates (Agilent). For the glycolysis stress test, cells were incubated in DMEM with 1 mM L-glutamine (Sigma-Aldrich); for the mito-stress test, with 2 mM L-glutamine, 5 mM D-glucose (Sigma-Aldrich), and 1 mM pyruvate with the pH adjusted to 7.4. Cells were first rested in a non-CO2 incubator at 37 $^0$C for 1 hour. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using a Seahorse XF Glycolysis Stress Test Kit and Mito Stress Test Kit in an XFp Analyzer (Agilent). The following compounds were introduced to the culture during the assays: 11 mM glucose, 1 µM oligomycin (Sigma-Aldrich), 22 mM 2-DG (Sigma-Aldrich), 1 µM FCCP (Sigma-Aldrich), and 1.25 µM rotenone and 2.5 µM antimycin A (Sigma-Aldrich).

### Statistical Analyses

GraphPad Prism 8 was used for statistical analyses and visualization. Wilcoxon matched-pairs signed rank test was employed to compare resveratrol groups with

their corresponding DMSO controls. Friedman test, followed by Dunn's multiple comparison test, was used to compare multiple groups to one reference group.

# RESULTS

## Resveratrol potentiates BCG-induced trained immunity in human monocytes

To assess resveratrol's long-term effects on human monocytes, we used an *in-vitro* trained immunity model (Figure 1A). Briefly, monocytes isolated from healthy donors were treated with resveratrol in the presence or absence of varying concentrations of BCG for 24 hours. Then, the stimuli were washed away, and monocytes were left to differentiate for five days in control culture medium. Cells were restimulated with LPS as the heterologous stimulus to assess the trained immunity response after resting.

Resveratrol alone led to the tolerization of monocytes in a dose-dependent manner (Figure 1B). At the end of the training protocol, resveratrol-treated cells could not respond to LPS by producing IL-6 and TNFα compared to DMSO-treated control cells. Of note, these concentrations of resveratrol did not increase cell death (Supplementary Figure 1). When combined with low-dose BCG (1 µg/ml), 25 and 50 µM resveratrol tended to improve the degree of trained immunity induction, indicated by fold changes of IL-6 and TNFα production, but statistical significance was not reached (Supplementary Figure 2). With 5 µg/ml BCG, which caused greater amplification of cytokine responses, 25 and 50 µM concentrations of resveratrol significantly boosted BCG's trained immunity effect (Figure 1C). All resveratrol concentrations inhibited the IL-6 and TNFα production induced by BCG in the first 24 hours (Supplementary Figure 3). However, after LPS restimulation, 50 µM resveratrol improved BCG-induced IL-6 production 2.9-fold and TNFα production 5.6-fold on average. For further experiments, 50 µM was chosen as the resveratrol concentration to study.

Next, we tested if resveratrol could also boost trained immunity induction by BCG in cells from older individuals (Figure 1D). The ages of these healthy donors ranged between 65 and 76. Resveratrol did not significantly alter the IL-6 response, but the TNFα response was improved (2.2-fold on average). These results indicate that despite being tolerogenic on its own, resveratrol can potently boost trained immunity induction by BCG in young and elderly individuals.

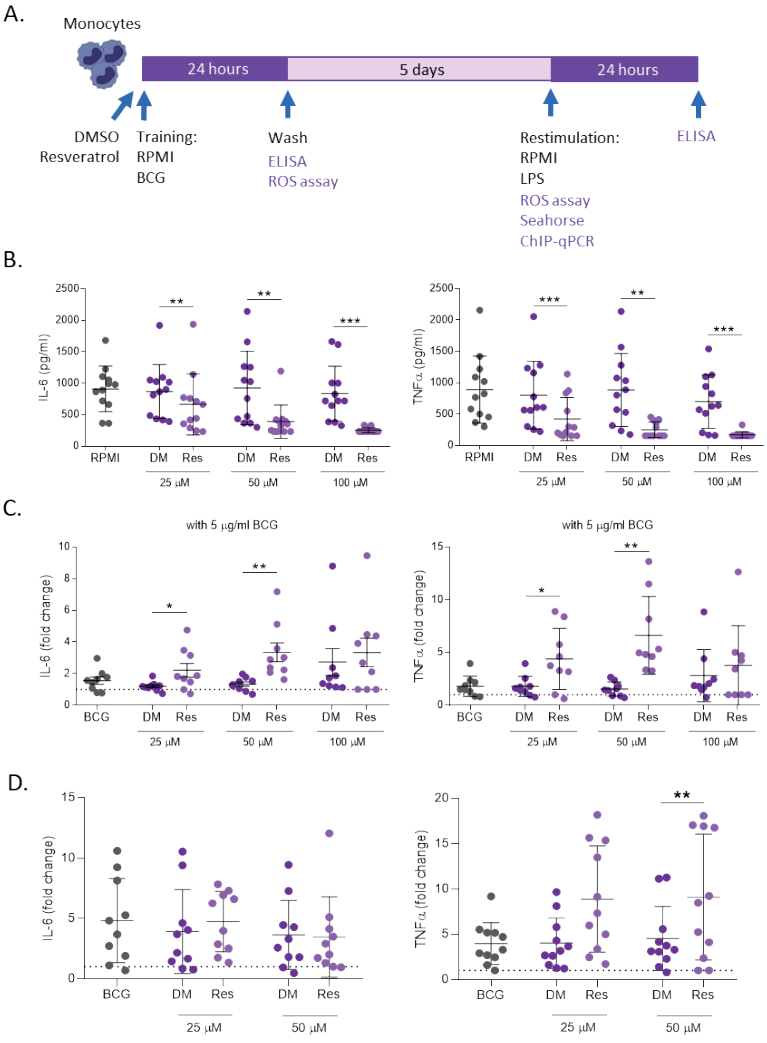



**Figure 1. Resveratrol's impact on pro-inflammatory cytokine production by healthy human monocytes in the presence or absence of BCG.** A. Schematic representation of the in-vitro trained immunity model and the readouts from each time point. Monocytes from healthy donors were incubated for 24 hours with resveratrol or DMSO as the solvent control in the absence or presence of BCG. After 24 hours, cells were washed and left to rest for 5 days in RPMI with 10% pooled human serum. At the end of the resting period, cells were restimulated with 10 ng/ml LPS. B. IL-6 and TNFα levels after restimulation of cells treated with DMSO or resveratrol alone (n=12 pooled from 4 independent experiments). C. Fold changes of IL-6 and TNFα production over non-trained controls after restimulation of cells trained with 5 μg/ml BCG with or without resveratrol (n=9 pooled from 3 independent experiments). D. Fold changes of IL-6 and TNFα production after restimulation of cells from healthy adults over 65 years old, trained with 5 μg/ml BCG with or without resveratrol (n=10-11 pooled from 4 independent experiments). DM: DMSO, Res: resveratrol. Dashed lines represent the fold change of 1. *p<0.05, **p≤0.01, ***p≤0.001, Wilcoxon matched-pairs signed rank test.

## Resveratrol promotes H3K27 acetylation of IL-6 and TNFα promoters

Next, we investigated the effects of resveratrol on epigenetic marks previously associated with BCG-induced trained immunity. The permissive H3K27 acetylation mark at the IL-6 promoter was enriched after BCG training and further increased with the addition of 50 μM resveratrol (Figure 2A). BCG did not increase the deposition of H3K27Ac on the TNFα promoter for all donors; however, resveratrol enriched the presence of the mark.

When the suppressive mark H3K9me3 was assessed on IL-6 and TNFα promoters, no statistically significant effect of BCG was observed, although most donors partially lost the mark after BCG training (Figure 2B). Resveratrol did not significantly impact the H3K9me3 presence; however, it tended to enrich the mark for some donors. These data suggest that resveratrol epigenetically modulates pro-inflammatory cytokine transcription and could improve BCG-induced cytokine production through increased H3K27 acetylation.

## Resveratrol limits BCG-induced ROS production, glycolysis, and oxidative phosphorylation

We also assessed the ROS response of monocytes against zymosan after training monocytes with resveratrol alone or combined with BCG. 50 μM, but not 25 μM, resveratrol suppressed ROS production by monocytes after 24 hours and on day 6 of the trained immunity protocol after resting of the cells (Figure 3A). BCG caused only a minimal effect on ROS production after 6 days, which was prevented by the presence of 50 μM resveratrol (Figure 3B).

Since BCG-induced trained immunity involves metabolic reprogramming, we assessed the metabolic effects of concurrent exposure of cells to resveratrol and BCG. BCG potently increased both glycolysis and oxidative phosphorylation in adherent PBMCs, consistent with previous reports (Figure 4). The presence of resveratrol limited both basal glycolysis and the maximum glycolytic capacity measured using the Seahorse XF Glycolysis Stress Test Kit (Figure 3A). Similarly, basal and maximal oxygen consumption rates, measured with the Seahorse XF Cell Mito Stress Test Kit, were both reduced by resveratrol compared to BCG challenge alone (Figure 3B). On the other hand, cells treated with BCG in the presence of resveratrol had higher glycolytic and oxidative phosphorylation rates on average than RPMI-treated control cells.

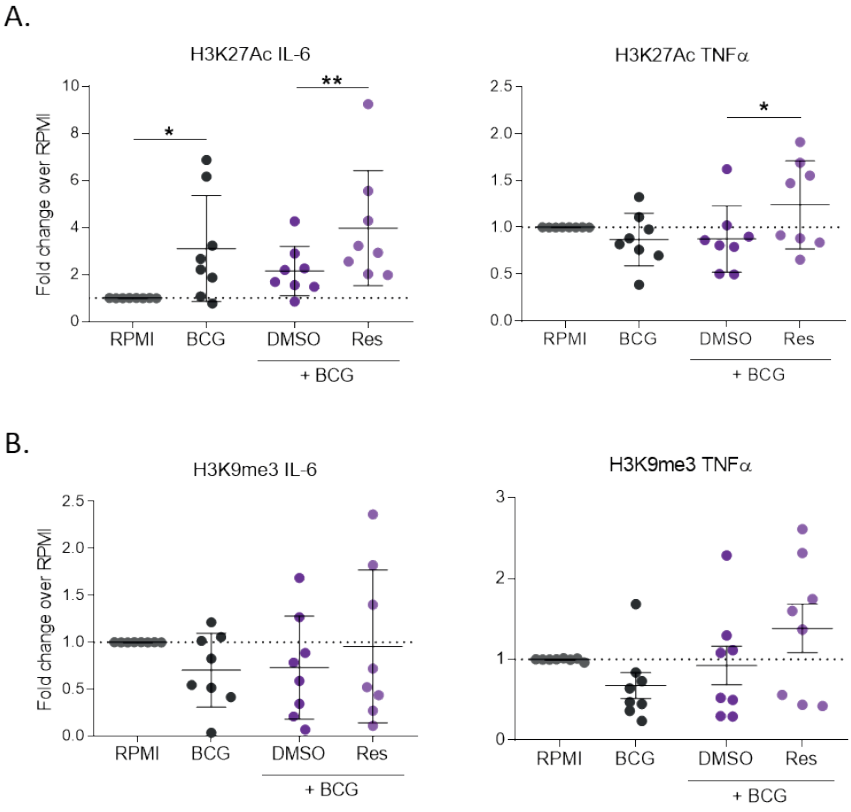



**Figure 2. Resveratrol's impact on epigenetic marks linked to BCG-induced trained immunity.** Enrichment of A. H3K27Ac and B. H3K9me3 marks on the IL-6 and TNFα promoters after BCG (5 μg/ml) training with or without DMSO control or resveratrol (50 μM) (n=8 pooled from 3 independent experiments). Res: resveratrol, Ac: acetylation, me3: trimethylation. Dashed lines represent the fold change of 1. *p<0.05, **p≤0.01, Wilcoxon matched-pairs signed rank test.

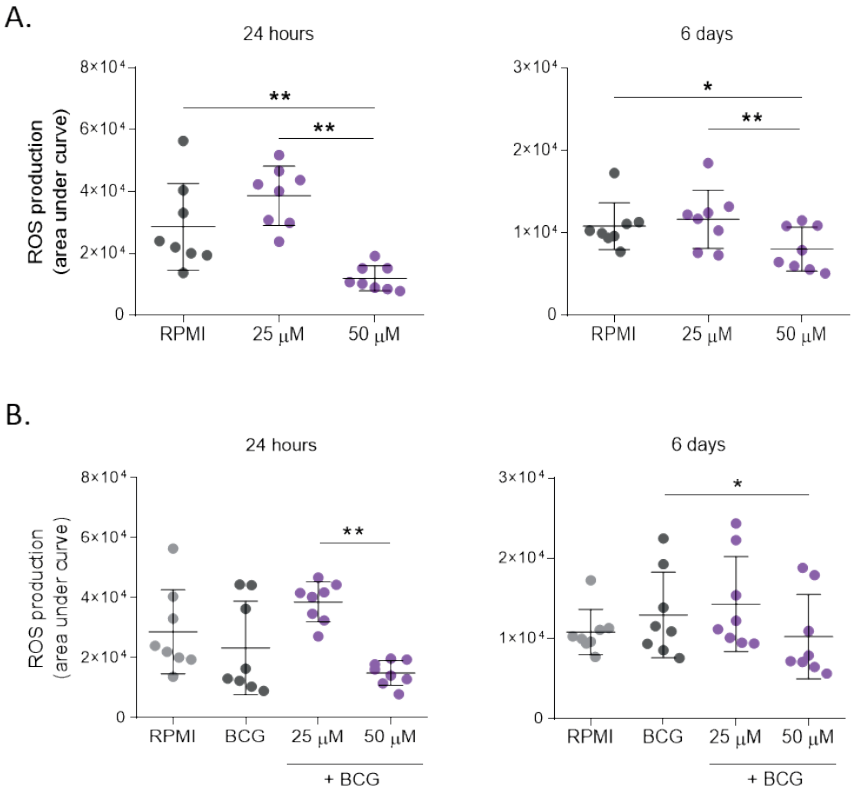



**Figure 3. Resveratrol's short- and long-term effects on ROS production by human monocytes.** Area under the curve (AUC) obtained from a luminescence-based assay representing the total ROS produced over an hour upon zymosan stimulation at days 1 and 6 of the training protocol with resveratrol (25 or 50 μM) in the A. absence or B. presence of 5 μg/ml BCG (n=8 pooled from 3 independent experiments). *p<0.05, **p≤0.01, Wilcoxon matched-pairs signed rank test.

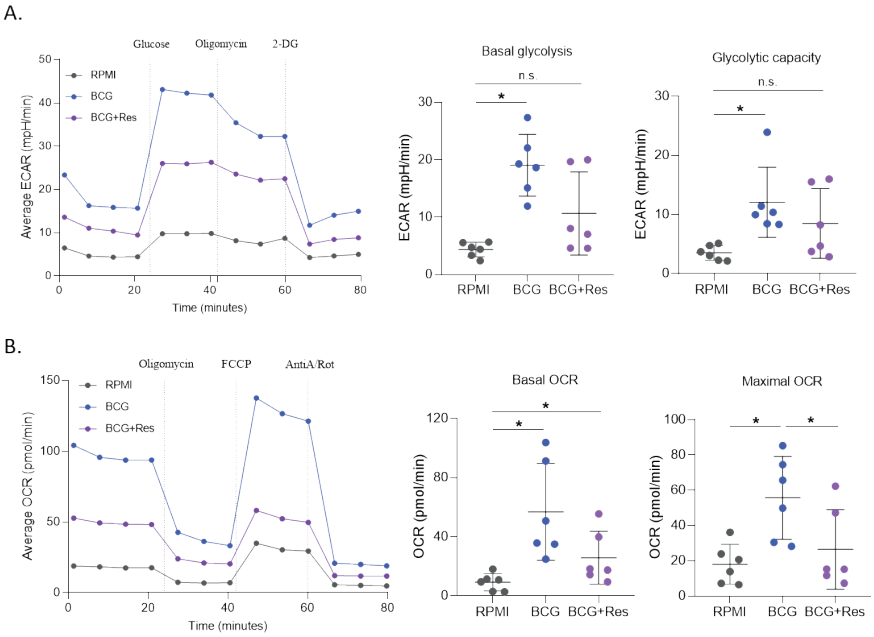



**Figure 4. Resveratrol's impact on BCG-induced glycolysis and oxidative phosphorylation.** A. Real-time average extracellular acidification rate (ECAR) measured with the Seahorse XF Glycolysis Stress Test Kit (left), basal glycolysis rate (center), and glycolytic capacity (right). Cells were trained with 5 µg/ml BCG with or without 50 µM resveratrol (n=6 pooled from 2 independent experiments). B. Real-time average oxygen consumption rate (OCR) measured with the Seahorse XF Cell Mito Stress Test Kit (left), basal OCR (center), and maximal OCR (right). Cells were trained with 5 µg/ml BCG with or without 50 µM resveratrol (n=6 pooled from 2 independent experiments). DM: DMSO, Res: resveratrol. n.s.: not significant, *p<0.05, Wilcoxon matched-pairs signed rank test.

## Resveratrol is a BCG-specific, SIRT1-dependent, amplifier of trained immunity

To assess whether resveratrol could broadly improve trained immunity responses, we tested it in combination with several other known trained immunity inducers: β-glucan, heat-killed *C. albicans*, oxLDL, and muramyl dipeptide (MDP), which is a component of BCG that induces trained immunity. Irrespective of the training stimulus, resveratrol suppressed the trained immunity response represented by IL-6 and TNFα production after restimulation with LPS (Figure 5).

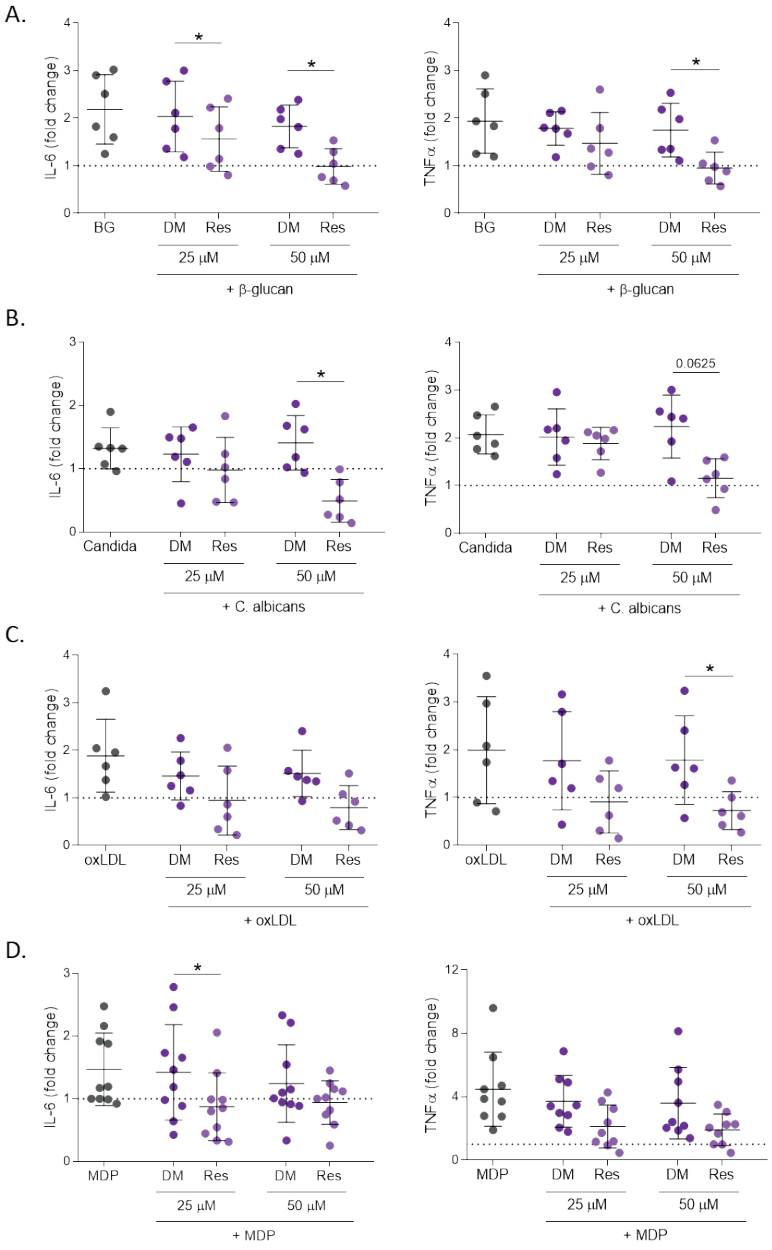



**Figure 5. Resveratrol's impact on trained immunity induction by β-glucan, *C. albicans*, oxLDL, and MDP.** Fold changes of IL-6 and TNFα production over non-trained controls after restimulation of cells trained with A. 1 μg/ml β-glucan (BG), B. 105/ml heat-killed *C. albicans*, C. oxidized low-density lipoprotein (oxLDL) or D. muramyl dipeptide (MDP) with or without resveratrol (25 or 50 μM) (n=6-10 pooled from 2-3 independent experiments). Dashed lines represent the fold change of 1. DM: DMSO, Res: resveratrol. *p<0.05, Wilcoxon matched-pairs signed rank test.

Based on these observations, we hypothesized that resveratrol's unique effect on BCG might be related to the fact that BCG is a live organism, unlike the other trained immunity inducers tested. To investigate this, we compared resveratrol's effect on γ-irradiated BCG to standard live BCG. Resveratrol could only significantly boost IL-6 and TNFα production when combined with live BCG and not irradiated BCG (Figure 6A). Furthermore, BCG was pre-incubated with varying doses of rifampicin to prevent RNA synthesis and proliferation during the training protocol. Rifampicin dose-dependently reduced the trained immunity response (as measured by IL-6 production) induced by the combination of BCG and resveratrol but not DMSO control (Figure 6B).

In another set of experiments, we investigated whether resveratrol's effect, when combined with BCG, depended on SIRT1 activation in monocytes by resveratrol. The addition of SIRT1 inhibitor EX527 during the first day of training dose-dependently reduced the improvement of cytokine responses by resveratrol (Figure 5C). However, the IL-6 and TNFα production capacities only decreased to the levels of the BCG-alone condition; EX527 did not block the BCG training.

Taken together, these results indicate that resveratrol amplifies the capacity of live BCG to induce trained immunity in a SIRT1-dependent manner.

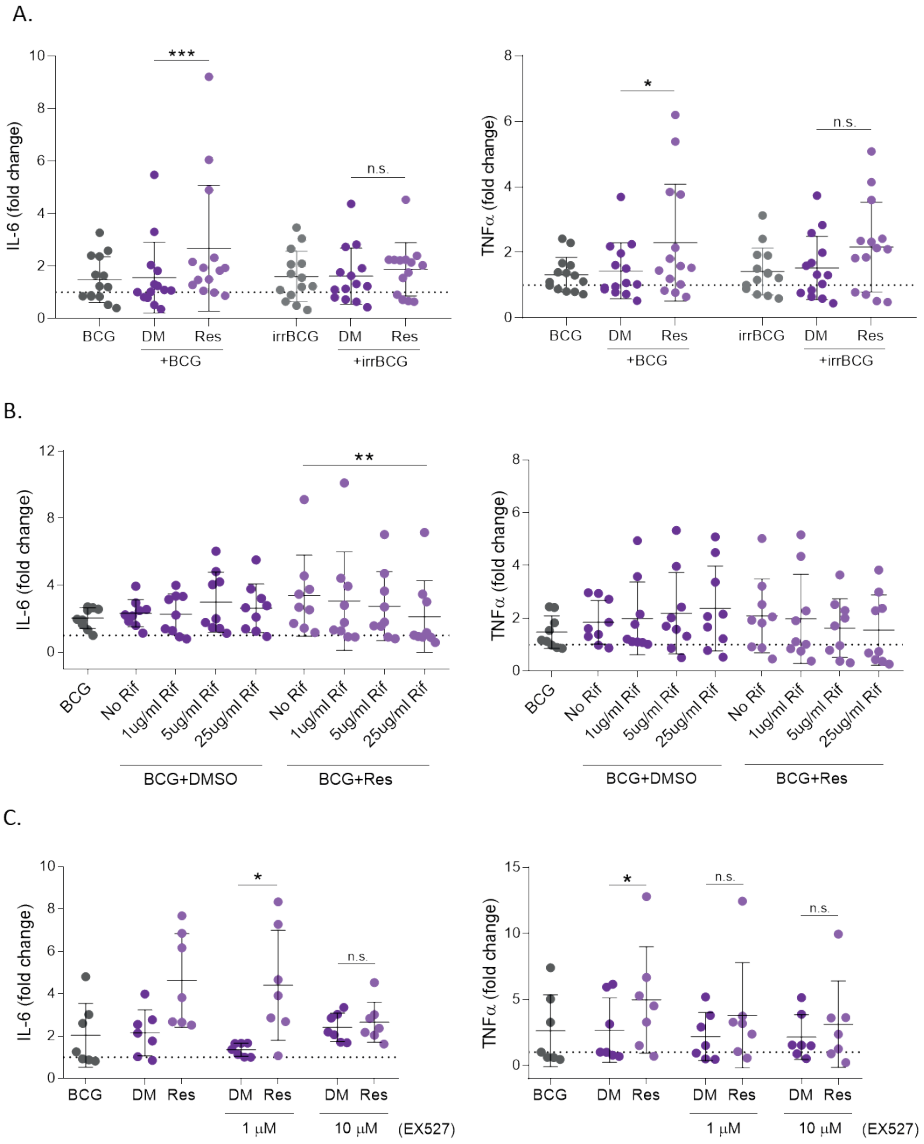



**Figure 6. Contributions of BCG viability, transcription, and SIRT-1 activation to the potentiation of BCG training by resveratrol.** Fold changes of IL-6 and TNFα production over non-trained controls after restimulation of cells trained with A. 5 μg/ml live or γ-irradiated BCG (n=14 pooled from 5 independent experiments), B. 5 μg/ml live BCG pre-incubated with rifampicin in varying concentrations (n=9 pooled from 3 independent experiments), and C. 5 μg/ml live BCG with or without 2 hour pre-incubation with 1 μM or 10 μM EX527 (n=7 pooled from 2 independent experiments). The resveratrol concentration was 50 μM for all experiments. DM: DMSO, Res: resveratrol, irrBCG: γ-irradiated BCG, Rif: rifampicin. *p<0.05, **p≤0.01, ***p≤0.001, n.s.: not significant, Wilcoxon matched-pairs signed rank test or Friedman test followed by Dunn's multiple comparison test.

## DISCUSSION

Resveratrol addition to the BCG vaccine augmented the in-vitro trained immunity response in primary human monocytes. While metabolic biomarkers of trained immunity (ROS production, glycolysis, and oxidative phosphorylation) were not impacted or even limited by resveratrol, the increased deposition of H3K27ac at the IL-6 and TNFα promoters suggests epigenetic regulation that leads to the improved cytokine response.

Resveratrol has been reported to have broad anti-inflammatory activities. These include inhibition of cyclooxygenase-2 (COX-2) activity, NF-κB and activator protein 1 (AP-1) phosphorylation, and their DNA binding capacity [29]. Studies focusing on monocytes or macrophages reported reduced nitric oxide generation, cytokine production, ROS production, and inflammasome activity [30-34]. Furthermore, resveratrol acts as an estrogen receptor (ER) agonist, and some of its immunomodulatory effects might be attributable to ERα signaling [35, 36]. In humans, resveratrol can also reduce systemic inflammation [37, 38]. The molecular mechanisms that mediate these effects of resveratrol include the activation of the deacetylase SIRT1. SIRT1/2 inhibition in human monocytes and macrophages increases the H3K27ac mark at several pro-inflammatory loci [39]. SIRT1/2 interact with DNA methyltransferase 3B (DNMT3B) to promote DNA methylation and loss of H3K4me3 and H3K27ac. In myeloid-specific SIRT1 knockout mice, NF-κB is hyperacetylated, resulting in increased TNFα, IL-6, and IL-1β [40]. Furthermore, resveratrol can inhibit NF-κB nuclear translocation, activity, and the expression of NF-κB-dependent genes [41, 42]. Our findings of long-term increased responsiveness after exposure of human monocytes to resveratrol and BCG, along with H3K27ac enrichment at pro-inflammatory gene promoters, suggest other possible targets of resveratrol that regulate inflammatory response at the epigenetic level or another level of regulation through Sirt1 that needs to be characterized.

Interestingly, however, trained immunity induced by other ligands such as β-glucan, heat-killed *C. albicans*, oxLDL, and MDP (a component of BCG) was inhibited by resveratrol. This led to the hypothesis that resveratrol's effect might depend on live BCG being able to metabolize resveratrol. Indeed, resveratrol could not improve cytokine production if BCG was inactivated by γ-irradiation or its RNA polymerase was inhibited. Pre-treating the human cells with a SIRT1 inhibitor also dose-dependently prevented the amplifying effect of resveratrol. These results suggest that resveratrol might be a specific amplifier of BCG-induced trained immunity. The

molecular mechanisms through which resveratrol exerts its effect on BCG remain to be investigated in future studies.

In contrast to the effects on BCG-induced trained immunity, our study showed that resveratrol inhibits oxLDL-induced trained immunity in human monocytes. SIRT1 activator SRT1720 was previously reported to augment oxLDL training [43], and this may suggest that resveratrol's inhibitory effect on oxLDL-induced trained immunity involves targets other than SIRT1. Resveratrol was reported to prevent LDL oxidation [44], and this may interfere with its capacity to induce trained immunity [45]. Interestingly, resveratrol and its metabolites can be detected in the LDL fraction of healthy humans after moderate wine intake [46, 47]. Resveratrol inhibits oxLDL-induced cytotoxicity and ROS production in endothelial cells [48] and blocks the oxLDL-induced activation of the PI3K/Akt/mTOR pathway in smooth muscle cells [49]. In a clinical study comparing daily administration of placebo, resveratrol-deficient grape extract, and resveratrol-enriched grape extract in statin-treated patients with stable coronary artery disease, the extract containing 8 mg resveratrol led to significantly reduced oxLDL and ApoB levels after 6 months, and elevated adiponectin levels alongside reduced plasminogen activator inhibitor-1 (PAI-1) levels after 1 year [50, 51]. Prevention of trained immunity, which plays an important role in cardiovascular disease [52], might be one of the many mechanisms behind resveratrol's cardioprotective actions.

While in this study, we described for the first time resveratrol's impact on trained immunity in primary human cells, some questions remain to be studied. Resveratrol specifically amplified trained immunity induced by BCG while suppressing β-glucan, *C. albicans*, and oxLDL-induced training. Although we demonstrated that BCG viability and transcriptional activity are essential for resveratrol's effect, future studies should identify the molecular mechanisms through which BCG utilizes and is altered by resveratrol. In addition, validating these effects of resveratrol in experimental in-vivo models and randomized controlled human trials would pave the way for potential applications as a BCG vaccine amplifier and/or a prophylactic or therapeutic agent for cardiovascular disease.

In conclusion, this study identifies resveratrol as a potential amplifier to improve BCG-induced trained immunity. Resveratrol is also a modulator of trained immunity induced by β-glucan, *C. albicans*, and oxLDL. Combining resveratrol with the BCG vaccine might result in an efficient immune response while limiting oxidative damage and overactivation of inflammatory pathways, which would especially be critical for individuals who are older or with co-morbidities.

## Funding

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## Conflict of interest

M.G.N. is a scientific founder and member of the scientific advisory board of Trained Therapeutix Discovery (TTxD), and is a scientific founder of Lemba and Biotrip. The other authors declare that they have no conflicts of interest.

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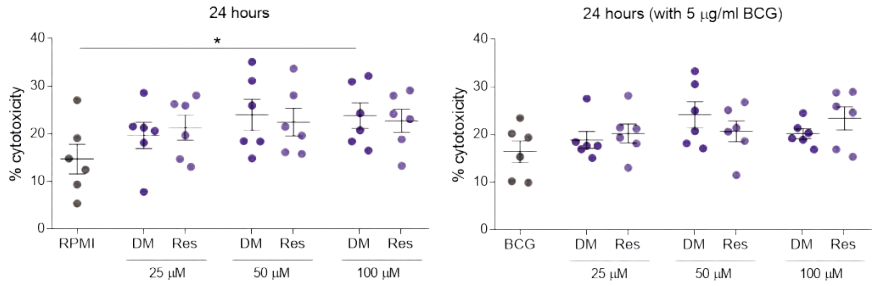
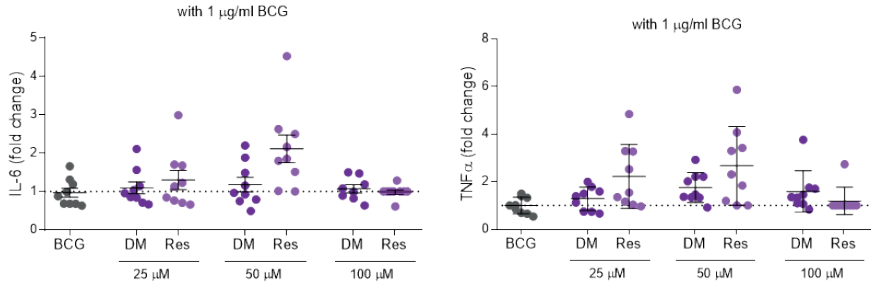
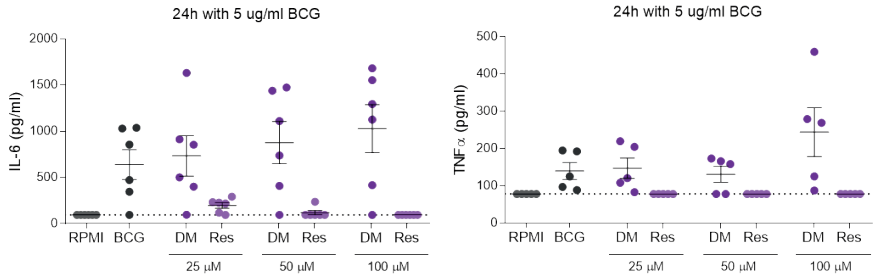
# SUPPLEMENTARY FIGURES



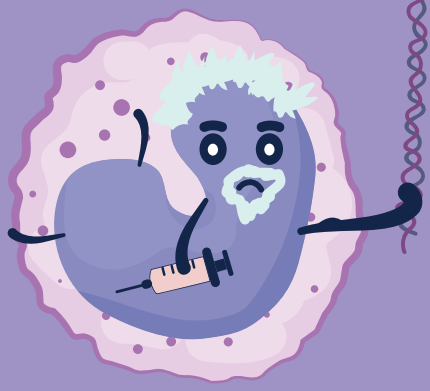

**Supplementary Figure 1. Resveratrol's impact on trained immunity induction by low-dose BCG.** Percent cytotoxicity was assessed with a colorimetric lactate dehydrogenase assay. Monocytes were incubated with varying doses of DMSO and resveratrol with or without 5 μg/ml BCG for 24 hours (n=6 pooled from 2 independent experiments). DM: DMSO, Res: resveratrol. *p<0.05, Friedman test followed by Dunn's multiple comparison test.




**Supplementary Figure 2. Resveratrol's impact on trained immunity induction by low-dose BCG.** Fold changes of IL-6 and TNFα production over non-trained controls after restimulation of cells trained with 1 μg/ml BCG with or without resveratrol (25, 50 or 100 μM) (n=9 pooled from 3 independent experiments). Dashed lines represent the fold change of 1. DM: DMSO, Res: resveratrol.




**Supplementary Figure 3. Resveratrol's impact on 24-hour cytokine production induced by BCG.** IL-6 and TNFα levels after monocytes were incubated with 5 μg/ml BCG with or without resveratrol (25, 50 or 100 μM) (n=6 pooled from 2 independent experiments). Dashed lines represent the lower detection limit of the assay. DM: DMSO, Res: resveratrol.



# CHAPTER 8

# BCG VACCINATION HAS SEX-SPECIFIC LONG-TERM EFFECTS ON TELOMERE LENGTH AND TELOMERASE ACTIVITY

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Submitted

## ABSTRACT

Vaccination with Bacillus Calmette-Guérin (BCG) induces long-term innate immune memory, also called *trained immunity*, characterized by metabolic and epigenetic changes leading to enhanced responsiveness upon exposure to heterologous pathogens. BCG vaccination also reduces systemic inflammation. Thus, BCG counteracts two significant immunological changes associated with aging: impaired responsiveness and elevated systemic inflammation. However, if and how BCG impacts other aging-related processes in immune cells, such as telomere shortening, remains unexplored.

In this study, we investigated the transcriptional impact of BCG training on telomere maintenance-related genes through RNA sequencing and determined average telomere length in whole blood via RT-qPCR before and three months after BCG vaccination in two independent human cohorts. Trained immunity response was measured by *ex-vivo* cytokine production induced by a heterologous stimulus three months after vaccination compared to baseline. In addition, we examined the effects of BCG on telomerase activation using an *in vitro* trained immunity model.

*In vitro* BCG training upregulated processes related to telomere maintenance and telomerase localization. *In vivo*, both studies showed shorter telomeres three months after BCG vaccination. Interestingly, the induction of a trained immunity response by BCG was correlated to the change in telomere length: more telomere shortening was observed in trained immunity non-responder individuals, particularly in males. Higher testosterone concentrations before vaccination were linked to more telomere loss. *In vitro,* BCG training of human monocytes activated the telomerase enzyme, predominantly in females, an effect blocked by exogenous testosterone treatment.

Overall, this study reports sex-specific long-term impacts of BCG vaccination on telomerase activity and telomere maintenance. These data add to the arguments that BCG vaccination impacts aging mechanisms, which warrants further investigation.

# INTRODUCTION

Innate immune cells are capable of mounting memory-like responses, a phenomenon termed 'trained immunity' [1]. The heightened response against non-specific pathogens after exposure to certain vaccines, pathogen- or damage-associated molecular patterns (PAMPs and DAMPs) is orchestrated through epigenetic and metabolic reprogramming. One of the vaccines investigated in the context of trained immunity is Bacillus Calmette-Guérin (BCG) [2], a live-attenuated *Mycobacterium bovis*, the pathogen that causes tuberculosis in cattle. Besides its use for tuberculosis prevention through the induction of Th1-based adaptive immune memory, BCG is studied as a safe inducer of trained immunity in humans. Epidemiological studies and clinical trials have shown that BCG vaccination reduces the incidence, severity, and mortality of heterologous infections [3, 4].

Aging of the immune system poses drastic problems as the world population rapidly ages. Older individuals suffer from higher morbidity and mortality due to their inability to mount a robust immune response during infection while responding poorly to vaccination [5]. Moreover, metabolic and inflammatory cellular aging processes lead to or exacerbate many diseases, such as diabetes, cardiovascular disease, obesity, cancer, and dementia [6, 7]. Over half of the individuals older than 65 have multiple age-related comorbidities [8, 9]. Importantly, recent studies have shown that immune aging is also directly related to the aging processes of solid organs [10]. Tackling immune aging is, therefore, critical to reducing the disease burden on individual lives and healthcare systems. An aged immune system is characterized by persistent low-grade systemic inflammation and suboptimal cellular responses [11]. Interestingly, BCG has been reported to improve cellular immune responses while reducing systemic inflammation [12], and we hypothesized that it may impact other hallmarks of aging, one of which is telomere shortening.



Telomeres are repetitive TTAGGG sequences at the ends of eukaryotic chromosomes [13]. A group of proteins bound to telomeres called the shelterin complex protects the chromosome ends from being recognized as DNA damage. However, shelterin and telomeres must unfold during replication to make the free end accessible to the replication machinery. With each replication cycle, telomeres are gradually eroded, and critical telomere shortening leads to cell cycle arrest and is one of the hallmarks of cellular aging [14]. Telomere length is affected by many extrinsic factors, including certain infections and total infectious burden [15-18]. Oxidative stress, which often accompanies inflammation, can aggravate telomere shortening [19]. Shorter telomeres are associated with many age-related conditions, including

neurodegenerative diseases, atherosclerosis, and metabolic syndrome [20]. However, abnormally long telomeres can also lead to clonal hematopoiesis and cancer through prolonged cell survival and accumulation of mutations [21]. Proper regulation of telomere length at the cellular level is thus required for homeostasis of the organism.

Telomerase, a ribonucleoprotein consisting of an RNA template and a reverse transcriptase, can extend the telomeric repeats. The transcription of telomerase reverse transcriptase (TERT) is repressed in most adult human cells but is active in many cancers, allowing cancer cells to replicate excessively [22]. Hematopoietic stem cells and T lymphocytes are among the rare cell types that can upregulate telomerase activity during homeostasis [23, 24]. Telomerase can modulate NF-κB transcription [25], and NF-κB-p65 facilitates the nuclear translocation of TERT, which is also regulated by TNFα [26].

In the present study, we revealed transcriptional regulation of telomere maintenance-related genes by *in vitro* BCG training and assessed telomere length before and three months after vaccination in two independent human BCG vaccination cohorts. In addition, we investigated the effects of BCG on telomerase activation.

# METHODS

## Study subjects and interventions

In the 300BCG study [27, 28], 323 healthy adult volunteers were recruited between April 2017 and June 2018 at Radboudumc in the Netherlands and vaccinated with a standard dose of BCG (Bulgaria strain, InterVax) administered intradermally. Venous blood was collected before, two weeks after, and three months after vaccination.

As the validation cohort, we used the BCG-Booster randomized placebo-controlled trial assessing different BCG vaccination regimens [29]. Recruitment took place between October 2019 and February 2020 at Radboudumc. Participants were allocated to receive a placebo vaccination (BCG vaccine diluent), a single standard dose of BCG (0.1 ml, 0.75 mg/ml, Denmark strain, AJ Vaccines), a high dose of BCG (0.1 ml 1.5 mg/ml), or revaccination with a single standard dose three months after the first vaccination. The single vaccination groups first received a placebo three months before the BCG vaccination to align the time points with the revaccination group. Only the participants who received the standard dose BCG or placebo vaccination were assessed for this manuscript. Blood was collected before and three months after each intervention.

The studies were approved by the Arnhem-Nijmegen Medical Ethical Committee (NL58553.091.16 and NL58219.091.16 in the Dutch trial registry). Written informed consent was obtained from all participants. Previous BCG vaccination, any vaccination in the 3 months before enrolling in the study, any febrile illness two weeks before the intervention, and medication use except for oral contraceptives were exclusion criteria shared by both studies.

### Telomere length measurements from whole blood

DNA from whole blood was isolated using QIAamp Blood kit (Qiagen), and concentrations were measured at 260 nm with a NanoDrop (Thermo Scientific) spectrophotometer. The average telomere length of the DNA samples was assessed using the Absolute Human Telomere Length Quantification qPCR Assay Kit (ScienCell) according to the manufacturer's protocol. The kit includes a telomere-specific primer set and a single-copy reference gene primer set for data normalization. The reference genomic DNA sample provided with a known telomere length was used to calculate the average telomere length of the samples.

### Hormone measurements in plasma

Testosterone, androstenedione, dehydroepiandrosterone sulfate, and 17-hydroxy-progesterone levels in plasma samples from 300BCG participants before vaccination were measured by liquid chromatography with tandem mass spectrometry (LC-MS/MS) after protein precipitation and solid-phase extraction as described in detail previously [30].



### Peripheral blood mononuclear cells (PBMCs) isolation and stimulation

PBMCs from whole blood were isolated using Ficoll-Paque (GE Healthcare) density gradient separation and washed twice with phosphate-buffered saline (PBS). Cells were resuspended in Dutch-modified RPMI 1640 medium (Invitrogen), supplemented with 50 µg/mL gentamycin, 2 mM Glutamax (Gibco), and 1 mM pyruvate (Gibco). $5 \times 10^5$ PBMCs/well were cultured in round-bottom 96-well plates (Greiner) and incubated for 24 hours at 37 °C with either RPMI control or $1 \times 10^6$ CFU/mL heat-killed *S. aureus*. Supernatants were collected and stored at -20 °C until analysis. IL-1β, IL-6, and TNF-α concentrations were measured using DuoSet ELISA kits (R&D Systems) according to the manufacturer's protocol. Different time point samples belonging to the same participant were measured on the same plate to minimize variation in the calculated cytokine fold changes.

### *In vitro* trained immunity model

The *in vitro* trained immunity model was described in detail by Dominguez-Andres et al. [31]. Instead of the enriched monocyte fraction in the aforementioned protocol, $3 \times 10^6$ PBMCs/well from healthy adult volunteers were incubated on flat-bottom 6-well plates (Greiner) in RPMI medium and incubated for one hour at 37 °C. After one hour, cells were washed with warm PBS to remove the non-adherent cells, enriching for adherent cells such as monocytes. Cells were incubated with 1, 5, or 10 µg/mL BCG or RPMI control for 24 hours, washed with PBS, and rested for five days in RPMI supplemented with 10% pooled human serum. After resting, cells were scraped and counted for telomerase activity assay.

### Telomerase activity assay

Telomerase activity in control or BCG-trained cells was assessed using the Telomerase Activity Quantification qPCR Assay Kit (ScienCell) following the manufacturer's protocol. Briefly, equal amount of cells were lysed in the lysis buffer to release intact telomerase and incubated in the reaction buffer to allow the synthesis of new telomeric DNA. Subsequently, the telomeric DNA was amplified and quantified by RT-qPCR (QuantStudio Real-Time PCR, Applied Biosystems) using a telomere-specific primer set. Therefore, a lower Ct value for telomeric DNA amplification represents higher telomerase activity.

### Statistical analysis

Wilcoxon matched-pairs signed rank test was used for statistical comparison between two groups with paired samples such as different time points of the same participants. Mann-Whitney U test was used to compare two non-paired groups such as males and females. Correlations were performed using Spearman's rank correlation. p values below 0.05 were considered statistically significant. Analyses and plotting were performed on GraphPad Prism 8.

### RNA sequencing and analyses

Adherent PBMCs of 5 healthy individuals were trained with 5 µg/mL BCG or RPMI control according to the *in vitro* trained immunity protocol mentioned above. At day 6, cells were harvested by scraping, centrifuged, and resuspended in 350 µl of Lysis Buffer RA1 from the NucleoSpin RNA kit (Macherey-Nagel) and saved at -80°C until RNA isolation. RNA was isolated using the NucleoSpin RNA kit following the manufacturer's instructions.

Total RNA was used to prepare the RNA sequencing libraries using the KAPA RNA HyperPrep Kit with RiboErase (KAPA Biosystems). Oligo hybridization and rRNA

depletion, rRNA depletion cleanup, DNase digestion, DNase digestion cleanup, and RNA elution were performed according to protocol. Fragmentation and priming were performed at 94°C for 6 min. First strand synthesis, second strand synthesis, and A-tailing were performed according to the protocol. For the adaptor ligation, a 1.5 µM stock was used (NextFlex DNA barcodes, Bio Scientific). The first and second post-ligation cleanup was performed according to protocol. A total of 11 PCR cycles were performed for library amplification. The library amplification cleanup was done using a 0.8x followed by a 1.0x bead-based cleanup. Library size was determined using the High Sensitivity DNA bioanalyzer kit, and the library concentration was measured using the dsDNA High Sensitivity Assay (Denovix). Paired-end sequencing of reads of 50 bp was generated using an Illumina NextSeq 500.

Gene set enrichment analysis was performed using the gseGO function of the clusterProfiler package in R (4.1.3). PANTHER over-representation enrichment analysis was performed using the rba_panther_enrich function of the rbioapi package [32]. For this analysis, ENSEMBL gene IDs were converted to ENTREZ gene IDs. However, 7.18% of the upregulated IDs and 17.97% of the downregulated IDs could not be matched, therefore, were excluded from the analysis.

# RESULTS



## BCG transcriptionally regulates genes required for telomere maintenance in immune cells

To investigate if BCG affected telomere maintenance, we first explored RNA sequencing data generated from 5 *in vitro* BCG-trained and control adherent PBMCs of healthy donors. 3384 transcripts were significantly upregulated, and 2271 were downregulated upon BCG training (adjusted p-value < 0.05). Gene set enrichment analysis revealed many immunological processes activated by BCG, including response to interferon-gamma, response to virus, response to lipopolysaccharide, positive regulation of cytokine production, and cytokine-mediated signaling pathway (Figure 1A). Additionally, cell cycle-related processes such as DNA replication, mitotic sister chromatid segregation, nuclear division, and regulation of mitotic cell cycle were enriched by BCG-induced training. Processes downregulated by BCG included positive regulation of osteoblast proliferation, positive regulation of megakaryocyte differentiation, positive regulation of glycogen biosynthetic process, positive regulation of glycogen metabolic process, response to potassium iron, G protein-coupled serotonin receptor signaling pathway, regulation of skeletal muscle differentiation, and negative regulation of neuroinflammatory responses.

Overrepresentation analysis using the PANTHER database revealed that multiple biological processes related to telomere maintenance were regulated by BCG training, along with immunological and metabolic processes (Figure 1B). Telomere maintenance processes such as 'telomere trimming', 't-circle formation', and 'positive regulation of establishment of protein localization to telomere' were significantly enriched in the genes upregulated by BCG training. Differentially expressed genes belonging to these processes and their fold changes are provided in Table 1. Among the genes downregulated by BCG, enriched processes included Rho protein signal transduction, regulation of osteoblast proliferation, regulation of myeloid cell apoptotic process, regulation of guanyl-nucleotide exchange factor activity, and neuroendocrine cell differentiation (Figure 1C).

These transcriptional analyses supported our hypothesis that BCG vaccination may regulate telomere dynamics. To confirm this, we assessed the average telomere length before and up to 3 months after BCG vaccination in two cohorts of healthy adults.

## Sample selection from the 300BCG cohort

We selected equal numbers of trained immunity responders and non-responders from the 300BCG cohort to investigate BCG vaccination's effect on telomere length. Trained immunity responders were defined as displaying at least a 20% increase in all three pro-inflammatory cytokines measured three months after BCG vaccination: IL-6, TNFα, and IL-1β, upon *ex vivo* challenge with *S. aureus*. Non-responders were defined as having no increase for at least two pro-inflammatory cytokines 3 months after BCG vaccination. To minimize the effect of age, we only considered young participants between 18 and 35. 40 young individuals were analyzed from both groups. To investigate the effect of sex, similar numbers of males and females were included in the responder and non-responder groups.

We also analyzed an older group of individuals between the ages of 50 and 71. The 300BCG cohort included only 24 individuals older than 50, and 20 had material available for telomere length measurements from all time points. Due to the smaller sample size, no responder/non-responder differentiation was performed for the individuals in the older group.

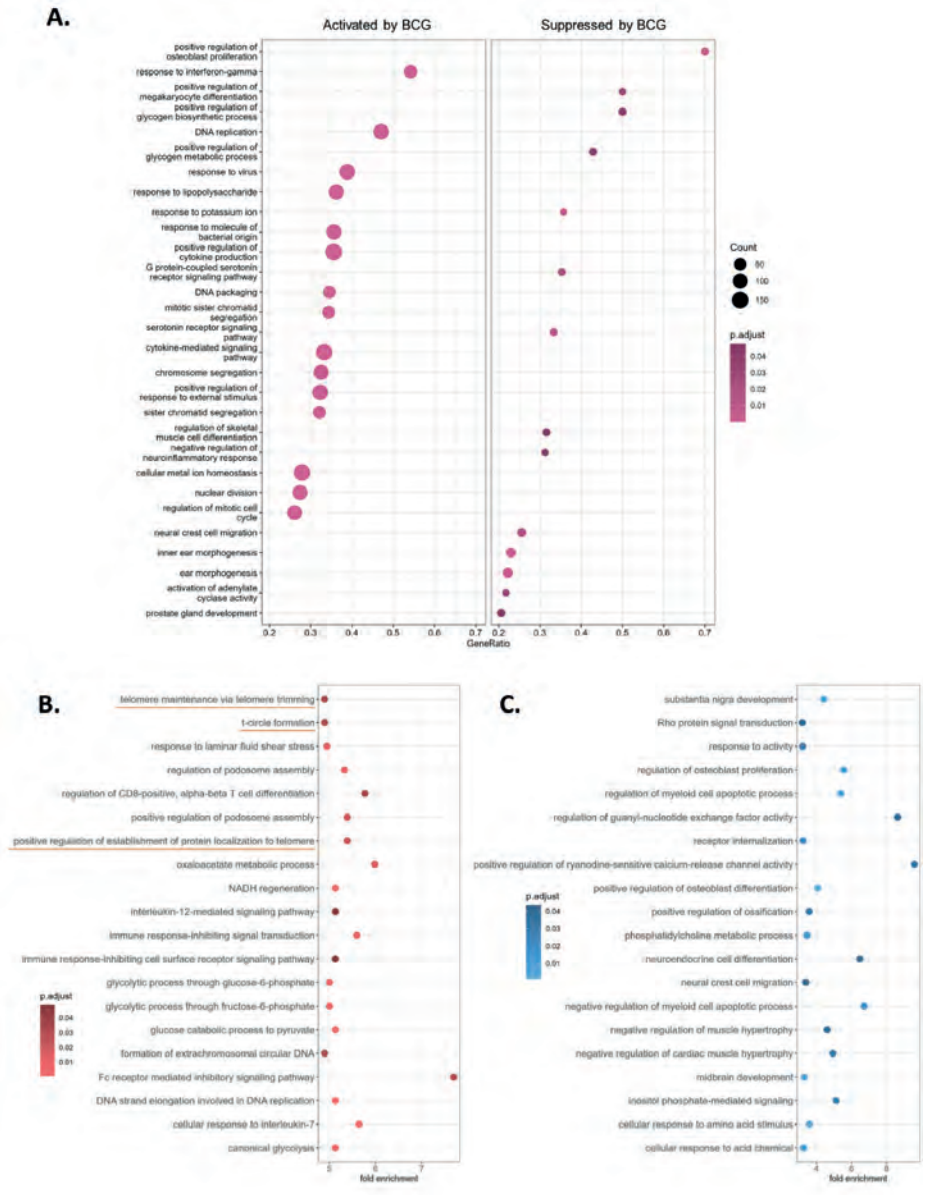


**Figure 1. Transcriptional changes upon *in vitro* BCG training revealed an impact on telomere maintenance.** A. Gene set enrichment analysis performed using RNA sequencing data upon *in vitro* BCG training of healthy PBMCs (n=5) in comparison to non-trained condition. B-C. Overrepresentation analysis using the 3384 genes that were upregulated (B) and 2271 genes that were downregulated (C) by BCG. The top 20 significant (adjusted p < 0.05) biological processes with the highest fold enrichment are depicted. Processes related to telomere maintenance are underlined.

**Table 1. Differentially expressed genes belonging to the telomere-related biological processes that were enriched by BCG training *in vitro*.**

| positive regulation of protein localization to telomere | | | | |
|---|---|---|---|---|
| **Gene** | **Full name** | **log2FoldChange** | **p value** | **adjusted p value** |
| CCT5 | Chaperonin Containing TCP1 Subunit 5 | 0,970563693 | 8,86028E-15 | 9,85354E-13 |
| CCT6A | Chaperonin Containing TCP1 Subunit 6A | 0,629685302 | 4,33981E-05 | 0,000499539 |
| CCT3 | Chaperonin Containing TCP1 Subunit 3 | 0,626906133 | 0,000151407 | 0,001410401 |
| CCT8 | Chaperonin Containing TCP1 Subunit 8 | 0,626826405 | 0,00014633 | 0,001372544 |
| CCT7 | Chaperonin Containing TCP1 Subunit 7 | 0,56957536 | 0,000186107 | 0,00167674 |
| CCT2 | Chaperonin Containing TCP1 Subunit 2 | 0,563903167 | 0,000392256 | 0,003094388 |
| TCP1 (CCT1) | T-complex Protein 1 Subunit Alpha | 0,536711745 | 0,000151239 | 0,001409439 |
| CCT4 | Chaperonin Containing TCP1 Subunit 4 | 0,501968204 | 4,60535E-05 | 0,000525364 |
| DKC1 | Dyskerin Pseudouridine Synthase 1 | 0,381099519 | 0,003041035 | 0,015956421 |
| LARP7 | La Ribonucleoprotein 7 | 0,25189321 | 0,005768843 | 0,026555537 |
| **telomere maintenance via telomere trimming / t circle formation** | | | | |
| **Gene** | **Full name** | **log2FoldChange** | **p value** | **adjusted p value** |
| EXO1 | Exonuclease 1 | 2,811564988 | 5,75955E-08 | 1,6327E-06 |
| NBN | Nibrin | 1,477106431 | 5,94306E-08 | 1,67414E-06 |
| DNA2 | DNA Replication Helicase/Nuclease 2 | 0,745845858 | 0,000604757 | 0,004383278 |
| BLM | Bloom Syndrome Protein | 0,718765946 | 3,77065E-06 | 6,26113E-05 |
| XRCC3 | X-Ray Repair Cross Complementing 3 | 0,571177134 | 0,000358893 | 0,00286876 |
| SLX4 | SLX4 structure-specific endonuclease subunit | 0,384836849 | 6,1397E-05 | 0,000668066 |
| SLX1A | SLX1 homolog A, structure-specific endonuclease subunit | 0,273331202 | 0,012927803 | 0,049672633 |
| SMARCAL1 | SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A Like 1 | 0,262165174 | 0,0032438 | 0,016824932 |

## BCG leads to shorter telomeres in young individuals three months after vaccination

When responders and non-responders were assessed together, the average telomere length of circulating cells was significantly lower for both sexes at three months (Figure 2A). For females, this was already the case after two weeks. 20 out of 42 males and 15 out of 38 females experienced more than 10% telomere shortening over three months (mean shortening 20.0 ± 13.0% for males, 23.7 ± 19.2% for females). In the older group, no significant change was observed in participants of either sex at any time (Figure 2B).

Notably, females had longer telomeres before vaccination than males, but the telomere length change in the 3 months after BCG was comparable between males and females (Figure 2C). Furthermore, older individuals had shorter average telomere lengths than young individuals before vaccination, which aligns with age-dependent telomere shortening (Figure 2D). The mean telomere length per chromosome end of old participants was 30.3% shorter than the mean of young participants. Overall, these results suggest that BCG vaccination leads to telomere shortening in young individuals but not in individuals over 50 who have shorter telomeres at baseline.

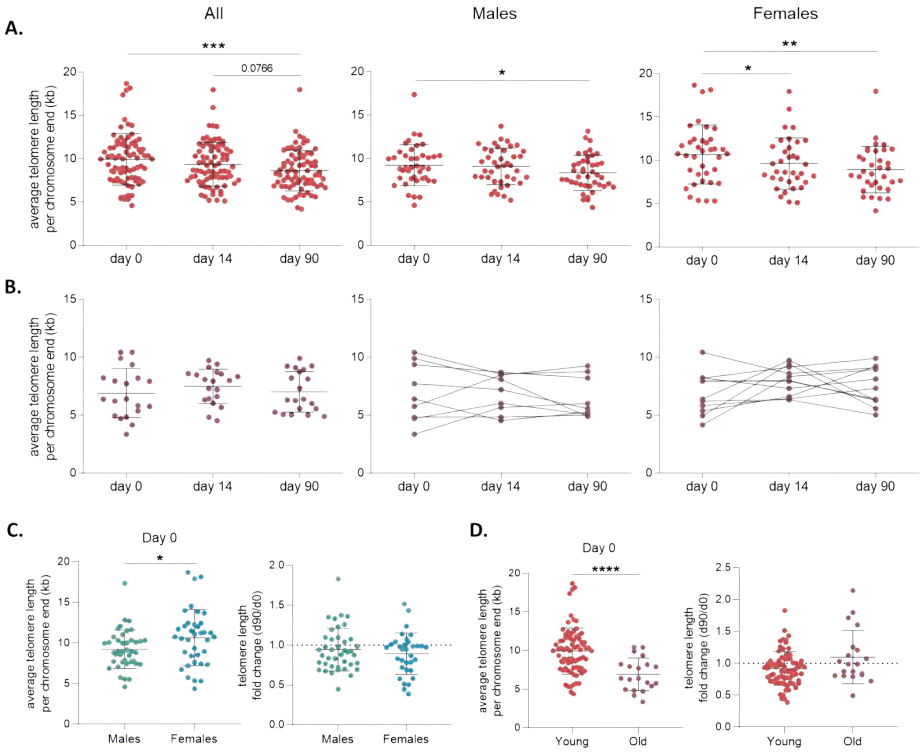


**Figure 2. BCG vaccination led to shorter telomeres in young, healthy individuals 3 months after vaccination.** A. Average telomere length per chromosome end in whole blood, quantified by qPCR, in all young individuals (aged 18-35, n=80), young males (n=42), or young females (n=38) before, 14 days or 90 days after vaccination. B. Average telomere length in all older individuals (aged 50-71, n=20), older males (n=9), or older females (n=11) before, 14 days, or 90 days after vaccination. C. Sex comparisons of average telomere length before vaccination and fold change in telomere length in three months. D. Age group comparisons of average telomere length before vaccination and fold change in telomere length in 3 months. Statistical analyses were performed using Wilcoxon matched-pairs signed rank test (A-B) or Mann-Whitney test (C-D). * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001.

## BCG-induced telomere shortening is linked to the trained immunity response

Next, we investigated the effect of BCG on telomere length depending on the trained immunity responder status. Interestingly, stronger telomere shortening three months after vaccination was observed in non-responders (mean change 24.3%) compared to responders (mean change 16.3%) (Figure 3A). When non-responder males and females were analyzed separately, telomere length change did not reach statistical significance for females, while male non-responders had shorter telomeres after three months (Figure 3B). However, the tendency of telomere shortening was similar in both sexes, and the lack of significance in women may be only due to a lack of statistical power. Telomere length change 3 months after vaccination differed significantly for male non-responders and responders but not for females (Figure 3C). Of note, the average telomere length of responders and non-responders was not different before vaccination (Figure 3D). For responders, improvement in ex vivo IL-6 response, but not TNFα and IL1β, was associated with the telomere length change, and this trend was clearer in males (Supplementary Figure 1). These results suggest that telomere shortening after BCG vaccination is linked to the trained immunity response, possibly stronger in males.

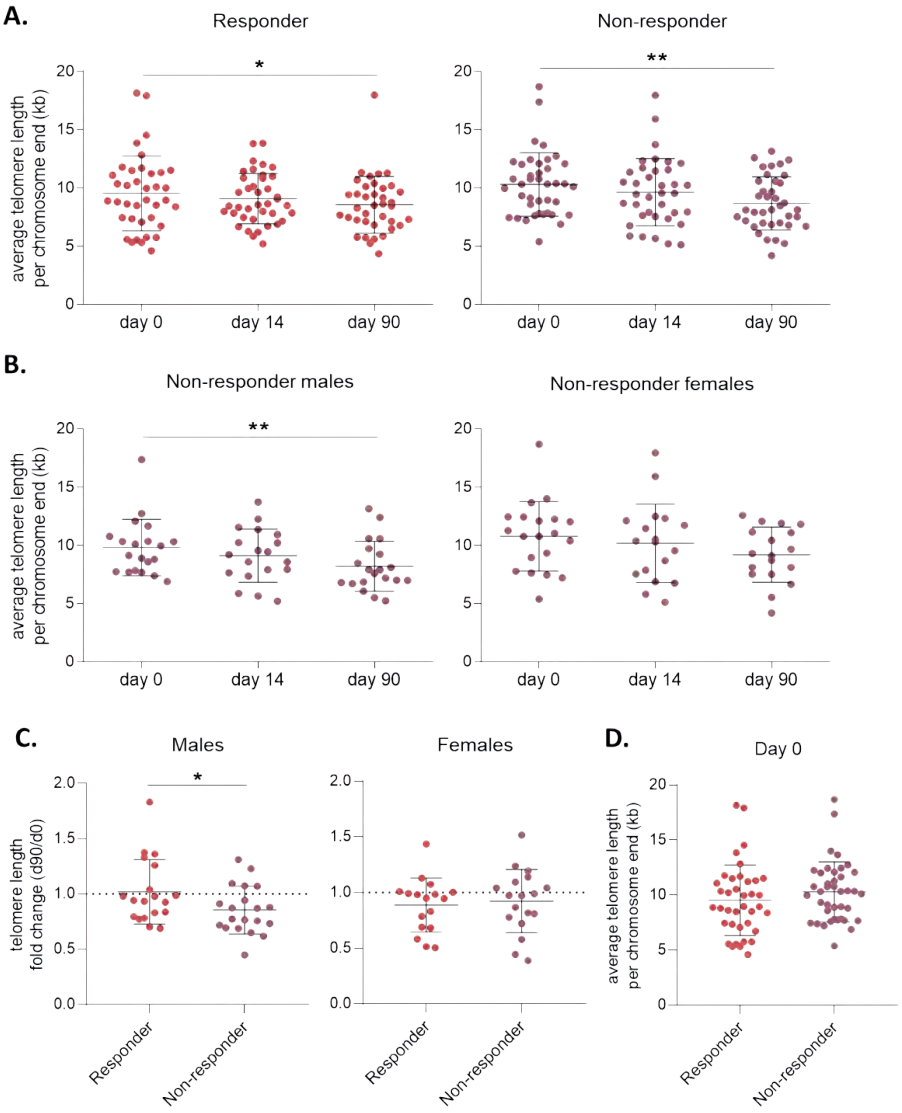


**Figure 3. Telomere shortening was linked to the trained immunity response, particularly in males.** A. Average telomere length per chromosome end in whole blood in young (aged 18-35) responders (n=40) and non-responders (n=40) before, 14 days, or 90 days after vaccination. B. Average telomere length of male (n=20) and female (n=20) non-responders before, 14 days, or 90 days after vaccination. C. Comparisons of the fold change in telomere length in three months for responders and non-responders in male and female groups. D. Average telomere length comparison of responders and non-responders before vaccination. Statistical analyses were performed using Wilcoxon matched-pairs signed rank test (A-B) or Mann-Whitney test (C-D). * $p \leq 0.05$, ** $p \leq 0.01$.

## Placebo-controlled validation cohort confirms BCG-induced telomere shortening

In order to validate our observations, we used samples from an independent clinical study in which participants were vaccinated with BCG or placebo, and blood was collected before and 3 months after vaccination. The study aimed to test different dosing regimens of BCG, including a double-dose vaccination [29]. Only the participants vaccinated with the standard BCG dose were assessed for our purposes.

Significantly shorter average telomere length was observed three months after vaccination, validating the results of the 300BCG cohort (Figure 4A). The effect was similar in men and women, although it did not reach statistical significance for men, most likely due to lack of statistical power in this smaller study: only 11 male participants were vaccinated with the standard dose of BCG, compared to 18 females.

Thanks to the placebo control in the validation study, we could distinguish if the observation of telomere shortening is due to BCG vaccination and not due to the assessment time. Among 18 placebo-vaccinated individuals, no change in average telomere length was observed three months later (Figure 4B). 13 of these participants belonged to a group that received placebo at first and a BCG vaccination three months later, after which they were followed for another three months. It was evident in this group that placebo vaccination had no such effect, while BCG vaccination caused shorter telomeres after three months (Figure 4C).

## BCG activates telomerase and this effect is counteracted by testosterone

To assess BCG's potential effect on telomerase activation, we used the *in vitro* trained immunity model. Telomere shortening was also observed in the *in vitro* setting, even to a greater extent, validating the observations *in vivo* (Figure 5A). Surprisingly, BCG dose-dependently promoted telomerase activation, and this effect was more apparent for female blood donors.

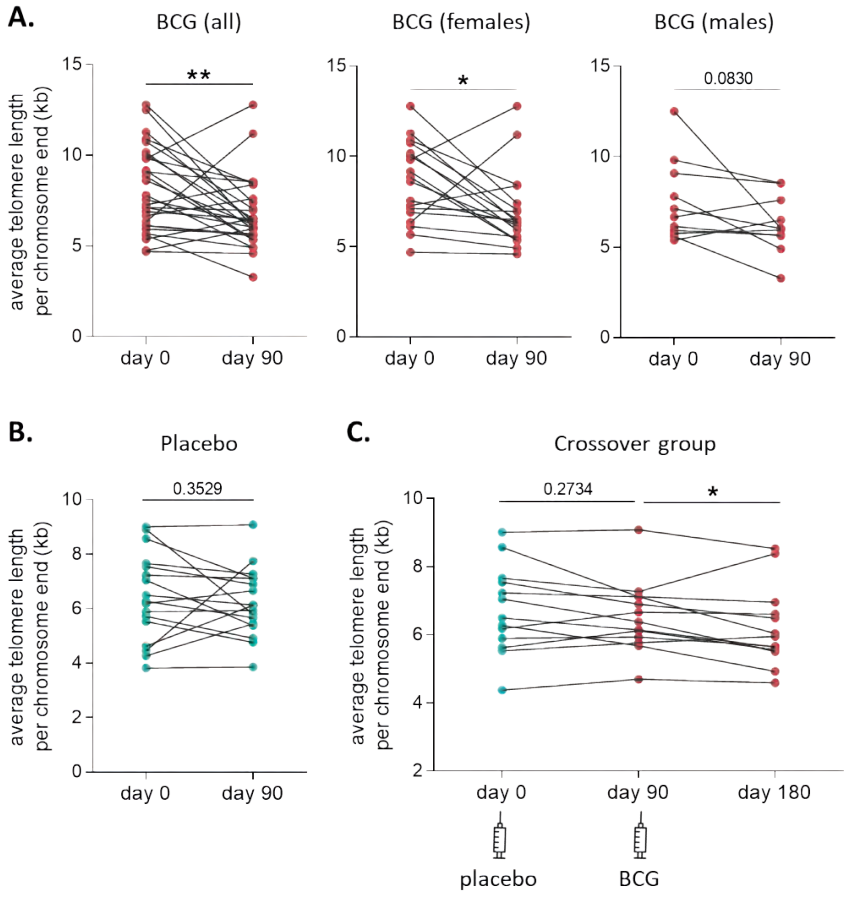


**Figure 4. Telomere shortening 3 months after BCG vaccination in an independent validation cohort.** A. Average telomere length per chromosome end in whole blood in participants vaccinated with BCG (aged 19-31, n=29), only males (n=11), and only females (n=18) before and 90 days after vaccination. B. Average telomere length of participants vaccinated with placebo (n=18) before and 90 days after vaccination. C. Average telomere length of the participants first vaccinated with placebo and 3 months later with BCG (n=13). Statistical analyses were performed using Wilcoxon matched-pairs signed rank test. * p≤0.05, ** p≤0.01.

Hypothesizing that the differences between sexes might be related to sex hormones, we correlated the telomere length change to hormone concentrations at baseline in the 300BCG cohort, for which these measurements were available. Female participants had no or very low detectable levels of testosterone. Both with and without the female testosterone concentrations considered, baseline testosterone concentrations were strongly negatively correlated with the fold-change of telomere length after BCG vaccination (Figure 5B). No association was observed between telomere length change and androstenedione, dehydroepiandrosterone

sulfate, or 17-hydroxyprogesterone concentrations (Supplementary Figure 2). Importantly, testosterone concentrations before BCG vaccination were not different between male responders and non-responders (Figure 5C).

To further validate the role of testosterone, we added exogenous testosterone to PBMCs from healthy female donors during BCG training and assessed telomerase activation on day 6. Pre-incubation of the female innate immune cells with testosterone partially, and for some donors almost completely, prevented BCG-induced telomerase activation (Figure 5D). Notably, testosterone also suppressed the trained immunity response induced by BCG *in vitro*, measured as IL-6 and TNFα secretion upon secondary challenge with LPS (Figure 5F). Measuring the BCG-induced telomerase activity at different time points during training revealed that the activation mainly occurred between days 3 and 6 post-BCG (Figure 5E). These results suggest a testosterone-dependent inhibition of the effects of trained immunity induction on telomerase activation.

## DISCUSSION

Our data reveal that BCG vaccination leads to telomere shortening sustained for at least 3 months after vaccination in adults below 35 years of age but not in adults over 50 who have shorter baseline telomeres. We validated BCG-induced telomere shortening in an independent *in vivo* placebo-controlled study and in an *in vitro* model. Notably, the two in-vivo clinical studies used different strains of BCG: Bulgaria and Denmark. Different BCG strains differ in immunostimulatory properties [33]; however, telomere shortening was observed with both BCG strains.

Possible mechanisms through which BCG leads to telomere loss include enhanced TNFα and reactive oxygen species (ROS) production known to be induced by BCG-stimulated innate immune cells. TNFα can cause telomere shortening [34], and ROS can damage the G-rich telomeric sequence, which is more vulnerable to oxidative damage than non-telomeric DNA [35]. In addition, in this study, we showed the regulation of telomere dynamics by BCG at the transcriptional level.

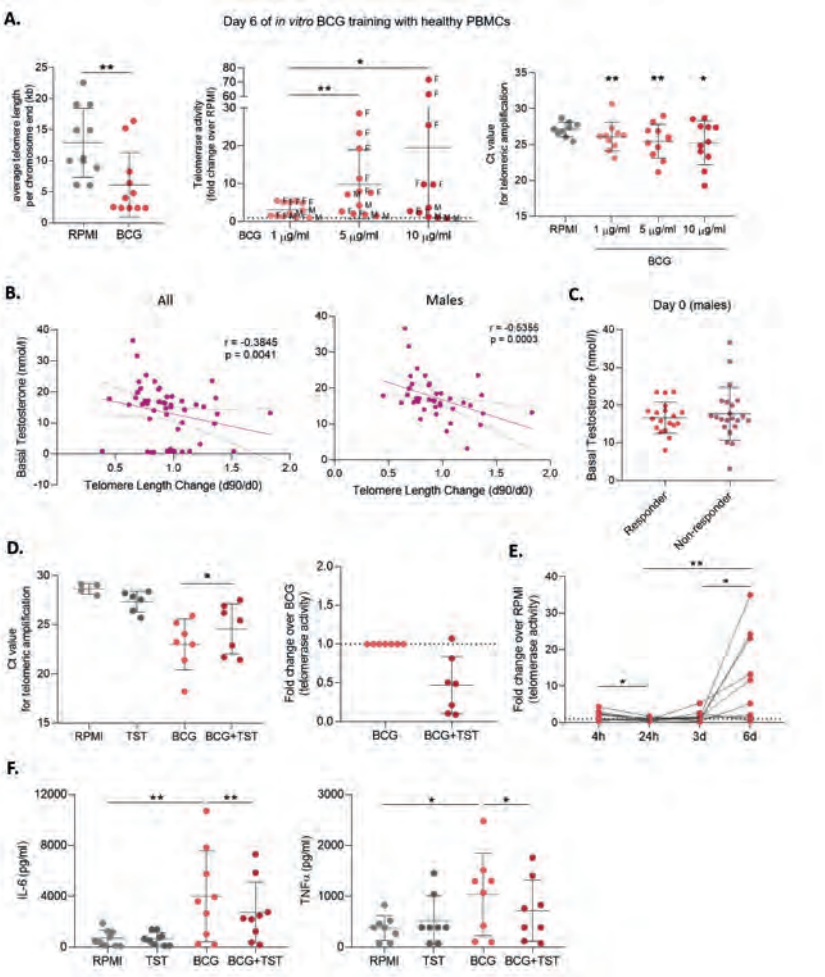


**Figure 5. BCG-induced telomerase activation that can be inhibited by testosterone.** A. Average telomere length per chromosome end (left) and telomerase activity, represented as relative activity (center) and raw Ct values of telomeric amplification (right), at day 6 of *in vitro* BCG training of healthy PBMCs (n=11-12). BCG concentration was 5 μg/ml for the left graph. B. Correlation of telomere length change in three months after BCG vaccination and basal testosterone levels in all young participants of the 300BCG cohort with available hormone measurements and only male participants. C. Basal testosterone levels of male responders and non-responders of the 300BCG cohort. D. Telomerase activity of PBMCs from healthy female donors trained with 5 μg/ml BCG with or without 50 μM testosterone (left) and the relative effect of testosterone on BCG-induced telomerase activation (right) (n=7). E. Relative telomerase activity of BCG-trained female PBMCs compared to non-trained controls at different time points during the training protocol (n=9). F. IL-6 and TNFα production by female PBMCs trained with BCG with or without testosterone upon restimulation with 10 ng/ml LPS (n=8-9). Statistical analyses were performed using Wilcoxon matched-pairs signed rank test (A, C, D, F) and Spearman's rank correlation (B). In the graphs depicting Ct values, some data points from RPMI and TST groups are missing due to undetected amplification. F: female, M: male, r: Spearman's rank correlation coefficient, TST: testosterone, h: hour, d; day. * p≤0.05, ** p≤0.01.

BCG upregulated multiple subunits of the chaperone TCP-1 Ring Complex (TRiC) (Table 1). TRiC is required for the folding of TCAB1, an essential component of the telomerase holoenzyme complex, and proper localization of telomerase [36, 37]. Dyskerin Pseudouridine Synthase 1 (DKC1) was also upregulated upon BCG training. This protein is another component of the telomerase complex. It binds to the RNA component of telomerase (hTR) and prevents its degradation [38]. LARP7, upregulated by BCG, also protects against hTR degradation and is necessary to stabilize the interaction of hTR with hTERT [39]. Thus, BCG transcriptionally activates processes that regulate telomerase assembly, localization, and stabilization.

When telomeres are over-elongated, such as in cancer cells, a trimming process occurs to control telomere length [40]. This leads to the generation of circular telomeric repeats called t-circles. Lymphocytes were shown to activate telomere trimming to counter the heightened telomerase activity induced by phytohemagglutinin stimulation [41]. BCG training upregulated several proteins implicated in this process including exonuclease 1 (EXO1) and helicase bloom syndrome protein (BLM) which are important for the resolution of telomeric G-quadruplex structures [42, 43], nibrin (NBN), part of the MRE11-RAD50-NBN complex regulating telomere homeostasis [44], SLX1A and SLX4 whose recruitment at the telomeres helps maintain telomere integrity [45], and SMARCAL which protects telomere integrity during replication [46]. Overall, in addition to telomerase regulation, BCG transcriptionally modulates telomere trimming and maintenance.

In the *in vitro* model of trained immunity, BCG dose-dependently activated telomerase, in line with the transcriptional results. Strikingly, this effect was primarily observed in PBMCs from female donors. To assess whether sex hormones impact this effect of BCG, we correlated the circulating sex hormone concentrations with telomere length. Concentrations of testosterone but not of other hormones before BCG vaccination correlated with the change in telomere length three months later. This strong inverse correlation suggests that the higher basal testosterone concentrations are, the more telomere loss occurs after BCG vaccination. Higher testosterone concentrations in men were previously linked to shorter telomeres in a large study [47]. Furthermore, treatment of PBMCs from female donors with exogenous testosterone significantly prevented BCG-induced telomerase activation, supporting a role for testosterone in telomere attrition.

The degree of trained immunity induction is heterogeneous among people [28]. In this study, we considered the group of people who displayed 20% or more improvement in cytokine production three months after vaccination as

"responders". Despite having similar baseline telomere lengths, trained immunity responders could maintain telomere length better compared to the non-responders. Individuals with lower baseline testosterone levels also experienced less telomere shortening. Notably, testosterone partially inhibited the trained immunity response induced by BCG *in vitro*. In addition, the amplification of telomerase activity needed several days in order to be induced, similar to trained immunity [48], possibly owing to the epigenetic and metabolic reprogramming induced by BCG. Together, these support the hypothesis that the impact of BCG vaccination on telomere dynamics is linked to the capacity to establish trained immunity.

Our study has some limitations to address. First, the low number of study participants over 50 years of age prevented sex-specific analyses in this sub-cohort of individuals, and future studies should re-assess these effects in older individuals. Second, replicative telomere attrition does not have a linear relationship with the number of cell divisions. The erosion rate accelerates as cells age and depends on the initial telomere length [49]. Therefore, further assessing BCG's effects in older individuals with shorter baseline telomeres is warranted. Third, the shortest telomere is a better determinant of senescence than the average telomere length [50] but only the average telomere length was measured in this study as a proxy for cellular aging. Multiple telomere length measurements such as qPCR, quantitative fluorescence in situ hybridization (Q-FISH), and telomere restriction fragment (TRF) analysis [51] need to be performed in future studies. We preferred the widely used qPCR method due to the protocol's high throughput and easy nature. However, with this method, it is only possible to determine an average telomere length, disregarding the heterogeneity between cell types and differentiation stages.



A working model of BCG's effects on telomere length and telomerase activity is depicted in Figure 6. Through acute activation of inflammation and ROS production, BCG leads to telomere loss. In parallel, however, BCG exerts long-term effects on trained immunity and telomerase activity and counteracts loss of telomere length. Post-transcriptional mechanisms responsible for long-term telomerase activation by BCG vaccination remain to be elucidated. *In vitro*, testosterone can inhibit BCG-induced telomerase activation and the trained immunity response. Together with *in vivo* observations of higher testosterone levels being linked to shorter telomeres, this points to the regulation of telomerase transcription or activity by testosterone. In the 300BCG study, male non-responders experienced more telomere loss than responders, and improvement of IL-6 response after BCG vaccination correlated with a positive telomere length change after BCG. Thus, the epigenetic and metabolic

rewiring responsible for the induction of trained immunity may be involved in BCG's effects on telomerase activation. Non-responders might be unable to maintain telomere length due to low telomerase activity, and male non-responders would be less likely to activate telomerase due to endogenous testosterone. Whether telomerase activation persists and eventually restores telomere loss *in vivo* remains to be studied.

Another intriguing possibility is that sustained effects of BCG on telomere dynamics are also induced at the level of bone marrow progenitors. This would explain our observations in circulating blood cells three months after vaccination, considering that most innate immune cells are shorter-lived. BCG has already been shown to transcriptionally reprogram hematopoietic stem and progenitor cells (HSPCs) in the bone marrow [52]. Therefore, assessing telomere length in HSPCs before and after BCG vaccination is warranted.

In conclusion, this study reports the long-term sex-specific effects of BCG vaccination on telomere maintenance and telomerase activity. Single-cell analyses are necessary to understand which types of cells in circulation are most affected and the potential consequences of telomere shortening in these cells. Future studies should also address the impact of BCG vaccination on other hallmarks of aging, such as mitochondrial dysfunction and DNA methylation. Our results highlight an interesting relationship between BCG-induced trained immunity and cellular aging that warrants deeper exploration.

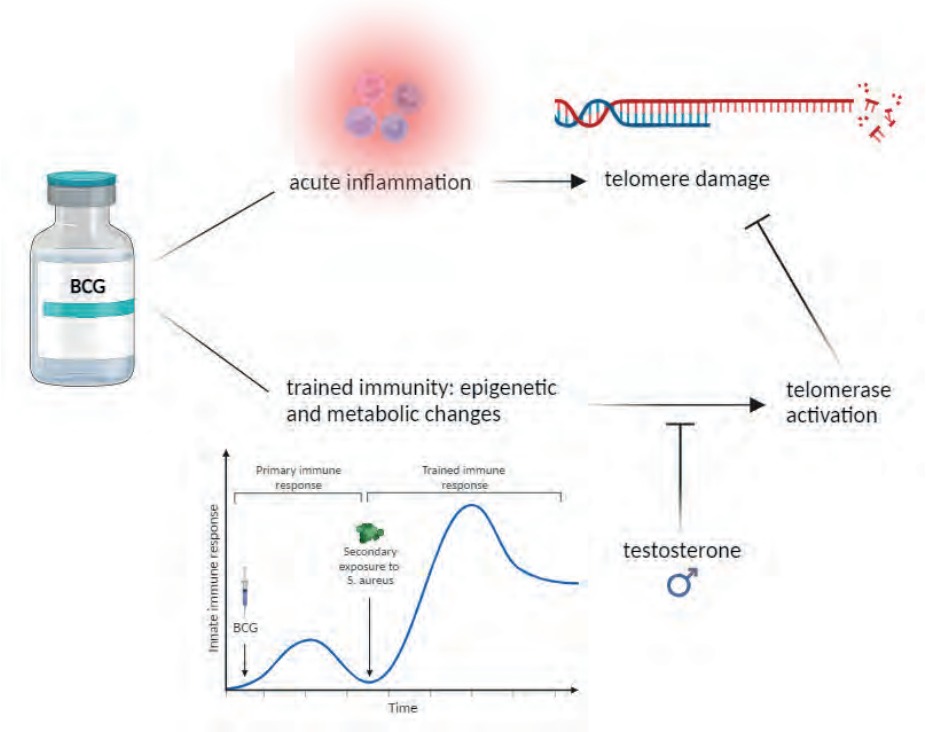


**Figure 6. Schematic representation of telomeric regulation by BCG vaccination.** BCG leads to telomere damage due to the induction of initial acute inflammation, potentially through increased oxidative stress. On the other hand, BCG can induce long-term telomerase activation, likely linked to the establishment of trained immunity, and limit the telomeric damage induced by acute inflammation. Testosterone inhibits BCG-induced telomerase activation.



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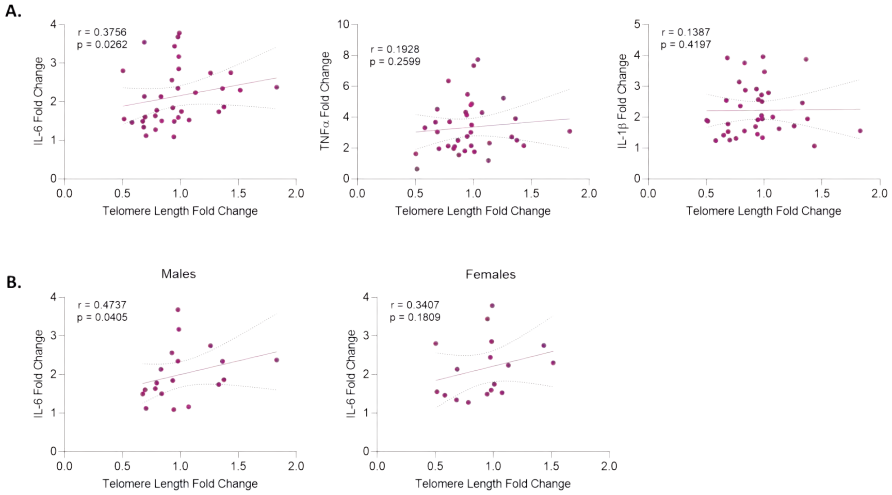
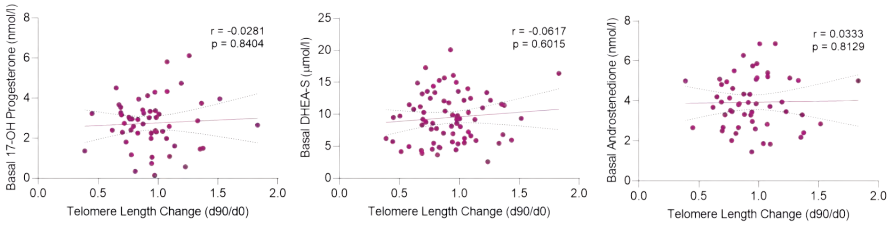
# SUPPLEMENTARY FIGURES

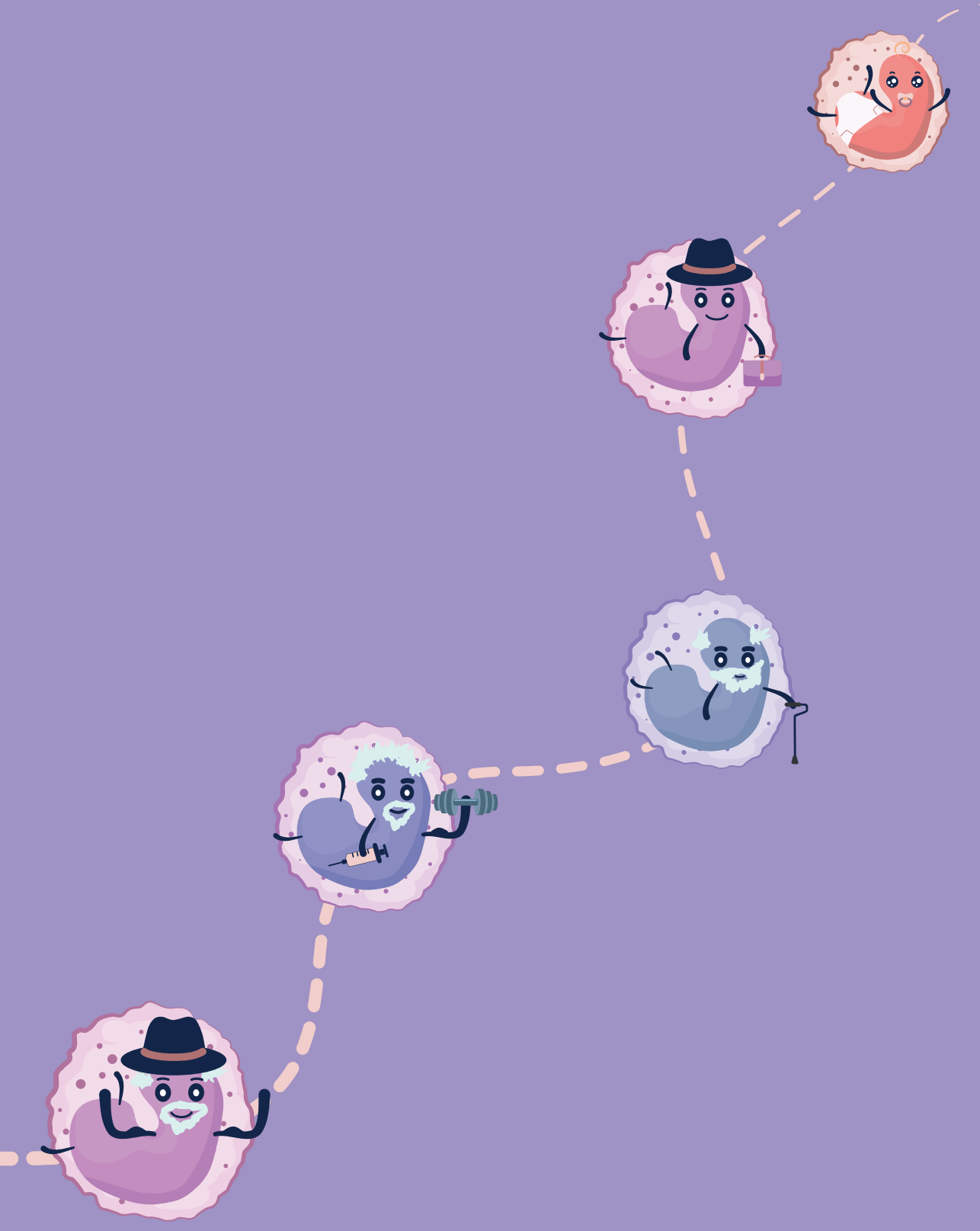



**Supplementary Figure 1. Correlations of fold-change cytokine response with the telomere length change three months after BCG vaccination in trained immunity responders.** A. Analysis of all young responders. B. Separate analysis of male and female responders for IL-6. Cytokines were measured upon 24 hours *ex-vivo* stimulation with *S. aureus* before and three months after BCG vaccination. Statistical analyses were performed using Spearman's rank correlation. r: Spearman's rank correlation coefficient.






**Supplementary Figure 2. Non-significant correlations of basal hormone levels with the telomere length change three months after BCG vaccination.** Data includes the young participants of the 300BCG cohort with available hormone measurements. Statistical analyses were performed using Spearman's rank correlation. DHEA-S: dehydroepiandrosterone sulfate, r: Spearman's rank correlation coefficient.



# CHAPTER 9

# SUMMARY AND DISCUSSION

# SUMMARY


## PART I - Mechanisms and biomarkers of immune aging

The epigenetic and metabolic changes that occur in immune cells during aging and their consequences on immune memory generation and maintenance are discussed in Chapter 2. In this chapter, I also propose that the immune system is central for the process of aging due to its complex interactions with all other physiological processes, and may be an important target in designing anti-aging strategies. This was illustrated by detailing the interdependencies between an aging immune system, the central nervous system, and the gut microbiota. Finally, two behavioral and two pharmacological interventions that target multiple aspects of aging and potentially slow or reverse aging-associated phenotypes were highlighted: caloric restriction, physical exercise, metformin, and resveratrol.

Chapter 3 compared the age-dependent changes in immune response and it identified the metabolic factors that probably contribute to these changes in two cohorts of healthy adults: one from the Netherlands and the other from Tanzania. We observed a common age-dependent decline in the IFNγ response. However, only Tanzanians experienced a weakening of innate immune responses. To determine the mechanisms behind the common age-related changes, we identified the metabolites whose concentrations were significantly different in individuals over 50 in both cohorts. Dehydroepiandrosterone (DHEA), DHEA sulfate, and androsterone glucuronide of the steroid hormone biosynthesis pathway, whose levels decreased with age, displayed age-independent associations with lower systemic inflammation and higher cytokine response against pathogens. Hippurate and 2-phenylacetamide, on the other hand, were more abundant in older individuals and inversely associated with innate and adaptive cytokine responses. They were also linked to shorter telomeres and faster epigenetic aging in males. We also identified several metabolites that might contribute to the declining innate immunity in individuals from Tanzania, with traumatic acid, d-erythro-l-galacto-nonulose, and epinephrine glucuronide showing the strongest associations. This chapter underscored the importance of studying diverse populations to deepen our understanding of the universal aging mechanisms, as well as the particular processes in each population linked to genetics and non-genetic factors such as diet.

Chapter 4 investigated age-related changes in healthy adults, explicitly focusing on circulating markers and immune cell types linked to COVID-19 severity. During the pandemic, it was evident early on that age and male sex were risk factors for COVID-19 severity and mortality. We investigated the sex- and age-dependent

differences in the circulating proteome and immune cell composition of two healthy adult cohorts from the Netherlands that were sampled before the pandemic. The severe COVID-19 profile, characterized by elevated circulating concentrations of various pro-inflammatory proteins and chemokines, along with reduced numbers of $CD56^{bright}$ NK cell, naïve B and T cell numbers, largely overlap with age- and sex-associated changes. This chapter proposed that these intrinsic immunological characteristics of old age and male sex observed in healthy individuals could impact their differential disease severity upon SARS-CoV-2 infection. When PBMCs of some participants were stimulated *in vitro* with SARS-CoV-2, older people produced more IL-1β and IL-1RA, but less IFNγ against the virus. Their IL-1RA production was also higher than that of younger participants without stimulation. An unbalanced IL1 axis and deficient production of IFNγ might be implicated in the failure to contain SARS-CoV-2 infection.

## PART II - BCG vaccination as a strategy to promote immune fitness

Age-dependent changes in innate and adaptive immune cells were briefly explained again in Chapter 5, followed by past and current strategies to improve vaccine efficacy for older people. The conventional ways of vaccine design mostly rely on efficient adaptive immune responses. However, T and B lymphocytes are the cells most heavily impacted by aging. This chapter provided a steppingstone for the subsequent chapters by laying out the promise of trained immunity induction, for instance, by BCG vaccination, in preventing or reverting immune aging. Although innate immune cells display age-related functional defects such as reduced phagocytosis, antigen presentation, cytokine production, and chemotaxis, these could be alleviated by inducing trained immunity. Furthermore, the benefits are antigen-agnostic and would aid in more than one infection or vaccination. Besides boosting the function of innate immune cells, BCG vaccination reduces systemic inflammation, another hallmark of immune aging. Understanding BCG's impact on other aging hallmarks and exploring strategies to improve its training ability were the main focuses of Part II of this thesis.



Alendronate (alendronic acid) is a medication used at older age to prevent and treat osteoporosis. This class of medications, called bisphosphonates, also inhibits cholesterol synthesis, which is critical for trained immunity. Chapter 6 investigated the impact of alendronate use on trained immunity induction by BCG vaccination in young, healthy volunteers. Simultaneous administration of a single alendronate tablet and BCG vaccine reduced ex-vivo cytokine production against heterologous bacterial and viral stimuli one month after vaccination compared to BCG vaccination alone. Alendronate also transcriptionally suppressed the immune

response pathways upregulated by BCG alone. These results show that alendronate inhibits the induction of trained immunity induced by BCG.

Chapter 7 explored the impact of resveratrol on BCG-induced trained immunity *in vitro*. Resveratrol is a natural phenolic compound and one of the anti-aging interventions highlighted in Chapter 2. It has various cardioprotective, neuroprotective, immunomodulatory, and anti-oxidant effects. In many model organisms, resveratrol also extends lifespan. Our study revealed that resveratrol inhibits *C. albicans*, β-glucan, and oxLDL-induced trained immunity, while amplifying BCG-induced trained immunity. Metabolically, resveratrol combined with BCG limited the enhanced glycolysis and oxidative phosphorylation, but maintained it higher than non-treated cells. It also suppressed ROS production, but strongly boosted cytokine production, accompanied by increased H3K27Ac deposition at TNFA and IL6 promoters. Improved cytokine production could also be observed with cells from individuals over 65 years of age. The distinct effect of resveratrol on BCG-induced trained immunity depended on BCG being alive and transcriptionally active. Inhibition of Sirtuin 1 in monocytes also partially blocked resveratrol's effect. Overall, this chapter suggested that resveratrol might be used to improve BCG-induced trained immunity response, while limiting excessive oxidative stress and metabolic overload.

BCG's ability to improve the functions of innate immune cells and reduce systemic inflammation has been already reported. However, its impact on other aging hallmarks remained unknown. Chapter 8 investigated the consequences of BCG vaccination on telomere length maintenance. BCG transcriptionally regulated genes that are critical for telomere maintenance mechanisms and telomerase function. In two independent human cohorts of BCG vaccination, a shorter average telomere length was observed 3 months after vaccination, which was not the case for placebo-vaccinated individuals. Telomere shortening was found in young participants between 18 and 30, but there was no significant change in participants over 50 with shorter baseline telomeres. Participants whose cytokine production capacity was not improved by BCG exhibited more telomere shortening, which was more evident for males. Moreover, we revealed that BCG could activate the telomerase enzyme that can extend the telomeric repeats. This was observed mainly with cells from female donors and was prevented by adding exogenous testosterone. In short, this chapter reported the long-term sex-specific impact of BCG vaccination on telomere dynamics and telomerase activity, and this was linked to the strength of trained immunity induction. Studies with longer follow-ups are essential to understand if the telomerase activity in females will result in recovery

of the telomere length. This novel relationship between BCG-induced trained immunity and cellular aging requires further investigation.

In conclusion, this thesis contributed with novel insights to the understanding of the mechanisms of immune aging in different populations, revealed the interference of alendronate with BCG-induced trained immunity, proposed resveratrol as an amplifier of BCG-induced trained immunity, and reported sex-specific, long-term effects of BCG vaccination on telomere maintenance.

## DISCUSSION AND PERSPECTIVES FOR FUTURE RESEARCH

### 1. Targeting innate immunity for healthier aging

As detailed in Chapters 2 and 5, immunosenescence involves changes in innate and adaptive immunity. However, most hallmarks of immunosenescence have been described to impact the adaptive arm of immunity: reduced thymic output, shrinking naïve B and T cell pools, and accumulation of terminally differentiated memory cells [1]. Moreover, with advancing age, hematopoiesis in bone marrow is skew towards myelopoiesis [2]. With the limited and exhausted lymphocyte populations, modulating the function of innate immune cells is an attractive approach to improve response to infections and vaccinations in old age.

Trained immunity induction boosts the effector functions of innate immune cells and provides a more effective immune response in an antigen-independent manner [3]. BCG vaccination offers protection against infections, mainly respiratory, and reduces all-cause mortality [4]. The non-specific effects of BCG also involve heterologous activation of Th1 and Th17 responses [5] and improved antibody production [6, 7] besides innate immune memory. Promoting more robust non-specific innate and adaptive responses to infections and vaccines, while reducing systemic inflammation [8, 9], makes BCG a fascinating candidate for overcoming immunosenescence.



If BCG is to be used in old age to boost immune responses, it is essential to assess the interactions between BCG vaccination and frequently-used vaccinations and medications in older populations. Although the study in Chapter 6 was conducted with young participants and involved one single alendronate ingestion, it revealed that alendronate, used in old age for osteoporosis, interferes with BCG-induced trained immunity. A BCG vaccination study in older adults chronically using alendronate or other bisphosphonates would give an even more clinically relevant

indication regarding their impact on the immunological effects of BCG. In case of significant interference, temporarily discontinuing alendronate treatment might be needed before BCG instillations in patients with bladder cancer.

Chapter 7 showed that resveratrol could potently improve the cytokine production capacity of human monocytes when combined with BCG, also for individuals over 65, compared to training with BCG alone. At the same time, resveratrol limits the upregulation of glycolysis, oxidative phosphorylation, and ROS production by BCG. The consequences of combining BCG and resveratrol in terms of susceptibility to infections *in vivo* remain to be studied. Nonetheless, resveratrol's beneficial effects on metabolic health [10] and its ability to reduce systemic inflammation markers [11] could further support BCG's potential to counteract immunosenescence.

However, as presented in Chapter 8, BCG leads to shorter telomeres in humans up to three months after vaccination. Telomere shortening is one of the hallmarks of aging and can lead to cellular senescence [12]. Shorter telomeres are also associated with the incidence and severity of various age-related disorders. Of note, BCG did not cause further erosion of telomeres in people older than 50 years with shorter baseline telomeres. Our study also showed that BCG transcriptionally regulates telomere maintenance and telomerase activity, and it can activate telomerase in female PBMCs *in vitro*. Telomerase activity started peaking 3 days after BCG exposure, suggesting that it possibly results from the long-term epigenetic and metabolic reprogramming induced by BCG. Assessing telomere length in BCG vaccination cohorts with longer follow-ups would reveal if telomere shortening progresses or is perhaps counteracted by BCG-induced telomerase activation in women.

How the establishment of trained immunity and telomere damage or maintenance are linked should be studied in more depth, considering sex differences such as the impact of hormones. Since resveratrol improves the induction of trained immunity by BCG and limits oxidative stress, it might reduce telomere damage. Future *in vivo* studies combining BCG and resveratrol should assess telomere length, among other outcomes. Studying the impact of BCG on other aging hallmarks, such as mitochondrial dysfunction and disrupted macroautophagy, would also be essential in understanding the cellular aging-related outcomes of BCG vaccination.

## 2. Sex matters: impact on immunity

Sex chromosomes and hormones result in distinct immunological profiles for males and females. In humans, women have stronger interferon and antibody responses to infections and vaccinations, but are also more likely to experience

autoimmune disorders [13]. Membrane-bound or intracellular sex steroid receptors directly regulate immune responses at the transcriptional level and alter disease outcomes [14]. Estrogens have anti-inflammatory effects on macrophages, but enhance T and B cell responses. On the other hand, androgens inhibit adaptive responses. Moreover, hormone concentrations fluctuate throughout our lifespan. After menopause, concentrations of sex hormones drop, and this is accompanied by rapidly increasing systemic inflammation and diminishing lymphocyte numbers and cytotoxicity, which result in altered susceptibility to cancer, infections, and autoimmunity for post-menopausal women [15]. Chapter 3 revealed age-independent negative correlations between systemic inflammation markers and steroid hormones DHEA, DHEA-S, and testosterone metabolite androsterone glucuronide. These results support the hypothesis that variation in sex hormone concentrations contribute to immunosenescence. Sex hormones, precursors, or metabolites may be considered as potential supplements to improve immune function in old age. Effects of DHEA(S) supplementation on older men and women should be assessed through randomized controlled trials measuring immune cell function and systemic inflammation.

Besides weaker effector responses and higher systemic inflammation, oxidative and DNA damage markers are higher in men than women [16]. Men also have shorter telomeres on average, a faster rate of telomere attrition, and a shorter lifespan [17, 18]. Chapter 8 found a strong association between basal testosterone levels and telomere shortening after BCG vaccination. Testosterone also inhibited telomerase activation. Our results suggest that, in addition to baseline differences in immunity, hormone concentrations could lead to further sex discrepancies in response to vaccinations. Telomere loss in males was linked to the degree of trained immunity induction by BCG, hinting at a complex mechanism involving inflammation, epigenetic reprogramming, androgen signaling, DNA damage, and repair that needs to be uncovered in future studies.



Analyses in Chapter 4 showed that women had similar or higher numbers of most immune cell types (both naïve and memory), except for CD8+ effector memory T cells that were more abundant in men. Despite lower cell numbers, circulating concentrations of cytokines and chemokines were higher in men of both cohorts. These results align with other reports of higher systemic inflammation in males. This chapter also reported sex differences in age-associated immunological changes. For instance, $CD56^{bright}$ NK and plasmablast numbers significantly declined with age only in women, while naïve Treg numbers only declined in men. Age-associated elevation of IL-6 and IL-18 was also sex-dependent. Decreasing TRANCE, also called

RANKL, and increasing OPG concentrations with age were only observed in men. TRANCE regulates cell proliferation and apoptosis by binding to receptor RANK, while OPG acts as a decoy receptor for TRANCE, preventing its binding to RANK [19]. TRANCE-deficient mice lack lymph nodes and show defects in lymphocyte differentiation [20], and an age-dependent reduction in TRANCE-RANK signaling might contribute to lymphocyte-related immune deficits in older males.

Sex differences have historically been understudied, but lately attracted the attention of many research fields, including immunology. In this thesis, all studies considered biological sex an important factor and explored sex differences in the circulating proteome, metabolome, immune cell composition, cytokine response, trained immunity, and telomere maintenance. Thorough understanding of the impact of sex chromosomes and hormones on immunity would allow better-targeted treatment and vaccination approaches for cisgender men and women, as well as transgender individuals undergoing gender-affirming hormone therapy.

## 3. Proposed biomarkers and potential interventions

BCG vaccination is the main intervention this thesis investigated in relation to immune aging. The section 'Targeting innate immunity for healthier aging' already discussed the implications of BCG vaccination in this regard. However, most BCG vaccination studies have been on newborns, children, or healthy adults. During the COVID-19 pandemic, many studies assessing whether BCG vaccination could protect against SARS-CoV-2 were initiated, some recruiting only older participants. A placebo-controlled study in 2014 individuals older than 60 years showed no significant effect of BCG vaccination on infection or hospitalization rates due to COVID-19, although it improved *ex-vivo* cytokine production and antibody titers after SARS-CoV-2 infection [21]. However, a smaller study with 301 participants reported a significant risk reduction at 6-month follow-up [22]. Outside of the COVID-19 context, one study followed 198 individuals with a mean age of 80 for 12 months after receiving a placebo or BCG vaccination [23]. This research uncovered a 45% reduction in the risk of any new infection and a 79% reduction in all respiratory infections. Similar studies with larger sample sizes and longer follow-ups will be essential in understanding the non-specific protective effects of BCG in older individuals. These studies should also measure systemic inflammation markers to validate previous findings of reduced cytokine and chemokine concentrations one month after BCG vaccination in individuals over 60 [9].

Besides BCG, several endogenous or food-derived metabolites with pro- or anti-aging associations were identified in Chapter 3. Shared age-related changes

between the Dutch and Tanzanian cohorts were described, including lower steroid hormone levels, higher systemic inflammation, and poorer cytokine response. One of these changing factors, DHEA, is already popular as an anti-aging supplement claimed to help with skin aging, cognitive function, and osteoporosis. However, the data currently available on the effects of DHEA on aging are insufficient. Randomized controlled trials assessing immunological outcomes, among other aging parameters, are necessary to justify DHEA's use as a supplement.

KB2, ximenoylacetone, and 14,16-Nonacosanedione, all food-derived metabolites, were associated with improved innate and adaptive cytokine response in Tanzanians. Europeans had lower concentrations of these metabolites, likely due to dietary differences, and no association with immune parameters was observed. Further research on these metabolites and their effects on immune function could pave the way for diet interventions to boost immune response in old age.

Chapter 3 also proposed hippurate and 2-phenylacetamide as metabolites linked to immune aging. They were more abundant in people older than 50 years in both cohorts, and their concentrations correlated with lower cytokine response, faster epigenetic aging, and shorter telomeres. Validation of these associations in other metabolomics studies would strengthen their potential as biomarkers for immune aging. *In vitro* studies with primary human cells would also help in understanding if these metabolites directly influence immune response.

Lastly, TRANCE and OPG emerge from Chapter 4 as intriguing biomarker candidates. In both cohorts analyzed in the study, a strong age-dependent decline in TRANCE and an increase in OPG was observed, but only in men. T and B cells are major sources of TRANCE and OPG, respectively [19, 24]. Reduced lymphocyte numbers in old age might partially be responsible for the declining TRANCE and OPG levels. However, low concentrations of these proteins can, in turn, influence immune response. Although these TNF superfamily members are known for their roles in bone remodeling [25], TRANCE is important for DC survival and function [26], while OPG regulates DC and B cell maturation and function [27]. These proteins have not been studied in the context of immunosenescence. However, a study reported higher circulating OPG levels in HIV-infected individuals on antiretroviral therapy compared to healthy controls [28]. Among HIV+ individuals, people with multiple comorbidities had higher OPG concentrations. Furthermore, COVID-19 patients in ICU had lower TRANCE concentrations than patients not requiring ICU treatment [29]. These results suggest a role for TRANCE and OPG in immunosenescence, and immunological consequences of a high OPG/TRANCE ratio in old age should be investigated, especially in men.

## 4. Concluding remarks

This thesis underscores the importance of the immune system in biological aging and in designing anti-aging strategies, sheds new light on the mechanisms of immune aging, and proposes potential interventions to mitigate its effects. BCG vaccination is an exciting strategy to promote immune fitness and merits further exploration. Additionally, the proposed metabolite and protein biomarkers provide valuable insights into immune aging and offer potential therapeutic targets. Moving forward, research should focus on validating these findings in larger cohorts of different origins, understanding the impact of BCG vaccination on other aging hallmarks, and further elucidating the sex differences and the role of sex hormones in immune aging. Overall, this work contributes significantly to our understanding of immune aging and paves the way for developing targeted strategies to promote healthier aging and improve immune resilience in older populations.

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9

# APPENDICES

# NEDERLANDSE SAMENVATTING

Leeftijd is een van de grootste risicofactoren voor veel ziektes, waaronder kanker, diabetes en hart- en vaatziekten. Dit komt deels doordat ouder worden gepaard gaat met veranderingen in het immuunsysteem. Naarmate het menselijk lichaam ouder wordt, ondergaan alle cellen en weefsels veranderingen en passen ze zich aan om met ouder worden om te gaan. Het immuunsysteem vormt daarop geen uitzondering. De hoeveelheid immuuncellen en hun voorlopers nemen af, ook werken de cellen minder goed. Dit wordt collectieve immunosenescentie genoemd. Tegelijkertijd treedt een leeftijdsgebonden toename van systemische ontsteking op (de zogenaamde inflammaging). Hoewel inflammaging ook wordt waargenomen bij gezond ouder worden zonder comorbiditeiten, kan inflammaging bijdragen aan de ontwikkeling van leeftijdsgebonden aandoeningen. Een suboptimaal immuunsysteem leidt tot verminderde immunosurveillance tegen kanker, een hogere vatbaarheid voor infecties en ziekten die verband houden met verhoogde systemische ontsteking, zoals atherosclerose en neurodegeneratieve ziekten.

Het aantal mensen ouder dan 65 jaar zal naar verwachting meer dan verdubbelen en in 2050 1,6 miljard bereiken. Een snel groeiende oudere bevolking leidt tot een afname van de kwaliteit van leven, en een enorme druk op de gezondheidszorg en economie als gevolg van leeftijdsgebonden aandoeningen en een hoog risico op infecties. Meer dan de helft van de personen ouder dan 65 jaar heeft meerdere chronische leeftijdsgebonden aandoeningen. Bovendien leiden verstoringen in het adaptieve immuunsysteem, veroorzaakt door veroudering, tot een lage vaccinatie-efficiëntie op oudere leeftijd. Het is daarom belangrijk om nieuwe manieren te vinden om een robuuste immuunreactie te bevorderen op oudere leeftijd. Dit proefschrift richt zich primair op hoe de functie en het metabolisme van immuuncellen gekoppeld zijn aan verouderingsprocessen, en identificeert verschillende manieren om homeostase te bevorderen binnen het immuunsysteem.

## Immuungeheugen en het ouder wordende immuunsysteem

Het immuunsysteem is verdeeld in twee armen. De eerste arm is het niet-specifieke aangeboren immuunsysteem dat de snelle reactie in de eerste uren en dagen na infectie coördineert. De tweede arm is het antigeen-specifieke adaptieve immuunsysteem dat het geheugen van eerdere ontmoetingen met een ziekteverwekker herbergt om deze efficiënt te bestrijden bij een volgende infectie. Een almaar groeiende hoeveelheid onderzoek heeft het laten zien dat niet alleen het adaptieve immuunsysteem immunologisch geheugen kunnen opbouwen. Aangeboren immuuncellen hebben ook herinneringen aan eerdere

blootstellingen aan infecties, dit geheugen van het aangeboren immuunsysteem noemen we "getrainde immuniteit". Het wordt veroorzaakt door metabole en epigenetische herprogrammering van aangeboren immuuncellen en hun voorlopers bij blootstelling aan bepaalde ziekteverwekkers of microbële liganden. Dit resulteert in een sterkere daaropvolgende reactie op niet-gerelateerde ziekteverwekkers. Het eeuwen oude Bacillus Calmette–Guérin (BCG) vaccin is een bekende en veilige inductor van getrainde immuniteit bij mensen. Hoewel BCG oorspronkelijk ontwikkeld is voor tuberculosepreventie, kan het beschermen tegen niet-gerelateerde infecties en de algehele mortaliteit significant verminderen in omgevingen met een hoge infectiedruk. Een gedetailleerde uitleg van de moleculaire mechanismen van BCG-geïnduceerde getrainde immuniteit en gunstige niet-specifieke effecten tegen andere infecties wordt gegeven in andere hoofdstukken van dit proefschrift.

T en B cellen zijn lymfocyten, ze behoren tot het adaptieve immuunsysteem en zijn de cellen die adaptief immunologisch geheugen ontwikkelen en behouden. Daarom zijn deze cellen het primaire doelwit voor vaccinontwikkeling. Echter, met het ouder worden, ontstaan er vanuit voorlopercellen relatief meer cellen die bij de aangeboren arm van het immuunsysteem horen. Daarnaast neemt de productie van naïeve T-cellen in de thymus af gedurende het leven als gevolg van thymische involutie. Bestaande lymfocyten kunnen senescent worden en niet meer effectief reageren op een uitdaging. Leeftijdsgebonden veranderingen in lymfocyten leiden tot een verminderde adaptieve immuunreactie en verminderde vaccinatie-efficiëntie.

Hoewel immunosenescentie voornamelijk wordt gedefinieerd door het verlies van naïeve lymfocytenpools, ophoping van terminaal gedifferentieerde senescente lymfocyten, en bijbehorende inflammaging, ondergaan ook aangeboren immuuncellen leeftijdsgebonden veranderingen. Ondanks die leeftijdsgebonden veranderingen van het aangeboren immuunsysteem, weegt immunosenescentie veel zwaarder op het adaptieve immuunsysteem. Daarom is het verbeteren van de functies van aangeboren immuuncellen door het stimuleren van getrainde immuniteit een interessante benadering om de gezondheid op oudere leeftijd te verbeteren.

# Samenvatting van het proefschrift

## Deel I - Mechanismen en biomarkers van immuunveroudering

De epigenetische en metabole veranderingen die optreden in immuuncellen tijdens veroudering en de gevolgen daarvan voor het immuungeheugen worden besproken in hoofdstuk 2. In dit hoofdstuk laat ik zien dat het immuunsysteem centraal staat in het verouderingsproces vanwege zijn complexe interacties met andere fysiologische processen. Het immuunsysteem kan daarom een belangrijk doelwit zijn bij het ontwikkelen van anti-verouderingsstrategieën. Dit werd geïllustreerd door de associaties tussen een verouderend immuunsysteem, het centrale zenuwstelsel en de darmmicrobiota in kaart te brengen. Ten slotte hebben we in dit hoofdstuk twee gedrags- en twee farmacologische interventies belicht die zich richten op meerdere aspecten van veroudering en potentieel verouderingsgerelateerde fenotypen kunnen vertragen of omkeren: calorische beperking, lichaamsbeweging, metformine en resveratrol.

In hoofdstuk 3 vergeleek ik de leeftijdsafhankelijke veranderingen in immuunreactie en identificeerde de metabole factoren die waarschijnlijk bijdragen aan deze veranderingen in twee cohorten van gezonde volwassenen: één uit Nederland en de andere uit Tanzania. We observeerden een gemeenschappelijke leeftijdsafhankelijke afname in de T-cel IFNγ-respons. Echter, alleen in Tanzanianen vonden we een verzwakking van de aangeboren immuunreactie. Om de mechanismen achter de gemeenschappelijke leeftijdsgerelateerde veranderingen te bepalen, identificeerden we metabolieten waarvan de concentraties significant verschilden bij individuen ouder dan 50 in beide cohorten. We identificeerde drie metabolieten (dehydroepiandrosteron (DHEA), DHEA sulfaat, en androsteron glucuronide), waarvan de niveaus afnamen bij een hogere leeftijd. Deze metabolieten vertoonden leeftijdsonafhankelijke associaties met lagere systemische ontsteking en een hogere cytokinerespons tegen pathogenen. Hippuraat en 2-fenylacetaamid, daarentegen, was hoger op oudere leeftijd en omgekeerd geassocieerd met de aangeboren en adaptieve cytokinerespons. Ze werden ook gekoppeld aan kortere telomeren en snellere epigenetische veroudering bij mannen. We identificeerden ook verschillende metabolieten die mogelijk bijdragen aan de afnemende aangeboren immuniteit bij individuen uit Tanzania, waarbij traumatisch zuur, d-erythro-l-galacto-nonulose en epinefrine glucuronide de sterkste associaties vertoonden. Dit hoofdstuk benadrukte het belang van het bestuderen van diverse populaties om ons begrip van de universele verouderingsmechanismen te vergroten, evenals de specifieke processen in elke populatie die zijn gekoppeld aan genetica en niet-genetische factoren zoals voeding.

In hoofdstuk 4 onderzocht ik leeftijdsgebonden veranderingen bij gezonde volwassenen, waarbij specifiek werd gefocust op circulerende markers en immuunceltypes die gekoppeld zijn aan de ernst van COVID-19. Tijdens de pandemie werd het al snel duidelijk dat leeftijd en mannelijk geslacht risicofactoren zijn voor COVID-19 ernst en mortaliteit. We onderzochten de geslachts- en leeftijdsafhankelijke verschillen in de circulerende proteoom- en immuuncelsamenstelling van twee gezonde volwassen cohorten uit Nederland die vóór de pandemie waren verzameld. Het profiel van ernstige COVID-19, gekenmerkt door verhoogde circulerende concentraties van verschillende pro-inflammatoire eiwitten en chemokines, samen met verminderde aantallen CD56heldere NK-cellen, naïeve B- en T-celnummers, overlapt grotendeels met leeftijds- en geslachtsgebonden veranderingen. Dit hoofdstuk stelde voor dat deze intrinsieke immunologische kenmerken van ouderdom en mannelijk geslacht die worden waargenomen bij gezonde individuen, hun differentiële ziekteernst bij infectie met SARS-CoV-2 kunnen beïnvloeden. Toen PBMC's van sommige deelnemers *in vitro* werden gestimuleerd met SARS-CoV-2, produceerden oudere mensen meer IL-1β en IL-1RA, maar minder IFNγ tegen het virus. Hun IL-1RA-productie was ook hoger dan die van jongere deelnemers zonder stimulatie. Een onevenwichtige IL1-as en deficiënte productie van IFNγ zouden betrokken kunnen zijn bij de verminderde capaciteit om een SARS-CoV-2-infectie op te lossen.

## Deel II - BCG-vaccinatie als strategie om een gezond immuunsysteem te bevorderen

In hoofdstuk 5 heb ik leeftijdsgebonden veranderingen in aangeboren en adaptieve immuun cellen kort opnieuw uitgelegd, gevolgd door strategieën om de vaccinatie-effectiviteit voor oudere mensen te verbeteren. De conventionele manieren van vaccinontwerp focust voornamelijk op een efficiënte adaptieve immuunreactie. Echter, T- en B-lymfocyten zijn de cellen die het meest worden beïnvloed door veroudering. Dit hoofdstuk biedt een opstapje voor de volgende hoofdstukken door een potentiële rol van getrainde immuniteit, bijvoorbeeld door BCG-vaccinatie, te beschrijven om veroudering van het immuunsysteem te voorkomen of terug te draaien. Hoewel aangeboren immuuncellen leeftijdsgebonden functionele defecten vertonen zoals verminderde fagocytose, antigeenpresentatie, cytokineproductie en chemotaxis, kunnen deze defecten worden verminderd door het induceren van getrainde immuniteit. Bovendien zijn de voordelen antigeen-agnostisch en zouden ze helpen bij meer dan één infectie of vaccinatie. Naast het stimuleren van de functie van aangeboren immuuncellen, vermindert BCG-vaccinatie systemische ontsteking, een ander kenmerk van immuunveroudering. Het begrijpen van de impact van BCG op andere verouderingskenmerken en het

verkennen van strategieën om de trainbaarheid ervan te verbeteren, waren de belangrijkste focus van Deel II van deze scriptie.

Alendronaat (alendroninezuur) is een medicijn dat gebruikt wordt om osteoporose te voorkomen en te behandelen op oudere leeftijd. Deze klasse van medicijnen, bisfosfonaten genaamd, remt ook de cholesterol synthese, wat essentieel is voor getrainde immuniteit. In hoofdstuk 6 heb ik de impact van het gebruik van alendronaat op inductie van getrainde immuniteit door BCG-vaccinatie bij jonge, gezonde vrijwilligers onderzocht. Gelijktijdige toediening van een enkele alendronaat tablet en BCG-vaccin verminderde ex vivo cytokineproductie tegen heterologe bacteriële en virale stimuli een maand na vaccinatie in vergelijking met alleen BCG-vaccinatie. Alendronaat onderdrukte ook transcriptioneel de immuunreactiepaden die door alleen BCG werden opgereguleerd. Deze resultaten tonen aan dat alendronaat de inductie van getrainde immuniteit geïnduceerd door BCG remt.

In hoofdstuk 7 heb ik de impact van resveratrol op BCG-geïnduceerde getrainde immuniteit *in vitro* onderzocht. Resveratrol is een natuurlijk fenolisch verbinding en een van de anti-verouderingsinterventies die in Hoofdstuk 2 zijn belicht. Het heeft verschillende cardiobeschermende, neuroprotectieve, immunomodulerende en antioxidante effecten. In veel modelorganismen verlengt resveratrol ook de levensduur. Onze studie onthulde dat resveratrol C. albicans, β-glucan en oxLDL-geïnduceerde getrainde immuniteit remt terwijl het BCG-geïnduceerde getrainde immuniteit versterkt. Metabool gezien beperkte resveratrol gecombineerd met BCG de verbeterde glycolyse en oxidatieve fosforylering maar handhaafde deze hoger dan bij niet-behandelde cellen. Resveratrol onderdrukte ook ROS-productie maar stimuleerde sterk de cytokineproductie, wat gepaard ging met een toegenomen H3K27Ac-depositie op TNFA- en IL6-promotors. Verbeterde cytokineproductie kon ook worden waargenomen bij cellen van individuen ouder dan 65 jaar. Het verschillende effect van resveratrol op BCG-geïnduceerde getrainde immuniteit was afhankelijk van BCG dat levend en transcriptioneel actief was. Remming van Sirtuin 1 in monocyten blokkeerde ook gedeeltelijk het effect van resveratrol. Over het algemeen suggereerde dit hoofdstuk dat resveratrol kan worden gebruikt om de reactie van BCG-geïnduceerde getrainde immuniteit te verbeteren terwijl het overmatige oxidatieve stress en metabole overbelasting beperkt.

De mogelijkheid van BCG om de functies van aangeboren immuuncellen te verbeteren en systemische ontsteking te verminderen, is al gerapporteerd. Echter, de impact ervan op andere verouderingskenmerken was nog onbekend. In hoofdstuk 8 heb

ik de gevolgen van BCG-vaccinatie voor het onderhoud van telomeerlengte onderzocht. BCG reguleerde genen die belangrijk zijn voor mechanismen van telomeeronderhoud en telomerasefunctie. In twee onafhankelijke menselijke cohorten van BCG-vaccinatie werd een kortere gemiddelde telomeerlengte waargenomen 3 maanden na vaccinatie. Dit gold niet voor personen die een placebo vaccinatie kregen. Telomeerverkorting werd gevonden bij jonge deelnemers tussen 18 en 30 jaar, maar er was geen significante verandering bij deelnemers ouder dan 50 met kortere basale telomeerlengtes. Deelnemers waarvan de capaciteit voor cytokineproductie niet werd verbeterd door BCG vertoonden meer telomeerverkorting, wat duidelijker was voor mannen. Bovendien toonden we aan dat BCG het telomerase-enzym kan activeren dat de telomeerherhalingen kan verlengen. Dit werd voornamelijk waargenomen bij cellen van vrouwelijke donoren en werd voorkomen door exogeen testosteron toe te voegen. Kortom, in dit hoofdstuk rapporteerden we de langdurige, geslachtsspecifieke impact van BCG-vaccinatie op telomeerdynamiek en telomeraseactiviteit, en koppelden we dit aan de sterkte van inductie van getrainde immuniteit. Studies met langere follow-ups zijn essentieel om te begrijpen of de telomeraseactiviteit bij vrouwen zal resulteren in herstel van de telomeerlengte. Deze nieuwe relatie tussen BCG-geïnduceerde getrainde immuniteit en cellulair verouderen vereist verder onderzoek.

Tot slot onderstreept dit proefschrift het belang van het immuunsysteem in biologisch verouderen en bij het ontwerpen van anti-verouderingsstrategieën. Dit proefschrift werpt nieuw licht op de mechanismen van immuunveroudering in verschillende populaties, en stelt potentiële interventies voor om de effecten ervan te verminderen. Dit proefschrift onthult ook de interferentie van alendronaat met BCG-geïnduceerde getrainde immuniteit, stelt resveratrol voor als een versterker van BCG-geïnduceerde getrainde immuniteit, en rapporteert geslachtsspecifieke, langdurige effecten van BCG-vaccinatie op telomeeronderhoud.

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# TÜRKÇE ÖZET

## SAĞLIKLI YAŞLANMAYA İLİŞKİN İMMÜNOLOJİK ANLAYIŞLAR: BCG AŞISI VE DİĞER STRATEJİLER

Yaşlanma; kanser, diyabet ve kalp hastalıkları gibi birçok hastalığın en büyük risk faktörlerinden biridir. Bu kısmen yaşlanmanın bağışıklık sisteminde değişikliklere yol açmasından kaynaklanmaktadır. İnsan vücudu yaşlandıkça, tüm hücreler ve dokular değişir ve yaşlanmayla başa çıkmak için uyum sağlar. Bağışıklık sistemi de bu değişimlerden etkilenir. İmmün hücrelerin ve öncüllerinin sayısı azalır ve hücreler daha az etkili çalışır. Bu duruma bağışıklık yaşlanması (immunosenescence) denir. Aynı zamanda, sistemik enflamasyonda yaşa bağlı bir artış (inflammaging olarak da bilinir) meydana gelir. Bu sağlıklı yaşlanmada da gözlemlenir, ancak yaşla ilgili hastalıkların gelişimine katkıda bulunabilir. Zayıf bir bağışıklık sistemi, kansere karşı bağışıklık gözetimini azaltır, enfeksiyonlara ve sistemik enflamasyonla ilişkili hastalıklara, örneğin ateroskleroz ve nörodejeneratif hastalıklara yatkınlığı artırır.

65 yaş üstü insan sayısının 2050 yılında 1,6 milyara ulaşarak iki kattan fazla artması bekleniyor. Hızla büyüyen yaşlı nüfus, yaşla ilgili hastalıklar ve enfeksiyon riski nedeniyle yaşam kalitesinin düşmesine ve sağlık hizmetleri ile ekonomi üzerinde büyük bir baskıya neden olmaktadır. 65 yaş üstü kişilerin yarısından fazlası birden fazla kronik yaşa bağlı hastalığa sahiptir. Ayrıca, yaşlanmanın neden olduğu kazanılmış bağışıklık sistemindeki bozulmalar, yaşlılıkta aşıların etkinliğinin düşmesine yol açar. Bu nedenle, yaşlılıkta güçlü bağışıklığı teşvik edecek yeni yollar bulmak önemlidir. Bu tez, bağışıklık hücrelerinin fonksiyonu ve metabolizmasının yaşlanma süreçleri ile ilişkilerini inceler ve bağışıklık sisteminde dengeyi sağlamak için çeşitli yöntemler ortaya koyar.

### Bağışıklık hafızası ve yaşlanan bağışıklık sistemi

Bağışıklık sistemi geleneksel olarak iki kola ayrılır. İlk kol, enfeksiyondan sonraki ilk saatler ve günlerde hızlı tepkiyi koordine eden, herhangi bir patojene (enfeksiyona sebep olan etmen) özel olmayan, doğuştan gelen bağışıklık sistemidir ve “doğal bağışıklık” olarak isimlendirilir. İkinci kol ise, bir patojenle önceki karşılaşmaların hafızasını tutan ve sonraki enfeksiyonlarda onunla etkili bir şekilde savaşmak için kullanılan, antijene özel “kazanılmış bağışıklık” sistemidir.

Son yıllardaki araştırmalar, sadece kazanılmış bağışıklık sisteminin değil, aynı zamanda doğal bağışıklık hücrelerinin de önceki enfeksiyonlara dair hafıza oluşturup saklayabileceğini göstermiştir. Bu yeni tip bağışıklık hafızasına "eğitilmiş bağışıklık" denir. Belirli patojenlere veya mikrobiyal ligandlara maruz kalındığında, doğal

bağışıklık hücrelerinde ve öncüllerinde metabolik ve epigenetik açıdan yeniden programlama gerçekleşir. Bu, daha sonra alakasız patojenlere karşı daha güçlü bir tepkiye yol açar. 100 yıldan fazla zamandır kullanılan Bacillus Calmette–Guérin (BCG) aşısı, insanlarda eğitilmiş bağışıklığın bilinen ve güvenli bir indükleyicisidir. BCG başlangıçta tüberkülozun önlenmesi için geliştirilmiş olmasına rağmen, eğitilmiş bağışıklık sayesinde alakasız enfeksiyonlara karşı da koruma sağlayabilir ve yüksek enfeksiyon riski olan ortamlarda genel ölüm oranını önemli ölçüde azaltabilir. BCG'nin neden olduğu eğitilmiş bağışıklığın moleküler mekanizmaları ve diğer enfeksiyonlara karşı yararlı etkileri bu tezin diğer bölümlerinde detaylı bir şekilde açıklanmaktadır.

Kazanılmış bağışıklık sistemine ait T ve B hücreleri (lenfositler), kazanılmış bağışıklık hafızasını geliştiren ve sürdüren hücrelerdir. Bu nedenle, bu hücreler aşı geliştirme için ana hedeflerdir. Ancak, yaşlandıkça, öncül hücreler kazanılmış bağışıklık hücrelerinden ziyade yeni doğal bağışıklık hücreleri oluşturur. Ayrıca, timus involüsyonu nedeniyle yaşam boyunca timusta yeni T hücrelerinin üretimi azalır. Mevcut lenfositler yaşlanabilir ve bir tehdide karşı etkili şekilde yanıt veremez hale gelebilir. Bu değişiklikler, kazanılmış bağışıklık tepkisinin azalmasına ve aşılama etkinliğinin düşmesine yol açar. Bağışıklık yaşlanması genellikle naif lenfositlerin kaybı, terminal olarak farklılaşmış yaşlanmış lenfositlerin birikmesi ve artan sistemik enflamasyon ile tanımlanır. Doğal bağışıklık hücreleri de yaşlanmayla birlikte değişiklikler geçirse de, yaşlanma kazanılmış bağışıklık sistemi üzerinde daha ağır bir etki yapar. Bu nedenle, eğitilmiş bağışıklığı teşvik ederek doğal bağışıklık hücrelerinin işlevlerini geliştirmek, yaşlılıkta sağlığı iyileştirmek için umut vaat eden bir yaklaşımdır.

## Tezin özeti

### 1. Kısım - Bağışıklık yaşlanmasının mekanizmaları ve biyobelirteçleri

Bağışıklık hücrelerinde yaşlanma sırasında meydana gelen epigenetik ve metabolik değişiklikler ve bunların bağışıklık hafızası üzerindeki etkileri 2. Bölüm'de tartışılmaktadır. Bu bölümde, bağışıklık sisteminin diğer fizyolojik süreçlerle olan karmaşık etkileşimleri nedeniyle yaşlanma sürecinde merkezi bir rol oynadığını iddia ettim. Bu nedenle, bağışıklık sistemi, yaşlanma karşıtı stratejiler geliştirmede önemli bir hedef olmalıdır. Bu, yaşlanan bağışıklık sistemi ile merkezi sinir sistemi ve bağırsak mikrobiyotası arasındaki ilişkilerin haritasını çıkararak örneklendirildi. Son olarak, bu bölümde yaşlanmanın birden fazla yönünü hedefleyen ve yaşlanmayla ilişkili değişiklikleri potansiyel olarak geciktirebilecek veya tersine çevirebilecek iki

davranışsal ve iki farmakolojik müdahaleyi vurgulandı: kalori kısıtlaması, fiziksel egzersiz, metformin ve resveratrol.

3. Bölüm'de, Hollandalı ve Tanzanyalı sağlıklı yetişkinlerden oluşan iki kohortta bağışıklık tepkisindeki yaşa bağlı değişiklikleri karşılaştırdım ve bu değişikliklere muhtemelen katkıda bulunan metabolik faktörleri belirledim. T hücrelerinin IFNγ tepkisinde ortak bir yaşa bağlı azalma gözlemlendi. Ancak, sadece Tanzanyalılarda doğal bağışıklık tepkisinde zayıflama bulundu. Yaşla ilişkili ortak değişikliklerin mekanizmalarını anlamak için, her iki kohortta 50 yaş üstü bireylerde önemli ölçüde farklı konsantrasyonlara sahip olan metabolitleri belirledik. DHEA, DHEA sülfat ve androsteron glukuronidin yaşla birlikte azaldığını ve bu metabolitlerin daha düşük sistemik enflamasyon ve patojenlere karşı daha yüksek sitokin tepkisi ile ilişkili olduğunu belirledik. Hipurat ve 2-fenilasetamid ise yaşla birlikte artmış ve hem doğal hem kazanılmış sitokin tepkisi ile ters orantı göstermiştir. Ayrıca, bu iki metabolit daha kısa telomerlerle ve erkeklerde daha hızlı epigenetik yaşlanma ile ilişkilendirildi. Tanzanya'daki bireylerde yaşla azalan doğal bağışıklığa katkıda bulunabilecek çeşitli metabolitler de belirledik ve en güçlü ilişkiler travmatik asit, d-eritro-l-galakto-nonüloz ve epinefrin glukuronid ile gösterildi. Bu bölüm, evrensel yaşlanma mekanizmalarını anlamak için farklı popülasyonları incelemenin önemini ve bunun yanında her popülasyondaki kendine özgü süreçleri vurgulamaktadır.

4. Bölüm'de, COVID-19 şiddeti ile ilişkililendirilmiş proteomik biyobelirteçler ve bağışıklık hücre türlerine odaklanarak sağlıklı yetişkinlerde yaşa bağlı değişiklikleri inceledim. Pandemi sırasında, yaş ve erkek cinsiyetin COVID-19 şiddeti ve ölüm oranı için önemli risk faktörleri olduğu hızla anlaşıldı. Pandemi öncesinde toplanan, Hollanda'dan iki sağlıklı yetişkin kohortunun dolaşımdaki proteom ve bağışıklık hücresi bileşimindeki cinsiyet ve yaşa bağlı farklılıkları inceledik. Çeşitli proenflamatuar proteinler ve kemokinlerin dolaşımdaki miktarlarının artmasıyla ve naif lenfositler ile $CD56^{parlak}$ NK hücrelerinin azlığıyla ilişkilendirilen şiddetli COVID-19 profili, incelenilen sağlıklı kohortlardaki daha yaşlı bireylerin ve özellikle yaşlı erkeklerin profiliyle örtüşmektedir. Bu bölüm, sağlıklı bireylerde gözlemlenen yaşlılık ve erkek cinsiyetine özgü bağışıklık özelliklerinin, SARS-CoV-2 enfeksiyonunda hastalık şiddetini etkileyebileceğini öne sürdü. Bazı katılımcılardan izole edilen bağışıklık hücreleri laboratuvarda SARS-CoV-2 ile uyarıldığında, yaşlı bireylerin daha fazla IL-1β ve IL-1RA, ancak daha az IFNγ ürettiklerini gördük. Ayrıca, uyarılmamış durumda bile yaşlıların IL-1RA üretimi gençlere göre daha yüksekti. Dengesiz bir IL1 ekseni ve yetersiz IFNγ üretimi, SARS-CoV-2 enfeksiyonunu çözme kapasitesinin azalmasında rol oynayabilir.

## 2. Kısım - Sağlıklı bir bağışıklık sistemi için BCG aşılaması

5. Bölüm'de, yaşa bağlı doğal ve kazanılmış bağışıklık hücrelerindeki değişiklikleri kısaca yeniden ele aldım ve yaşlılar için aşılama etkinliğini artırma stratejilerini inceledim. Geleneksel aşı tasarımı, genellikle güçlü bir kazanılmış bağışıklık tepkisi yaratmaya odaklanır. Ancak, lenfositler yaşlanmadan en çok etkilenen hücrelerdir. Bu bölüm, bağışıklık sisteminin yaşlanmasını önlemek veya tersine çevirmek için eğitilmiş bağışıklığın, örneğin BCG aşılaması yoluyla, potansiyel rolünü tanımlayarak sonraki bölümler için bir temel sunar. Doğal bağışıklık hücreleri yaşlandıkça fagositoz, antijen sunumu, sitokin üretimi ve kemotaksis gibi işlevlerde bozukluklar gösterir, ancak bu bozukluklar eğitilmiş bağışıklık ile azaltılabilir. Ayrıca, eğitilmiş bağışıklığın faydaları antijenden bağımsızdır ve birden fazla enfeksiyon veya aşılama durumunda yardımcı olabilir. Doğal bağışıklık hücrelerinin işlevini artırmanın yanı sıra, BCG aşılaması bağışıklık yaşlanmasının bir diğer özelliği olan sistemik enflamasyonu da azaltır. BCG'nin diğer yaşlanma özellikleri üzerindeki etkisini anlamak ve eğitilmiş bağışıklık kapasitesini artırma stratejilerini keşfetmek, bu tezin 2. kısmının ana odak noktalarıdır.

Alendronat, ileri yaşlarda kemik erimesini önlemek ve tedavi etmek için kullanılan bir ilaçtır. Bu ilaç sınıfı bisfosfonatlar, aynı zamanda eğitilmiş bağışıklık için gerekli olan kolesterol sentezini de engeller. 6. Bölüm'de, genç ve sağlıklı gönüllülerde BCG aşılaması ile eğitilmiş bağışıklığın indüklenmesi üzerinde alendronat kullanımının etkisini araştırdım. BCG aşısı ile eşzamanlı olarak verilen tek bir alendronat tableti, sadece BCG aşılamasına kıyasla aşılamadan bir ay sonra farklı bakteriyel ve viral uyaranlara karşı sitokin üretimini azalttı. Alendronat ayrıca BCG tarafından aktive edilen bağışıklık yolaklarını transkripsiyonel olarak baskıladı. Bu sonuçlar, alendronatın BCG tarafından indüklenen eğitilmiş bağışıklığı engellediğini göstermektedir.

Bitkilerde bulunan doğal bir fenolik bileşik olan resveratrol, çeşitli kardiyokoruyucu, nörokoruyucu, bağışıklık düzenleyici ve antioksidan etkileri olan, ve 2. Bölüm'de vurgulanan yaşlanma karşıtı müdahalelerden birisidir. Resveratrol birçok model organizmada yaşam süresini de uzakmaktadır. 7. Bölüm'de, resveratrolun BCG tarafından indüklenen eğitilmiş bağışıklık üzerindeki etkisini araştırdım. Bu çalışma, resveratrolün BCG tarafından indüklenen eğitilmiş bağışıklık yanıtını epigenetik ve sitokin üretimi açısından güçlendirirken bir taraftan aşırı oksidatif stresi azalttığını ortaya koydu. Bu, resveratrolün BCG aşısının faydalarını artırarak özellikle yaşlı bireylerde bağışıklık fonksiyonunu iyileştirmekte faydalı olabileceğini göstermektedir.

8. Bölüm'de, BCG aşılamasının hücresel yaşlanmanın bir göstergesi olan telomer uzunluğu üzerindeki etkilerini inceledim. BCG, telomer bakımı ve telomeraz

fonksiyonu için önemli olan birçok geni transkripsiyonal seviyede düzenlemektedir. BCG aşılamasından üç ay sonra genç yetişkinlerde ortalama telomer uzunluğunda kısalma gözlemlendi. Ancak bu durum 50 yaşın üstündeki yetişkinlerde gözlenmedi. BCG'nin sitokin üretim kapasitesini artırmadığı katılımcılar, özellikle erkekler, daha fazla telomer kısalması gösterdi. Ayrıca, BCG'nin kadınlarda telomeraz enzimini aktive edebildiğini ve potansiyel olarak telomer kısalmasına karşı gelebilecegini gördük. Bu durum, hücresel yaşlanma ile BCG tarafından indüklenen eğitilmiş bağışıklık arasında cinsiyete bağlı yeni bir ilişkiyi ortaya koymaktadır ve daha fazla araştırma gerektirmektedir.

Sonuç olarak bu tez, bağışıklık sisteminin biyolojik yaşlanmadaki önemini ve yaşlanma karşıtı stratejileri tasarlamadaki rolünü vurgulamaktadır. Farklı popülasyonlarda bağışıklık yaşlanmasının mekanizmalarına yeni bir ışık tutmakta ve bu etkileri azaltmak için potansiyel müdahaleler önermektedir. Önerilen yaşlanma karşıtı müdahalelerden başlıcası BCG aşısı ile doğal bağışıklık hücrelerinde eğitilmiş bağışıklığı tetiklemektir. Tez, ayrıca, alendronat kullanımının BCG tarafından indüklenen eğitilmiş bağışıklık ile olan etkileşimini ortaya koymakta, resveratrolün bu bağışıklığı güçlendirici rolünü öne sürmekte ve BCG aşılamasının telomerlere yönelik cinsiyete özgü etkilerini rapor etmektedir.

# RESEARCH DATA MANAGEMENT

## Ethics and privacy

This thesis is largely based on the results of research with human participants. Studies were subject to the Medical Research Involving Human Subjects Act (WMO) and were conducted in accordance with the ICH-GCP guidelines (Good Clinical Practice). The medical ethical review committee 'METC Oost-Nederland' has given approval for the studies conducted in the Netherlands (file numbers NL42561.091.12, NL58553.091.16, NL74082.091.20, NL58219.091.16). The cohort study from Tanzania (Chapter 3) was approved by the ethical committees of Kilimanjaro Christian Medical University College and the National Institute for Medical Research in Tanzania. Informed consent was obtained from participants for the collection and processing of their data and the sharing of pseudonymized data for follow-up research. Pseudonymization, restricted access authorization, and secure data storage were employed to secure the privacy of study participants.

## Data collection and storage

The research presented in this thesis was conducted according to the FAIR (findability, accessibility, interoperability and reusability) principles. Data for Chapter 6 was collected through electronic Case Report Forms (eCRF) using CASTOR EDC. Pseudonymized data were stored and analyzed on the Internal Medicine department server, only accessible by project members working at the Radboudumc. *in vitro* and ex vivo data were also stored digitally on the local department server. Paper (hardcopy) data was stored in locked cabinets in the department.

## Availability of data

Published studies in this thesis are open-access. Links or accession codes for publicly available datasets are included in the chapters. The rest of the anonymized datasets used for analysis will be made available upon reasonable request. The data from human studies will be archived for 15 years after the studies are terminated.

# PHD PORTFOLIO

Department: **Internal Medicine**
PhD period: **01/10/2019 – 31/08/2024**
PhD Supervisor(s): **Prof. M.G. Netea**
PhD Co-supervisor(s): **Dr J. Dominguez-Andres**

| Training activities | Hours |
|---|---|
| **Courses** | |
| • Course on laboratory animal science (LAS) (2019) | 84.00 |
| • R programming for absolute beginners (2020) | 28.00 |
| • Statistics with R - Beginner level (2020) | 8.40 |
| • Statistics with R - Intermediate level (2020) | 8.40 |
| • E-BROK (2020) | 42.00 |
| • How to write a medical scientific paper (2020) | 8.40 |
| • Panel design workshop (2020) | 5.60 |
| • Scientific integrity for PhD candidates (2020) | 28.00 |
| • How to sell your science? (2020) | 8.40 |
| • Science journalism and communication (2020) | 84.00 |
| • Design and illustration (2020) | 28.00 |
| • RIMLS - Introduction course "In the lead of my PhD" (2021) | 21.00 |
| • Mindfulness-based stress reduction (2021) | 28.00 |
| • Open science for PhD candidates (2021) | 28.00 |
| • RU - Career guidance for international PhD's (2021) | 14.00 |
| • Intensive ChIP workshop (2022) | 2.80 |
| • Leiden University - Science communication summer school (2022) | 40.00 |
| **Seminars** | |
| • Workshop: anxiety & stress at work (2019) | 2.80 |
| • Radboud research rounds: translational research in animal models (2020) | 2.80 |
| • Research integrity rounds: recognition and rewards for Radboudumc academics (2020) | 2.80 |
| • Research integrity rounds: the dark side of science (2021) | 2.80 |
| • Online college tour: mental health (2021) | 2.80 |
| • RIMLS meet the expert: FAIR research (2021) | 2.80 |
| • Radboud research rounds: eLife (2021) | 2.80 |
| • Olink proteomics & gene expression profiling (2021) | 2.80 |
| • Radboud research rounds: inflammation and chronic disease, meta-inflammation (2021) | 2.80 |
| • RIMLS meet the expert: how to write a rebuttal (2021) | 2.80 |
| • Radboud research rounds: inflammation in cardiovascular disease (2022) | 2.80 |
| • Research integrity rounds: research integrity in times of crisis (2022) | 2.80 |
| • DNA methylation workshop (2022) | 4.00 |
| • Research integrity round: college tour (2023) | 2.80 |

| | |
|---|---|
| **Conferences** | |
| • 4th International Conference on Innate Immune Memory (participation) (2019) | 14.00 |
| • Optimmunize: Improving the beneficial effects of vaccines (poster presentation) (2020) | 35.00 |
| • ESCMID Conference on Coronavirus Disease (poster presentation) (2020) | 35.00 |
| • RIMLS PhD Retreat (participation) (2020) | 21.00 |
| • Biomarker Meeting of Paris 2020 (poster presentation) (2020) | 21.00 |
| • 31st European Congress of Clinical Microbiology & Infectious Diseases (ECCMID) (poster presentation) (2021) | 42.00 |
| • 6th European Congress of Immunology 2021 (oral presentation) (2021) | 35.00 |
| • RIMLS PhD Retreat (participation) (2021) | 21.00 |
| • RIMLS PhD Retreat (oral presentation) (2022) | 21.00 |
| • Summer Innate Immunology Conference (poster presentation) (2022) | 21.00 |
| • Optimmunize 2022 (oral presentation) (2022) | 28.00 |
| • 5th International Symposium on Trained Immunity (poster presentation) (2023) | 28.00 |
| • ImmunoMetNet 2023 (participation) (2023) | 7.00 |
| • ImmunoMetNet 2024 (participation) (2024) | 7.00 |
| **Other** | |
| • Blog for the Research Newsletter (2020) | 2.80 |
| • Vlog for RIMLS Annual Report 2020 (2020) | 5.60 |
| • Blog for the Research Newsletter (2021) | 2.80 |
| • Blog for the Research Newsletter (2021) | 2.80 |
| • Blog for the Research Newsletter (2021) | 2.80 |
| • PON PhD Day (2021) | 5.60 |
| • Blog for the Research Newsletter (2021) | 2.80 |
| • Trained Immunity Journal Club (x45) (2021-2024) | 3.60 |
| **Teaching activities** | |
| **Lecturing** | |
| • Host-Microbe Interactions (session Aging Alongside Microbes) (2022) | 2.80 |
| • Host-Microbe Interactions (session Aging Alongside Microbes) (2023) | 2.80 |
| **Supervision of internships / other** | |
| • Supervision of MSc student for 6 months (2022) | 56.00 |
| • Supervision of MSc student for 6 months (2023) | 56.00 |
| **Total** | **1,024.80** |

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Dear **Jorge**, you are much more than a supervisor and mentor (and the harem boss :P). You are a big brother to me and many others. Without your daily support, academic or personal, I would not have survived this PhD (at least not in this shape). You strike a rare balance as a supervisor. Strong scientific guidance is the obvious part of the job. But you're also incredibly attentive, understanding, supportive, and ridiculously fun to be around. When things are not fun, and I'm crying in the office, you listen and provide honest support. Another big thank you for encouraging and guiding my unusual career goals the whole time. Speaking of my career goals, I admire the public speaker and science communicator that you are. Thank you for being a close example of "scientists CAN do this well; they don't have to be boring and incomprehensible". I'm fortunate to have fallen into your orbit, and I intend to stay there with non-academic titles.

## Unofficial supervisors

Dear **Niels**, our roads did not cross much for most of my PhD, but you were an influential presence and support for me during the final year while I wrapped up the projects and wrote this book. I've enjoyed working with you immensely. I learned a lot from you and your group, doing research at a different pace with a slightly different approach. Thank you dearly for your understanding, patience, and trust during this process.

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## The core support team

**Gizem**, you've been my "lab wife" since 2016, and we've been inseparable to the point where people confuse us with each other. It's not inaccurate to say that I ended up in NL following you, and I'm glad you chose to come here. You are one of the pillars of my life that keep the whole thing going. We used to dream of running a lab together, just like Mihai and Leo, besties ruling the field. Then, life nudged us towards different plans. We'll soon part ways as lab wives and co-authors :'( But I wouldn't want anyone else to have spent my last 8 years with. Not only were you generously sharing one brain cell with me during this PhD (and MSc), but you also kept me alive and functioning. Some know that I'm not exaggerating when I say you, together with a few others, kept me alive. I'll always support you for whatever comes next, and we'll keep building our lives here in NL together.

**Büş**, it feels insane that I've only known you for 4 years, considering the gigantic place you now hold in my life. You came to this lab a year after I did, stayed with me

for your first week, and soon, we were three potatoes rolling through life together. Thank you for being my big sister, a.k.a. the voice of sanity and maturity in my life, without judgement. Thank you for all the silliness and laughs that take my breath away. I cannot wait to be on the same continent again so that we can have frequent adventures, regular tarot nights, and lots of cuddles <3.

**İlaylay**, the latest addition to my little harem of smart and gorgeous Turkish girls, my go-to person for Spanish dramas, volleyball matches, and D&B parties. Also, my anxiety buddy (critical to have in this job so you don't feel insane). The times we were constantly in the lab together were some of the most fun I had at work. Being with you was the only thing that made looong ChIP days and the Greece madness actually fun. Let's keep laughing, dancing, and lifting each other up!

**Andy**, bebişim, my solid ground and safety net. I always thought working with one's partner was a recipe for disaster, and I would quickly get bored seeing them 24/7. But boy, was I wrong. I absolutely love being in the lab with you and will be pretty sad when that ends. Having you around as a source of smiles, kisses, hugs, and constant support is priceless. Beyond the visible help of the involved and hardworking colleague that you are, there are so many loving, selfless little daily actions that make up your giant impact on this PhD. I've gotten so lucky with you and cannot wait to see how we take on the next stages of life together.

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## The journal club crew

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Dear **Sasha** and **Joppe**, I was so lucky to have students as intelligent, interested, capable, and responsible as you. Thank you for the valuable supervision experiences. I wish you the best of luck with whatever you do, and please know that you can always reach out to me about anything. Sasha, special thanks for selecting me to give your laudation at your MSc graduation. That was a unique experience for me and I was such a proud mama!

## The research newsletter team

I've been generally very fortunate during my time at Radboudumc. One more example is the group of fantastic people who are or were involved in preparing the weekly Radboudumc Research Newsletter, which was a great outlet for me for a significant part of my PhD. Thank you for allowing me to figure out new strengths to focus on and run wild with writing blogs, interviews, basically whatever I could get my hands on or what I believed was needed. **Hasan**, I cherish the person you are and how every conversation with you enriches my mind. **Clasien** and **Dagmar**, thank you for all you do behind the scenes to make Radboudumc a better place for all PhDs and postdocs.

**Estel**, I did not expect to gain a lifelong friend when I joined this team. I remember our first Skype call when I was supposed to briefly interview you, but we ended up talking for hours. Soon after, it moved from online to real life, and you were breaking the pandemic curfew for me <3 Around you, somehow, I become a version of myself that I like more. We both jump great heights, chasing profound and complicated moments and feelings in life. Whenever I spend time with you, I leave inspired. You're one phenomenal woman, and I'm lucky to have you.

## The oldtimers

**Saygın**, my favorite concert buddy, thanks for sharing some of the best trips, cathartic moments (special thanks to Lorde), and memories of these past years. We've been through so much together for almost 15 years now (whaatttt?!), and the last few inevitably included weekends of meeting up in whichever country and crying over our PhDs. But we've done it! It's over! I love you so much, and please never move farther away from just across the border.

**Said**, being away from you is one of the suckiest things about my life in the Netherlands. You still managed to be there for me when it mattered the most, and I can't possibly thank you enough for that. I should've been there for you way more, but I can't deny that I disappointed myself these past years, getting too caught up in daily stresses and not making enough time for the most important things. You mean the world to me Pato; this never changes <3

**Dilara**, Cems to my Baboli, you are a true sister (the complete package with growing up sharing a room, jealousy, fighting, but eventually being unable to stay apart for long and having continuous unconditional love throughout). When I feel like a deranged weirdo, and no one else mentioned in these pages would understand me, you are the one I go to. Thanks for being a constant in my life.

## Family away from home

Dear **Annelies** and **Donne**, you might be the only landlords acknowledged in a PhD thesis and that's because you are very special people. You treated me like your own kid since day one, before even knowing me well. With my family far away, you truly were a second family for the two and a half years I lived next to you. I appreciate all the care, support, and forest walks with dear Zappa.

Dear **Carmen** and **Freddy**, thank you for immediately accepting me into your family and being there for both of us. It actually feels wrong to write 'Carmen' because you just became 'Mama' to me right away. I see my own family once or twice a year, and I really appreciate being able to have big lovely family meals and warm mama hugs here.

## Family / Aile

My dear brother **Önder**, you were my idol growing up, and I followed your lead in my own way. Thank you for sparking my curiosity and interest in science from an early age and always guiding me to make sure I achieve my dreams. Having a fellow academic in the family gave me confidence in my pursuit. It also made my

parents understand better when I could not spare too much time to go visit them or why I still needed to work when I was there. Whether the gains are worth it is a question I've struggled with for years, and I concluded that for me, it's not worth it. I hope the new life I'm trying to build means more visits and trips with you (and Duygu <3), more connection, and lots of late nights philosophizing over life like we used to.

**Babacığım**, bu doktora süresince maddi manevi (en önemlisi manevi) hep arkamda olduğun ve neyin peşinden koşuyorsam desteklediğin için teşekkür ederim. İşime gösterdiğim önem ve özen (ben sık sık fazla abartsam da) bana senden geçen ve bütün başarılarımda katkısı olan şeyler. Çocukluğumdan beri bilim insanı olma hayalimi destekledin. Maalesef bu pek sık rastlanan bir şey değil, özellikle bizim ülkemizde. Bunun için minnettarım. Doktora için Hollanda'ya taşındığımdan beri oğluşum Emerson'ı benim kadar sevip benimsediğiniz ve içimi rahat tuttuğunuz için de sana da anneme de çok teşekkür ederim.

**Canım annem**, kendimde gurur duyduğum birçok şeyi sana borçluyum ve senin kızın olduğum için çok şanslıyım. Küçük ya da büyük bütün fedakarlıkların için minnettarım (her gün ben ders çalışırken meyve soyup getirmek kadar küçük, istediğim eğitimi alabileyim diye daha çocukken ülkenin başka bir ucuna taşınmama katlanmak kadar büyük). Senin yaşamana izin veya olanak verilmeyen ve benim sahip olduğum her güzel şeyi, her başarıyı senin için de yaşıyorum. Bu doktora tezini de tüm kalbimle sana ithaf ediyorum.

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*This piece was originally published on the Radboudumc website and the Research Newsletter on 25 March 2021. The data is, at this point, slightly outdated. However, the main points and my calls to action are as relevant as ever, if not increasingly more.*

# PHD CANDIDATES ARE AT RISK, AND WE NEED TO TALK ABOUT IT

by Özlem Bulut

Stress is a word almost synonymous with doing a PhD. Scientific endeavour always pushes one to move forward, which can be an excellent motivator for curious minds. Sadly, the state of constant running also takes a toll on people. 36% of graduate students reported that they have sought help for PhD-related anxiety and depression in a large multicultural survey[1] done by the journal Nature in 2019. This serious mental health risk needs to urgently attract the attention of institutions and group leaders.

## The Netherlands is not doing better

From my conversations with friends doing their PhDs in various countries, I know that we have relatively good conditions as PhD candidates here in the Netherlands. However, studies about mental health lay out a different picture. PhD council of the University of Amsterdam conducted a survey[2] in 2015 and found out that 36.5% of the PhD candidates are at risk for clinical depression. This risk was 41% for women. A 2018 study[3] by Leiden University supported these findings and found 38.3% of PhD candidates at serious mental health risk. Many reported frequent loss of sleep and concentration problems, which could seriously affect their work performance.

## Why are we not talking about this?

With all these studies ringing the alarm bells, I am very frustrated that we are not talking about this enough. Whether or not PhD-related, having mental health problems is still a secret whispered in coffee room corners to that one colleague who seems non-judgemental. Sometimes not even that. Some just quietly suffer until they collapse with burnout. I've been dealing with my fair share of anxiety disorder and depression for years. My research career is not the source of these problems, but it can certainly be a trigger at times. On top of that, my mental health struggles frequently affect my performance, making me feel guilty and even more anxious. You see, it turns into a vicious cycle. Unless we talk about it and work together to manage it.

## You are not alone!

Why should we be open about our mental health? Firstly, to tell the quiet sufferers they are not alone. I often feel like I'm the only one having problems while everyone seems to be doing just fine. This can't be the reality, though. Just look at the statistics! Being affirmed that we are not alone would take so much pressure off our shoulders. It's really frustrating not being able to tell your colleagues or supervisor about your suffering simply because you don't want them to see you as incompetent. Especially if there's no guarantee that the response would be empathetic.

## Dear group leaders, you should help us start this conversation

I believe that the people with the most power to change this status-quo are the group leaders. They are usually unaware of the problems junior researchers are facing. Undoubtedly, managing a team while simultaneously teaching, doing administrative tasks, and hunting for grants is challenging. However, this is a crisis worth directing time and attention to. My belief is that we need frequent conversations about mental health. Asking about it once a year as a formality for the annual performance appraisal is definitely not enough.

It would be great to have regular group discussions about the work atmosphere and the difficulties people experience. The supervisors might lead the way by being open and sharing some of their own past and current challenges. It would signal that our struggles are not to be ashamed of and that the people we idealize struggle too. In one-to-one meetings, supervisors should be clear about their expectations from each person to prevent anxiety stemming from uncertainty and perceived pressure to be fast. Of course, some tasks might really be urgent. We have been doing work at lightning speed in my department during the COVID-19 pandemic. However, it's important to be flexible about what to expect from each person at a certain time, depending on their particular situation.

I believe that if group leaders get more open and encouraging to discuss these issues, junior colleagues would also naturally start to communicate and create a more comfortable and compassionate environment among themselves.

## Institutions are not helping enough

Although communication with colleagues and leaders is critical, institutions are certainly not off the hook. I'm lucky enough to have an amazing daily supervisor with whom I can share my problems and who always has my back. Unfortunately, not everyone is that lucky. If the immediate work environment is not able or willing

to help, one needs other doors to knock on. Nature's survey did not just reveal the mental health risks of graduate students but also the inability of institutions to take care of them. Out of those who sought help, only 26% were able to get assistance at their institution. Another 18% have tried it but weren't supported. The figure below from the survey illustrates the sad situation better than I can put into words.

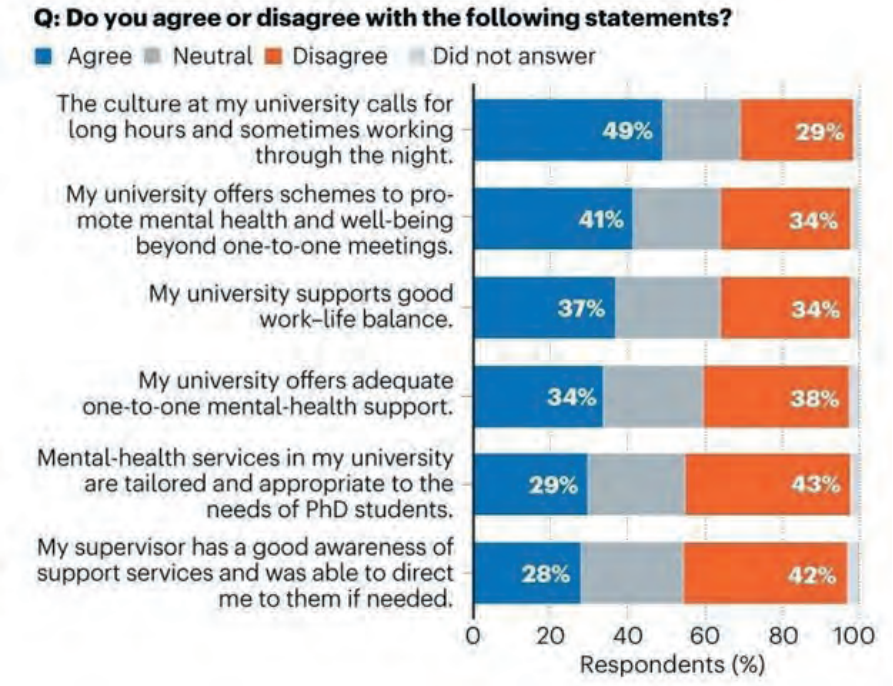


Image Source: Nature PhD Survey 2019

## How can Radboudumc help?

Radboudumc has just published a new Intranet page called "A vibrant workplace". On this site, you can find information on who you can turn to other than your colleagues and supervisors, supporting activities and events like peer coaching groups, and some helpful course offerings, including mindfulness-based stress reduction. Even though there is still much to be done, this is a good step to guide the people seeking support.

To anyone struggling: Please know that you are not alone, and you can speak up. Seek help. Use the resources that Radboudumc offers and demand more if you think they are not enough. To group leaders and all other colleagues: Please try to start a conversation and don't shy away from leading the way. A comfortable, safe,

and healthy work environment would make everyone more productive, creative, and collaborative.

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# AUTHOR BIOGRAPHY

Özlem Bulut was born in Afyonkarahisar, Türkiye on August 1, 1995. Early on, she was fascinated by science, particularly biology, and grew up devouring encyclopedias and popular science magazines. Her curiosity led her to the prominent Aydın Science High School across the country. She then earned her bachelor's degree in molecular biology and genetics from Bilkent University in Ankara, where she also completed her Master's under the guidance of Prof. İhsan Gürsel. Her research there focused on characterizing the immunomodulatory potential of mesenchymal stem cell exosomes and testing them in various pre-clinical models.

In 2019, Özlem joined Prof. Mihai Netea's lab at Radboudumc, where she was introduced to the complex world of aging and produced this PhD thesis. She found herself intrigued by trained immunity, a paradigm-shifting concept described and characterized by Netea's group, and its potential to help healthier aging. Now, as a postdoctoral researcher with Prof. Niels Riksen and Mihai Netea, Özlem is trying to understand the complicated dance between immune aging and cardiometabolic health.

It was during the pandemic in 2020 that Özlem discovered a new passion: science communication. After taking a science journalism course, she got completely hooked on bringing the incredible science produced every day to the masses in a correct, nuanced, and gripping way. In addition to her PhD work, she spent the last few years training and volunteering, preparing to jump into the media world. While she continues to love doing scientific research, Özlem's future plans center around making science more accessible, inspiring future scientists, and showing everyone just how cool biology can be.


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