Mechanisms Underlying Dementia and Stroke

an immunity and inflammation perspective

Lana Fani

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Mechanismen onderliggend aan dementie en hersenberoertes
vanuit het perspectief van het immuunsysteem en inflammatie

Mechanisms Underlying Dementia and Stroke
an immunity and inflammation perspective

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Voor mijn lieve ouders

Stairwells

Thousands of scientists
are trying to comprehend
the billions of connections
that relentlessly extend

through different layers
of distinct cells
passing electrical signals
like pouring water from stairwells.

The source of our behavior,
our thinking,
our entire mind.
A super construction, of which the blueprint we must find

to cure people who forget,
cannot speak,
are paralysed.
Their independence severely compromised.

L. Fani

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*Both authors contributed equally to this study

PROLOGUE

I remember well the image of a real brain during a neurosurgical procedure I attended as a medical student. It was pulsating calmly to the rhythm of the heart, yet looking so vulnerable as it was exposed when the piece of skull was removed. A little scratch to this soft pink organ could have devastating consequences as I learned. I was intrigued by this organ. How could it be so powerful, controlling our entire body, yet be so prone to damage? And damage could occur in a vast variety of forms: from tumours to vascular damage, to trauma. Moreover, in contrast to for example trauma, where it is clear where the damage comes from, we often do not know why damage to the brain occurs in the first place. Why do some people develop brain tumours? Like my mom, who fortunately recovered. Why do others have extensive vessel disease in their brain? Why does the brain shrink so fast in some people? All these phenomena have devastating consequences. I fell in love with the brain, with its mystery, its complexity and its weaknesses.

The cover of my thesis depicts the grey matter of a normal brain with 'resting' microglia cells stained with Rio Hortega`s silver carbonate method. Microglia are the guardians that react to potential danger to the brain. But the question is, could they also become traitors? While my thesis does not specifically focus on microglia, it does attempt to answer this question, among others, by viewing inflammation or the immune system as a potential mechanism that may cause dementia or stroke.

Chapter 1
General Introduction

The brain is a fascinating and unique organ which orchestrates our entire behaviour. The earliest reference to the brain anywhere in human records is in an ancient Egyptian medical treatise called the Edwin Smith Surgical Papyrus (**Figure 1**).[1] Since then, our understanding of the brain has come a long way, however many mysteries still remain.[2] One of those mysteries is the neural basis underlying psychiatric and neurological disease. With an ageing population projected to include 152 million people with dementia by 2050[3], along with the rising global burden of disability attributed to stroke[4], these two diseases play a key role in the worldwide health burden.[5]

Figure 1. Hieroglyphic for "Brain". *From Principles of Neural Science.*

DEMENTIA: SENILE INSANITY

The word dementia derives from the Latin root *demens*, which means being out of one's mind. The term "dementia" has been used since the 13th century, but its mention in the medical community was only reported in the 18th century.[6] Dementia was recognized as having two types of brain changes. In some of these brains, numerous healed infarcts could be seen, which were clearly the result of arteriosclerosis; in the others, the only evident change was a generalized atrophy as a consequence of aging.[7] Alzheimer wrote his first major article in 1899 on the nature of senile insanity or dementia in which he maintained that those cases of atrophy without recognizable infarcts also were due to vascular disease, but that this involved arterioles rather than arteries, so the infarcts were microscopic.[8] By 1906, when Alzheimer's disease (AD) was discovered, Alzheimer had moved to Munich where he became the chief of pathology in the lavish new institute of brain research headed by Emil Kraepelin, the foremost psychiatrist in the world.[9] Kraepelin was an organist, believing that all mental disease was due to some alteration of the brain as an organ, opposing the Freudian concept of psychoanalysis in which mental disease was due to a conflict between the consciousness and subconscious mind.[10] Kraepelin lay the foundation of the modern classification system for mental disorders, and remarkable similarities remain in the now widely used Diagnostic and Statistical Manual of Mental Disorders (DSM), of which the latest, fifth edition appeared in 2013.

STROKE: APOPLEXIA

As far as 2,500 years ago the term *apoplexia* has been found in writings, meaning "struck down with violence", describing a disorder in which "a person suddenly falls, without consciousness or motion, retaining pulse or respiration".[11] This characteristic picture was well known to the ancient Greeks, and it was Hippocrates who was responsible for the first recorded appearance of the term "apoplexy".[12] During The Renaissance, the promotion of human dissection allowed society to use autopsy for forensic, health and scientific purposes, paving the way for modern medicine.[13] During this modern era from the 17th century onwards, "apoplexy" began to lose its unitary (umbrella) meaning,[14] marking the start of studies on vascular diseases of the brain or "cerebrovascular disease(s)"[11], after which Cole (1689) first used the term "stroke".[15] This term then appeared only much later in the ICD-9 in 1968.[16] The definition of the concept by the World Health Organization (WHO) appeared soon after in 1971 and 1980, and more recently, a new definition was proposed by the American Heart Association-American Stroke Association (AHA-ASA) in 2013, incorporating clinical and tissue criteria.[17] Stroke is nowadays widely recognized as the appearance of acute focal neurological symptoms or signs that result from diseases involving cerebral blood vessels.[18]

WHY STUDYING DEMENTIA AND STROKE?

The etiological similarity between dementia and stroke was already suggested in Alzheimer's first major article on dementia mentioned earlier, describing that those cases of atrophy without recognizable infarcts were due to microscopic infarcts[8]. This idea was further supported by Walter Alvarez, a gastroenterologist, whose interest in AD was stimulated by the presence of this condition in his father-in-law.[7] The episodic aggravation of his father-in-law's condition led him to the conclusion that these changes were the results of small episodes of brain damage, ultimately known as little strokes.[19] Indeed, it is now recognized that all major dementias have a vascular component, ranging from 61% in frontotemporal dementia to 80% in AD.[20] In fact, the presence of a vascular component doubles the probability that the neurodegenerative pathology will have manifested as dementia later in life.[21] At the same time, not all dementias can be prevented by preventing stroke and there are growing genetic, transcriptomic and proteomic data pointing to the complexity of AD pathogenesis.[22] It is thus essential to gain a better understanding of the underlying pathophysiology that may lead to both these diseases.[23]

DEMENTIA AND STROKE: AN IMMUNITY AND INFLAMMATION PERSPECTIVE

When Alois Alzheimer first described the histopathology of AD, he noted, "The glia have developed numerous fibers".[24] It was only in the late 1980s that scientists began to study this feature.[25] It included the involvement of the immune system in AD, and was shown by the attachment of complement proteins to diseased tissue, and by the activation of cells associated with the immune system.[26,27] Over the last 10 years, this field of research saw a steep increase[25] after the discovery of AD-associated variants in the triggering receptor expressed on myeloid cell 2 (*TREM2*) gene that is only expressed on immune cells of myeloid origin. Further support came from a large-scale genome-wide association study that identified multiple additional immune risk factors[28], but to date, it is still not clear whether immunity and inflammation in fact lead to AD or become only present in a later stage, when the disease already started. In order to shed some light on this issue, **chapter 2** explores the effects of the immune system on the risk of dementia using population-based data. **Chapter 2.1** considers immune cells in relation to dementia risk. These immune cells consist of granulocyte and platelet count as measures of the innate immune system and lymphocyte count as measure of the adaptive immune system. In the field of cancer research it has been suggested that taking the ratio of these innate and adaptive immune cells results in new white blood-cell based inflammatory indices as markers of low-grade inflammation.[29] These are the neutrophil-to-lymphocyte ratio (NLR), the platelet-to-lymphocyte ratio (PLR) and the systemic immune-inflammation index (SII), which are thought to better reflect the relative balance between the innate and adaptive immune system (**Figure 2**).

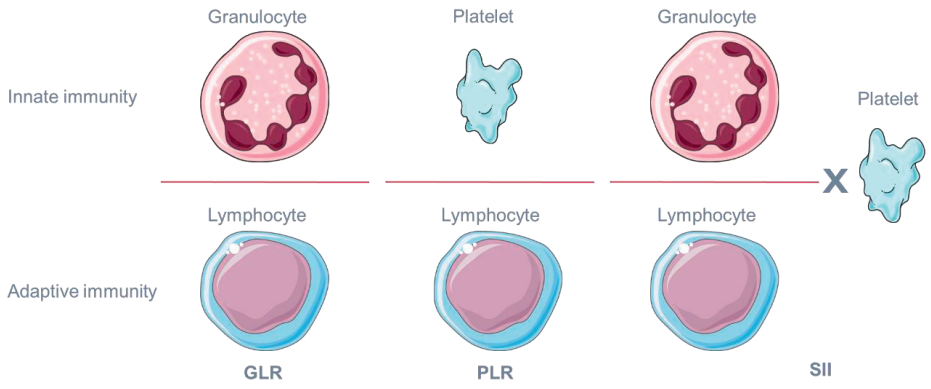

Figure 2. White blood-cell based inflammatory indices reflecting the relative balance between the innate and adaptive immune system: Granulocyte-to-lymphocyte ratio (GLR), the platelet-to-lymphocyte ratio (PLR) and the systemic immune-inflammation index (SII).

To follow-up on the findings of **chapter 2.1**, I performed a two-sample Mendelian randomization study (**chapter 2.2**) examining immune cells and signaling molecules in relation to the risk of AD and hippocampal volume. I further study whether common infectious diseases could be a trigger of low-grade inflammation by studying the relation between *Helicobacter pylori* (**chapter 2.3**) and Herpes Simplex Virus type 1 (**chapter 2.3**) in relation to the risk of dementia.

The immune system is not only a potential determinant for dementia, but also for stroke.[30] Indeed, studies have shown that gene expression changes in the peripheral blood and immune cells of stroke patients can serve as potential diagnostic biomarkers in acute stroke.[31,32] Moreover, randomized clinical trials have also shown potential for anti-inflammatory treatment in reducing cardiovascular risk including stroke.[33,34] **Chapter 3** therefore examines immunity components, the same immune cells and derived ratios as studied in **chapter 2.1**, in relation to stroke. In **chapter 3.1**, I determine the association between immunity components and the risk of atherosclerotic cardiovascular disease, including stroke and coronary heart disease. I furthermore relate these immunity components to arterial calcifications as measures of subclinical atherosclerosis and examine to what extent arterial calcifications serve as a potential mediator in the relation between immunity components and atherosclerotic cardiovascular disease. Finally, in **chapter 3.2** I examine immunity components with carotid atherosclerotic plaque characteristics to study whether immunity components relate to the plaque's vulnerability to rupture.

BRAIN PATHOLOGY

In addition to immunity and inflammation being potentially important factors for AD and stroke, Alois Alzheimer already reported early on plaques and neurofibrillary tangles, after which Kraepelin included AD in his book *Pschiatrie*.[9] To date, amyloid plaques and neurofibrillary tangles are still considered the hallmark features of AD (**Figure 3**).[35] Amyloid plaques contain toxic peptides known as β-*amyloid*, while neurofibrillary tangles contain micro-tubule-associated proteins, known as *tau.*[18]

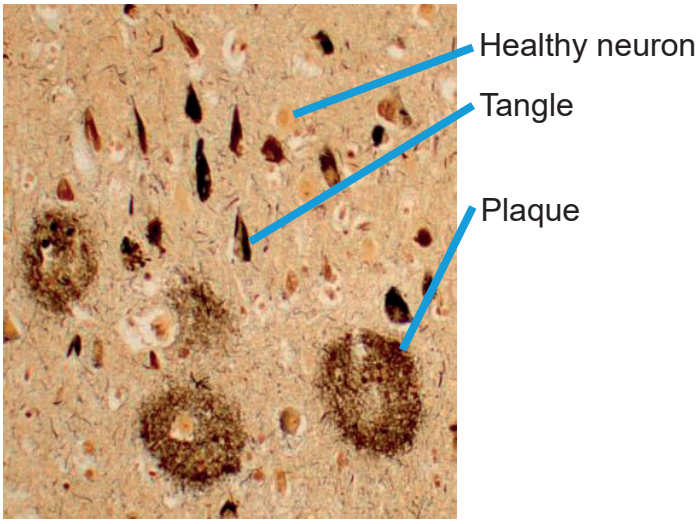

Figure 3. A section of a neocortex from a patient with Alzheimer's disease treated with a silver stain. The tissue shows neuronal cell bodies containing neurofibrillary tangles and neuropil containing amyloid plaques. *Modified from Principles of Neural Science.*

While in AD, extracellular deposits of polymerized β-amyloid peptides create an amyloid plaque, β-amyloid deposition can also occur in the vascular walls, which leads to loss of vessel integrity and can result in cerebral amyloid angiopathy, a condition that increases the risk for stroke caused by bleeding.[36] **Chapter 4.1** of this thesis describes how these biomarkers of AD-related brain pathology as measured in plasma relate to stroke risk in the general population. I furthermore describe the immune system as potential determinant of these markers (**chapter 4.2**) by studying their relation with immune cells. Some of these immune cells, the leukocytes, own a nucleus and thus contain DNA from which we can measure telomere length.[37] As an indicator of oxidative stress and senescence, telomere length is hypothesized to be a biomarker of aging.[38] In **chapter 4.3** I study telomere length as measured from leukocytes as another potential biomarker of AD.

Brain hemodynamics

The brain is highly vulnerable to disturbance of its blood supply.[18] Therefore, blood flow to the central nervous system must efficiently deliver oxygen, glucose, and other nutrients and remove carbon dioxide, lactic acid, and other metabolites.[39] The major vessels that provide the brain of its blood are the carotid arteries and the vertebrobasilar arterial system (**Figure 4**).[18] The brain vasculature has special anatomical and physiological properties that protect the brain from hypoperfusion. Autoregulation involves the alteration of small vessel diameters and is observed in virtually every vascular bed, but it is most pronounced in the brain and kidney.[39] When this protective mechanism fails, ischemia could occur which may lead to neurological dysfunction. When this episode of neurological dysfunction is brief

(<24 hours), we refer to this as a *transient ischemic attack* or TIA. Although most occlusive strokes are caused by atherosclerosis and thrombosis, there are also several other causes, of which subclinical hypoperfusion remains relatively unknown.[40] **Chapter 5** of this thesis is dedicated to studying measures of brain hemodynamics within the general population. First, I describe the association between global brain perfusion and the risk of TIA and ischemic stroke (**chapter 5.1**). In **chapter 5.2**, I then present thyroid function as a potential determinant of brain hemodynamics.

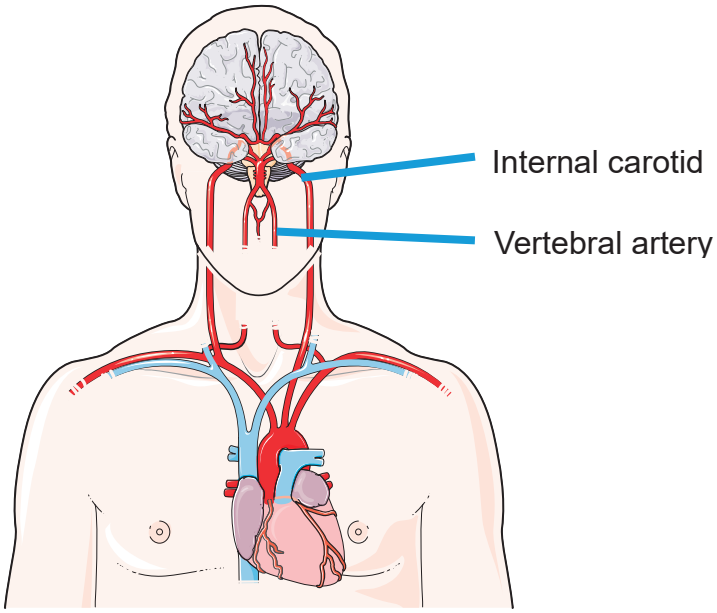

Figure 4. Major arteries supplying the brain of blood. *Modified from smart servier medical art.*

Studying the role of various etiological entities including brain pathology and brain hemodynamics in dementia and stroke provides new opportunities to connect the two diseases. Moreover, I show that targeting immunity and inflammation also point to new directions, and sheds new light on pathological and hemodynamical changes that occur in the brain. In **chapter 6**, the findings described in this thesis are therefore reflected upon from an immunity perspective. I conclude with a discussion of the implications for future research.

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Chapter 2
Immunity and dementia

2.1 Balance between innate versus adaptive immune system

ABSTRACT

Background: Immunity has been suggested to be important in the pathogenesis of dementia. However, the contribution of innate versus adaptive immunity in the development of dementia is not clear. In this study we aimed to investigate 1) the association between components of innate immunity (granulocytes and platelets) and adaptive immunity (lymphocytes) with risk of dementia and 2) the association between their derived ratios (granulocyte-to-lymphocyte ratio [GLR], platelet-to-lymphocyte ratio [PLR], and systemic immune-inflammation index [SII]), reflecting the balance between innate and adaptive immunity, with risk of dementia.

Methods: Blood cell counts were measured repeatedly between 2002-2015 in dementia-free participants of the prospective population-based Rotterdam Study. Participants were followed-up for dementia until 1st January 2016. Joint models were used to determine the association between granulocyte, platelets, and lymphocyte counts, and their derived ratios with risk of dementia.

Results: Of the 8313 participants (mean [standard deviation] age 61.1 [7.4] years, 56.9% women), 664 (8.0%) developed dementia during a median follow-up of 8.6 years. Doubling of granulocyte and platelet counts tended to be associated with an increased risk of dementia (HR [95%CI]: 1.22 [0.89-1.67] and 1.45 [1.07-1.95], respectively). Doubling of the derived ratios GLR, PLR, and SII were all associated with an increased dementia risk (HR [95%CI]: 1.26 [1.03-1.53], 1.27 [1.05-1.53], and 1.15 [0.98-1.34], respectively).

Conclusions: GLR, PLR, and SII are associated with an increased risk of dementia in the general population. This supports the role of an imbalance in the immune system towards innate immunity in the pathogenesis of dementia.

INTRODUCTION

Dementia poses a huge burden on societies in terms of financial costs as well as on individual patients and their caregivers regarding suffering and grief [1]. Dementia is a multifactorial disease, in which various pathologies interact during the long pre-clinical phase, ultimately resulting in its clinical manifestations of cognitive decline and loss of independence. While amyloid depositions, neuronal loss, and vascular damage have long been established as key pathologies underlying dementia[2], recent findings point towards a key role for the immune system [3-5]. The immune system is a highly complex system involving multiple synergistic and antagonistic substrates, yet broadly can be classified into two components, i.e. innate immunity and adaptive immunity [6]. Innate immunity refers to immune responses present at birth, forming a first line of defence, whereas adaptive immunity is acquired during life by exposure to specific antigens [7]. High activity of innate immunity can lead to disrupted neuronal integrity, and ultimately in cell death [8]. Although these components of the immune system work closely together, adaptive immunity is considered to be more neuroprotective than innate immunity, presumably by stimulating phagocytosis of amyloid fibrils [9, 10].

Exact quantification of these opposing components of the immune system is challenging and focus of ongoing research, but recent work from the field of cancer research suggests that easily obtainable laboratory measurements may in fact capture their relative activity levels to a reliable degree [11]. Measuring granulocytes, including the most abundant subtype neutrophils, and platelets provides important markers of the innate immunity, whereas measuring lymphocytes yields information on the adaptive immunity [12, 13]. Furthermore, combining these measurements into ratios, i.e. the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII), is thought to even better reflect the relative balance between innate and adaptive immunity [11, 14-16]. Previous work on the link between innate versus adaptive immunity and dementia showed higher NLR and PLR in dementia patients compared to healthy individuals [17-19]. Yet, to really understand the role of the immune system in the risk of developing dementia, it is pivotal to study how these markers change during the pre-clinical phase of the disease.

We thus investigated the longitudinal association of markers of the innate versus adaptive immune system with the risk of dementia. The underlying hypothesis was that higher activity of the innate versus adaptive immune system would be associated with an increased risk of dementia. A further methodological novelty of our study was the use of joint modelling that enabled us to study the longitudinal evolution of the various markers during the pre-clinical phase in conjunction with survival analyses.

METHODS

Study population

The present study is embedded in the Rotterdam Study, a prospective population-based cohort study in Rotterdam, the Netherlands. The Rotterdam Study started in 1990 with 7983 persons (response of 78%) aged ≥55 years and residing in the district Ommoord, a suburb of Rotterdam. This first subcohort (RS-I) was extended with a second subcohort (RS-II) in 2000, consisting of 3011 persons (response of 67%) and with a third subcohort (RS-III) in 2006, composed of 3932 persons aged ≥45 years (response of 65%). The design of the Rotterdam Study has been described in detail previously [20]. In brief, participants were examined in detail at study entry and at follow-up visits every three to five years. They were interviewed at home by a trained research nurse, followed by two visits at the research facility for additional interviewing, laboratory assessments, imaging, and physical examinations.

The Rotterdam Study was approved by the Medical Ethics Committee of Erasmus Medical Center and by the board of The Netherlands Ministry of Health, Welfare, and Sports. A written informed consent was obtained from all participants.

Laboratory tests for granulocytes, platelets, and lymphocytes were introduced from 2002 onwards, corresponding with the following assessment rounds in the Rotterdam Study (baseline in this study): i.e. fourth round of RS-I, second round of RS-II, and first round of RS-III, comprising 9994 participants. From these 9994 eligible participants, we excluded those without complete baseline blood measurements (n=1288). Of the remaining participants, we excluded those with a history of dementia (n=52), participants who were insufficiently screened for dementia (n=62), and those without informed consent to assess medical records during follow-up (n=39). Lastly, we excluded participants with missing apolipoprotein E (*APOE*) genotype (n=240), resulting in 8313 participants for analysis (Flowchart in **Figure 1**).

Assessment of blood cell counts and their derived ratios

Fasting blood samples were taken during each visit at the research center with a maximum of three visits during follow-up. Full blood count measurements were performed using the COULTER® Ac·T diff2™ Hematology Analyzer (Beckman Coulter, San Diego, California, USA) directly after blood sample drawn. Laboratory measurements included absolute granulocyte, platelet, and lymphocyte counts in 10^9 per liter. Since neutrophil counts were not available, we used granulocyte count as a reliable proxy given that these are the most abundant subtype of neutrophils [21, 22].

The GLR and PLR were calculated as the ratio of granulocyte count to lymphocyte count, and as the ratio of platelet count to lymphocyte count, respectively. The SII was defined as platelet count times the GLR.

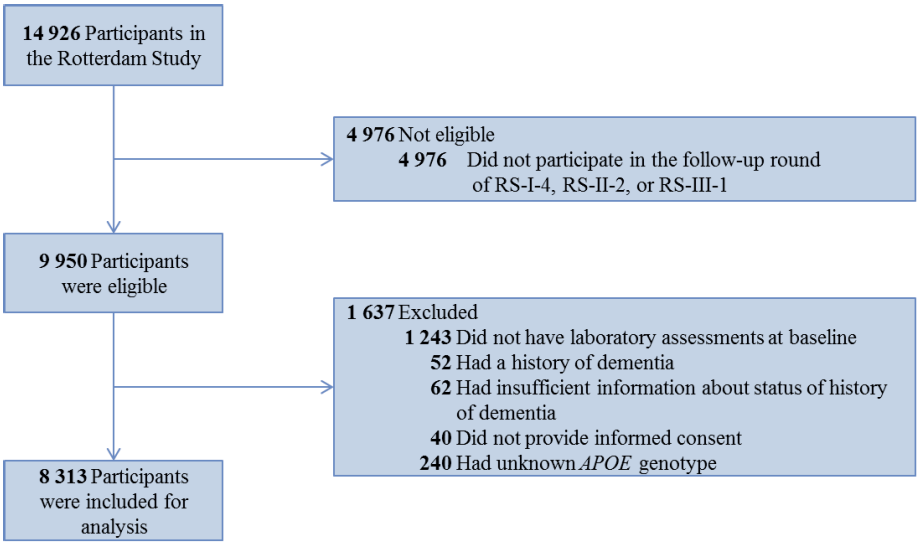

Figure 1 *Flowchart participants for analysis association between blood cell counts and their derived ratios, and dementia.*
Abbreviations: *APOE* = apolipoprotein E.

Assessment of dementia

Participants were screened for dementia at baseline and subsequent center visits with the Mini-Mental State Examination and the Geriatric Mental Schedule organic level [23]. Those with a Mini-Mental State Examination score <26 or Geriatric Mental Schedule score >0 underwent further investigation and informant interview, including the Cambridge Examination for Mental Disorders of the Elderly. The entire cohort was continuously under surveillance for dementia through electronic linkage of the study database with medical records from general practitioners and the regional institute for outpatient mental health care. Available information on clinical neuroimaging was used when required for diagnosis of dementia subtype. A consensus panel led by a consultant neurologist established the final diagnosis according to standard criteria for dementia (Diagnostic and Statistical Manual of Mental Disorders III-revised), Alzheimer's disease (AD, National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association), and vascular dementia (National Institute of Neurological Disorders and Stroke and Association Internationale pour la Recherché et l'Enseignement en Neurosciences). Follow-up until 1st January 2016 was virtually complete (93.8% of potential person-years observed).

Other measurements

We assessed education and smoking by interview. Education level was classified into primary education, lower (lower general education, intermediate general education, or lower vocational education), intermediate (intermediate vocational education or higher general education), or higher (higher vocational education or university). Smoking status was categorized as never, former, or current smoker. Body mass index (BMI) was computed from measurements of height and weight (kg/m^2). Diabetes mellitus was defined as use of antidiabetic medication, fasting serum glucose level ≥7.1 mmol/L, or random serum glucose level ≥11.1 mmol/L [24]. History of stroke was assessed by interview and verified by reviewing medical records [25]. *APOE* genotype was determined using polymerase chain reaction on coded DNA samples in RS-I and with a bi-allelic TaqMan assay in the two extensions (RS-II and RS-III) [26, 27]. *APOE-ε4* carrier status was defined as carrier of one or two *APOE-ε4* alleles.

Statistical analysis

We associated the different blood cell counts and their derived ratios with the risk of all-cause dementia using the framework of joint models for longitudinal and survival data. In this way we are able to account for the endogenous nature (i.e. blood cell counts can be measured with error during follow-up and their values at any time point can be affected by an event occurring at an earlier time point) [28] and the correlations in the repeated measurements of granulocyte, platelet, and lymphocyte counts [29].

In order to normalize the skewed distribution of granulocyte, platelet, and lymphocyte counts, and their derived ratios we used a natural logarithmic transformation. Hazard ratios (HRs) with 95% confidence intervals (CIs) were obtained from the joint models using the piecewise-constant baseline hazard, and multiplied with log(2), providing a HR for doubling of the blood cell counts and their ratios. We computed two nested models: Model I was adjusted for baseline age (continuous, centered as age minus mean age) and sex; Model II was additionally adjusted for education, smoking status, BMI (continuous), diabetes mellitus, history of stroke, and *APOE-ε4* carrier status. For assessment of the association between the individual components of the ratios and dementia, we repeated analyses with adjustment for the baseline blood cell counts of the remaining two blood cell types (for instance, the association of granulocyte count with dementia was adjusted for platelet and lymphocyte counts). Follow-up time was used as timescale and started at the first laboratory assessment until date of all-cause dementia diagnosis, death, loss to follow-up, or 1st January 2016, whichever came first. Censoring participants at date of death allowed us to compute cause-specific HRs.

In sensitivity analyses, we repeated all analyses using age as timescale instead of follow-up time to account for potential residual confounding by age and to minimize potential effects of left truncation. We additionally censored for stroke events during follow-up to preclude

that the observed effect may be driven by incident strokes that occurred before dementia diagnosis. Moreover, we investigated the association between the ratios and AD or vascular dementia separately. Lastly, we explored effect modification by stratifying by median age, sex, smoking status, diabetes mellitus, and *APOE-ε4* carrier status.

Multiple imputation was used for missing covariates (maximum of 0.99%), with five imputed datasets based on other covariates and the outcome. Rubin's method was used for pooled HRs and 95% CIs [30]. Two-sided P <.05 was considered statistically significant. Statistical analyses were performed using the R packages 'survival', 'nlme', 'JM', and 'JMbayes' in RStudio Version 3.3.2 [28, 29, 31, 32].

RESULTS

Characteristics of included and excluded study participants are presented in **Table 1**. An overview of the median blood cell counts and blood cell-based ratios per assessment round is shown in Supplementary Table 1. Mean age of included study participants was 61.1 years and 56.9% were women. During a median follow-up of 8.6 years (70273 person-years), 664 participants developed all-cause dementia (543 AD, 31 vascular dementia) with an incidence rate of 9.4 (95% CI, 8.7-10.2) per 1000 person-years.

Higher levels of granulocytes reflecting higher innate immunity were associated with an increased risk of dementia, but only after correcting for the platelet and lymphocyte counts (HR for doubling granulocyte count [95% CI] = 1.33 [0.99-1.79], **Table 2**). Doubling of platelets was associated with an increased risk of dementia (HR [95% CI] = 1.48 [1.11-1.96]). Regarding adaptive immunity, higher levels of lymphocytes were associated with a decreased risk of dementia (HR for doubling lymphocyte count [95% CI] = 0.80 [0.64-0.99]).

Higher levels of GLR, PLR, and SII were associated with an increased dementia risk (HR [95% CI] for doubling GLR = 1.34 [1.10-1.63]; for PLR = 1.29 [1.08-1.55]; for SII = 1.18 [1.02-1.39], respectively [**Table 2**]). Risk estimates were comparable when using the adjusted model and when using age as timescale instead of follow-up time.

Censoring for stroke did not meaningfully change the risk estimates (**Table 3**). Higher levels of platelets showed a slightly stronger association with AD compared with all-cause dementia, whilst the association with granulocytes was less pronounced for AD. Risk estimates for all-cause dementia and AD were comparable for the ratios. For vascular dementia, risk estimates regarding the individual blood cell components and their derived ratios were more pronounced than for all-cause dementia, but small numbers led to wider confidence intervals (n=31).

Table 1 Baseline characteristics of the included and excluded study participants.

Characteristic	Included participants (N = 8313)	Excluded participants (N = 1528)#	
		No blood measurements (N = 1288)	Unknown APOE genotype (N = 240)
Age, year, mean (SD)	61.1 (7.4)	72.6 (11.8)	61.7 (8.2)
Women	4729 (56.9)	845 (65.6)	160 (66.7)
Education			
Primary	908 (11.0)	233 (18.4)	25 (11.7)
Lower	3329 (40.3)	537 (42.4)	95 (44.4)
Intermediate	2429 (29.4)	336 (26.5)	57 (26.7)
Higher	1588 (19.2)	161 (12.7)	37 (17.3)
Body mass index, kg/m^2, mean (SD)	27.6 (4.3)	27.6 (4.5)	28.2 (4.8)
Smoking status			
Current	1595 (19.3)	308 (24.4)	57 (24.5)
Former	4191 (50.7)	550 (43.6)	106 (45.5)
Diabetes mellitus	501 (6.0)	136 (10.7)	15 (6.3)
History of stroke	305 (3.7)	54 (4.2)	11 (4.6)
APOE-ε4 carrier status	2328 (28.0)	244 (30.8)	
Blood cell types, 10^9/L, median (IQR)			
Granulocytes	3.8 (1.6)		4.0 (1.7)
Platelets	263 (84)		277 (87)
Lymphocytes	2.2 (0.8)		2.3 (0.9)
Blood cell-based ratios, median (IQR)			
Granulocyte-to-lymphocyte ratio	1.7 (0.9)		1.7 (0.8)
Platelet-to-lymphocyte ratio	120 (55)		119 (54)
Systemic immune-inflammation index	455 (280)		473 (312)

Abbreviations: *APOE*, apolipoprotein E; IQR, interquartile ratio; N = number of participants; SD, standard deviation.

Values are shown before multiple imputation and therefore not always add up to 100%.

Data are presented as number (percentage) of participants unless otherwise indicated.

Excluded participants in this table only include those participants who were excluded due no complete blood measurements or unknown *APOE-ε4* carrier status.

Table 2 Association between blood cell counts and derived ratios, and risk of all-cause dementia.

Laboratory assessment#	All-cause dementia (n/N = 664/8313)	
	Model I	**Model II**
	HR (95% CI)	**HR** (95% CI)
Granulocytes	1.14 (0.87-1.50)	1.07 (0.80-1.43)
Corrected for platelets and lymphocytes	1.33 (0.99-1.79)	1.22 (0.89-1.67)
Platelets	1.48 (1.11-1.96)*	1.43 (1.08-1.90)*
Corrected for granulocytes and lymphocytes	1.48 (1.10-2.00)*	1.45 (1.07-1.95)*
Lymphocytes	0.80 (0.64-0.99)*	0.81 (0.64-1.03)
Corrected for granulocytes and platelets	0.76 (0.61-0.96)*	0.78 (0.62-1.00)
Granulocyte-to-lymphocyte ratio	1.34 (1.10-1.63)*	1.26 (1.03-1.53)*
Platelet-to-lymphocyte ratio	1.29 (1.08-1.55)*	1.27 (1.05-1.53)*
Systemic immune-inflammation index	1.18 (1.02-1.39)*	1.15 (0.98-1.34)

Abbreviations: CI = confidence interval; HR = hazard ratio; n = number of incident dementia events; N = number of participants for analysis. Model I is adjusted for age and sex. Model II is adjusted for age, sex, education, smoking status, body mass index, diabetes mellitus, history of stroke, and *APOE4* ε4 carrier status.

[#] All types of blood cells and their derived ratios were natural logarithmic transformed.

[*] Indicates statistically significant result.

Stratified analyses showed that the association between the ratios and dementia was particularly pronounced in participants aged below the median age of 65.4 years, women, and non-smokers (**Figure 2**). However, formal interaction terms did not reach statistical significance. Also, no significant effect modification was observed across different strata of these variables for the association between granulocyte, platelet, and lymphocyte counts, and risk of dementia (**Figure 3**).

Table 3 Association between blood cell counts derived ratios, and risk of all-cause dementia and dementia subtypes.

Laboratory assessment#	All-cause dementia, censored for stroke (n/N = 579/8008)	Alzheimer's disease (n/N = 543/8313)	Vascular dementia (n/N = 31/8313)
	HR (95% CI)	HR (95% CI)	HR (95% CI)
Granulocytes	1.13 (0.83-1.56)	1.03 (0.75-1.42)	1.99 (0.52-7.55)
Corrected for platelets and lymphocytes	1.36 (0.96-1.93)	1.12 (0.79-1.58)	1.92 (0.44-8.41)
Platelets	1.45 (1.07-1.96)*	1.59 (1.17-2.17)*	3.86 (1.02-14.6)*
Corrected for granulocytes and lymphocytes	1.47 (1.07-2.02)*	1.63 (1.18-2.27)*	3.39 (0.84-13.7)
Lymphocytes	0.80 (0.62-1.02)	0.85 (0.66-1.10)	0.76 (0.25-2.30)
Corrected for granulocytes and platelets	0.76 (0.58-0.98)*	0.81 (0.62-1.06)	0.64 (0.20-2.03)
Granulocyte-to-lymphocyte ratio	1.33 (1.07-1.65)*	1.17 (0.95-1.46)	1.85 (0.74-4.62)
Platelet-to-lymphocyte ratio	1.31 (1.07-1.60)*	1.30 (1.06-1.60)*	1.99 (0.82-4.81)
Systemic immune-inflammation index	1.19 (1.01-1.41)*	1.15 (0.97-1.37)	1.77 (0.87-3.63)

Abbreviations: CI = confidence interval; HR = hazard ratio; n = number of incident dementia events; N = number of participants for analysis. Models are adjusted for age, sex, education, smoking status, body mass index, diabetes mellitus, history of stroke, and *APOE4* ε4 carrier status.

[#] All types of blood cells and their derived ratios were natural logarithmic transformed.

[†] Number of participants for analysis is 8313 minus participants with a history of stroke (n=305).

[*] Indicates statistically significant result.

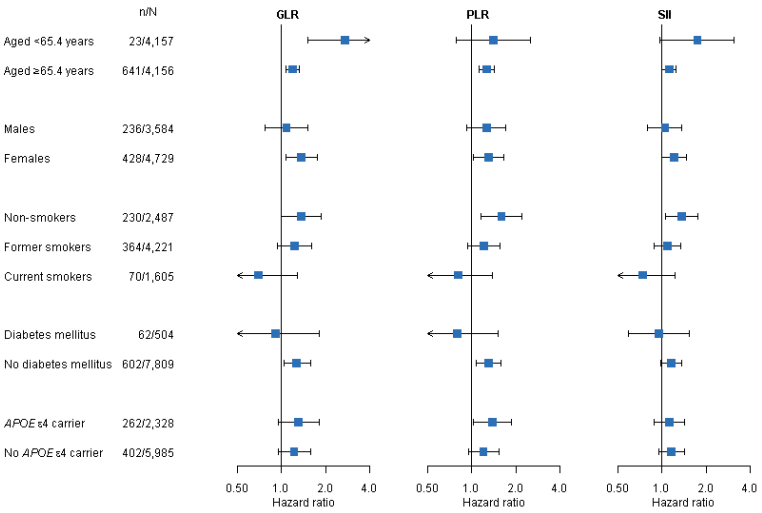

Figure 2 *Forest plots of the association of the GLR, PLR, and SII, and risk of dementia.* Hazard ratios are shown in logarithmic scale with stratification by median age, sex, smoking status, diabetes mellitus, and *APOE-ε4* carrier status.
Abbreviations: APOE, apolipoprotein E; n = number of incident dementia events; N = number of participants for analysis.

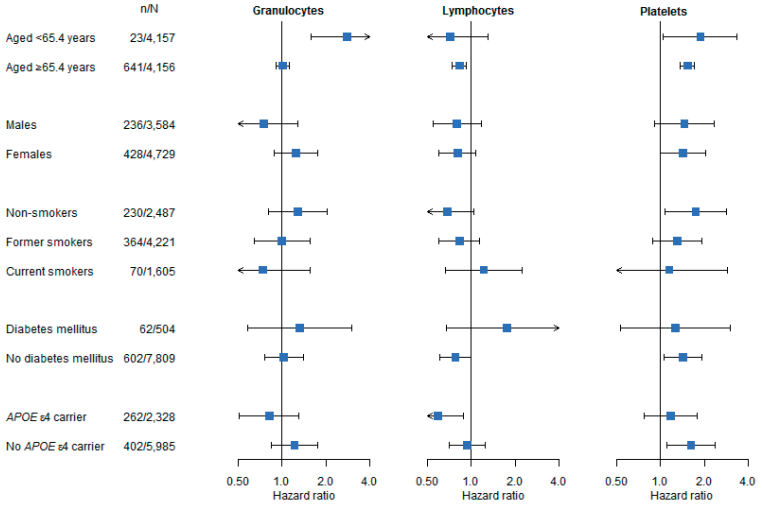

Figure 3 *Forest plots of the association of granulocytes, platelets, and lymphocytes, and the risk of dementia.* Hazard ratios are shown in logarithmic scale with stratification by median age, sex, smoking status, diabetes mellitus, and *APOE-ε4* carrier status. Abbreviations: APOE, apolipoprotein E; n = number of incident dementia events; N = number of participants for analysis.

DISCUSSION

In this population-based study, we found that higher levels of granulocyte and platelet counts are related to an increased risk of dementia, whereas a higher lymphocyte count is associated with a decreased dementia risk. Furthermore, higher levels of their derived ratios, i.e. GLR, PLR, and SII are associated with an increased risk of all-cause dementia, including its subtype AD and even more with vascular dementia.

Activation of the immune systems can result in inflammation by production of different cytokines [33]. These cytokines can act as a link between the innate and the adaptive immune system, having pro- or anti-inflammatory effects depending on the type of cytokine [34]. A recent meta-analysis of 175 studies suggests that AD is accompanied by an inflammatory response and that this can be reflected by a variety of systemic cytokines, for instance interferon-γ, interleukin(IL)-2, and in particular IL-6, of which dysregulation has been associated with multiple chronic inflammatory diseases [35, 36]. It is now recognized that systemic inflammation can trigger or exacerbate the inflammatory environment of the brain, thereby contributing to chronic neuroinflammation and neurodegeneration [37]. A plausible explanation for the occurrence of this chronic neuroinflammation in (pre)demented individuals involves a disruption of a process called resolution [38]. Resolution is an active process that halts the acute phase of inflammation and restores tissue homeostasis. The acute inflammatory phase is usually initiated in response to infection, neoplasia, tissue injury, or other major homeostatic stressors. This phase is accompanied by the increased release of pro-inflammatory mediators such as prostaglandins, leading to leukocyte recruitment. Normally, resolution would clear the recruited granulocytes [39]. However, it has been shown that failure of resolution, induced by any chronic inflammatory state, is associated with an over-active innate immune system, resulting in the development of chronic inflammation, which could subsequently lead to AD [38, 40, 41]. Our finding that an increase in the granulocyte count, resulting in a higher GLR and SII, is associated with an increased risk of dementia could therefore support the role of insufficient resolution in the pathogenesis of dementia.

Only few studies examined the interplay between the innate and adaptive immunity by studying levels of these blood cell-based ratios in dementia patients. Two cross-sectional studies showed that NLR and PLR were elevated in AD patients compared to dementia-free controls [17, 18]. In contrast, a longitudinal study assessing the trajectory of NLR found no significant difference in its longitudinal evolution between AD patients and dementia-free participants [19]. Although they examined differences between AD patients and dementia-free controls, they did not investigate the risk of developing dementia in dementia-free participants in relation to their levels of NLR. In the present study, we did take the time until dementia into account by a joint modelling approach and were therefore able to assess the risk of dementia in relation to the change of blood cell counts and their derived ratios.

Interestingly, recent evidence shows that the NLR and PLR are partly genetically determined with 36% estimated heritability for NLR and 64% for PLR in a healthy population [42]. Moreover, different single nucleotide polymorphisms (SNPs) identified through genome-wide association study (GWAS) were significantly related to the PLR phenotype, but not to NLR [43]. Importantly, some but not all of these SNPs were also related to platelet, indicating that these SNPs capture the interplay between platelets and lymphocytes. Thus far no GWAS for SII has been performed. Exploring the dementia risk by genetically predicted blood cell-based ratios may provide more insight in the causal role of immunity in dementia.

Strengths of our study include the population-based setting and the thorough follow-up for dementia. Another strength is the prospective design of this study, with the blood cell counts being measured at multiple time points. Using an innovative statistical method, we combined these repeated measurements with dementia as survival outcome. Moreover, we used blood cell counts and their derived ratios, which low-cost and easy to implement in the clinic and other research settings. Although these ratios are proven to be associated with chronic systemic inflammation, we need to emphasize that it is unknown whether higher levels of GLR, PLR, and SII are functional and cause higher levels of pro-inflammatory cytokines. To identify the actual involved immune cell populations determination of different cytokines is still needed. Furthermore, the innate and adaptive immune systems are overlapping, making it difficult to completely distinguish their separate effects. In addition, we used the granulocyte count as proxy for the neutrophil count. Although the relative proportion of neutrophils compared to eosinophils and basophils may be lower in persons with several specific diseases such as parasitic infections, asthma, or immune diseases, neutrophils are generally the most important subtype of granulocytes. If anything, misclassification of the granulocytes would be non-differential and would therefore lead to underestimation of the estimates [11]. In addition, we cannot rule out reversed causality, i.e. that dementia is subclinical at time of the laboratory assessments and causes higher levels of GLR, PLR, and SII. Lastly, we did not have the power to study other neurodegenerative diseases beyond dementia such as Parkinson's disease or amyotrophic lateral sclerosis. It would be interesting for future studies to also investigate the relation between inflammation and these diseases.

In conclusion, higher levels of the ratios GLR, PLR, and SII are associated with an increased risk of developing dementia in the general population. Higher activation of the innate immune system reflected by higher levels of granulocytes and platelets, is associated with an increased dementia risk, while the adaptive immune system is suggested to be more neuroprotective. These findings support the role of dysregulation of the immune systems in the pathogenesis of dementia. Further studies are warranted to assess during which phase of the pathogenesis of dementia immunity is involved and to assess causality in order to develop prevention and therapeutic strategies.

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SUPPORTING INFORMATION

Supplementary Table 1 Overview of median blood cell counts and blood cell-based ratios measured per Rotterdam Study assessment round.

Laboratory assessment	First assessment round# (N=8313)	Second assessment round† (N=5663)	Third assessment round* (N=1886)
Blood cell types, 10^9/L, median (IQR)			
Granulocytes	3.8 (1.6)	4.0 (1.6)	3.6 (1.5)
Platelets	263 (84)	262 (83)	224 (75)
Lymphocytes	2.2 (0.8)	2.2 (0.8)	1.9 (0.9)
Blood cell-based ratios, median (IQR)			
Granulocyte-to-lymphocyte ratio	1.7 (0.9)	1.8 (0.9)	1.9 (1.1)
Platelet-to-lymphocyte ratio	120 (55)	116 (53)	117 (59.1)
Systemic immune-inflammation index	455 (280)	461 (283)	421 (290)

Abbreviations: IQR, interquartile ratio; N = number of participants; RS = Rotterdam Study.

[#] The first measurement corresponds with the fourth round of RS-I, second round of RS-II, and first round of RS-III.

[†] The second measurement corresponds with the fifth round of RS-I, third round of RS-II, and second round of RS-III.

[*] The third measurement corresponds with the sixth round of RS-I and the fourth round of RS-II.

2.2 A Mendelian randomization study

ABSTRACT

Importance: Observational studies have shown associations of circulating biomarkers of immunity and inflammation on Alzheimer's disease risk, but it is uncertain whether the observed associations are causal.

Objective: To explore the association between genetically predicted circulating levels of immunity and inflammation and the risk of Alzheimer's disease (AD) and hippocampal volume.

Design: A two-sample Mendelian Randomization Study using large-scale genomic data.

Setting: Genetic variants associated with immunity and inflammation were selected from publicly available summary statistics.

Participants: Associations with AD were evaluated in The International Genomics of Alzheimer's Project (IGAP) GWAS dataset (21,982 cases; 41,944 controls of European ancestry). For hippocampal volume, we extracted data from a GWAS meta-analysis on 33,536 participants of European ancestry.

Exposures: We identified 12 markers of immune cells and derived ratios (platelet count, eosinophil count, neutrophil count, basophil count, monocyte count, lymphocyte count, platelet-to-lymphocyte ratio, monocyte-to-lymphocyte ratio, CD4 count, CD8 count, CD4-to-CD8 ratio, CD56) and 5 signaling molecules (IL-6, fibrinogen, CRP, Lp-PLA2 activity and mass) as primary exposures of interest. Other genetically available immune biomarkers with a weaker a priori link to AD were considered secondary exposures.

Main Outcomes and Measures: Clinically diagnosed AD and hippocampal volume as measured by high-resolution MRI brain scans.

Results: None of the primary or secondary genetically predicted circulating levels of immunity and inflammation showed statistically significant associations with AD or with hippocampal volume following *P*-value correction for multiple comparisons using false discovery rate<5% (*Q*-value<0.05). CD4 count showed the strongest suggestive association with AD (Odds Ratio 1.32, $P<0.01$, $Q>0.05$). There was evidence for heterogeneity in the MR inverse variance–weighted meta-analyses as measured by Cochran *Q* and weighted median and weighted mode for multiple exposures. Further cluster analyses did not reveal clusters of variants that could influence the risk factor in distinct ways.

Conclusions and Relevance: This study suggests that genetically predicted circulating biomarkers of immunity and inflammation are not associated with AD risk or hippocampal volume. Future studies should assess competing risk, explore in more depth the role of adaptive immunity in AD, in particular T cells and the CD4 subtype, and confirm these findings in other ethnicities.

INTRODUCTION

The immune system is increasingly recognized to be involved in the pathogenesis of Alzheimer's disease.[1,2] Recent genome-wide association studies (GWASs) have established AD risk loci within or near genes that are expressed in microglia.[3] This led to the concept of the innate immune system being involved in the early steps of the disease, and thus much effort has been dedicated in studying innate immunity in relation to AD. A recent meta-analysis of observational studies revealed that the immune-related signaling molecules C-reactive protein (CRP), interleukin (IL)-6, α1-antichymotrypsin, lipoprotein-associated phospholipase A2 (Lp-PLA2) activity, and fibrinogen were each associated with risk of all-cause dementia.[4] Less is known about the contribution of the adaptive immune system in relation to AD, but a recent observational study discovered clonally expanded $CD8^+$ T effector memory cells in the cerebrospinal fluid of AD patients, revealing an adaptive immune response in AD.[5] Moreover, we previously found that higher levels of innate immune cells lead to a higher dementia risk, whereas higher levels of adaptive immune cells are protective for developing dementia.[6] Given the observational design of the majority of available studies and the difficulty of studying the effect of the immune system on AD in a trial, it is uncertain whether the observed associations are causal, i.e. independent of other risk factors, and not biased by reverse causation.[7]

Mendelian randomization (MR) exploits genetic variants influencing the exposure of interest as unbiased proxies for the exposure, i.e. instruments, to infer causality.[8] To our knowledge, there are only few MR studies performed where the association between circulating biomarkers of immunity and inflammation and dementia was studied.[9-13] Moreover, a large GWAS meta-analysis on hippocampal volume[14] allows exploration of these biomarkers with hippocampal volume as imaging endophenotype of AD, which to date has not been performed. Furthermore, since GWAS studies are increasing in size, the number of instruments that can be used to estimate the causal effect of a risk factor on an outcome also increases. This could lead to more heterogeneity among the causal estimates obtained from multiple genetic variants, pointing to a possible violation of the necessary instrumental variable assumptions, but also to a scenario in which causal estimates based on each variant in turn differ more strongly than expected by chance alone. These variants could then be divided into distinct clusters, such that all variants in the cluster have similar causal estimates. There are now novel techniques available that allow for cluster analyses of variants, which can capture distinct causal mechanisms by which a risk factor influences an outcome with different magnitudes of causal effect.[15]

Here, by leveraging data from large-scale genomic studies on circulating biomarkers of immunity and inflammation and the large AD dataset from The International Genomics of Alzheimer's Project (IGAP) GWAS[3] and hippocampal volume GWAS[14], we implemented a two-sample MR study to (1) explore the associations between genetic predisposition to

higher or lower circulating levels of immune cells[12,16] and signaling molecules[13,17-19] with risk of AD; (2) explore the associations between genetic predisposition to these biomarkers with hippocampal volume; (3) explore the associations between genetic predisposition to circulating biomarkers of immunity and inflammation with limited a-priori evidence with AD and hippocampal volume; and (4) explore whether different genetic variants influence the exposures and thus AD and hippocampal volume in distinct ways by performing cluster analyses.

METHODS

Study Design, Data Sources and Genetic Instrument Selection

Table 1 summarizes the data sources used in the current MR study. The genetic instruments were taken from publicly available summary statistics. For each of the circulating immunity traits, we selected single-nucleotide polymorphisms (SNPs) associated with their circulating levels at a genome-wide threshold of significance ($P<5\times10^{-8}$). After extracting the summary statistics for significant SNPs, we pruned all SNPs in linkage disequilibrium (LD) ($r^2<0.01$ in the European 1000 Genomes Project reference panel), retaining SNPs with the lowest P value as independent instrument. For some exposures (IL-6, CRP, Lp-PLA2, 23 cytokines and IL-1) we used previously selected instruments (**Table 1**). We identified independent SNPs significantly associated with circulating biomarker levels of immunity and inflammation and merged these with the outcome datasets; the SNPs that were also available in the outcome datasets were used as final instruments for analysis. As all analyses are based on publicly available summary statistics and not individual-level data, no ethical approval from an institutional review board was required.

Primary exposures (biomarkers of immunity and inflammation with strong a-priori evidence)

To minimize weak instrument bias and maximize power, we carefully selected our primary exposures prior to data analysis based on the underlying GWAS size, population characteristics and a priori evidence for the associations with AD (**Table 1**).[20] We identified 12 immune cells and derived ratios (platelet count, eosinophil count, neutrophil count, basophil count, monocyte count, lymphocyte count, platelet-to-lymphocyte ratio, monocyte-to-lymphocyte ratio, CD4 count, CD8 count, CD4 to CD8 ratio, CD56) and 5 signaling molecules (IL-6, fibrinogen, CRP, Lp-PLA2 activity and mass) as primary exposures of interest.

Table 1. Study design and data sources MR.

Discovery GWAS	Phenotype	Sample size	Ancestry	Adjustments	
Primary exposures (biomarkers of immunity and inflammation with strong a-priori evidence**)**					Instruments
Immune cells					
UK Biobank/ UK BiLEVE/ INTERVAL	Lymphocyte count, granulocyte count, platelet count, monocyte count, basophil count, eosinophil count	171,643 individuals	European	Age, sex, body mass index, alcohol consumption, and smoking status	As performed by Astle et al. [12]
NTR	Neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR)	5,901 individuals	European	age, sex and genotype platform	Only instruments for PLR were available[16]
NTR	Monocyte to lymphocyte ratio	5,892 individuals	European	Age, sex, principal components, genotype platform	As described by Lin et al.[35]
International HIV Controllers study	CD4:CD8 lymphocyte ratio, CD3, CD4, CD8-positive T and CD19-positive B lymphocytes	2,538 individuals	European	age and sex effects	Derived from summary statistics from Ferreira et al.[36]
Signaling molecules					
INTERVAL study/ CHARGE Inflammation Working Group	IL-6	204,402 individuals	European	age, sex, duration between blood draw and processing, population structure	As selected by Georgakis et al.[17]
CHARGE Inflammation Working Group [18]	Fibrinogen levels	120,246 individuals	European	age, sex, population structure	as selected by Ward-Caviness et al.[37]
CHARGE Inflammation Working Group	CRP levels	204,402 individuals	European	age, sex, population structure	As selected by Georgakis et al.[17]

Table 1. Study design and data sources MR. (continued)

Discovery GWAS	Phenotype	Sample size	Ancestry	Adjustments	
CARDIoGRAM consortium [38]	Lp-PLA2 activity/ mass	12,126 individuals	European	age, sex	As selected by Casas et al.[19]
Secondary exposures (biomarkers of immunity and inflammation with limited a-priori evidence)					Instruments
FINRISK/ CRYFS	Circulating levels of 41 cytokines and growth factors	8,293 individuals	Finnish	age, sex, BMI	For 23 cytokines as selected by Georgakis et al.[22]
Cardiovascular Health Study (>65 years)**/ InCHIANTI** [39]	IL-1	4,500 individuals	European	age, gender and the first 10 principal components reflecting background ancestry.	As selected by Freitag et al.[40]
CHARGE [41]	ICAM-1	9813 individuals	European	Age and sex, sampling design	From genome-wide significant SNPs
Women's Genome Health Study	ICAM-1	22,435 women	European	sub-Caucasian ancestry, age, menopause, smoking, BMI	Used only the 4 novel loci that have been replicated in CHARGE[42]
CHARGE [41]	P-selectin	4,115 Genome-wide significant SNPs	European	Age and sex, sampling design	From genome-wide significant SNPs
Primary Outcomes					Source
IGAP	Alzheimer's disease	21,982 cases, 41,944 controls	European	sex and age	[43]
ENIGMA and the CHARGE consortia	Hippocampal volume	33,536 individuals	European	4 MDS components, age, age^2, sex, intracranial volume and diagnosis (when applicable)	[14]

GWAS names: UK BiLEVE, UK Biobank Lung Exome Variant Evaluation; NTR, Netherlands Twin Register; CHARGE, Cohorts for Heart and Aging Research in Genomic Epidemiology; CARDIoGRAM, Coronary ARtery DIsease Genome-wide Replication and Meta-analysis; CRYFS, Cardiovascular Risk in Young Finns Study; InCHIANTI, aging in the Chianti area; IGAP, International genomics of Alzheimer's project.
Phenotypes: CD, cluster of differentiation; IL, interleukin; CRP, C-reactive protein; Lp-PLA2, lipoprotein-associated phospholipase A2; ICAM-1, Intercellular Adhesion Molecule 1.

Secondary exposures (biomarkers of immunity and inflammation with limited a-priori evidence)

Other immune-related exposures, for which there are less validated biomarkers of immunity and inflammation or less valid instruments available, were selected as secondary exposures (**Table 1**). More specifically, a GWAS identified multiple common genetic variants that influence circulating levels of 41 cytokines and growth factors[21], of which we used pre-selected instruments for 23 cytokines or growth factors that were not in linkage disequilibrium and not associated with levels of >1 cytokine.[22] These instruments have a weaker a priori link to AD and were therefore selected as secondary exposures. Furthermore, we used genetic instruments for IL-1, ICAM-1 and P-selectin as additional secondary exposures due to smaller powered underlying GWASs.

Outcomes

The primary outcome for this study was AD defined by clinical diagnosis of AD. In addition, we looked at hippocampal volume as an imaging AD endophenotype as hippocampal degeneration is one of the pathological hallmarks of AD. We extracted estimates for the associations of the selected instruments with AD from The International Genomics of Alzheimer's Project (IGAP) GWAS dataset (21,982 cases; 41,944 controls of European ancestry).[3] For hippocampal volume, we extracted data from publicly available summary statistics of the Cohorts for Heart & Aging Research in Genomic Epidemiology - Enhancing Neuro Imaging Genetics through Meta Analysis (CHARGE-ENIGMA) GWAS meta-analysis on 33,536 participants of European ancestry.[14]

Our power calculations[23] revealed that based on the sample size of IGAP we had sufficient power for most biomarkers of immunity and inflammation to detect meaningful effect sizes. Specifically, we had >80% power to detect significant associations with AD for 8 out of 12 immune cells and for 3 out of 5 signaling molecules at an effect size (odds ratio [OR]) of 1.10 or 0.90 (**Table S1**). All markers were analysed, even when potentially underpowered, to guide future research.

Statistical analysis

We first extracted data and harmonized the effect alleles across GWASs. The MR association between each immune cell or signaling molecule and AD or hippocampal volume was then estimated using the Wald estimator and the delta method after pooling individual SNP MR estimates using inverse variance-weighted (IVW) meta-analysis.[24] Fixed-effect IVW was used in the absence of heterogeneity and random-effects in the presence of heterogeneity (Cochran *Q*-derived $P<0.05$). Statistical significance for the MR associations with AD and hippocampal volume were set at a *P*-value corrected for multiple comparisons using false discovery rate (FDR)<5%. A $P<0.05$ but above the FDR-corrected threshold, was considered as suggestive for an association. These analyses were repeated for the secondary exposures

with AD and hippocampal volume and we set a separate corrected *P*-value for multiple comparisons of secondary exposures using FDR<5%.

Cochran's *Q*-derived *P*<0.05 from the IVW MR was used as indicator of possible horizontal pleiotropy.[25] For markers with >2 SNPs showing either significant or suggestive associations or significant heterogeneity in the primary IVW MR analysis, we conducted additional sensitivity analyses that vary in their underlying assumptions regarding the presence of pleiotropic genetic variants that may be associated with the outcome independently of the exposure. In particular, we used the weighted median method, which requires that at least half of the information for the MR analysis comes from valid instruments.[26] We also used the weighted mode approach, which requires that the largest number of similar (identical in infinite samples) individual-instrument causal effect estimates comes from valid instruments, even if the majority of instruments are invalid.[27] For consistency with other literature and to further relax the IVW assumptions, we used MR-Egger regression, which provides a consistent estimate of the causal effect, under a weaker assumption—the InSIDE (Instrument Strength Independent of Direct Effect) assumption.[28] In addition, we used the contamination mixture method, which is implemented by constructing a likelihood function based on the variant-specific causal estimates. If a genetic variant is a valid instrument, then its causal estimate will be normally distributed about the true value of the causal effect, but if a genetic variant is not a valid instrument, then its causal estimate will be normally distributed about some other value.[29] We also tested for outlier SNPs using MR-Pleiotropy Residual Sum and Outlier.[30]

Finally, since it is possible that different genetic variants influence the risk factor in distinct ways, e.g. via distinct biological mechanisms, we further examined heterogeneity by performing cluster analyses using the MR-Clust package.[15] As recommended, we implemented this method conservatively, i.e. only assigning a variant to a cluster if the conditional probability of cluster assignment is ≥0.8, and only reporting a cluster if at least 4 variants satisfy this criterion. Variants that do not satisfy these criteria and that do not fit into a null cluster will be assigned to a 'junk' cluster. Immune cells or signaling molecules that showed suggestive associations and for which more genome-wide significant SNPs were available were also explored for potential clustering of variants.

Statistical analyses were conducted in RStudio (R version 3.6.3).

RESULTS

Primary exposures with AD

The primary results of the MR analyses for the genetic variants of immune cells and signaling molecules with AD are presented in **Figure 1**. Following *P*-value correction for multiple comparisons using FDR<5% (*Q*-value<0.05), none of the immune cells or signaling mol-

ecules showed statistically significant associations with AD. CD4 count showed the strongest suggestive association with AD by an Odds Ratio of 1.32, *P*=0.005, *Q*=0.170 (*P*<0.01, *Q*>0.05) with the next strongest suggestive association being between CRP and AD with *P*=0.029 (*P*<0.05, *Q*>0.05).

Primary exposures with hippocampal volume

The primary results of the MR analyses for the immune cells and signaling molecules with hippocampal volume are presented in **Figure 1** and **Table S1**. None of the immune cells or signaling molecules showed statistically significant associations with hippocampal volume following *P*-value correction for multiple comparisons. Only platelet-to-lymphocyte ratio (PLR) showed a suggestive *P*=0.037 (*P*<0.05, *Q*>0.05) association with hippocampal volume.

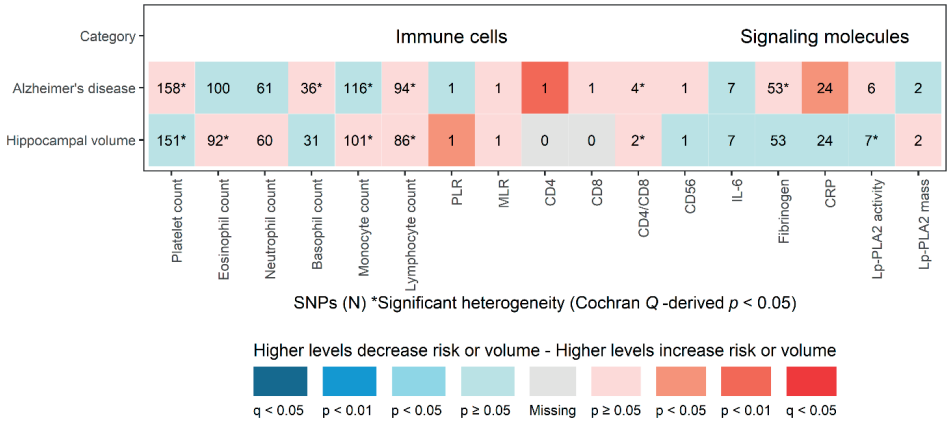

Figure 1. Primary Mendelian randomization associations of circulating immune cell and signaling molecule levels with Alzheimer's disease and hippocampal volume.
Shown are the results derived from the primary inverse variance-weighted meta-analysis. None of the immune cells or signaling molecules survived the multiple testing threshold of false discovery rate<5% (q<0.05). PLR indicates platelet-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; CD, cluster of differentiation; IL, interleukin; CRP, C-reactive protein; Lp-PLA2, Lipoprotein-associated phospholipase A2.

Secondary exposures with AD and hippocampal volume

The secondary results of the MR analyses are presented in **Figure 2** and **Table S2**. Similarly, none of the biomarkers of immunity and inflammation showed statistically significant associations with AD or hippocampal volume following *P*-value correction for multiple comparisons. MIP-1b showed a suggestive association with AD with *P*=0.024 (*P*-value<0.05, *Q*>0.05) while SCF (*P*=0.031) and ICAM-1 (*P*= 0.016) showed suggestive associations with hippocampal volume (*P*<0.05 level, *Q*>0.05).

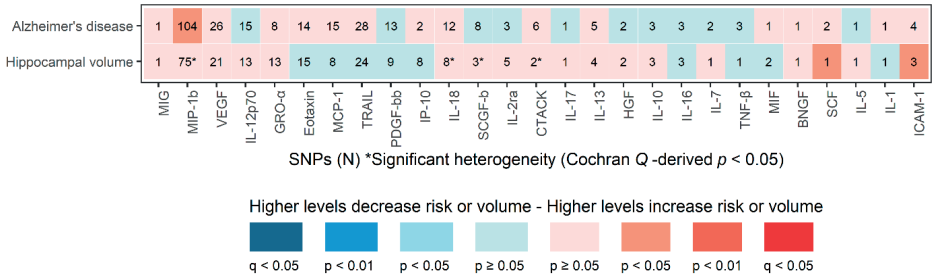

Figure 2. Secondary Mendelian randomization associations of circulating cytokines and growth factors with Alzheimer's disease and hippocampal volume.

Shown are the results derived from the secondary inverse variance-weighted meta-analysis. None of the immune traits survived the multiple testing threshold of false discovery rate<5% (q<0.05). MIG indicates monokine induced by gamma interferon indicates; MIP-1b, macrophage inflammatory protein 1 beta; VEGF, vascular endothelial growth factor; IL, interleukin; GRO-α, growth-regulated oncogene alpha; MCP-1, monocyte chemoattractant protein-1; TRAIL, TNF-related apoptosis-inducing ligand; PDGF-bb, platelet-derived growth factor-bb; IP-10, interferon gamma-induced protein 10; SCGF-b, stem cell growth factor beta; CTACK, cutaneous T-cell-attracting chemokine; HGF, hepatocyte growth factor; TNF-related apoptosis-inducing ligand; MIF, macrophage migration inhibitory factor; BNGF indicates beta nerve growth factor; SCF, stem cell factor; ICAM-1, Intercellular Adhesion Molecule 1.

Sensitivity analyses

There was evidence for heterogeneity in the primary as well as the secondary MR IVW analyses as measured by Cochran *Q*. Alternative tests furthermore revealed varying estimates changing direction for multiple exposures (**Table S3**). Cluster analyses did not reveal clusters of variants (**Figure 3**).

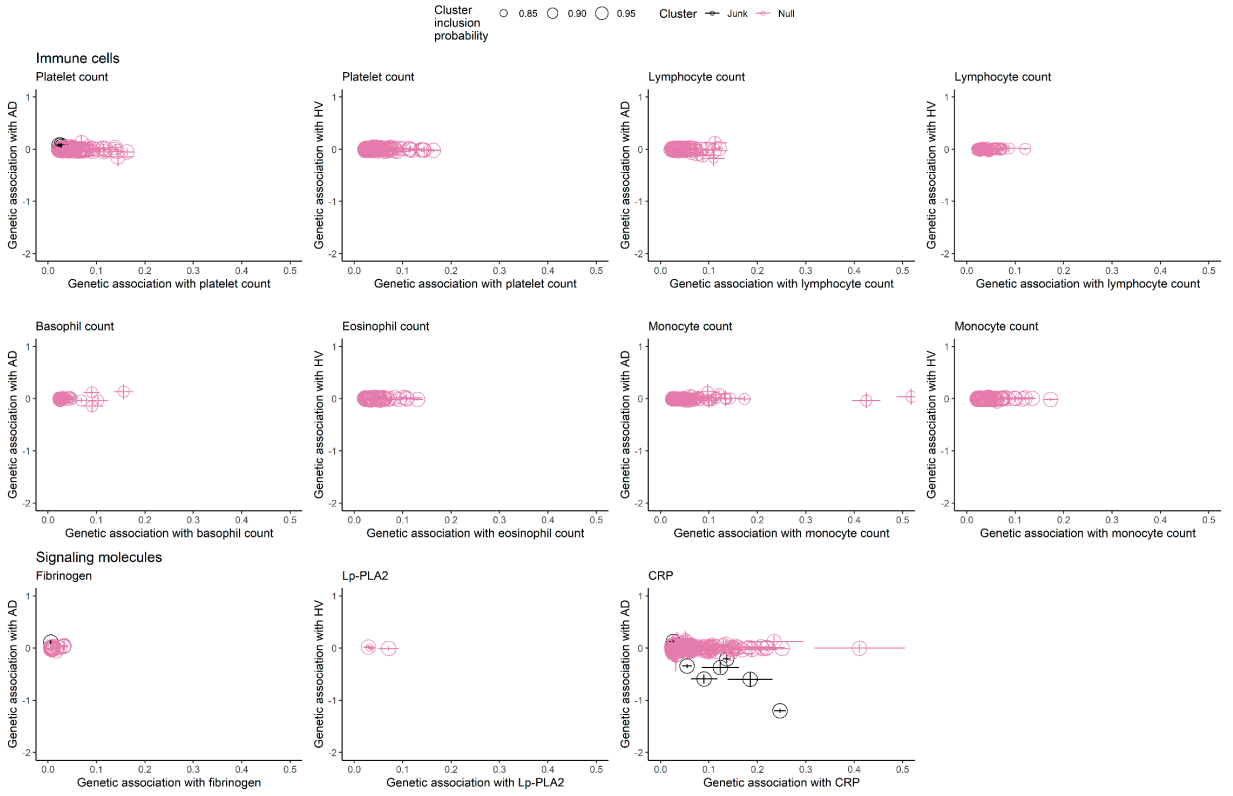

Figure 3. Exploration of heterogeneity by cluster analyses. Shown are the genetic associations for the individual variants with the exposure and outcome; lines indicate confidence intervals. When restricting to a cluster probability assignment of ≥0.8 and ≥4 variants per cluster, no clusters of variants were identified. CRP, C-reactive protein; Lp-PLA2, Lipoprotein-associated phospholipase A2; AD, Alzheimer's disease; HV, hippocampal volume. The junk cluster denotes variants with estimates that do not fit in any cluster.

DISCUSSION

Exploring genetically predicted circulating biomarkers of immunity and inflammation in an adequately powered two-sample MR approach involving the largest GWAS datasets available, we found no association between genetically predicted circulating levels of immune cells and signaling molecules as primary exposures and AD or hippocampal volume. Similarly, none of the secondary exposures including genetically predicted levels of biomarkers of immunity and inflammation showed statistically significant associations with AD or hippocampal volume. Sensitivity analyses showed evidence for heterogeneity, but we found no clustering of variants.

Our findings are in contrast with observational studies that reported on significant associations between several circulating blood biomarkers of signaling molecules, immune cells and AD.[4,6] For example, we expected to find an association between higher platelet count and higher AD risk, since we previously found that an increase of circulating platelets as measured in blood over time increased AD risk.[6] However, if the risk factor is a protein biomarker, such as CRP, we can select genetic variants located in or around the coding region for that protein as instruments. This is more difficult for polygenic risk factors such as platelets, as the influence of genetic variants on such a risk factor is unlikely to be specific.[31] Indeed, we found substantial heterogeneity when studying immune cells and signaling molecules, but could not find meaningful clusters of genetic variants that could have a distinct effect on the risk factor, supporting the conclusion that our findings are truly null. On the other hand, our power calculations revealed that some analyses were underpowered to detect significant associations, for example for platelet count and AD. However, these exposures in particular did not even show suggestive associations with AD, implying that estimates are very small and probably – even if sufficiently powered - would not have survived correction for multiple testing

The strongest suggestive association we found in our study was between CD4 cell count and AD, where higher levels of CD4 cell count increase AD risk, although only one SNP could be used as an instrument. This suggestive association is unexpected, since HIV-associated dementia is accompanied by a lower CD4 cell count.[32] However, it is recognized that T lymphocytes play a central role in the pathogenesis of multiple sclerosis (MS), with CD4+ T cells predominating in acute MS lesions.[33] Combined with the recent finding that clonally expanded $CD8^+$ T effector memory cells have been found in the cerebrospinal fluid of AD patients[5], the role of adaptive immunity in AD, in particular T cells and the CD4 subtype, is worthwhile investigating further in relation to AD.

In contrast to our findings, one MR study[13] found a protective effect of CRP on AD. An explanation for this difference could be the selection of instruments for CRP. In our study we used 24 SNPs as instruments that are gene-specific for CRP, thereby reducing pleiotropy. When examining CRP further by performing a cluster analysis including all genome-wide

significant SNPs, we found no clusters of variants, in particular no cluster forming a biologically meaningful protective pathway of CRP on AD.

Our study has limitations. First, we could not assess competing risk by for example mortality in this study, which could generate paradoxical MR estimates.[34] Second, we cannot exclude that the additional adjustments for body mass index, alcohol consumption and smoking status performed in the blood cell trait GWAS[12] led to collider bias (i.e. a collider between a genetic variant and confounders of the risk factor-outcome association) during instrument selection. However, the potential impact of such collider bias is likely to be less than other biases.[34] Third, since we used multiple proposed instruments where effect heterogeneity is likely, effect estimates need to be interpreted with caution. Fourth, for some exposures, especially those reflecting adaptive immunity, we were limited by the few known genome-wide significant genetic variants influencing these traits, potentially leading to weak instrument bias. Targeted studies incorporating further GWAS data on individual circulating adaptive immune biomarkers might reveal additional associations not captured by our approach. Finally, the underlying study populations were of European ancestry, limiting generalizability to other ethnicities

In conclusion, this study suggests that genetically predicted circulating biomarkers of immunity and inflammation are not associated with AD risk or hippocampal volume. Future studies should assess competing risk, explore in more depth the role of adaptive immunity in AD, in particular T cells and the CD4 subtype and confirm these findings in other ethnicities.

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SUPPORTING INFORMATION

Table S1. Characteristics of the genetic instruments selected for the circulating levels of immune cells and signaling molecules, variance explained by the selected instruments and power calculations for the Mendelian randomization study based on the sample sizes of the IGAP dataset.

Exposure	SNPs (N)	Variance explained (R^2)	Odds Ratio found in this study	Odds Ratio * for α <0.05 and [1-β] >0.8
Immune cells				
Platelet count	158	0.124	1.005	1.010
Eosinophil count	100	0.064	0.950	0.930
Neutrophil count	61	0.038	0.941	0.911
Basophil count	36	0.015	1.172	1.161
Monocyte count	116	0.100	0.967	0.944
Lymphocyte count	94	0.055	1.035	1.081
PLR	1	0.119	0.999	0.948
MLR	1	0.000	1.423	12.000
CD4 count	1	0.016	1.317	1.156
CD8 count	1	0.022	1.015	1.131
CD4 to 8 ratio	4	0.130	1.043	1.052
CD56 count	1	0.316	1.102	1.033
Signaling molecules				
IL-6	7	0.008	0.926	0.816
Fibrinogen	53	0.002	1.614	1.505
CRP	24	0.061	1.106	1.077
Lp-PLA2 activity	6	0.003	1.145	1.400
Lp-PLA2 mass	2	0.001	0.610	0.751

*Shown are the Odds Ratios per 1 unit increase in circulating levels of the immune cells/signaling molecule, for which there is power (1-β) ≥80% to detect an existed association at a type I error of α <0.05. $R2 = (beta\ x\ \sqrt{2}\ x\ MAF(1 - MAF))2$, where MAF is the minimum allele frequency and beta is the effect of the SNP on the respective immune cell or molecule levels (Park et al 2010, Nat. Genet. 42, 570–575). Total variance was calculated in an additive model assuming no interaction between the individual SNPs.

Table S2. Results of secondary IVW MR analyses of the associations between cytokines and growth factors with Alzheimer's disease and hippocampal volume.

Outcome	Exposure	IVW Estimate	95% CI		P-value	Heterogeneity	Cochran Q-derived P	SNPs	FDR-corrected P
Alzheimer's disease	MIG	0.157	-0.044	0.359	0.126	-	-	1	0.906
Hippocampal volume	MIG	0.087	-0.035	0.210	0.161	-	-	1	0.906
Alzheimer's disease	MIP-1b	0.026	0.003	0.049	0.024	102.599	0.493	104	0.552
Hippocampal volume	MIP-1b	0.003	-0.014	0.020	0.744	98.840	0.029	75	0.946
Alzheimer's disease	VEGF	0.017	-0.022	0.055	0.405	19.211	0.787	26	0.906
Hippocampal volume	VEGF	0.003	-0.021	0.027	0.805	20.223	0.444	21	0.953
Alzheimer's disease	IL-12p70	-0.025	-0.078	0.028	0.360	9.125	0.823	15	0.906
Hippocampal volume	IL-12p70	0.010	-0.023	0.042	0.558	14.847	0.250	13	0.906
Alzheimer's disease	GRO-α	0.011	-0.032	0.054	0.612	11.118	0.134	8	0.906
Hippocampal volume	GRO-α	0.012	-0.014	0.038	0.383	8.691	0.276	13	0.906
Alzheimer's disease	Eotaxin	0.015	-0.047	0.077	0.637	14.952	0.310	14	0.906
Hippocampal volume	Eotaxin	-0.021	-0.058	0.016	0.273	14.299	0.282	15	0.906
Alzheimer's disease	MCP-1	0.009	-0.052	0.070	0.771	21.518	0.089	15	0.946
Hippocampal volume	MCP-1	-0.016	-0.052	0.020	0.386	14.787	0.393	8	0.906
Alzheimer's disease	TRAIL	0.029	-0.010	0.068	0.143	23.521	0.657	28	0.906
Hippocampal volume	TRAIL	-0.016	-0.038	0.007	0.181	13.184	0.948	24	0.906
Alzheimer's disease	PDGF-bb	-0.021	-0.091	0.049	0.550	13.461	0.336	13	0.906
Hippocampal volume	PDGF-bb	-0.028	-0.070	0.014	0.189	2.512	0.961	9	0.906
Alzheimer's disease	IP-10	0.060	-0.087	0.206	0.426	2.461	0.117	2	0.906
Hippocampal volume	IP-10	-0.041	-0.122	0.041	0.329	0.017	0.896	8	0.906
Alzheimer's disease	IL-18	0.025	-0.027	0.077	0.353	13.792	0.245	12	0.906
Hippocampal volume	IL-18	0.010	-0.038	0.059	0.672	14.975	0.036	8	0.911

Table S2. Results of secondary IVW MR analyses of the associations between cytokines and growth factors with Alzheimer's disease and hippocampal volume. (continued)

Outcome	Exposure	IVW Estimate	95% CI		P-value	Heterogeneity	Cochran Q-derived P	SNPs	FDR-corrected P
Alzheimer's disease	SCGF-b	-0.012	-0.078	0.053	0.712	7.217	0.407	8	0.937
Hippocampal volume	SCGF-b	0.033	-0.025	0.091	0.265	14.800	0.039	3	0.906
Alzheimer's disease	IL-2ra	-0.009	-0.067	0.049	0.761	5.898	0.052	3	0.946
Hippocampal volume	IL-2ra	0.013	-0.022	0.048	0.483	5.175	0.075	5	0.906
Alzheimer's disease	CTACK	0.019	-0.044	0.081	0.553	10.021	0.075	6	0.906
Hippocampal volume	CTACK	0.000	-0.074	0.075	0.994	13.631	0.009	2	0.996
Alzheimer's disease	IL-17	-0.018	-0.297	0.262	0.902	-	-	1	0.977
Hippocampal volume	IL-17	0.134	-0.031	0.300	0.111	-	-	1	0.906
Alzheimer's disease	IL-13	0.021	-0.037	0.080	0.478	1.420	0.841	5	0.906
Hippocampal volume	IL-13	0.009	-0.027	0.045	0.620	4.670	0.198	4	0.906
Alzheimer's disease	HGF	-0.038	-0.188	0.113	0.626	0.078	0.780	2	0.906
Hippocampal volume	HGF	0.087	-0.007	0.181	0.070	0.047	0.828	2	0.906
Alzheimer's disease	IL-10	-0.034	-0.122	0.054	0.454	1.897	0.387	3	0.906
Hippocampal volume	IL-10	0.018	-0.034	0.071	0.494	4.515	0.105	3	0.906
Alzheimer's disease	IL-16	-0.014	-0.077	0.050	0.675	0.275	0.871	3	0.911
Hippocampal volume	IL-16	-0.004	-0.042	0.035	0.855	5.397	0.067	3	0.975
Alzheimer's disease	IL-7	-0.054	-0.141	0.033	0.226	0.304	0.581	2	0.906
Hippocampal volume	IL-7	0.003	-0.051	0.056	0.926	-	-	1	0.977
Alzheimer's disease	TNF-β	-0.041	-0.110	0.028	0.241	1.276	0.528	3	0.906
Hippocampal volume	TNF-β	-0.004	-0.072	0.065	0.919	-	-	1	0.977
Alzheimer's disease	MIF	0.022	-0.163	0.208	0.812	-	-	1	0.953

Table S2. Results of secondary IVW MR analyses of the associations between cytokines and growth factors with Alzheimer's disease and hippocampal volume. (continued)

Outcome	Exposure	IVW Estimate	95% CI		P-value	Heterogeneity	Cochran Q-derived P	SNPs	FDR-corrected P
Hippocampal volume	MIF	-0.043	-0.155	0.068	0.446	-	-	2	0.906
Alzheimer's disease	BNGF	0.016	-0.175	0.208	0.867	-	-	1	0.975
Hippocampal volume	BNGF	0.072	-0.044	0.188	0.222	-	-	1	0.906
Alzheimer's disease	SCF	0.047	-0.147	0.241	0.635	2.067	0.151	2	0.906
Hippocampal volume	SCF	0.135	0.013	0.257	0.031	3.456	0.063	1	0.552
Alzheimer's disease	IL-5	-0.105	-0.294	0.084	0.278	-	-	1	0.906
Hippocampal volume	IL-5	0.004	-0.106	0.114	0.941	-	-	1	0.977
Alzheimer's disease	IL-1	0.085	-0.140	0.310	0.461	-	-	1	0.906
Hippocampal volume	IL-1	-0.037	-0.173	0.099	0.596	-	-	1	0.906
Alzheimer's disease	ICAM-1	0.002	-0.845	0.850	0.996	5.174	0.160	4	0.996
Hippocampal volume	ICAM-1	1.066	0.200	1.932	0.016	2.2641	0.322	3	0.552

Shown are the results derived from the secondary inverse variance-weighted meta-analysis. Estimate denotes log odds ratio for Alzheimer's disease and mean difference for hippocampal volume. MIG indicates monokine induced by gamma interferon indicates; MIP-1b, macrophage inflammatory protein 1 beta; VEGF, vascular endothelial growth factor; IL, interleukin; IL, interleukin; GRO-α, growth-regulated oncogene alpha; MCP-1, monocyte chemoattractant protein-1; TRAIL, TNF-related apoptosis-inducing ligand; PDGF-bb, platelet-derived growth factor-bb; IP-10, interferon gamma-induced protein 10; SCGF-b, stem cell growth factor beta; CTACK, cutaneous T-cellattracting chemokine; HGF, hepatocyte growth factor; TNF-related apoptosis-inducing ligand; MIF, macrophage migration inhibitory factor; BNGF indicates beta nerve growth factor; SCF, stem cell factor; ICAM-1, Intercellular Adhesion Molecule 1.

Table S3. Alternative tests for markers showing either significant or suggestive associations or significant heterogeneity in the primary IVW MR analysis.

Exposure	Outcome	MR test	Estimate*	95% CI		P-value	Heterogeneity	Cochran Q-derived P	SNPs	FDR-corrected P
Platelet count	Alzheimer's disease	Random-effect IVW	0.005	-0.068	0.077	0.895	24	0.000	158	1.00
		Weighted median	-0.043	-0.134	0.048	0.358				
		Weighted mode	-0.062	-0.174	0.050	0.279				
		MR-Egger	-0.126	-0.289	0.037	0.131				
		ConMix	-0.060	-0.110	0.000	0.083				
		MR-PRESSO raw	0.014	-0.063	0.092	0.720				
		MR-PRESSO corrected	-0.011	-0.078	0.055	0.737				
	Hippocampal volume	Random-effect IVW	-0.018	-0.059	0.023	0.393	204	0.002	151	0.84
		Weighted median	-0.016	-0.077	0.044	0.594				
		Weighted mode	-0.035	-0.114	0.044	0.390				
		MR-Egger	-0.013	-0.102	0.076	0.778				
		ConMix	-0.010	-0.050	0.020	0.513				
		MR-PRESSO raw	-0.019	-0.061	0.024	0.385				
		MR-PRESSO corrected	-0.013	-0.054	0.029	0.549				
Eosinophil count	Hippocampal volume	Random-effect IVW	0.016	-0.041	0.073	0.579	117	0.034	92	0.86
		Weighted median	0.018	-0.062	0.099	0.659				
		Weighted mode	-0.063	-0.277	0.150	0.563				
		MR-Egger	0.024	-0.113	0.162	0.730				
		ConMix	0.040	-0.040	0.090	0.633				

Table S3. Alternative tests for markers showing either significant or suggestive associations or significant heterogeneity in the primary IVW MR analysis. (*continued*)

Exposure	Outcome	MR test	Estimate*	95% CI		P-value	Heterogeneity	Cochran Q-derived P	SNPs	FDR-corrected P
		MR-PRESSO raw	0.015	-0.043	0.073	0.613				
		MR-PRESSO corrected	0.033	-0.021	0.087	0.231				
Basophil count	Alzheimer's disease	Random-effect IVW	0.158	-0.066	0.383	0.167	6	0.013	36	0.71
		Weighted median	0.226	-0.067	0.519	0.131				
		Weighted mode	0.527	-0.046	1.100	0.080				
		MR-Egger	0.265	-0.357	0.887	0.404				
		ConMix	0.300	0.030	0.560	0.028				
		MR-PRESSO	No significant outliers							
Monocyte count	Alzheimer's disease	Random-effect IVW	-0.033	-0.112	0.045	0.406	15	0.008	116	0.84
		Weighted median	-0.043	-0.155	0.07	0.457				
		Weighted mode	-0.034	-0.176	0.109	0.646				
		MR-Egger	0.012	-0.141	0.165	0.878				
		ConMix	-0.040	-0.120	0.040	0.340				
		MR-PRESSO raw	-0.034	-0.114	0.046	0.406				
		MR-PRESSO corrected	-0.051	-0.128	0.026	0.198				
	Hippocampal volume	Random-effect IVW	0.011	-0.039	0.060	0.671	125	0.047	101	0.93
		Weighted median	0.044	-0.019	0.106	0.169				
		Weighted mode	0.032	-0.053	0.117	0.463				

Table S3. Alternative tests for markers showing either significant or suggestive associations or significant heterogeneity in the primary IVW MR analysis. (*continued*)

Exposure	Outcome	MR test	Estimate*	95% CI		P-value	Heterogeneity	Cochran Q-derived P	SNPs	FDR-corrected P
		MR-Egger	0.016	-0.095	0.127	0.780				
		ConMix	0.010	-0.030	0.050	0.703				
		MR-PRESSO raw	0.012	-0.039	0.063	0.651				
		MR-PRESSO corrected	0.020	0.067	0.067	0.401				
Lymphocyte count	Alzheimer's disease	Random-effect IVW	0.034	-0.070	0.138	0.521	13	0.009	94	0.84
		Weighted median	0.039	-0.119	0.197	0.629				
		Weighted mode	0.081	-0.106	0.267	0.398				
		MR-Egger	0.179	-0.106	0.463	0.218				
		ConMix	0.010	-0.090	0.110	0.898				
		MR-PRESSO	No significant outliers							
	Hippocampal volume	Random-effect IVW	0.023	-0.041	0.086	0.483	117	0.012	86	0.84
		Weighted median	0.048	-0.031	0.127	0.237				
		Weighted mode	0.058	-0.060	0.175	0.340				
		MR-Egger	0.045	-0.129	0.22	0.613				
		ConMix	0.060	0.000	0.120	0.061				
		MR-PRESSO	No significant outliers							
CD4 to 8 ratio	Alzheimer's disease	Random-effect IVW	0.042	-0.092	0.176	0.539	12	0.006	4	0.84

Table S3. Alternative tests for markers showing either significant or suggestive associations or significant heterogeneity in the primary IVW MR analysis. (*continued*)

Exposure	Outcome	MR test	Estimate*	95% CI		P-value	Heterogeneity	Cochran Q-derived P	SNPs	FDR-corrected P
		Weighted median	0.036	-0.05	0.122	0.410				
		Weighted mode	0.002	-0.099	0.102	0.977				
		MR-Egger	0.027	-0.426	0.48	0.906				
		ConMix	0.000	-0.130	0.280	1.000				
		MR-PRESSO raw	0.052	-0.089	0.194	0.520				
		MR-PRESSO corrected	0.003	-0.105	0.112	0.959				
Fibrinogen	Alzheimer's disease	Random-effect IVW	0.479	-0.18	1.139	0.154	88	0.001	53	0.71
		Weighted median	0.789	0.001	1.576	0.050				
		Weighted mode	0.717	-0.187	1.621	0.126				
		MR-Egger	0.452	-1.230	2.134	0.598				
		ConMix	0.780	0.140	1.460	0.019				
		MR-PRESSO raw	0.608	-0.184	1.401	0.138				
		MR-PRESSO corrected	0.321	-0.472	1.113	0.268				
CRP	Alzheimer's disease	Random-effect IVW	0.101	0.010	0.191	0.029	16	0.852	24	0.42
		Weighted median	0.070	-0.050	0.189	0.253				
		Weighted mode	0.071	-0.060	0.202	0.285				
		MR-Egger	0.086	-0.104	0.234	0.452				
		ConMix	0.100	0.020	0.190	0.028				
		MR-PRESSO	No significant outlier							

Table S3. Alternative tests for markers showing either significant or suggestive associations or significant heterogeneity in the primary IVW MR analysis. (*continued*)

Exposure	Outcome	MR test	Estimate*	95% CI		P-value	Heterogeneity	Cochran Q-derived P	SNPs	FDR-corrected P
Lp-PLA2 activity	Hippocampal volume	Random-effect IVW	-0.316	-0.715	0.084	0.121	19	0.004	7	0.71
		Weighted median	-0.285	-0.620	0.049	0.094				
		Weighted mode	-0.133	-0.668	0.401	0.642				
		MR-Egger	-0.882	-2.194	0.430	0.188				
		ConMix	-0.180	-0.440	0.170	0.427				
		MR-PRESSO raw	-0.343	-0.751	0.066	0.151				
		MR-PRESSO corrected	-0.089	-0.410	0.233	0.612				

Methods used to test for associations and for violations of the Mendelian randomization assumptions. IVW, inverse variance–weighted; MR, Mendelian randomization; ConMix, contamination mixture method; MR-PRESSO, Mendelian Randomization Pleiotropy Residual Sum and Outlier; SNP, single-nucleotide polymorphism; FDR, false-discovery rate, CRP, C-reactive protein; Lp-PLA2, Lipoprotein-associated phospholipase A2. *Log odds ratio for Alzheimer's disease and mean difference for hippocampal volume.

2.3 *Helicobacter pylori*

ABSTRACT

Background: *Helicobacter pylori* infection might increase risk of dementia, but available evidence is inconsistent and longitudinal studies are sparse. We investigated the association between *H. pylori* serology and dementia risk in a population-based cohort.

Methods: Between 1997-2002, we measured *H. pylori* serum IgG titers in 4215 non-demented participants of the Rotterdam Study with a mean age of 69 years. We determined the association between *H. pylori* at baseline and dementia incidence until 2015, per natural log(U/mL) increase in titer, and for seropositive/seronegative, using Cox models adjusting for cohort, sex, age, education and cardiovascular risk factors.

Results: During a median follow-up of 13.3 years, 529 participants developed dementia of whom 463 had Alzheimer's disease (AD). *H. pylori* was not associated with risk of dementia (hazard ratio [95% confidence interval] for antibody titer: 1.04 [0.90-1.21]; for seropositivity 1.03 [0.86-1.22]), nor AD.

Discussion: In this community-dwelling population, *H. pylori* was not associated with dementia risk.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative bacillus that colonizes the stomach and is estimated to infect half of the world's population [1]. The bacterium is generally acquired during childhood by oral ingestion. In adults, the infection is usually chronic and will not heal without specific therapy. The clinical course, however, is highly variable. While in some individuals infections remain asymptomatic, others may develop serious gastric complications, such as ulcers or gastric carcinoma [2].

In addition, recent evidence suggests that *H. pylori* infection might be associated with extra-gastric diseases including dementia [3-5]. This may be due to detrimental consequences of *H. pylori* associated anemia, inflammation and hyperhomocysteinemia on vascular and neuronal health [6, 7]. A recent systematic review and meta-analysis [8] showed a 71% increased risk of dementia with *H. pylori* infection, but heterogeneity across studies was high. This may relate to differences in (geographical) setting and study design, with current evidence mainly arising from cross-sectional studies. We aimed to investigate the association of *H. pylori* with dementia in a prospective population-based cohort study.

METHODS

Design and study population

This study was embedded within the Rotterdam Study, a prospective population-based cohort study among middle-aged and elderly individuals in the Netherlands [9]. A detailed description is provided in the online supplement. Established in 1990, participants were invited every 4-5 years to undergo follow-up examinations at the research center. Between 1997 and 2002, a total of 7444 participants from two subcohorts visited the research center, of whom 4215 dementia-free subjects provided blood samples for measurement of *H. pylori* titer (Figure S1).

The Rotterdam Study has been approved by the medical ethics committee according to the Population Study Act Rotterdam Study and written informed consent was obtained.

Assessment of anti-H. pylori antibodies

Blood was drawn at baseline. To obtain serum and plasma, tubes were centrifuged according to a protocol standardising time and conditions from the drawing of blood to centrifugation. All samples were snap frozen at –196°C using liquid nitrogen and stored at –80°C. Anti–*H. pylori* serum IgG antibody titers were measured in 2011 using commercial enzyme immunoassays (Pyloriset EIA-G III ELISA; Orion) as described earlier [10]. We used anti-*H. pylori* serum IgG antibody titers primarily as a continuous variable. In addition, seropositivity was

defined as an anti–*H. pylori* IgG titer equal to or greater than 20 U/mL, according to the manufacturer's recommendation.

Ascertainment of incident dementia

Participants were screened for dementia at baseline and subsequent center visits with the Mini-Mental State Examination and the Geriatric Mental Schedule organic level. Those with a Mini-Mental State Examination score <26 or Geriatric Mental Schedule score >0 underwent further investigation and informant interview, including the Cambridge Examination for Mental Disorders of the Elderly. In addition, the entire cohort was continuously under surveillance for dementia through electronic linkage of the study center with medical records from general practitioners and the regional institute for outpatient mental health care. A consensus panel headed by a consultant neurologist established the final diagnosis according to standard criteria for dementia (DSM-III-R) and AD (National Institute of Neurological and Communicative Disorders and Stroke– Alzheimer's Disease and Related Disorders Association). Follow-up until 1st January 2015, was virtually complete (99.1% of potential person-years in the original cohort and for 97.0% of potential person-years in the extended cohort). Within this period, participants were censored at date of dementia diagnosis, death, loss to follow-up, or administrative censoring date, whichever came first.

Covariates

Potential confounding factors for dementia were chosen on the basis of previous literature [8, 11]. In all models, we adjusted for cohort, sex and age at baseline. In multivariate adjusted models, we additionally adjusted for education, smoking, systolic and diastolic blood pressure, anti-hypertensive drug use, body mass index (BMI), cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, APOEε carrier status, stroke, diabetes mellitus, ethnicity and serum lipid reducing agents at baseline. APOEε carrier status was corrected for since a study has shown that *H. pylori* and ApoE 4 polymorphism may be associated with dysphagic symptoms in older adults [12]. Assessment methods of the covariates are described in the supplemental materials. Blood samples for determination of levels of hemoglobin, homocysteine, CRP, interleukin-6, α1-antitrypsin (α1-AT), lipoprotein-associated phospholipase A2 activity and fibrinogen were obtained at the research center.

Statistical analysis

Because of a skewed distribution of anti-*H. pylori* serum IgG antibody titers, we performed a natural log transformation. Differences in baseline characteristics between the *H. pylori* positive and negative groups were assessed using Student's *t*-test and chi-squared test. We determined the association between *H. pylori* (continuously as well as dichotomized for seroprevalence) and risk of dementia, using Cox regression models, adjusting for age, sex,

study cohort, education and cardiovascular risk factors. The proportional hazards assumption was met.

We repeated analysis for Alzheimer's disease only and used higher cut-offs for seroprevalence (in steps of 5 U/mL from the manufacturer's recommended cut-off) to explore differential effects among individuals with more profound antibody response. We assessed interaction by age, sex or medication use by stratification and testing for multiplicative interaction. In addition, we tested for multiplicative interaction between *H. pylori* titer and CRP to assess if there is a difference in dementia risk between more severe infected and less severely infected groups. Finally, we computed Pearson correlation coefficients for the association of *H. pylori* titer with levels of hemoglobin, homocysteine and CRP, with homocysteine and CRP both natural log transformed. In addition to CRP, which was available for all participants, we also determined the correlation between *H. pylori* and four other inflammatory biomarkers that have previously been related to dementia risk [13], and were available in a subsample of approximately 500 participants with *H. pylori* measurement.

All analyses were performed using IBM SPSS Statistics version 21.0 (IBM Corp., Armonk, NY).

RESULTS

Baseline characteristics of the study population are presented in Table 1. During a mean follow-up of 10.6 (±4.5) years and median follow-up of 13.3 years (interquartile range of 5.9) with 47,664 person-years, 529 individuals were diagnosed with dementia of whom 463 had AD.

Serum antibody titer of *H. pylori* was not associated with risk of dementia (hazard ratio (HR), 95% confidence interval (CI), per log unit increase: 1.04, 0.90-1.21). Similarly, *H. pylori* seroprevalence was not associated with dementia risk (HR 1.03, 0.86-1.22). These results were similar for AD (Table 2) and robust for different antibody titer cut-offs defining seroprevalence (Online supplement Figure S2). Risk estimates tended to be higher in older participants and in men, albeit interaction terms were not statistically significant (p = 0.383 and p = 0.142, respectively; Figure S3). We observed no significant interaction between *H. pylori* serum IgG titer or use of antibiotics and anti-acids on dementia risk (p = 0.449) (Figure S3), nor with CRP (p = 0.974). Antibody titers of *H. pylori* showed only very weak correlation with concurrent levels of hemoglobin, homocysteine and CRP (r = 0.004 and p = 0.835, r = 0.086 and p < 0.01 and r = 0.053 and p = 0.001, respectively). Finally, interleukin-6 (IL6), α1-AT, lipoprotein-associated phospholipase A2 activity and fibrinogen showed no meaningful correlations with *H pylori* (r = 0.039 for IL6; r = 0.080 for α1-AT; r = -0.067 for lipoprotein-associated phospholipase A2 and r = 0.004 for fibrinogen; all P >0.05).

Table 1. Baseline characteristics of the Rotterdam Study cohort

	Study population for analysis (N = 4215)	H. pylori positive (N = 2088)	H. pylori negative (N = 2127)	P-value for difference
Age, years	68.4 (8.6)	66.4 (7.5)	65.1 (7.4)	<0.001
Female	2288 (54.3)	1093 (52.3)	1195 (56.2)	0.012
Caucasian ethnicity	4140 (98.2)	2056 (98.5)	2081 (97.8)	0.161
Education				<0.001
Primary education	537 (12.7)	325 (15.6)	215 (10.1)	
Lower/intermediate general education	1873 (44.4)	944 (45.2)	928 (43.6)	
Intermediate vocational education	1244 (29.5)	602 (28.8)	642 (30.2)	
Higher vocational education	561 (13.3)	217 (10.4)	342 (16.1)	
Smoking				0.006
Never smoker	1335 (31.7)	615 (29.5)	719 (33.8)	
Former, now non-smoker	2130 (50.5)	1077 (51.6)	1051 (49.4)	
Current smoker	759 (17.8)	396 (19.0)	357 (16.8)	
Systolic blood pressure, mm Hg	143.4 (21.2)	143.8 (21.2)	143.1 (21.2)	0.284
Diastolic blood pressure, mm Hg	76.9 (11.2)	76.6 (11.3)	77.1 (11.1)	0.117
Anti-hypertensive medication	1488 (35.3)	757 (36.3)	728 (34.2)	0.178
Body mass index, kg/m^2	27.0 (3.9)	27.0 (3.8)	27.0 (4.0)	0.495
Diabetes mellitus	579 (13.7)	276 (13.2)	304 (14.3)	0.333
Cholesterol, mmol/L	5.8 (1.0)	5.8 (1.0)	5.8 (1.0)	0.939
High-density lipoprotein, mmol/L	1.4 (0.4)	1.3 (0.4)	1.4 (0.4)	<0.001
Triglycerides, mmol/L	1.6 (0.8)	1.6 (0.8)	1.6 (0.8)	0.537
Lipid lowering medication	572 (13.6)	291 (13.9)	288 (13.5)	0.742
History of stroke	163 (3.9)	85 (4.1)	78 (3.7)	0.549
Apolipoprotein E genotype				0.701
ε3 homozygote	2478 (58.8)	1218 (58.3)	1264 (59.4)	
ε2/ε2 or ε2/ε3	587 (13.9)	299 (14.3)	288 (13.5)	
ε4/ε4, ε3/ε4 or ε2/ε4	1149 (27.3)	571 (27.3)	575 (27.0)	
H. pylori serum IgG antibody titers, U/mL	19.6 (11.5; 92.2)	94.0 (36.0; 238.9)	11.5 (10.2; 14.2)	<0.001
H. pylori positive seroprevalence	2088 (49.5)			

Abbreviations: N = number of subjects; *H. pylori* = Helicobacter pylori.

Data presented as mean (standard deviation) for continuous variables and number (percentages) for categorical variables. *H. pylori* serum IgG antibody titers is presented as median (interquartile range). Differences in baseline characteristics between the *H. pylori* positive and negative groups were assessed using Student's *t*-test and chi-squared test.

Table 2. *H. pylori* infection and the risk of dementia: longitudinal results

	All dementia n/N = 529/4215 HR, 95% CI	Alzheimer n/N = 463/4215 HR, 95% CI
Model I		
H. pylori serum IgG, per log(U/mL)* **increase**	1.07, 0.92-1.23	1.11, 0.94-1.30
H. pylori seroprevalence, positive versus negative	1.03, 0.87-1.23	1.08, 0.89-1.31
Model II		
H. pylori serum IgG, per log(U/mL)* **increase**	1.04, 0.90-1.21	1.06, 0.90-1.25
H. pylori seroprevalence, positive versus negative	1.03, 0.86-1.22	1.06, 0.87-1.29

Abbreviations: n = number of cases; N = number of persons at risk; HR = hazard ratio; CI = confidence interval; *H. pylori* = Helicobacter pylori.

Cox regression model I: Adjusted for age, sex and study cohort.

Cox regression model II: Adjusted for age, sex, study cohort, education, smoking, systolic and diastolic blood pressure, anti-hypertensive drug use, body mass index (BMI), cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, APOEε carrier status, stroke, diabetes mellitus, ethnicity and serum lipid reducing agents.

*Natural log transformed because of right skewed distribution.

DISCUSSION

In this prospective cohort study, we found no association between *H. pylori* infection and risk of all-cause dementia or AD.

There are only two other longitudinal studies performed on this association, which show a hazard ratio of 1.46 (CI 1.01-2.11) [14] and 1.51 (CI 1.25-1.82) [15] for developing dementia. These findings are in contrast with our results, but several potential explanations, including methodological differences, may clarify this contrast. For instance, the community-based PAQUID cohort study performed in France [14] had an older study population (65 years and older) compared with our cohort of 55 years and over. Since we show a tendency for an increased risk in older age groups (Figure S3), an older cohort may indeed show an effect of *H. pylori* on dementia. Also, the PAQUID cohort selected their study sample on being followed-up for 20 years, thereby selecting the survivors, which is more susceptible to selection bias. The other longitudinal study is a Taiwanese registry study [15] using medical information from a nationwide population-based dataset. The study population was aged ≥40 years and followed during 1998–2010. In this retrospective cohort study, dementia diagnosis was therefore based on registries instead of multiple-stage designs with in-person screening for dementia diagnosis, making dementia diagnosis less reliable. In addition, results are prone to confounding due to the inherent shortcomings of the NHRI database. Also, participants with newly diagnosed *H. pylori* were identified with ICD-9 codes, potentially including the symptomatic *H. pylori* cases which may be

more virulent and thereby observing a stronger effect of *H. pylori* on dementia risk in the overall population.There are also potential biological explanations for the differences across studies, such as geographical differences. Studies have found that the rate of colonization with cagA(+) *H. pylori* strains has become very low in the Netherlands [16, 17]. CagA is a highly antigenic protein that is associated with a prominent inflammatory response [18]. This finding suggests that the *H. pylori* infections in the Netherlands may have low virulence in general compared with other countries, leading to less complications. For example, in studies comparing dyspeptic patients, studies indeed report higher prevalence rates of cagA+ strains in France (88%) [19] and Taiwan (99%) [20] compared to 78% in The Netherlands in 1996 [21] with a Dutch study from 2013 showing a sharp age specific decline in CagA+ from 38% to 14% in randomly selected blood donors [17]. This might be reflected in the higher risk estimates in older participants in our study, who would have been exposed more to these highly virulent strains in earlier years, suggesting a cohort effect. Thus, infection-induced cognitive decline can be supported by this finding.

Mechanistically wise, there are several hypotheses regarding the *H. pylori* and dementia association. Prior studies have suggested altered tau phosphorylation [18], vitamin B deficiency (and consequent hyperhomocysteinemia) [7, 22], systemic inflammation [23, 24], and anemia [25] as potential mechanisms by which *H. pylori* could increase risk of dementia. Although no studies have directly assessed these factors as potential intermediates, the lack of any correlation between *H. pylori* and homocysteine, inflammatory markers (CRP, IL6, α1-AT, lipoprotein-associated phospholipase A2 activity, fibrinogen) and hemoglobin levels in our study might also support the notion of a less virulent *H. pylori* strain that is currently prevalent in The Netherlands. This in turn could explain why we did not observe an association of *H. pylori* with risk of dementia.

The role of infectious diseases in the development of dementia has been longer known with diseases such as neurosyphilis and acquired immunodeficiency syndrome (AIDS) being accepted infectious causes [26].However, also more frequently occurring diseases such as herpes virus, toxoplasmosis and cryptococcus have been shown to have a relationship with dementia [27]. It is postulated that since systemic infection can provoke the enhanced synthesis of inflammatory mediators in the brain, infectious diseases may promote the onset of dementia [28]. Thus, even more common infections which an individual is unable to resolve adequately could lead to chronic inflammation and subsequently neuroinflammation [29]. It is therefore important for future studies to identify such sources of inflammation and eventually identify individuals with increased susceptibility for infections for treatment and prevention purposes.

Several limitations of this study should be taken into account. First, the diagnostic test for detecting *H. pylori* infection has the disadvantage that antibodies are present in blood even after eradication of *H. pylori*. Therefore, current and past infection cannot be distinguished, so we cannot rule out the presence of information bias in the form of misclassification of

the exposure. However we do not expect that the association with incident dementia would be affected, since the timing of infection may only be shifted for several months or years. An alternative for future studies would be the stool antigen test, which proves to be more accurate and still non-invasive compared to the serology test [30]. Second, considering the differences in virulence factors of the bacterium and treatment strategies in different countries, our findings may not be generalizable to other geographical regions. To take differences in virulence factors into account, future studies could assess CagA phenotypes in *H. pylori.* Third, the invasiveness of lumbar puncture prevents cerebrospinal fluid (CSF) sampling in participants in the Rotterdam Study and therefore we have not been able to measure *H. pylori* IgG antibodies in CSF. Although CSF measurements are likely more reflective of pathology within the CNS, studies show a larger mean concentration of anti-*H. pylori* IgG concentrations both in CSF as well as in serum in AD patients compared to controls [31, 32]. This finding supports the systemic effect of the infection, which can be thus measured in serum. Finally, the length of time with the infection could not be accounted for in this present study, which may also contribute to the age-related trend for infection-induced cognitive decline that is observed.

In conclusion, we have found no evidence of an association between *H. pylori* infection and risk of all-cause dementia or AD in our Dutch population. This could be due to low virulence of *H. pylori* in the Netherlands. Future studies are warranted to further elucidate the role of common infectious diseases in the pathophysiology of Alzheimer's disease and thereby identifying novel targets for prevention and treatment.

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SUPPORTING INFORMATION

Methods

Study population

Of the 10,215 invited inhabitants, 7,984 agreed to participate in the baseline examinations. In 2000, the cohort was extended with 4,472 invitees of whom 3,011 agreed to participate. From the original cohort, we used the third examination cycle as baseline due to availability of *H. pylori* and homocysteine data. This cohort contained 1892 participants that were included in the analyses based on dementia and *H. pylori* data availability. In the extended cohort, 2408 participants were included in the analyses (Figure S1).

Covariates

All covariates were measured at baseline. Since the study population included 2 cohorts from the Rotterdam Study, cohort was used as a covariate. Educational level was categorized as low (primary education or lower vocational education), intermediate (secondary education or intermediate vocational education), and high educational level (higher vocational education or university). Smoking habits were categorized as current, former and never smoking. Blood pressure was measured twice at the right arm in sitting position at the research center and the average of 2 blood pressure readings was used. BMI was calculated as weight in kilograms per height in meters squared. Total cholesterol and HDL cholesterol were measured in serum in mmol/l. For the anti-hypertensive drugs and serum lipid reducing agents, participants presented all the medication they used in the past week during the home interview. Trained research assistants registered drug names, dose, and indication on a structured data entry form. APOE genotype was determined using polymerase chain reaction on coded DNA samples.

APOEε carrier status was defined as low risk for dementia (2 ε3 alleles), intermediate risk for dementia (2 ε2 alleles or 1 ε3 allele and 1 ε2 allele) and high risk for dementia (1 or 2 ε4 alleles). Diabetes mellitus was defined as a fasting serum glucose level ≥7.0 mmol/L, non-fasting serum glucose level ≥11.1 mmol/L, or use of anti-diabetic medication. Ethnicity was genetically determined and divided into two different categories: European and other ethnicities.

Total plasma homocysteine level was determined with high-performance liquid chromatography. The CRP levels were measured by kinetic nephelometry.

Statistical analysis

Medication use involved anytime use of antacids (H2 receptor antagonists, prostaglandins, PPIs, combinations of drugs for eradication of *H. pylori* and/or other anti-acids). Also, anytime use of *H. pylori* sensitive antibiotics were examined, which include clarithromycin,

amoxicillin and tetracycline. Anytime use involved medication use at baseline or during follow-up of the study.

Missing covariate data (maximum 11.2%) were imputed using 5-fold multiple imputation, based on included covariates. Distribution of covariates was similar in the imputed versus non-imputed dataset.

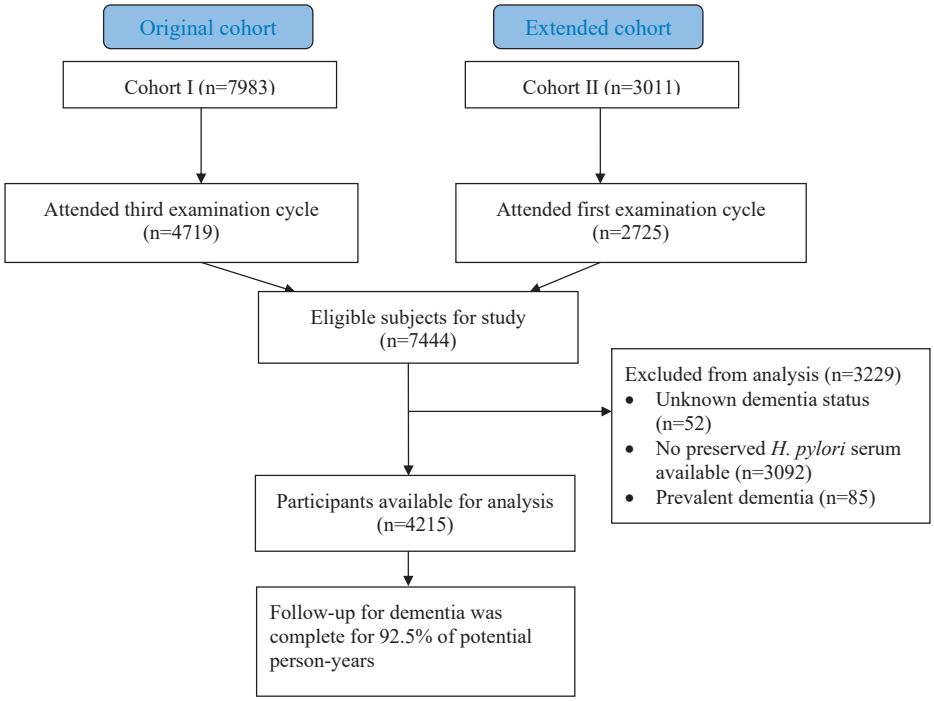

Figure S1. Flow-chart of study population

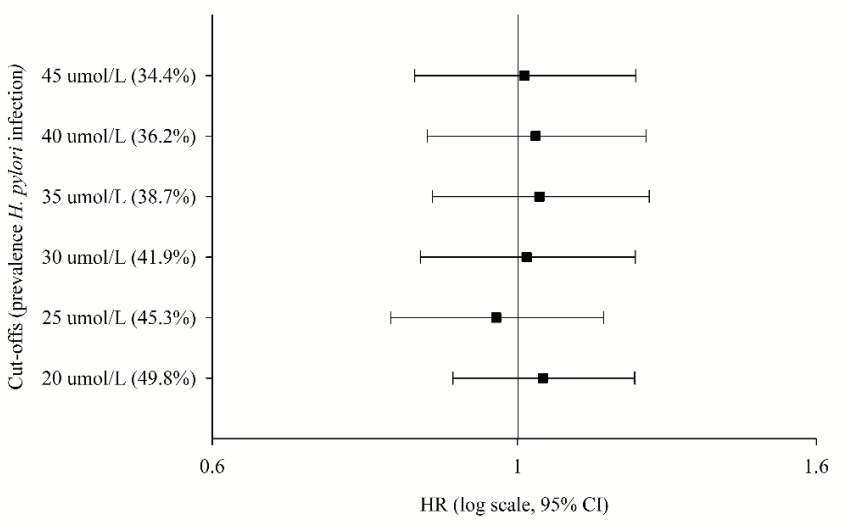

Figure S2. Sensitivity analyses for cut-off values for anti-Helicobacter pylori serum IgG antibody titers for obtaining seroprevalence states.

Abbreviations: H. pylori, helicobacter pylori; U/mL, units of activity per millilitre; HR, hazard ratio. Sensitivity analyses for the effect of *H. pylori* serum IgG (per log increase) on the dementia-free survival of persons in a Cox regression model with the following covariates: cohort, sex, age, education, smoking, blood pressure, anti-hypertensive drug use, body mass index, cholesterol, high-density lipoprotein cholesterol, triglycerides, APOEε carrier status, stroke, diabetes mellitus, ethnicity and serum lipid lowering agents. Error bars indicate 95% confidence intervals of the HR.

Number of persons at risk = 4215, number of cases = 529.

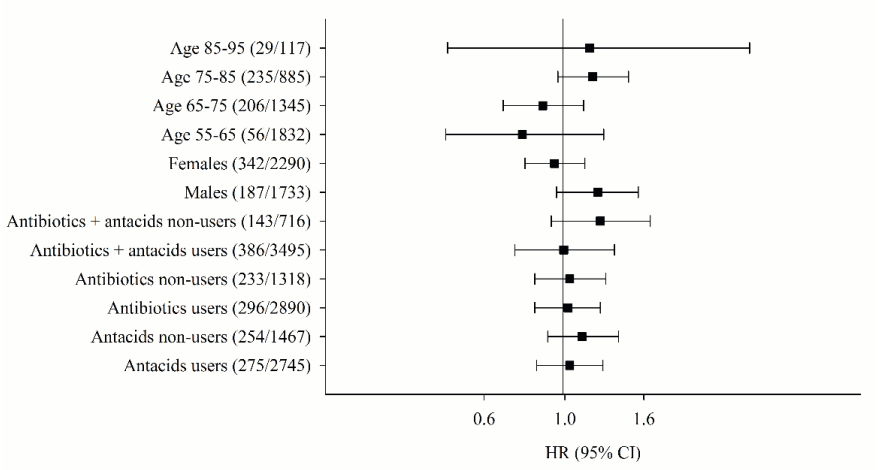

Figure S3. Sensitivity analyses for age, gender and medication use on the association between anti-Helicobacter pylori serum IgG antibody titers and the risk of dementia.

Abbreviations: n = number of cases; N = total number at risk; H. pylori = helicobacter pylori; PPI = proton pump inhibitor; U/mL = units of activity per millilitre; HR = hazard ratio; histamine-2 receptor antagonists = H2 antagonists.

Sensitivity analyses for the effect of *H. pylori* serum IgG antibody titers per log increase on the dementia-free survival of persons in a fully adjusted Cox regression model stratified for the following: age, gender and anytime use of medication (n/N). Medication use includes use of antibiotics (tetracycline, clarithromycin and/or amoxicillin) and antacids (H2 receptor antagonists, prostaglandins, PPIs, combinations of drugs for eradication of *H. pylori* and/or other anti-acids). Error bars indicate 95% confidence intervals of the HR.

2.4 Herpes Simplex Virus 1

ABSTRACT

Background: Herpes Simplex Virus 1 (HSV1) is a neuroinvasive virus capable of entering the brain which makes it a candidate pathogen for increasing risk of dementia Previous studies are inconsistent in their findings regarding the link between HSV1 and dementia, therefore, we investigated how HSV1 relates to cognitive decline and dementia risk using data from a population-based study.

Methods: We measured HSV1 immunoglobulin (IgG) antibodies in serum collected between 2002 and 2005 from participants of the Rotterdam Study. We used linear regression to determine HSV1 in relation to change in cognitive performance during 2 consecutive examination rounds on average 6,5 years apart. Next, we determined the association of HSV1 with risk of dementia (until 2016) using a Cox regression model. We repeated analyses for Alzheimer's disease. All models were adjusted for age, sex, cardiovascular risk factors, and apolipoprotein E genotype.

Results: Of 1,915 non-demented participants (mean age 71.3 years, 56.7% women), with an average follow-up time of 9.1 years, 244 participants developed dementia (of whom 203 Alzheimer's disease). HSV1 seropositivity was associated with decline in global cognition (mean difference per standard deviation -0.16; 95% confidence interval (95%CI), -0.26; -0.07), as well as separate cognitive domains, namely memory, information processing, and executive function, but not motor function. Finally, HSV1 seropositivity was not associated with risk of dementia (adjusted hazard ratio, 1.18, 95%CI, 0.83; 1.68), similar for Alzheimer's disease.

Discussion: HSV1 is associated with cognitive decline but not with incident dementia in the general population. These data suggest HSV1 to be associated only with subtle cognitive disturbances but not with greater cognitive disorders that result in dementia.

INTRODUCTION

Dementia is a problem that continues to rise as the elderly population increases, while the exact mechanisms of its cause are unknown. Immunity and inflammation have been implicated as possible pathways in the development of this disease[1]. A potential trigger for an immune response and inflammation is an infection, specifically, caused by Herpes Simplex Virus 1 (HSV1). HSV1 is a neuroinvasive and neurotoxic virus capable of entering the brain via the peripheral nerves, and it is thus a candidate pathogen for increasing dementia risk[2,3].

HSV1 is highly prevalent worldwide. Once infected, the virus remains latent in the body and can be reactivated throughout a person's lifetime[2]. This constant presence and reactivation of the virus has been implicated in the immune response and subsequent development of Alzheimer's disease.[4]. The virus initiates an innate immune system response that causes the production of amyloid beta[1]. The accumulation of amyloid beta due to increased production consequently causes neuroinflammation. The constant inflammation leads to damage to the brain cells (i.e. neurodegeneration) and eventually could result in dementia. Indeed, we have previously shown that an increase of various innate immune cells, in particular platelets, are associated with an increased risk of dementia in the general population[5]. Previous studies have looked at HSV1 and dementia, although mainly cross-sectionally or with short follow-up[6]. These studies have also been inconsistent on their findings on whether HSV1 is linked to dementia. Hence, there is still a gap in knowledge on how the virus affects the various stages of deteriorating cognition. In this study, we determined the association of HSV1 with cognitive decline and incident dementia. We hypothesized that HSV1 is associated with poorer (global) cognition and subsequently an increased risk of dementia.

METHODS

Study population

This study was embedded within the Rotterdam Study, a prospective population-based cohort study in Rotterdam, the Netherlands[7]. Within the study there are currently 4 cohorts; however, due to the availability of serum samples for HSV1 measurement this study used the first and second cohorts of the Rotterdam study. The first cohort began in 1990 and the second cohort began in 2000. Both cohorts started with participants of age 55 years or older at baseline. A total of 2,000 randomly selected serum samples, 1,000 from each cohort, were collected at examination rounds between 2002-2005. Baseline for this study was considered the ascertainment date of serum samples from participants (fourth visit for the first cohort, January 2002 through July 2004, and second visit for the second cohort, July 2002 and December 2005). Out of the 2,000 participants, 10 were excluded due to missing HSV1 data and 59 were excluded for missing Apolipoprotein E (*APOE*) carriership. Participants

with prevalent dementia were also excluded; therefore, the final study group consisted of 1,915 participants (**Figure 1**).

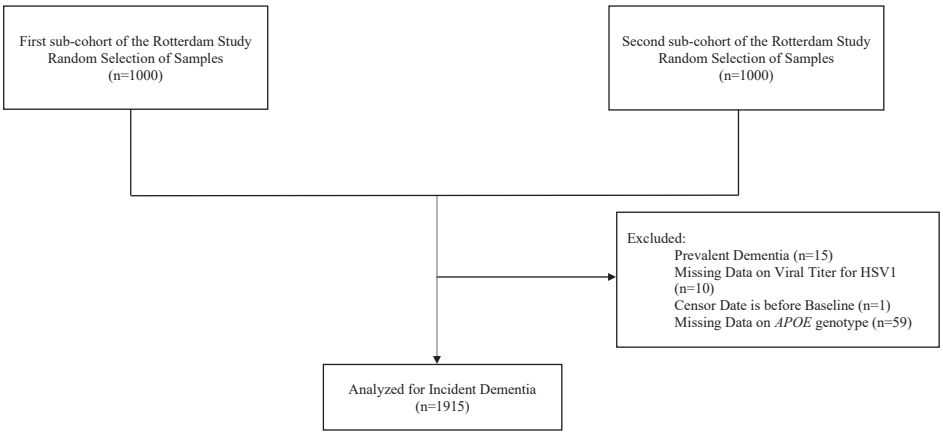

Figure 1. Flowchart for Selection of Participants for Incident Dementia Analysis

Assessment of HSV1 antibodies

The serum samples were collected and centrifuged in EDTA treated containers. The aliquoted plasma was frozen at -80ºC based on standard procedures. From the 2,000 serum samples, chemiluminescence immunoassay (CLIA) was done to determine if immunoglobulin G antibodies (IgG) to HSV1 were present. The DiaSorin LIASION® HSV1/2 IgG assay analyzer gave an index value ranging 0.5 to 30, based on the presence of the antibodies. Samples with an index value below 0.9 were considered negative for the virus. Index values ranging from 0.9 to 1.1 were considered equivocal[8]. Based on these ranges, a dichotomous variable for the virus was created. Values below 1.1 were defined as seronegative, while values of 1.1 and above were defined as seropositive for the HSV1 antibodies.

Assessment of global cognition and cognitive domains

Participants underwent a battery of cognition tests at the baseline examination visit as well as the next follow-up examination[9]. The tests included Mini Mental State Examination (MMSE), Word Learning Test (WLT immediate and WLT delayed recall), Letter-Digit Substitution Task (LDST), Verbal Fluency Task (VFT), Stroop Test (reading subtask, color naming subtask, and the color-word interference subtask), and Purdue Pegboard Task (PPB). For global cognition, we calculated the g-factor (general cognitive factor) as a standardized compound score using principle component analysis with LDST, Stroop color-word interference subtask, VFT, WLT delayed recall, and PPB sum of both hands. On each test, a higher score indicates a better performance apart from the Stroop test. Data on cognition

was collected at baseline and at subsequent examination rounds between 2009-2011 to track cognitive decline.

Assessment of incident dementia and Alzheimer's disease Assessment

Screening for dementia was done at baseline and throughout the study via examination visits and digital medical records. Participants underwent multiple steps before being diagnosed with dementia. The first step was the MMSE and the Geriatric Mental Schedule (GMS). If participants screened positive in either test (MMSE<26 or GMS>0) then it was followed by an examination with the Cambridge Examination for Mental Disorders of the Elderly (CAMDEX) and, if believed to have dementia, consequent neuropsychological examinations[10]. Any participant who was unable to visit the center was followed via digital medical records through general practitioners and the Regional Institute for Outpatient Mental Health Care. The final diagnosis was made by a consensus panel in accordance with standard criteria (DSM-III-R) Alzheimer's disease (NINCDS–ADRDA) and vascular dementia (NINDS-AIREN)[11,12]. Follow-up was until January 1, 2016 with 95.5% of participants. Participants were censored at date of dementia diagnosis, death, or loss to follow-up.

Covariates

Potential confounding variables were chosen based on previous literature[10]. Data on sex, age, and prevalent diseases were obtained at each examination visit. Information on smoking, alcohol consumption, and education was collected through interview. Body mass index (BMI) was calculated by weight in kilograms divided by height in meters squared. We grouped blood pressure and cholesterol into two categorical covariates, hypertension and hypercholesterolemia, due to the large number of covariates. Measurement of blood pressure was done while participants were in a sitting position with a random-zero sphygmomanometer. The average was taken from two measurements. Hypertension was then found if blood pressure was over ≥140/90 mmHg or if the participant was taking blood pressure lowering medication. Cholesterol and high-density lipoprotein (HDL)-cholesterol were collected in serum through an automated enzymatic procedure, the Boehringer Mannheim System. Hypercholesterolemia was categorized as having high cholesterol levels (>6.2 mmol/L) or use of lipid lowering medication. *APOE* genotype was found via serum sample. Additional information from the serum samples was collected on high sensitivity C-reactive protein (hs-CRP) and platelet count.

Statistical analysis

The continuous values of HSV1 IgG antibodies were standardized. We used both the continuous and the dichotomous values of HSV1 antibodies in the models. All models were corrected for sub cohort, sex, and age at baseline. We additionally adjusted for BMI, smok-

ing, alcohol consumption, education, hypercholesterolemia, hypertension, *APOE* genotype, coronary heart disease, diabetes mellitus, and stroke, all measured at baseline.

The Stroop subtask scores were inverted by multiplying with -1 to better match the other cognitive tests. In addition, the Stroop reading subtask scores were log transformed, the Stroop color naming and color-word interference subtask scores were square root transformed. All cognitive test results were standardized. Linear regression analysis was done for each cognition test performed during the fifth examination visit as outcome variable and the baseline (fourth examination visit) cognition test scores were adjusted for in the model. First the g-factor and MMSE were analyzed after which we explored each test separately.

A Cox regression analysis was performed to study the association between HSV1 and incident dementia and Alzheimer's disease (AD). For incident dementia, we performed a sensitivity analysis using varying cutoffs for seroprevalence to assess effects of varying antibody levels (index value of cutoffs for seroprevalence: 0.7 to 1.4 with 0.1 increment increase). In addition, we assessed stratification and multiplicative interaction by age (10-year intervals), sex, hs-CRP (threshold: >2 mg/L), *APOE*-ε4 carrier status, and platelet levels (based on the median; threshold: $>252\times10^9$ per liter)[5]. Interactions by hs-CRP and platelet levels were analyzed to check whether a higher innate immunity status affected the link of HSV1 with dementia compared to having a lower innate immunity status.

The assumptions for linear regression and Cox regression were checked for all analyses. The data was imputed 5 times using multiple imputation by chained equations (MICE) using the outcome and covariates as predictors to impute missing covariates. The pool of the 5 imputations was used. All analyses were completed using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk NY).

RESULTS

The baseline characteristics of the study population are in **Table 1**. The mean age at baseline was 71.3 years (±7.5). Of 1915 participants, 1518 were HSV1-seropositive (79.3%).

Of 1900 participants (99.6%) who underwent detailed cognitive assessment at baseline, 1249 (65.4%) had repeated assessment at follow-up (mean interval 6.5 years). Within HSV1 seropositive participants, the mean difference in g-factor score was lower by 0.16 (95% confidence interval [95% CI]: -0.26; -0.07) compared to HSV1 seronegative participants. Per standard deviation (SD) increase in HSV1 antibody titer the mean difference in g-factor [z-score] was -0.04 (-0.08; 0.002). For MMSE, the mean difference in z-score for HSV1 seropositive participants compared to seronegative participants was lower by 0.12 (-0.24; 0.002) compared to seronegative participants and per SD increase in HSV1 antibody titer -0.06 (-0.11; -0.01).

Table 1. Baseline Characteristics of the Study Population

Characteristics	Cohort undergoing cognitive examination* (N = 1249)	Dementia-free cohort (N=1915)
Females	719 (57.6%)	1086 (56.7%)
Age, years	69.0 ±6.3	71.3 ±7.5
Cohort		
First	671 (45.7%)	928 (48.5%)
Second	678 (54.3%)	900 (50.3%)
Education		
Primary education	95 (7.7%)	203 (10.7%)
Lower/intermediate general education	546 (44.1%)	849 (44.8%)
Intermediate vocational education	382 (30.9%)	564 (29.7%)
Higher vocational education	214 (17.3%)	281 (14.8%)
Apolipoprotein E4ε carriership		
Non-carrier (ε2/ε2, ε2/ε3 or ε3/ε3)	918 (73.5%)	1398 (73.0%)
Carrier (ε4/ε4, ε3/ε4 or ε2/ε4)	331 (26.5%)	517 (27.0%)
Smoking		
Current	171 (13.9%)	270 (14.3%)
Former	692 (56.1%)	1053 (55.7%)
Never	370 (30.0%)	568 (30.0%)
Body mass index, kg/m^2	27.7 ±3.9	27.6 ±4.1
Hypertension	913 (73.1%)	1485 (77.5%)
Hypercholesterolemia	607 (51.2%)	900 (47.2%)
Alcohol consumption	12.7 ±14.1	12.1 ±14.7
History of coronary heart disease	108 (8.7%)	206 (10.9%)
Diabetes mellitus type 2	142 (11.6%)	279 (15.2%)
History of stroke	35 (2.8%)	78 (4.1%)

Abbreviations: N, number of participants included in study. Data presented as mean (standard deviation) for continuous variables and number (percentages) for categorical variables. Data represent original data without imputed values.

*Participants underwent multiple cognitive tests.

HSV1 was significantly associated with several cognitive tests. WLT delayed had the largest difference with a z-score mean difference of -0.12 (-0.24; 0.004) for HSV1 seropositive compared to HSV1 seronegative. Seropositive HSV1 was associated with decreased z-scores in LDST. HSV1 seropositive was significant in both the VFT (-0.13, -0.24; -0.03) and the LDST (-0.12, -0.21; -0.04) compared to seronegative participants. Results were similar

Table 2. Association of serum HSV1 titer with Cognitive Decline in the Rotterdam Study

Mean change in cognition in standard deviation (95% CI)										
	G-factor	**MMSE**	**WLT Immediate**	**WLT Delayed**	**Stroop: Reading**	**Stroop: Color Naming**	**Stroop: Color-Word**	**LDST**	**VFT**	**PPB**
	n=901	**n=1249**	**n=1091**	**n=1091**	**n=1154**	**n=1152**	**n=1145**	**n=1159**	**n=1193**	**n=1029**
HSV1 serum seroprevalence	-0.16 (-0.26; -0.07)	-0.12 (-0.24; 0.002)	-0.03 (-0.14; 0.09)	-0.12 (-0.24; 0.004)	-0.10 (-0.21; 0.01)	-0.08 (-0.19; 0.04)	-0.11 (-0.23; 0.01)	-0.12 (-0.21; -0.04)	-0.13 (-0.24; -0.03)	-0.09 (-0.19; 0.01)
HSV1 serum IgG antibody titer	-0.04 (-0.08; 0.002)	-0.06 (-0.11; -0.01)	-0.02 (-0.07; 0.03)	-0.07 (-0.12; -0.02)	-0.04 (-0.08; 0.01)	-0.02 (-0.06; 0.03)	-0.02 (-0.07; 0.03)	-0.04 (-0.08; -0.01)	-0.05 (-0.10; -0.01)	-0.02 (-0.06; 0.03)

The Stroop subtask scores were inverted to better match the other cognitive tests. In addition, the Stroop reading subtask scores were log transformed, the Stroop color naming and color-word interference subtask scores were square root transformed. All cognitive test results were standardized. Linear regression analysis was done for each cognition test performed during the fifth examination visit as outcome variable and the baseline (fourth examination visit) cognition test scores were adjusted for in the model.

Abbreviations: MMSE, Mini Mental State Examination; WLT, Word Learning Test; LDST, Letter-Digit Substitution; VFT, Verbal Fluency Test; PPB, Purdue Pegboard Test: Sum of Both Hands.

Note: Linear Regression Model adjusted for sub cohort, sex, age, body mass index, smoking, alcohol consumption, education, hypertension, hypercholesterolemia, apolipoprotein E carrier status, coronary heart disease, diabetes mellitus, and stroke.

for HSV1 antibody titer. Neither HSV1 seropositivity nor HSV1 antibody titer showed a significant decrease in the PPB Sum test (**Table 2**).

During an average follow-up time of 9.1 years (±3.4), 244 participants were diagnosed with dementia, 203 of whom were diagnosed with Alzheimer's disease. HSV1 seropositivity was not associated with a higher risk of all-cause dementia (adjusted hazard ratio [HR] of 1.18 (95% CI: 0.83; 1.68)). Per SD increase of antibody titer of HSV1, the HR was 1.03 (0.90; 1.17). For Alzheimer's disease, the results were similar for HSV1 seropositive and HSV1 antibody titer (1.13, 95% CI: 0.77; 1.66 and 1.05, 95% CI: 0.90; 1.21, respectively) (**Table 3**).

Table 3. Association of serum HSV1 titer with Incident Dementia in the Rotterdam Study

	Hazard Ratio of dementia (95% CI)			**Hazard Ratio of Alzheimer's Disease** (95% CI)		
	n/N	**Model I**	**Model II**	**n/N**	**Model I**	**Model II**
HSV1 serum IgG seronegative	38/397	reference	reference	32/397	reference	reference
HSV1 serum IgG seropositive	206/1518	1.21 (0.86; 1.72)	1.18 (0.83; 1.68)	171/1518	1.18 (0.80; 1.72)	1.13 (0.77; 1.66)
HSV1 serum IgG antibody titer per standard deviation increase	244/1915	1.06 (0.93; 1.20)	1.03 (0.90; 1.17)	203/1915	1.08 (0.94; 1.25)	1.05 (0.90; 1.21)

Abbreviations: HR, hazard ratio; CI, Confidence Interval; n, number of cases; N, number of subjects; IgG, immunoglobulin G.

NOTE: Cox Model I, adjusted for sub cohort, sex, and age. Cox Model II, adjusted for sub cohort, sex, age, body mass index, smoking, alcohol consumption, education, hypertension, hypercholesterolemia, apolipoprotein carrier status, coronary heart disease, diabetes mellitus, and stroke.

Note: n/N represents the number of dementia cases. P-value is for the interaction term of the grouping variable and seroprevalence HSV1.

Abbreviations: HR, hazard ratio of incident dementia; CI, confidence interval.

The different cut-off levels for HSV1 seroprevalence showed similar results (**Figure S1**). Seropositive males had a larger hazard ratio of dementia than seropositive females (1.56, 95% CI: 0.84; 2.90 and 1.06, 95% CI: 0.69; 1.63, respectively), although the multiplicative interaction between sex and HSV1 seropositive was not significant. The hazard ratio for HSV1 seroprevalent dementia cases varied widely between age groups, with the interaction term for age and HSV1 seroprevalence being significant (p=0.034). The highest age category, 88-98 years old, was not analyzed due to lack of power. The group with higher hs-CRP levels (>2 mg/L) had a higher hazard ratio for seroprevalence (HR 1.66) compared to the group with lower hs-CRP levels (HR 1.05), with the multiplicative interaction between the virus and CRP being borderline significant (p=0.180). The group with higher platelet levels

(>252×10^9 per liter), had a higher HR of 1.32 (95% CI: 0.78; 2.26) compared to lower platelet levels for the seropositive group (HR 1.07), however we observed no significant interaction. *APOE*-ε4 carriers had no effect change for HSV1 seropositivity (**Figure 2**). All assumptions were met for all analyses.

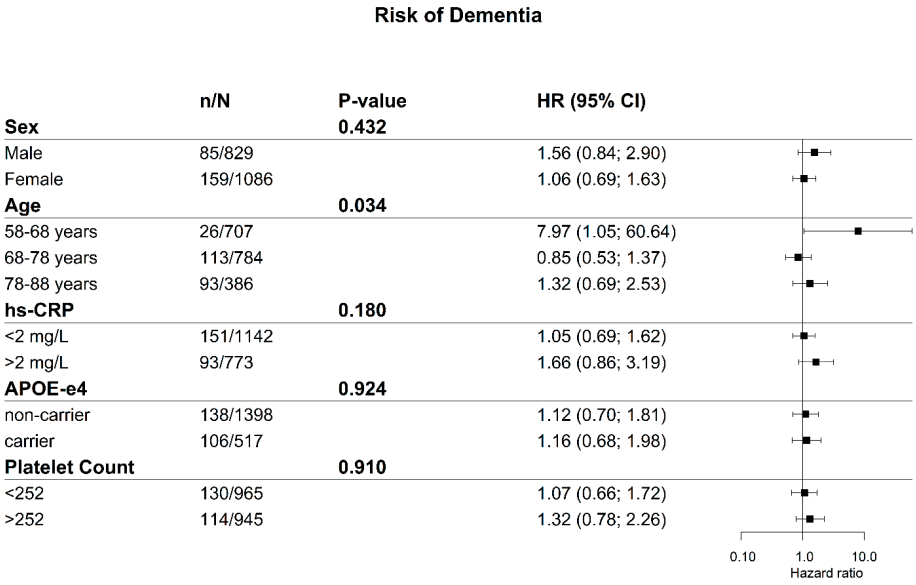

Figure 2. Stratification of Various Factors for the Relation of serum HSV1 titer to the Risk of Dementia

DISCUSSION

In this study, cognitive decline was more pronounced in HSV1 seropositive compared to seronegative participants in global cognition (g-factor and MMSE) and the cognitive domains of memory (WLT immediate and delayed recall), information processing (LDST and Stroop reading and color naming subtasks), and executive function (VFT, LDST, and Stroop color-word interference subtask)[13]. There was no association between HSV1 and the development of dementia.

The current literature mainly focuses on the final stage of deteriorated cognition: dementia[6] and only few studies have investigated cognition[12,13]. In the studies that found an association between declined cognition and HSV, it was only with high infectious burden (a combination of HSV1, HSV2, and Cytomegalovirus)[14] or in carriers of *APOE*-ε4[15]. One study found no association between HSV1 and cognitive decline [16].Previous studies found conflicting results regarding the association between HSV1 and dementia[6]. Two previous

cohort studies varied from our cohort in having a smaller study sample(the largest being n=1204)and a shorter follow-up time(the longest being 4 years)[17,18,]. In a Taiwanese cohort study they found an association, but all the HSV cases accounted for were more virulent infections requiring anti-viral medication which may have resulted in stronger effects[19]. Another study only saw the association in *APOE*-ε4 carriers[20], unlike our study. In one of the few longitudinal studies on the subject, an association was found only with HSV1 immunoglobulin M (IgM) antibodies and AD but not with HSV1 IgG antibodies and AD (HR: 0.99, 95% CI: 0.57; 1.72)[21] which is consistent with our study. Another study looking at HSV IgG antibodies and dementia revealed a HR of 1.67 (95% CI: 0.75; 3.73)[22], yet both of these studies failed to differentiate between HSV1 and HSV2 antibodies. A third prospective cohort studying the same association found a HR of 0.77 (95% CI: 0.58; 1.04) for HSV1 specific IgG antibodies[23]. It is possible that the presence of HSV1 IgG antibodies does not lead to constant inflammation; therefore, the latent virus is not associated with greater cognitive disturbances. Since our study comprised the general population, a high prevalence of latent HSV1 was expected as opposed to a reactivated infection.

A potential mechanism for an HSV1 infection to affect cognition is via a temporary production of amyloid beta to repress the infection. This leads to short-lived inflammation which affects cognition according to the recent antimicrobial protection hypothesis of AD[1]. Once the infection and subsequent inflammation are gone, cognition is no longer impaired.

In our study, the HSV1 infection as measured by IgG antibodies (latent infection) are associated with a subtle decrease in global cognition over time. Due to lack of information on cognition tests at later examination dates (after 2011) in our study, we do not know whether cognition stayed at the new decreased level or if it returned to the previous level measured at baseline. However, our results suggest that it did not decrease further; otherwise, we would have observed an association between HSV1 and dementia. The combination of multiple neurotoxic viruses may be required to cause permanent damage to cognitive function, therefore HSV1 alone is not enough to see a significant effect.

A major strength of this study was the availability of prospectively collected data and the length of follow-up time. Most of the previous studies on the matter only look cross-sectionally at HSV1 and dementia or had a short follow-up time. In addition, we were able to analyze both the subtle (i.e. cognition) and large changes in cognition (i.e. dementia) in one study.

It is also important to consider the limitations of this study. A major one is the availability of only HSV1 IgG antibodies which only gave us information on infection prior to baseline. Without data on the HSV1 IgM, we were unable to tell whether the participants had an active HSV1 infection at baseline. Since the reactivation of the virus is the proposed pathway to deteriorating cognition, we would need to investigate HSV1 IgM antibodies and each stage of deteriorating cognition to gain a better understanding. Another limitation

might be the lack of generalizability as the Rotterdam Study cohort predominantly consists of Caucasian participants living in a middle-income area in Rotterdam.

In conclusion, HSV1 is associated with a decrease in global cognition and cognitive domains, but not with dementia risk in the general population. These findings suggest that HSV1 IgG antibodies may lead to subtle cognitive disturbances but not greater cognitive disorders.

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Table S1. Mean Cognitive Scores at Baseline and Follow-up

Cognitive Test Score	Baseline Test			Follow-Up Test			
	N	Mean	Standard deviation	N	Mean	Standard deviation	Cognitive Domain
G-factor	901	-1.7	1	901	0.0	1.0	Global Cognition
Mini-Mental State Exam	1249	27.5	2.6	1249	27.4	2.8	Global Cognition
Word Learning Test: Immediate Recall	1091	6.8	2.1	1126	7.2	2.1	Memory
Word Learning Test: Delayed Recall	1091	6.5	2.8	1126	6.8	2.9	Memory
Stroop Test: Reading	1154	18.7	4.7	1199	18.3	4.1	Information Processing
Stroop Test: Color Naming	1152	24.9	6.3	1197	25.1	6.1	Information Processing
Letter-Digit Substitution Test	1159	27.2	7.1	1203	26.6	7.1	Information Processing, Executive Function
Stroop Test: Color-Word	1145	60.4	31.6	1190	60.0	27.2	Executive Function
Verbal Fluency Test	1193	20.7	5.4	1238	21.0	5.7	Executive Function
Purdue Pegboard Test: Sum of Both Hands	1029	33.5	5.2	1071	32.6	5.0	Motor Function

Note: n/N represents the number of individuals that underwent the cognitive tests.

Figure S1. Sensitivity Analysis of HSV1 Seroprevalence with Risk of Dementia using Different Seroprevalence Cut-offs

Note: Each row represents the hazard ratio of dementia for each index cut-off for HSV1 seroprevalence.

Chapter 3
Immunity and stroke

3.1 Subclinical atherosclerosis, and cardiovascular disease

ABSTRACT

Background: Atherosclerotic cardiovascular disease (ASCVD) is driven by multifaceted contributions of the immune system. However, the dysregulation of immune cells that leads to ASCVD is poorly understood. We determined the association of components of innate and adaptive immunity longitudinally with ASCVD, and assessed whether arterial calcifications play a role in this association.

Methods and findings: Granulocyte (innate immunity) and lymphocyte (adaptive immunity) counts were determined 3 times (2002–2008, mean age 65.2 years; 2009–2013, mean age 69.0 years; and 2014–2015, mean age 78.5 years) in participants of the population-based Rotterdam Study without ASCVD at baseline. Participants were followed-up for ASCVD or death until 1 January 2015. A random sample of 2,366 underwent computed tomography at baseline to quantify arterial calcification volume in 4 vessel beds. We studied the association between immunity components with risk of ASCVD and assessed whether immunity components were related to arterial calcifications at baseline. Of 7,730 participants (59.4% women), 801 developed ASCVD during a median follow-up of 8.1 years. Having an increased granulocyte count increased ASCVD risk (adjusted hazard ratio for doubled granulocyte count [95% CI] = 1.78 [1.34–2.37], $P < 0.001$). Higher granulocyte counts were related to larger calcification volumes in all vessels, most prominently in the coronary arteries (mean difference in calcium volume [mm^3] per SD increase in granulocyte count [95% CI] = 32.3 [9.9–54.7], $P < 0.001$). Respectively, the association between granulocyte count and incident coronary heart disease and stroke was partly mediated by coronary artery calcification (overall proportion mediated [95% CI] = 19.0% [–10% to 32.3%], $P = 0.08$) and intracranial artery calcification (14.9% [–10.9% to 19.1%], $P = 0.05$). A limitation of our study is that studying the etiology of ASCVD remains difficult within an epidemiological setting due to the limited availability of surrogates for innate and especially adaptive immunity.

Conclusions: In this study, we found that an increased granulocyte count was associated with a higher risk of ASCVD, whereas an increase in lymphocytes was protective for ASCVD in the general population. Moreover, higher levels of granulocytes were associated with larger volumes of arterial calcification. Arterial calcifications may explain a proportion of the link between granulocytes and ASCVD.

INTRODUCTION

Atherosclerotic cardiovascular disease (ASCVD) arises from various interacting pathophysiological processes [1]. Recent findings point towards a key role of the immune system, which can be broadly classified into innate and adaptive immunity [2]. Innate immunity refers to immune responses present at birth, whereas adaptive immunity is acquired during life by exposure to antigens [3]. The role of innate immunity in the pathophysiology of ASCVD has been recognized on the basis of evidence from experimental and observational data [4–8]. To date, however, no easy, accessible, low-cost treatment has been identified to effectively target the innate immune system for preventing ASCVD [8]. Given the mechanistic diversity of inflammatory pathways, considering other pathways could widen avenues for therapeutic targets, for example targeting the adaptive immune system. Adaptive immune cells may provide protective responses at atherosclerotic sites, as has been shown in neurodegenerative disorders [9,10] and also in cardiovascular disease in experimental and clinical studies [11]. Moreover, repeatedly measuring inflammation may better capture the biologically dynamic processes of these 2 immune systems than a single measurement, because of the assessment of changes over time.

Recent work from the field of cancer research suggests that measuring granulocytes and platelets provides important markers of innate immunity [12–14], whereas measuring lymphocytes yields information on adaptive immunity [15]. Furthermore, combining these measurements into ratios, i.e., the granulocyte-to-lymphocyte ratio (GLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII), is thought to even better reflect the relative balance between innate and adaptive immunity [16–18]. In this study, we first determined the longitudinal association of these immunity components with risk of ASCVD in the general population by using the framework of joint models for longitudinal and survival data, and hypothesized that an increase in innate immunity markers and decrease in adaptive immunity lead to increased risk of ASCVD. Second, we examined the association between the immunity components and arterial calcifications to explore atherosclerosis as a possible mediator.

METHODS

Study population

The present study is embedded within the Rotterdam Study, a prospective population-based cohort study in Rotterdam, the Netherlands. The Rotterdam Study started in 1989 with 7,983 persons (78% response rate) aged ≥55 years and residing in the district Ommoord, a suburb of Rotterdam. This first subcohort (RS-I) was extended with a second subcohort (RS-II) in 2000, consisting of 3,011 persons (67% response rate) aged ≥55 years, and with a

third subcohort (RS-III) in 2006, composed of 3,932 persons aged ≥45 years (65% response rate). The design of the Rotterdam Study has been described in detail previously [19]. In brief, participants were examined at study entry and at follow-up visits every 3 to 5 years. They were interviewed at home by a trained research nurse, followed by 2 visits at the research facility for additional interviewing, laboratory assessments, and imaging.

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act [WBO], license number 1071272-159521-PG). The Rotterdam Study personal registration data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands Trial Register (https://www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (https://www.who.int/ictrp/network/primary/en/) under the shared catalogue number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians.

For the current study, the analysis plan was drafted in January 2019 (S1 Analysis Plan). This study is reported as per the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guideline (S1 STROBE Checklist).

Laboratory tests for granulocytes, platelets, and lymphocytes were introduced from 2002 onwards, corresponding with the following assessment rounds in the Rotterdam Study (baseline in this study): fourth round of RS-I, second round of RS-II, and first round of RS-III (n = 9,996 in total).

Assessment of blood cell counts and their derived ratios

Fasting blood samples were drawn during each visit at the research center, with a maximum of 3 visits during follow-up. From 7,730 participants, 5,085 participants were available for a second blood cell assessment, and 270 participants were available for a third blood cell assessment. Full blood count measurements were performed using the Coulter AcT diff2 Hematology Analyzer (Beckman Coulter, Brea, CA, US) directly after the blood sample was drawn. Laboratory measurements included absolute granulocyte, platelet, and lymphocyte counts in 10^9 per liter. GLR and PLR were calculated as the ratio of granulocyte count to lymphocyte count and as the ratio of platelet count to lymphocyte count, respectively. SII was defined as platelet count times GLR [20].

Assessment of stroke and coronary heart disease

The clinical outcomes of interest included first fatal or nonfatal stroke or coronary heart disease (CHD) separately as well as combined into ASCVD as previously described. Follow-up data were collected through general practitioners and subsequent collection of information from letters by medical specialists and discharge reports, in cases of hospitalization. CHD

included (1) nonfatal myocardial infarction (MI) and (2) CHD mortality (mortality with definite MI, definite CHD, or possible CHD as underlying cause of death) [21]. Stroke was defined as a syndrome of rapidly developing symptoms of focal or global cerebral dysfunction lasting ≥24 hours or leading to death, with apparent vascular cause [22,23]. We categorized strokes as ischemic or hemorrhagic, based on neuroimaging reports and clinical symptoms, or as unspecified if we were unable to differentiate the stroke type further. Subarachnoid hemorrhages due to ruptured aneurysms were not considered as stroke. Follow-up was virtually complete until 1 January 2015 (96.6% of potential person-years observed). ASCVD furthermore included other ASCVD mortality.

Assessment of arterial calcification

Non-contrast computed tomography (CT) images were obtained using a 16-slice or 64-slice multidetector CT scanner (Somatom Sensation 16 or 64; Siemens, Forchheim, Germany). Using a cardiac scan and an extracardiac scan that reached from the aortic root to the intracranial vasculature (1 centimeter above the sella turcica), we visualized the following vessels: coronary arteries, aortic arch, extracranial internal carotid arteries, and intracranial internal carotid arteries. Radiation dose and other imaging parameters of both scans are described elsewhere [24,25].

The amount of calcification in the coronary arteries, aortic arch, and extracranial carotid arteries was quantified using widely used, commercially available dedicated software (Syngo. via; Siemens). Calcification in the coronary arteries comprised a summation of calcification in the left main, left anterior descending, left circumflex, and right coronary artery. Calcification in the aortic arch was scored from the origin of the aortic arch (defined as the image in which the ascending and descending aorta merge into the inner curvature of the aortic arch) to the first 1 cm of the branches originating from the arch. Calcification in the extracranial carotid arteries was assessed within 3 cm proximal and distal of the bifurcation on both sides and summed.

Intracranial internal carotid artery calcification was assessed from the horizontal segment of the petrous internal carotid artery to the top of the internal carotid artery. Scoring was done semi-automatically, because automated software is not yet available for this region (the close relationship between calcification and the skull base precludes the use of commercial software given a high false-positive rate). Regions of interest were manually drawn in the course of the intracranial internal carotid artery on both sides, after which the number of pixels within these regions above 130 Hounsfield units was summed and multiplied by the pixel size and the slice increment in order to obtain a volume in cubic millimeters [26,27].

Covariates

We assessed education, smoking, and use of antihypertensive, lipid-lowering, and antidiabetic medication at baseline by interview. Diastolic and systolic blood pressures were mea-

sured twice on the right arm with a random-zero sphygmomanometer, of which the mean was used. Total cholesterol and high-density lipoprotein (HDL) cholesterol were measured with a Coulter AcT diff2 Hematology Analyzer. Body mass index (BMI) was computed from measurements of height and weight (kg/m^2). Diabetes mellitus was defined as use of antidiabetic medication, fasting serum glucose level ≥ 7.1 mmol/l (≥127.9 mg/dl), or random non-fasting serum glucose level ≥ 11.1 mmol/l (≥200.0 mg/dl) [28]. High-sensitivity C-reactive protein (hs-CRP) was determined in serum that was drawn during the third assessment round in the Rotterdam Study, i.e., third round of RS-I (1996–1999), which was stored at −20 °C until performance of the hs-CRP measurements in 2003 to 2004. Hs-CRP was measured using Rate Near Infrared Particle Immunoassay (Immage Immunochemistry System, Beckman Coulter). History of cancer was obtained from general practitioners' medical records (including hospital discharge letters), the Dutch Hospital Data registry, and regional histopathology and cytopathology registries. Furthermore, we assessed history of immune-modulating medication use—nonsteroidal anti-inflammatory drugs (NSAIDs) (ATC code M01), immunosuppressives (ATC code L04), and methotrexate (ATC code L01BA01)—by number of prescriptions between 1 January 1995 and blood draw date.

Statistical analysis

Because the blood cell counts and their derived ratios have a skewed distribution and to adhere to the linearity assumptions, the analysis was based on the natural logarithmic (Ln) transformation of their values. We first determined the association of the granulocyte, platelet, and lymphocyte counts and their derived ratios with the risk of stroke and CHD separately as well as ASCVD, using the framework of joint models for longitudinal and survival data [29]. For the longitudinal data, a linear mixed effects model was used, in which we specified the immunity component as the dependent variable and time as the independent variable to assess the mean change in immunity components. Random intercepts and slopes were used to incorporate individual response trajectories. For modeling survival data, we used a Cox regression model in which we included the true underlying profile of the blood cell counts as estimated from the longitudinal model. Here the baseline risk function assumed a piecewise constant with 6 knots placed at equally spaced percentiles of the observed event times. Joint models allow accounting for (1) measurement error during follow-up, i.e., biological variation, (2) the effect of factors at an earlier time point on the values of blood cell measurements at a later time point [30], and (3) the correlations in the repeated measurements of the blood cell counts. Because we are using log-log models ($\log Y_i = \alpha + \beta \log X_i + \varepsilon$), we report hazard ratios (HRs) with 95% confidence intervals (CIs) obtained by exponentiating Ln(2) times the estimated coefficients from the Cox model. These HRs correspond to the increase in risk for a doubling of the blood cell count or ratio. We computed 2 nested models: Model I was adjusted for baseline age (continuous, centered as age minus mean age) and sex; model II was additionally adjusted for education, smoking

status, BMI, diabetes mellitus, systolic and diastolic blood pressure (continuous, centered as blood pressure minus mean blood pressure), antihypertensive medication, total cholesterol, HDL cholesterol, and lipid-lowering medication. For assessment of the association between individual blood cell counts and ASCVD, we performed the analyses with adjustment for the baseline blood cell counts of the remaining 2 blood cell types. Follow-up time was used as the time scale and started at the date of first laboratory assessment; follow-up continued until date of stroke, CHD, death, loss to follow-up, or 1 January 2015, whichever came first. We also investigated the association of the blood cell counts and their ratios with ischemic and hemorrhagic stroke separately. We assessed the joint model assumptions by examining the proportional hazards assumption using Schoenfeld residuals, and goodness of fit using Cox–Snell residuals for the survival part and marginal residuals for the longitudinal part.

In sensitivity analyses, we additionally excluded participants with a history of cancer or a history of immune-modulating medication at baseline. In addition, we performed additional adjustments for NSAID use. We explored effect modification by stratifying by age, sex, hs-CRP (at a cutoff of 2 mg/l), use of lipid-lowering medication, coronary artery calcification volume (at a cutoff of 10 mm^3), and smoking, all at baseline. We formally tested interaction between these factors and blood cell counts on the multiplicative scale by adding interaction terms to model II. Lastly, we repeated the analyses for the granulocytes and lymphocytes and risk of ASCVD using age as the time scale instead of follow-up time to account for potential residual confounding by age and to minimize potential effects of left truncation.

Next, in order to explore possible mechanisms, we performed a cross-sectional analysis assessing the association of the granulocyte, platelet, and lymphocyte counts and their derived ratios at baseline, per standard deviation increase, with calcification volume in each vessel bed (coronary arteries, aortic arch, extracranial carotid arteries, and intracranial carotid arteries) using linear regression models. To facilitate interpretation of these analyses, we chose to first standardize the blood cell counts only, to make results comparable, and keep the rest of the variables untransformed. We adjusted these analyses for age and sex (model I), and additionally for education, smoking status, BMI, diabetes mellitus, systolic blood pressure, diastolic blood pressure, antihypertensive medication, total cholesterol, HDL cholesterol, and lipid-lowering medication (model II). To adhere to the homoscedasticity and linearity assumptions, we repeated linear regression models after natural log-transforming exposure and outcome variables. As a sensitivity analysis, we looked at the association between the granulocyte, platelet, and lymphocyte counts and their derived ratios and continuous arterial calcification only in those with non-zero arterial calcification, since the magnitude of the increase from 0 to 1 for volume may not be the same as other incremental increases in arterial calcification.

Finally, we performed a mediation analysis to assess a possible mediating effect of calcification volume in the coronary arteries and intracranial carotid arteries on the association between innate immunity and the risk of CHD and stroke, respectively. We chose the innate

immunity marker displaying the strongest association with both calcifications and incident CHD or stroke for this analysis. We tested the overall proportion mediated by calcifications. To test for mediation effects, we used the decomposition analysis techniques as described by VanderWeele [31]. Participants with a history of CHD or stroke were excluded from the analysis. We analyzed the association of innate immunity with calcifications with linear regression models. The association of innate immunity or calcifications with CHD or stroke was investigated by Cox regression models.

Multiple imputation by chained equations was used for missing covariates (maximum of 1.5%), with 5 imputed datasets based on other covariates and the outcome. Rubin's method was used for pooling the 5 analyses to obtain pooled HRs and 95% CIs [32]. Two-sided $P < 0.05$ was considered statistically significant for all analyses. Statistical analyses were performed using the R packages *mice, survival, nlme, JM,* Results

From 8,712 participants, we excluded those with a history of ASCVD (n = 819) at baseline, those who had incomplete data regarding their history of ASCVD (n = 95), and those who did not provide informed consent to assess medical records during follow-up (n = 60). Lastly, we excluded participants with incomplete laboratory assessment (n = 8), resulting in 7,730 participants for analysis (flowchart in Fig 1). From the fourth round of RS-I and the second round of RS-II, a random sample was invited to undergo non-contrast CT to quantify arterial calcification. Of these participants, 2,366 had complete calcification and blood assessment.

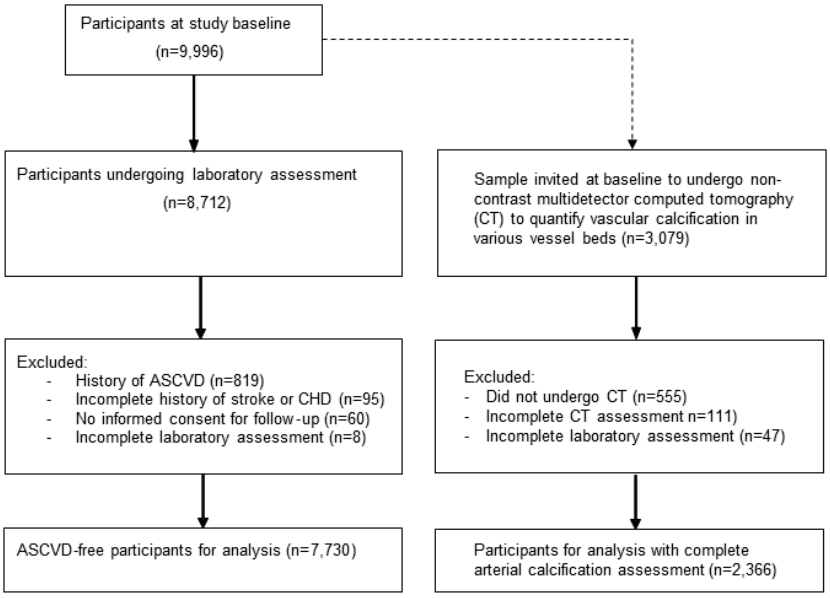

Fig 1. Flowchart of study population.
ASCVD, atherosclerotic cardiovascular disease; CHD, coronary heart disease.

Characteristics of the study participants are presented in Table 1. Mean age of included study participants was 65.2 years, and 59.4% were women. During a median (interquartile range) follow-up of 8.1 (3.4) years (62,095 person-years), 801 participants developed ASCVD, of whom 423 participants developed CHD and 378 stroke. ASCVD-free survival probability and average change of granulocytes and lymphocytes over time are presented in S1 Fig in the total population and in S2 Fig in younger and older individuals separately.

Table 1. Baseline characteristics.

Characteristic	Laboratory assessment cohort N = 7,730	Sample undergoing CT N = 2,366
Women	4,594 (59.4%)	1,236 (52.2%)
Age, years	65.2 (10.2)	69.1 (6.7)
Smoking		
Ever	5,278 (68.7%)	1,662 (71.2%)
Never	2,401 (31.3%)	670 (28.7%)
Diabetes mellitus	415 (5.4%)	129 (5.5%)
BMI, kg/m^2	27.6 (4.4)	27.6 (4.0)
Education		
Primary	830 (10.8%)	179 (7.7%)
Lower	3,124 (40.7%)	979 (42.1%)
Intermediate	2,205 (28.8%)	716 (30.8%)
Higher	1,509 (19.7%)	451 (19.4%)
Systolic blood pressure, mm Hg	142.3 (21.8)	146.7 (20.0)
Diastolic blood pressure, mm Hg	81.3 (11.0)	80.2 (10.7)
Antihypertensive drugs	2,571 (33.6%)	921 (39.5%)
Total cholesterol, mmol/l	5.7 (1.0)	5.6 (1.0)
HDL cholesterol, mmol/l	1.5 (0.4)	1.4 (0.4)
Lipid-lowering medication	1,429 (18.7%)	535 (23.0%)
Granulocyte count, ×10^3/µl	4.0 (1.4)	4.0 (1.3)
Platelet count, ×10^3/µl	270.9 (66.6)	259.0 (64.0)
Lymphocyte count, ×10^3/µl	2.3 (1.1)	2.3 (1.0)
GLR	1.9 (0.8)	1.9 (0.9)
PLR	127.9 (46.4)	124.9 (45.8)
SII	509.3 (270.7)	494.3 (272.2)

Data presented as mean (standard deviation) for continuous variables and number (percentage) for categorical variables. Number of missing values for the ASCVD-free cohort: 51 (0.7%) for smoking, 22 (0.3%) for diabetes, 25 (0.3%) for BMI, 62 (0.8%) for education, 41 for systolic and diastolic blood pressure (0.5%), 69 for blood pressure lowering medication (0.9%), 25 for cholesterol (0.3%), 27 for HDL cholesterol (0.3%), and 69 for lipid lowering medication (0.9%). Number of missing values for the sample undergoing CT are: 52 (0.6%) for smoking, 22 (0.3%) for diabetes, 26 (0.3%) for BMI, 41 (0.5%) for education, 14 for systolic and diastolic blood pressure (0.6%), 35 for blood pressure lowering medication (1.5%), 6 for cholesterol (0.3%), 6 for HDL cholesterol (0.3%) and 35 for lipid lowering medication (1.5%).

BMI, body mass index; CT, computed tomography; GLR, granulocyte-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index.

An increased granulocyte count, but not platelet count, was associated with an increased risk of ASCVD (adjusted HR [95% CI] for doubled granulocyte count = 1.78 [1.34–2.37], P < 0.001, and for doubled platelet count = 1.17 [0.90–1.51], P = 0.24, Table 2). With respect to adaptive immunity, an increased level of lymphocytes was associated with a decreased risk of ASCVD, although this result was not statistically significant (adjusted HR for doubled lymphocyte count [95% CI] = 0.87 [0.71–1.06], P = 0.17). Both an increased GLR and an increased SII were associated with increased ASCVD risk (adjusted HR for doubled GLR [95% CI] = 1. 37 [1.14–1.65], P = 0.001, and for doubled SII = 1.19 [1.03–1.36], P = 0.02), whereas an increased PLR was not associated with ASCVD risk. We observed no major violations of the joint model assumptions. Effect estimates did not materially change, and in fact became stronger, when we excluded participants with a history of cancer or history of immune-modulating medication at baseline (723 ASCVD cases among 7,063 remaining participants) (adjusted HR for doubled granulocyte count [95% CI] = 1.90 [1.41–2.57], P < 0.001, and for doubled lymphocyte count = 0.82 [0.65–1.04], P = 0.10). Additional adjustment for NSAID use did not change the effect estimates. We found that the association between higher levels of granulocytes and risk of ASCVD was more pronounced in participants aged younger than the median age of 64 years compared to those 64 years or older (adjusted HR for doubled granulocyte count [95% CI] = 2.32 [1.20–4.47], P < 0.001) ($P_{interaction}$ = 0.002). Results of all stratified analyses with interaction terms are shown in S1 Table. Lastly, risk estimates were comparable when using age as the time scale instead of follow-up time.

Table 2. Joint models for the association between repeated blood-based immunity markers and subsequent risk of ASCVD.

Model and immunity marker	ASCVD n/N = 801/7,730		Stroke n/N = 378/7,730		CHD n/N = 423/7,730	
	HR (95% CI)	**P value**	**HR** (95% CI)	**P value**	**HR** (95% CI)	**P value**
Model I						
Innate immunity*						
Granulocytes	2.63 (2.03–3.40)	<0.001	2.14 (1.48–3.11)	<0.001	3.10 (2.18–4.42)	<0.001
Platelets	1.10 (0.85–1.41)	0.47	1.27 (0.88–1.83)	0.21	0.96 (0.68–1.36)	0.83
Adaptive immunity*						
Lymphocytes	1.03 (0.85–1.25)	0.77	0.96 (0.72–1.29)	0.80	1.09 (0.83–1.43)	0.54
Balance between innate and adaptive immunity						
GLR	1.52 (1.27–1.82)	<0.001	1.47 (1.13–1.91)	0.004	1.56 (1.22–2.01)	<0.001
PLR	1.02 (0.87–1.19)	0.84	1.15 (0.91–1.46)	0.24	0.97 (0.78–1.20)	0.76
SII	1.25 (1.09–1.43)	0.002	1.27 (1.04–1.56)	0.02	1.24 (1.03–1.51)	0.03

Table 2. Joint models for the association between repeated blood-based immunity markers and subsequent risk of ASCVD. (continued)

Model and immunity marker	ASCVD n/N = 801/7,730		Stroke n/N = 378/7,730		CHD n/N = 423/7,730	
	HR (95% CI)	**P value**	**HR** (95% CI)	**P value**	**HR** (95% CI)	**P value**
Model II						
Innate immunity*						
Granulocytes	1.78 (1.34–2.37)	<0.001	1.50 (1.00–2.27)	0.05	1.98 (1.34–2.93)	0.001
Platelets	1.17 (0.90–1.51)	0.24	1.38 (0.95–2.01)	0.09	1.01 (0.71–1.44)	0.95
Adaptive immunity*						
Lymphocytes	0.87 (0.71–1.06)	0.17	0.85 (0.63–1.15)	0.29	0.88 (0.66–1.17)	0.37
Balance between innate and adaptive immunity						
GLR	1.37 (1.14–1.65)	0.001	1.31 (1.00–1.70)	0.05	1.37 (1.07–1.78)	0.01
PLR	1.17 (0.99–1.38)	0.06	1.25 (0.98–1.59)	0.07	1.10 (0.88–1.38)	0.41
SII	1.19 (1.03–1.36)	0.02	1.21 (0.99–1.48)	0.06	1.16 (0.96–1.41)	0.13

HR is per doubled blood cell count. Model I is adjusted for age and sex. Model II is adjusted for age, sex, education, smoking status, body mass index, diabetes mellitus, systolic and diastolic blood pressure, antihypertensive medication, high-density lipoprotein cholesterol, total cholesterol, and lipid-lowering medication=. All markers were logarithmically transformed.

*Analysis for each blood cell type adjusted for the baseline blood cell counts of the remaining 2 blood cell types.

ASCVD, atherosclerotic cardiovascular disease; CHD, coronary heart disease; CI, confidence interval; GLR, granulocyte-to-lymphocyte ratio; HR, hazard ratio; *n,* number of incident events; *N,* number of participants for analysis; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index (defined as platelet count times GLR).

When examining CHD and stroke as separate events, an increased granulocyte count was associated with an increased risk of both events (adjusted HR for doubled granulocyte count [95% CI] = 1.98 [1.34–2.93], *P* = 0.001, for CHD and 1.50 [1.00–2.27], *P* = 0.05, for stroke; Table 2). An increased platelet count was associated with higher risk of stroke only (adjusted HR for doubled platelet count [95% CI] = 1.38 [0.95–2.01], *P* = 0.09). An increased lymphocyte count showed similar protective effects for both CHD and stroke, although these effects were not statistically significant (adjusted HR for doubled lymphocyte count [95% CI] = 0.88 [0.66–1.17], *P* = 0.37, for CHD and 0.85 [0.63–1.15], *P* = 0.29, for stroke). With respect to the ratio measures, an increased GLR was associated with both increased CHD and stroke risk (adjusted HR for doubled GLR [95% CI] = 1.37 [1.07–1.78], *P* = 0.01, for CHD and 1.31 [1.00–1.70], *P* = 0.05, for stroke). When investigating ischemic and hemorrhagic stroke separately, an increased granulocyte count

was associated with an increased risk of ischemic stroke (HR for doubled granulocyte count [95% CI] = 1.44 [0.90–2.31], P = 0.13) and hemorrhagic stroke (2.48 [0.86–7.14], P = 0.09), whereas platelets only increased risk of ischemic stroke (HR for doubled platelet count [95% CI] = 1.44 [0.93–2.22], P = 0.10).

Regarding the association of immunity with arterial calcification, we found that higher levels of granulocytes at baseline related to larger calcification volumes in all arteries, but most prominently in the coronary arteries (mean difference in calcium volume [mm^3] per SD increase in granulocyte count [95% CI] = 32.3 [9.1 to 54.7], P < 0.001). Regarding adaptive immunity, higher levels of lymphocytes were not related to calcification volumes. Higher levels of GLR and SII were related to larger calcification volumes in the aortic arch only (mean difference in calcium volume [mm^3] per SD increase [95% CI] = 48.6 [2.5 to 94.8], P = 0.04, and 58.8 [13.8 to103.8], P = 0.03, respectively). In Table 3, the linear regression models after transforming the exposure and outcome variables are presented. Our sensitivity analyses with non-zero calcifications similarly showed associations between higher levels of granulocytes and larger calcification volumes in all arteries, but the analysis with extracranial internal carotid artery calcification as outcome did not reach statistical significance.

Table 3. Models for the association between blood-based immunity markers and arterial calcification volume.

Immunity marker[a]	Arterial calcification volume[b]							
	Coronary arteries		Aortic arch		Extracranial internal carotid		Intracranial internal carotid	
	Mean difference (95% CI)	P value	Mean difference (95% CI)	P value	Mean difference (95% CI)	P value	Mean difference (95% CI)	P value
Innate immunity*								
Granulocytes	0.26 (0.14; 0.39)	<0.001	0.20 (0.07; 0.32)	<0.001	0.22 (0.09; 0.35)	<0.001	0.18 (0.05; 0.32)	0.01
Platelets	−0.02 (−0.18; 0.13)	0.76	0.01 (−0.14; 0.17)	0.87	−0.05 (−0.21; 0.11)	0.57	−0.09 (−0.25; 0.07)	0.27
Adaptive immunity*								
Lymphocytes	0.15 (0.03; 0.27)	0.02	0.01 (−0.11; 0.13)	0.91	0.09 (−0.04; 0.21)	0.17	0.08 (−0.04; 0.21)	0.19
Balance between innate and adaptive immunity								
GLR	0.06 (−0.04; 0.15)	0.24	0.10 (0.00; 0.19)	0.04	0.06 (−0.03; 0.16)	0.18	0.04 (−0.05; 0.14)	0.36
PLR	−0.09 (−0.19; 0.01)	0.08	0.01 (−0.09; 0.11)	0.85	−0.06 (−0.17; 0.04)	0.25	−0.08 (−0.18; 0.03)	0.15
SII	0.06 (−0.01; 0.14)	0.10	0.09 (0.01; 0.16)	0.03	0.06 (−0.02; 0.13)	0.15	0.03 (−0.05; 0.11)	0.48

Adjusted for age, sex, education, smoking status, body mass index, diabetes mellitus, systolic and diastolic blood pressure, antihypertensive medication, high-density lipoprotein cholesterol, total cholesterol, and lipid-lowering medication.

[a]All markers were natural log-transformed (Ln[immunity component × 10^3/µl]).

[b]We added 1.0 mm^3 to the non-transformed values to deal with calcification volumes of 0 and used standardized natural log-transformed values (Ln[calcification volume + 1.0 mm^3]).

*Analysis for each blood cell type adjusted for the baseline blood cell counts of the remaining 2 blood cell types.

CI, confidence interval; GLR, granulocyte-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index.

The association between granulocytes and incident CHD was partly mediated by coronary artery calcifications (overall proportion mediated [95% CI] = 19.0% [−10% to 32.3%], P = 0.08), while the association between granulocytes and incident stroke was partly mediated by intracranial artery calcifications (14.9% [−10.9% to 19.1%], P = 0.05), albeit this finding was not statistically significant.

DISCUSSION

In this study, we found that an increase of innate immunity markers over time, as reflected by a doubling of the granulocyte count and the ratios GLR and SII, was robustly associated with a higher risk of ASCVD. Furthermore, higher levels of granulocytes at baseline were related to larger burden of subclinical atherosclerosis, as measured by arterial calcifications. Coronary artery calcifications mediated 19.0% of the association between granulocytes and CHD, while intracranial artery calcifications mediated 14.9% of the association between granulocytes and stroke.

To date, few population-based studies have investigated the association between innate immunity markers and cardiovascular outcomes. Elevated GLR has been linked to ischemic stroke incidence and cardiovascular disease [35,36]. A meta-analysis of 38 studies showed that elevated GLR was associated with coronary artery disease, acute coronary syndrome, stroke, and composite cardiovascular events [37]. However, most of these studies were cross-sectional or were performed in selected clinical populations of patients with cardiovascular disease. Besides observational studies, various clinical trials have targeted the immune system to reduce the risk of ASCVD. One of the largest is the CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study) randomized trial, in which patients with elevated hs-CRP and previous MI were assigned to receive canakinumab or placebo. Canakinumab selectively inhibits interleukin-1β, resulting in an inhibition of the innate immunity. The 150-mg dose resulted in a lower incidence of recurrent cardiovascular events, compared to placebo [38]. In addition to interleukin-1β, also hs-CRP, fibrinogen, and interleukin-6, all markers in the same cascade, have been shown to have an association with CHD [39–41]. Our findings also show an important role of innate immunity in first-ever stroke and CHD risk in the general population, independent of hs-CRP level or lipid-lowering medication use.

Interestingly, a more recent trial assessing low-dose methotrexate to reduce inflammation in order to prevent recurrent cardiovascular disease showed that methotrexate did not lower levels of interleukin-1β, interleukin-6, or hs-CRP, and that it did not result in a lower number of cardiovascular events compared to placebo [8]. Together with the results from the CANTOS trial, these studies indicate that reducing the risk of cardiovascular events through inflammation may depend on the pathway targeted. In addition to these established

pathways, our results indicate that the pathway of adaptive immunity may also affect the risk of cardiovascular disease. More specifically, lymphocyte count, an important player in adaptive immunity, showed protective effects on the risk of ASCVD, although this finding was not statistically significant. Indeed, mobilizing anti-inflammatory T cells to atherosclerotic sites may provide protective responses as has been shown in neurodegenerative disorders [9] and also in cardiovascular disease in experimental and clinical studies [11]. These cells thus additionally serve as a promising therapeutic target to control the proinflammatory processes of atherosclerosis. The significant association we found between GLR and SII and incident ASCVD, although weaker than with granulocytes, supports the hypothesis that ASCVD results from an imbalance between innate and adaptive immunity. Future studies may focus more specifically on targeting both innate and adaptive immunity, rather than only innate immunity, and refine insights into these pathways.

Although previous studies have established the inflammatory hypothesis of atherothrombosis in coronary artery disease, fewer studies have specifically investigated stroke. However, increasing evidence from experimental studies suggests an equally important role for the immune response in stroke [42]. Similarly, we demonstrated that higher levels of granulocytes were associated with stroke of any type, as well as with ischemic and hemorrhagic stroke separately. Especially the latter finding warrants further research since most studies so far have mainly focused on inflammatory responses after the occurrence of an intracerebral hemorrhage, and not before [43].

An interesting finding of our study is that the association between higher levels of granulocytes and risk of ASCVD was more pronounced in younger individuals than older individuals (age below the median [64 years]: adjusted HR [95% CI] for doubled granulocyte count = 2.32 [1.20–4.47], $P < 0.001$; age at or above the median: 1.67 [1.21–2.29], $P = 0.002$). Recently, an English and Dutch study found that while there has been a decline in mortality from acute stroke within 30 days, stroke event rates have increased and 15-year mortality risk in young stroke-survivors remained elevated [44,45]. This suggests that stroke awareness and stroke prevention need to start at much lower age to reduce the occurrence of stroke in younger people. Our results show that inflammation reduction could be a potential important strategy to achieve this.

Moreover, our study showed that coronary and intracranial carotid artery calcifications substantially mediate the association of granulocytes with CHD and stroke, respectively. The observed proportion of mediation for CHD was 19.0%, while for stroke the proportion mediated was 14.9%. This is meaningful given the multiple mechanisms through which innate immunity affects cardiovascular health, including atrial fibrillation (AF) and coagulation [46]. Indeed, the infiltration of immune cells and proteins that cause an inflammatory response in cardiac tissue and circulatory processes is associated with AF [47], and AF is in turn an important risk factor for CHD and stroke. Another possible mediator is blood coagulation, since inflammation results in activation of coagulation, due to tissue-factor-

mediated thrombin generation, downregulation of physiological anticoagulant mechanisms, and inhibition of fibrinolysis, increasing the risk of CHD and stroke [48]. Furthermore, besides calcifications, plaques may consist of intraplaque hemorrhage and a lipid core. Although calcifications are an adequate quantitative measure for atherosclerosis, vulnerable plaques without calcification, which are more strongly associated with ASCVD [49], are not quantified by this method. Therefore, it is plausible that the true proportion of inflammation mediated through atherosclerosis of all plaque components is actually higher than the measured proportion mediated through the calcification component.

Blood cell count measurements display short-term variability or measurement error, but we do not expect that this variability affected our results for several reasons. First, we expect to have captured a more chronic or stable effect of immune status because we used blood cell counts measured at multiple time points per participant. Although this longitudinal information was collected intermittently and with error, we could successfully reconstruct the complete longitudinal history by postulating a suitable mixed effects model to describe participant-specific time evolutions. The mixed model accounted for the measurement error problem by postulating that the observed level of the blood cell count measurements equaled the true level of the blood cell count measurements plus a random error term. Thus, the joint models we used accounted for measurement error or variability of the blood cell count measurements. Second, as a sensitivity analysis, we additionally excluded participants with a history of cancer or a history of immune-modulating medication at baseline, and we performed additional adjustments for NSAID use. We performed these sensitivity analyses specifically to address the issue of participants having significantly altered blood cell counts due to underlying disease processes. The results of these analyses did not show significant changes in our effect estimates. Lastly, by extensively correcting for potential confounders such as BMI, we attempted to rule out effects of determinants of blood cell counts as much as possible.

Our study has some limitations. First, studying the etiology of ASCVD, which is the focus of this study, remains difficult within an epidemiological setting due to the limited availability of surrogates for innate and especially adaptive immunity. Second, we could not directly relate the effect of hs-CRP with the different blood cell measurements and ASCVD risk since we did not have hs-CRP measurements available at study baseline, but only 1 round before. Third, the vast majority of our population is of European ancestry (97.6%), potentially limiting generalizability to other ethnicities. Fourth, it is important to note the possible influence of unmeasured confounders on our results. Fifth, due to the cross-sectional nature of the analyses regarding the association between immunity markers and calcifications, there is potential for reverse causality. However, according to the present evidence, it is very plausible that inflammation comes first since activation of endothelial cells results in increased expression of leukocyte adhesion molecules and the release of chemokines, which attract leukocytes that subsequently infiltrate the vascular wall and drive atherogenesis [50].

Finally, the measurement error for both subclinical ASCVD and immune cell subsets limits the mediation analysis in its reliability. However, since this measurement error introduces random error into the direct effect, it is expected to lead to underestimation of the mediation. Of course, we cannot rule out that it could bias the results in the opposite direction and inflate mediation estimates.

In conclusion, our study shows that increased granulocyte count, GLR, and SII are associated with a higher risk of ASCVD, whereas increased lymphocyte count is protective for ASCVD in the general population. Moreover, higher levels of granulocytes are associated with subclinical atherosclerosis, as measured by arterial calcifications. These calcifications explain a substantial proportion of the link between granulocytes and ASCVD. Our findings support the hypothesis that ASCVD results from an imbalance between innate and adaptive immunity.

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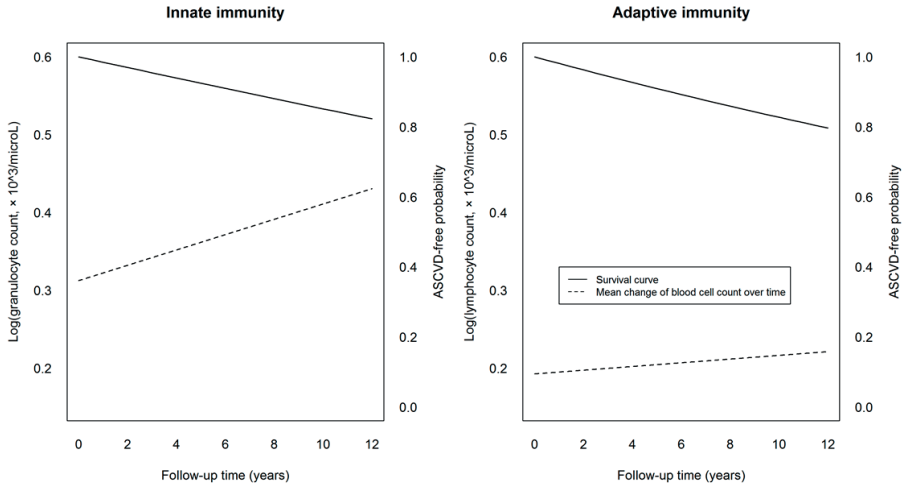
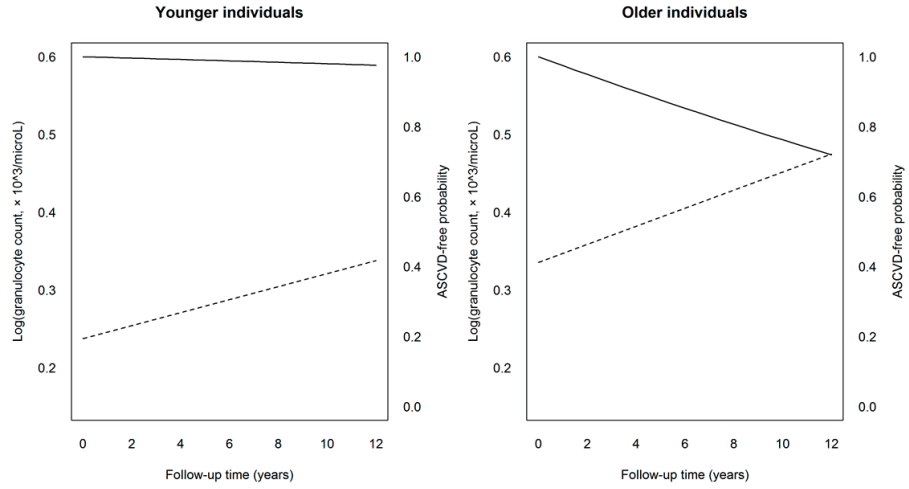
SUPPORTING INFORMATION



S1 Fig. ASCVD-free survival and mean change of blood cell count over time in the total population. The left axis in each panel denotes the blood cell count (dotted line), the right axis the ASCVD-free survival (solid line). All curves were adjusted for all covariates in model II.



S2 Fig. ASCVD-free survival and mean change of granulocyte count over time stratified by younger and older participants.

Younger participants are those below the median age of 64 years. Of these participants, 142 developed ASCVD, out of 3,799 persons at risk.

S1 Table. Models for the stratified analyses.

Laboratory assessmenta		Age		Sex		Smoking
Per doubling	P for interaction	HR (95% CI), P	P for interaction	HR (95% CI), P	P for interaction	
Granulocytes*	0.002	Low: 2.32 (1.20-4.47), <.001	0.245	Women: 1.30 (0.88-1.93), 0.18	0.565	Never: 1.49 (1.06-2.09), 0.02
		High: 1.67 (1.21-2.29), 0.002		Men: 2.32 (1.53-3.52), <.001		Ever: 2.19 (1.27-3.77), 0.005
Platelets*	0.867	Low: 1.65 (0.87-3.14), 0.13	0.167	Women: 1.11 (0.76-1.62), 0.6	0.784	Never: 1.34 (1.00-1.81), 0.05
		High: 1.08 (0.81-1.45), 0.6		Men: 1.26 (0.88-1.79), 0.21		Ever: 0.79 (0.47-1.31), 0.363
Lymphocytes*	0.694	Low: 0.97 (0.56-1.66), 0.9	0.209	Women: 0.83 (0.62-1.11), 0.2	0.028	Never: 0.78 (0.61-0.99), 0.04
		High: 0.87 (0.7-1.09), 0.23		Men: 0.91 (0.68-1.22), 0.55		Ever: 1.06 (0.73-1.55), 0.746
GLR	0.009	High: 1.3 (1.08-1.58), 0.007	0.945	Men: 1.49 (1.15-1.93), 0.003	0.172	Never: 1.38 (1.12-1.7), 0.003
		Low: 1.52 (0.93-2.48), 0.09		Women: 1.25 (0.96-1.62), 0.1		Ever: 1.28 (0.85-1.93), 0.241
PLR	0.8	High: 1.16 (0.97-1.38), 0.11	0.966	Men: 1.19 (0.95-1.49), 0.13	0.009	Never: 1.34 (1.1-1.62), 0.003
		Low: 1.29 (0.77-1.87), 0.41		Women: 1.17 (0.92-1.49), 0.21		Ever: 0.82 (0.61-1.1), 0.181
SII	0.641	High: 1.14 (0.98-1.32), 0.09	0.601	Men: 1.27 (1.05-1.53), 0.02	0.238	Never: 1.22 (1.04-1.43), 0.016
		Low: 1.40 (0.96-2.04), 0.08		Women: 1.11 (0.90-1.37), 0.32		Ever: 1.06 (0.79-1.43), 0.705
		Calcifications		CRP		Lipid-lowering medication
Granulocytes*	0.559	Low: 3.51 (1.14-10.83), 0.03	0.488	Low: 1.47 (0.8-2.71), 0.21	0.798	Yes: 1.77 (0.89-3.49), 0.10
		High: 1.86 (1.05-3.31), 0.03		High: 0.94 (0.5-1.76), 0.84		No: 1.97 (1.45-2.68), <.001
Platelets*	0.178	Low: 0.56 (0.19-1.65), 0.29	0.806	Low: 1.35 (0.76-2.41), 0.31	0.057	Yes: 0.69 (0.39-1.24), 0.21
		High 1.11 (0.68-1.81), 0.69		High: 1.07 (0.62-1.83), 0.82		No: 1.26 (0.95-1.67), 0.12
Lymphocytes*	0.073	Low: 2.22 (1.04-4.71), 0.04	0.176	Low: 0.79 (0.49-1.27), 0.33	0.605	Yes: 0.86 (0.53-1.40), 0.54
		High: 0.73 (0.50-1.09), 0.13		High: 0.79 (0.54-1.16), 0.22		No: 0.93 (0.74-1.16), 0.51

S1 Table. Models for the stratified analyses. (continued)

Laboratory assessmenta		Age		Sex		Smoking
GLR	0.354	Low: 1.03 (0.51-2.08), 0.93	0.925	Low: 1.33 (0.92-1.94), 0.13	0.707	Yes: 1.34 (0.88-2.06), 0.18
		High: 1.55 (1.08-2.24), 0.02		High: 1.15 (0.83-1.59), 0.40		No: 1.41 (1.15-1.74), 0.001
PLR	0.018	Low: 0.54 (0.29-1.02), 0.06	0.45	Low: 1.22 (0.9-1.65), 0.21	0.315	Yes: 0.97 (0.67-1.41), 0.88
		High: 1.29 (0.94-1.77), 0.12		High: 1.22 (0.9-1.65), 0.21		No: 1.17 (0.97-1.41), 0.09
SII	0.176	Low: 0.91 (0.51-1.62), 0.75	0.81	Low: 1.24 (0.92-1.68), 0.16	0.396	Yes: 0.99 (0.72-1.36), 0.94
		High: 1.25 (0.95-1.64), 0.11		High: 1.06 (0.81-1.37), 0.69		No: 1.23 (1.05-1.44), 0.009

Adjusted for age, sex, education, smoking status, body mass index, diabetes mellitus, systolic blood pressure, diastolic blood pressure, antihypertensive medication, HDL cholesterol, total cholesterol, and lipid-lowering medication. [a]All markers were natural log-transformed (Ln[immunity components × 10^3/μl]). *Analysis for each blood cell type adjusted for the baseline blood cell counts of the remaining 2 blood cell types. CI, confidence interval; GLR, granulocyte-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index.

3.2 Atherosclerotic plaque vulnerability

ABSTRACT

Objective: To determine the association of circulatory markers of innate and adaptive immunity with carotid atherosclerotic plaque characteristics.

Approach: In 1,602 participants from the population-based Rotterdam Study with subclinical carotid atherosclerosis, blood sampling was performed to determine granulocyte, platelet, monocyte (innate immunity) and lymphocyte (adaptive immunity) counts, from which we calculated the granulocyte-to-lymphocyte ratio[GLR], platelet-to-lymphocyte ratio[PLR], monocyte-to-lymphocyte ratio[MLR] and systemic immune-inflammation index[SII]. All participants underwent carotid MRI for evaluation of plaque characteristics. We assessed plaque size (stenosis >30%, maximum plaque thickness) and plaque composition (presence of intraplaque hemorrhage[IPH], lipid-rich necrotic core[LRNC], and calcification). Using linear and logistic regression models, we assessed the association of innate and adaptive immunity markers with plaque size and plaque components, adjusting for relevant confounders.

Results: Higher levels of granulocytes were significantly associated with larger plaque thickness (mean difference [Ln(mm)] per Ln increase granulocyte count [95% CI]: 0.06 [0.02;0.10]). Conversely, more lymphocytes related with smaller maximum plaque thickness (mean difference [Ln(mm)] per Ln increase lymphocyte count: -0.09 [-0.14;-0.04]) and a lower prevalence of IPH (odds ratio per Ln increase lymphocyte count: 0.60 [0.37;0.97]). Moreover, all ratio measures were associated with larger plaque thickness, of which the MLR also associated with more frequent LRNC (odds ratio per Ln increase MLR: 1.26 [1.02;1.56]).

Conclusions: The innate immunity links to larger plaques, whilst the adaptive immunity seems to relate to smaller plaques and a lower frequency of IPH. These results suggest that an imbalance in innate and adaptive immunity may play a role in the vulnerability of carotid atherosclerotic plaques.

INTRODUCTION

Atherosclerosis poses an enormous burden on healthcare by being the most important cause of cardiovascular events.[1] Over the last years, increasing attention has been pointed towards the role of inflammation in the development of atherosclerosis and its clinical sequelae, as a potential modifiable target for intervention.[2] Indeed, recent trials demonstrated protective effects of anti-inflammatory medication on the risk of clinical cardiovascular outcomes including cardiovascular death, myocardial infarction and ischemic stroke.[3-6] The mechanism through which inflammation acts on cardiovascular disease is less clear, but we previously found that higher levels of granulocytes were associated with a larger burden of arterial calcifications in the general population.[7] Similarly, other studies have shown that markers of innate immunity are related to plaque thickness and degree of stenosis.[8-10] Whereas these studies focussed on severity of atherosclerosis in general, less attention has been devoted to markers of plaque composition like lipid-rich necrotic core (LRNC) and intraplaque hemorrhage (IPH), which represent more vulnerable plaques.[11,12] In this context, the role of the different circulatory markers of immunity remains largely unknown. Preliminary work showed that neutrophils, the most abundant type of granulocytes, have been shown to be present in these rupture-prone atherosclerotic plaques.[13] In contrast to granulocytes, we earlier found protective effects of lymphocytes on the risk of cardiovascular events, suggesting different effects of the innate and adaptive immunity on atherosclerosis.[7]

Granulocytes, monocytes and platelets are important markers of the innate immunity[14,15], whereas lymphocytes yield information on the adaptive immunity.[16] Work from the field of cancer research suggests that combining these measurements into ratios, i.e., the granulocyte-to-lymphocyte ratio (GLR), monocyte-to-lymphocyte ratio (MLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII), is thought to even better reflect the relative balance between innate and adaptive immunity.[17,18] Therefore, we aimed to determine whether circulatory markers of immunity are related with vulnerable carotid plaque characteristics, taking into account the balance between innate and adaptive immunity. To relate the markers of immunity to plaque vulnerability, we use a selected population within the population-based Rotterdam Study, having carotid atherosclerosis.

METHODS

Setting

The present study was embedded within the Rotterdam Study, a prospective population-based cohort study in Rotterdam, the Netherlands. The Rotterdam Study started in 1989 with 7,983 persons (78% response rate) aged ≥ 55 years and residing in the district Ommoord, a suburb of Rotterdam. This first subcohort (RS-I) was extended with a second

subcohort (RS-II) in 2000, consisting of 3,011 persons (67% response rate) aged ≥ 55 years, and with a third subcohort (RS-III) in 2006, composed of 3,932 persons (65% response rate) aged ≥ 45 years. The design of the Rotterdam Study has been described in detail previously.[19] In brief, participants were examined at study entry and at follow-up visits every 3 to 5 years. They were interviewed at home by a trained research nurse, followed by 2 visits at the research facility for additional interviewing, laboratory assessments, and imaging.

Between the years 2007 and 2012, all participants with carotid atherosclerosis were invited to undergo an MRI scan of the carotid arteries. Participants were selected on the basis of carotid artery ultrasound examination (intima-media thickness ≥ 2.5 mm in one or both carotid arteries) performed in all participants of the Rotterdam Study. From the 2,666 invited participants, 684 did not undergo an MRI scan, because of claustrophobia (n=57), physical restrictions (n=191), contraindications (n=115), refusal to participate (n=272), no show or lost to follow-up (n=49). From the 1,982 (74%) patients that did undergo MRI, 242 participants were excluded due to poor image quality (n=95), if no plaques ≥2 mm were observed bilaterally (n=41) or if scanning was interrupted due to claustrophobia (n=106). Of the remaining 1,739 participants with carotid MRI scans, we excluded participants with incomplete information on history of coronary heart disease (CHD) (n=8) or stroke (n=48), leaving 1,740 participants for the present analyses. Of those, 1,602 had complete assessment of blood cell counts reflecting innate and adaptive immunity.

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act [WBO], license number 1071272-159521-PG). The Rotterdam Study personal registration data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands Trial Register (https://www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (https://www.who.int/ictrp/network/primary/en/) under the shared catalogue number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians.

Assessment of innate and adaptive immunity

Laboratory tests for granulocytes, monocytes, platelets and lymphocytes were performed during the fifth round of RS-I, third round of RS-II and second round of RS-III. Full blood cell count measurements were performed using the Coulter AcT diff2 Hematology Analyzer (Beckman Coulter, Brea, CA, US) directly after the fasting blood sample was drawn. Laboratory measurements included absolute granulocyte, platelet, monocyte and lymphocyte counts in 10^9 per liter. GLR, MLR and PLR were calculated as the ratio of granulocyte count to lymphocyte count, monocyte count to lymphocyte count and as the

ratio of platelet count to lymphocyte count, respectively. SII was defined as platelet count times GLR.[17]

Assessment of carotid atherosclerosis

MR imaging was performed using a 1.5 Tesla scanner (GE Healthcare, Milwaukee, WI, USA) with a dedicated bilateral phase-array surface coil (Machnet, Eelde, the Netherlands). Details of the scan protocol, scan reading procedure, and reproducibility are described in detail elsewhere.[20] In brief, we used the proton density weighted (PDw) fast spin echo (FSE) images to measure maximum carotid wall thickness and degree of luminal stenosis (dichotomized as above or lower than 30%) as measures of plaque size.[21] Subjects were categorized as having a degree of luminal carotid artery stenosis > 30% when this was the case in one or both carotid arteries. Next, we evaluated the presence of IPH, LRNC and calcification. IPH was defined as the presence of a hyperintense region in the atherosclerotic plaque on 3D-T1 weighted (T1w)-gradient echo sequence. LRNC was defined as the presence of a hypointense region, not classified as IPH or calcification, in the plaque on PDw- FSE-black blood (BB), PDw-echo planar imaging (EPI) and T2 weighted-EPI sequences, and with relative signal intensity drop on the T2w-EPI sequence. Calcification was defined as the presence of a hypointense region in the plaque on all sequences. All sequences were used in parallel to record the presence of plaque components. Subjects were recorded as positive for the presence of any plaque component if the component was identified in one or both carotid arteries.

Assessment of covariables

We assessed smoking, and use of antihypertensive, lipid-lowering, antidiabetic and antithrombotic medication by interview.[19] Diastolic and systolic blood pressures were measured twice on the right arm with a random-zero sphygmomanometer, of which the mean was used. Total cholesterol and high-density lipoprotein (HDL) cholesterol were measured with a Coulter AcT diff2 Hematology Analyzer. Diabetes mellitus was defined as use of antidiabetic medication, fasting serum glucose level ≥ 7.1 mmol/l (≥ 127.9 mg/dl), or random non-fasting serum glucose level ≥ 11.1 mmol/l (≥ 200.0 mg/dl). History of cardiovascular disease (CVD) was defined as prevalent CHD[22] or fatal or nonfatal stroke[23] at the time of MRI.

Statistical analysis

Because the blood cell counts, their derived ratios and maximum plaque thickness have a skewed distribution we performed natural logarithmic (Ln) transformations of their values in order to prevent non-linearity and outliers. We investigated the association of the granulocyte, monocyte, platelet and lymphocyte counts, and their derived ratios per Ln increase, with measures of plaque size (> 30% stenosis, maximum plaque thickness) and plaque

components (IPH, LRNC, and calcification), using linear- and logistic regression models. We adjusted these analyses for age and sex (model I), and additionally for current smoking, diabetes mellitus, systolic blood pressure, diastolic blood pressure, use of antihypertensive medication, total cholesterol, HDL cholesterol, and use of lipid-lowering medication (model II). The analyses on plaque components were additionally adjusted for maximum plaque thickness, to adjust for plaque size.

We explored effect modification of various factors on the association between blood cell counts and measures of plaque size and plaque components by stratifying by median age (74 years), sex, history of CVD and use of NSAIDs. We formally tested interaction between these factors and blood cell counts on the multiplicative scale by adding interaction terms to model II. Interaction terms were considered significant when *P*-interaction < 0.01.

Missing covariable data (maximum 3.2%) were imputed using 5-fold Multiple Imputation by Chained Equations based on determinant, outcome, and included covariates. All analyses were performed using RStudio version 1.0.153 (R version 3.6.3, RStudio, Inc., Boston, MA).

Results

Characteristics of the study participants are presented in Table 1. Mean age of included study participants was 72 years, and 45% were women. The prevalence of a stenosis 30% or higher was 20.1%, and the median (interquartile range) maximum plaque thickness was 3.4 (2.9-4.0) mm. IPH was present in 533 (33.3%) participants, an LRNC in 714 (44.6%), and calcification in 1,316 (82.1%) participants.

Table 1. Population characteristics

Variable	**Total** (n=1602)
Clinical characteristics	
Age, years	72 ± 9
History of CVD, n (%)	341 (21.3%)
Prevalent diabetes mellitus, n (%)	390 (24.3%)
Systolic blood pressure, mmHg	149 ± 23
Diastolic blood pressure, mmHg	84 ± 12
Use of antihypertensive medication, n (%)	875 (54.6%)
Total cholesterol, mmol/L	5.4 ± 1.1
HDL, mmol/L	1.4 ± 0.4
Use of lipid lowering medication, n (%)	621 (38.8%)
Current smoker, n (%)	265 (16.5%)
Use of antithrombotic medication, n (%)	622 (38.8%)
Blood biomarker characteristics	

Granulocytes, 10^9/L	4.2 [3.4-5.2]
Platelets, 10^9/L	262.0 [220.0-306.0]
Monocytes, 10^9/L	0.5 [0.3-0.6]
Lymphocytes, 10^9/L	32.9 [27.4-37.8]
Granulocyte-lymphocyte ratio	0.13 [0.09-0.18]
Platelet-lymphocyte ratio	8.1 [6.3-10.2]
Systemic immune-inflammation index	33.5 [22.8-49.8]
Monocyte-lymphocyte ratio	0.014 [0.010-0.019]
Plaque characteristics	
Stenosis degree > 30%, n (%)	322 (20.1%)
Maximum plaque thickness, mm	3.4 [2.9-4.0]
Lipid-rich necrotic core , n (%)	714 (44.6%)
Intraplaque haemorrhage, n (%)	533 (33.3%)
Calcification, n (%)	1316 (82.1%)

Continuous variables are denoted by mean ± standard deviation and skewed variables by median [interquartile range]. Number of missing values for history of CVD is 10 (0.6%), for prevalent diabetes mellitus 35 (2.2%), systolic blood pressure 9 (0.6%), diastolic blood pressure 9 (0.6%), use of antihypertensive medication 4 (0.2%), total cholesterol 6 (0.4%), HDL 7 (0.4%), use of lipid-lowering medication 4 (0.2%), current smoker 4 (0.2%), and use of antithrombotic medication 4 (0.2%).

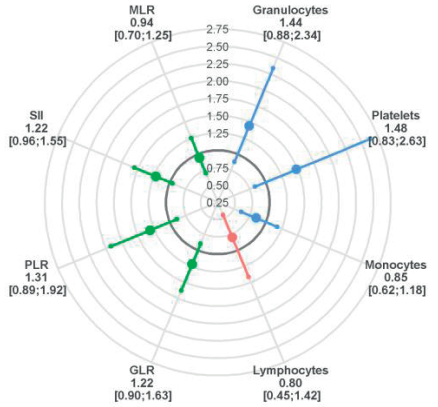
Immunity markers and plaque size

We found no association between blood cell counts and having a stenosis of > 30%. Higher levels of granulocytes associated with a statistically significantly larger maximum plaque thickness (mean difference in plaque thickness [Ln(mm)] per Ln increase in granulocyte count [95% CI] = 0.06 [0.02;0.10]). Conversely, higher levels of lymphocytes were associated with smaller maximum plaque thickness (mean difference in plaque thickness [Ln(mm)] per Ln increase in lymphocyte count [95% CI] = -0.09 [-0.14;-0.04]). Higher levels of GLR, PLR, SII and MLR were all associated with significantly larger maximum plaque thickness, this was most prominent for PLR (mean difference in plaque thickness [Ln(mm)] per Ln increase in PLR [95% CI] = 0.06 [0.02;0.09], **Figure 1**).

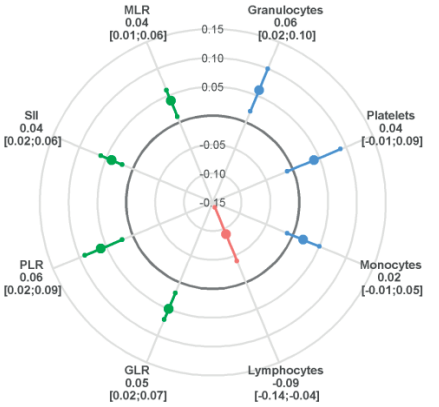
Immunity markers and plaque composition

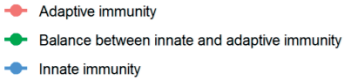
Only few immune markers showed significant associations with plaque components. There were no significant associations between granulocytes and plaque components. However, higher levels of platelets were associated with lower prevalence of IPH (odds ratio per Ln increase in platelet count [95% CI] = 0.62 [0.39;1.00]). Moreover, higher lymphocyte counts were also related with lower IPH prevalence (odds ratio per Ln increase in lymphocyte count [95% CI] = 0.60 [0.37;0.97]). Of all ratio measures, only the MLR displayed a significant

Stenosis degree

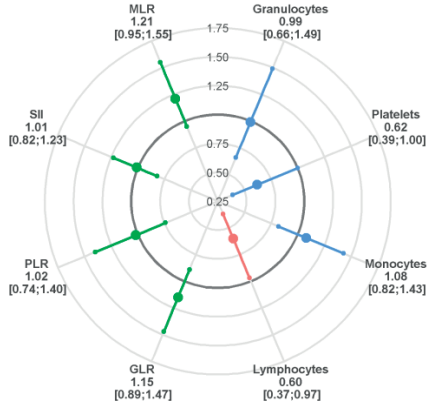


Maximum plaque thickness

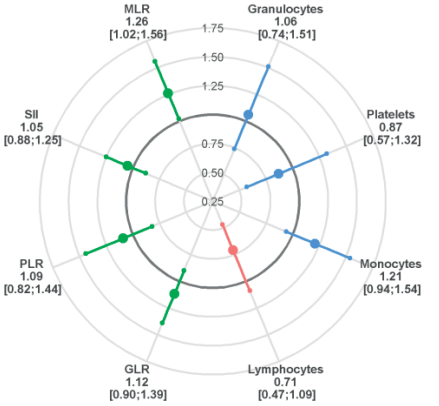




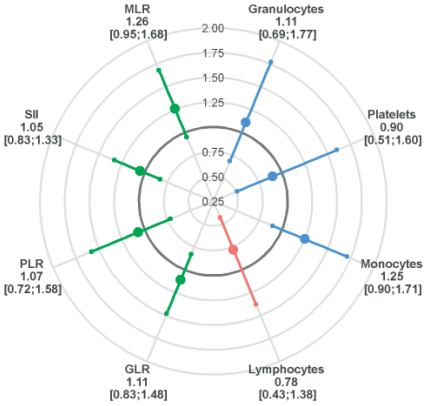
Intraplaque hemorrhage



Lipid-rich necrotic core



Calcifications



← **Figure 1.** Associations of immune cells with plaque characteristics
Values represent odds ratios [95% confidence interval] for stenosis degree, intraplaque hemorrhage, lipid-rich necrotic core and calcifications. For maximum plaque thickness values represent βs [95% confidence interval]. GLR = granulocyte-lymphocyte ratio; PLR = platelet-lymphocyte ratio; SII = systemic immune-inflammation index; MLR = monocyte-lymphocyte ratio. Maximum plaque thickness and all immunity markers vare Ln transformed. Bold grey circle denotes β = 0 for maximum plaque thickness and odds ratio = 1 for the remaining plaque characteristics. The models are adjusted for age, sex, maximum plaque thickness *, systolic blood pressure, diastolic blood pressure, use of antihypertensive medication, total cholesterol, HDL, use of lipid lowering medication, diabetes mellitus, and current smoking. *Except for maximum plaque thickness as dependent variable.

association with higher prevalence of LRNC (odds ratio per Ln increase in MLR [95% CI] = 1.26 [1.02;1.56], **Figure 1, Table S1)**.

The results of stratified analyses are presented in **Figure S2-4**. We found evidence for effect modification by age, where in younger participants (under the median age of 74 years), higher levels of platelets related to larger maximum plaque thickness. We found no evidence for effect modification by sex nor CVD history. Higher levels of monocytes related to more frequent IPH in NSAID users.

DISCUSSION

This study, performed in participants of the Rotterdam Study, showed that innate immunity was linked to larger plaques, whilst the adaptive immunity related to smaller plaques and a lower frequency of IPH. Higher levels of platelets were also associated with lower prevalence of IPH. These results suggest that an imbalance in innate and adaptive immunity may play a role in the vulnerability of the carotid atherosclerotic plaque.

We previously found in a CT-based study among participants in the general population, that an increase in granulocytes was associated with calcification in multiple vessel beds, including the extracranial internal carotid artery.[7] In the current MRI study performed in a population where subjects were included based on presence of carotid atherosclerotic plaques, we also found an association between granulocytes and maximum plaque thickness, but additionally we found that lymphocytes had the largest stabilizing effect on plaque thickness. We previously did not find an association between lymphocytes and arterial calcifications. This could be because in this study we selected participants with ultrasound-assessed carotid atherosclerotic plaques, whereas in our previous study we assessed arterial calcification in an unselected sample of the general population.

In the present study, we found primarily associations between the MLR, reflecting the balance between innate (monocytes) and adaptive (lymphocytes) immunity, with LRNC. Indeed, previous experimental and clinical studies have shown that both innate and adaptive

immune mechanisms can accelerate or curb atherosclerosis.[24] Several studies suggest that monocytes influence the clinical course of atherosclerosis, as patients with acute cardiovascular events present with increased circulating monocytes.[2,25] Other studies even showed that circulating monocyte chemoattract protein-1 (MCP-1), one of the key chemokines that regulate migration and infiltration of monocytes, increases stroke risk observationally as well as genetically[26,27], supporting a role for monocytes in plaque vulnerability. Whether MCP-1 itself increases stroke risk or whether this is mediated via monocytes remains to be elucidated.

The role of the adaptive immune system has become more clear in a recent study in which they used single-cell proteomic and transcriptomic analyses to map the immune microenvironment of atherosclerotic lesions. They found that carotid artery plaques from clinically symptomatic patients (recent stroke or transient ischemic attack) compared to asymptomatic disease were characterized by a distinct subset of CD4+ T cells and by T cells that were activated and differentiated.[2] But their analysis of symptomatic plaques was intrinsically limited to lesions following acute cardiovascular events, so it could be that T cell exhaustion is initiated by T cell overactivation triggered by plaque disruption. In our study we found that a higher lymphocyte count was associated with less frequent IPH. Although we could not assess lymphocyte subsets in our study, these results altogether do suggest a primary protective effect of circulatory lymphocytes, and provide further support for stimulating the adaptive immune system as potential therapeutic target for atherosclerosis.

Our study has limitations inherent to the cross-sectional design, which affects proper causal inferences. Additionally, we were not able to analyse subsets of immune cells. This can especially be relevant for identifying therapeutic strategies. Furthermore, we attempted to also stratify by colchicine use due to the recent results of the COLCOT trial[3], where colchicine at a dose of 0.5 mg daily led to a significantly lower risk of ischemic cardiovascular events than placebo. However, since the prevalence of colchicine use in our population was relatively low leading to underpowered analyses we could not perform these tests. Lastly, in terms of generalizability, this is a study among individuals selected on presence of ultrasound-assessed carotid atherosclerotic plaques, limiting its external generalizability to the general population.

In terms of clinical practice, our findings may have clinical implications given that these suggest that immune cells convey information on the atherosclerotic plaque composition. Interestingly, we found evidence for effect measure modification by age and use of NSAIDs. This underscores that immunomodulatory treatments must be tailored to specific individuals with specific immune defects.

In conclusion, we found that innate immunity was linked to larger plaques, whilst the adaptive immunity related to smaller plaques and a lower frequency of IPH. These results suggest that an imbalance in innate and adaptive immunity may play a role in the vulnerability of the carotid atherosclerotic plaque.

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SUPPORTING INFORMATION

Table S1. Associations of immune cells with plaque characteristics

	Stenosis > 30% OR [95% CI]	Maximum plaque thickness β [95% CI]	Intraplaque hemorrhage OR [95% CI]	Lipid-rich necrotic core OR [95% CI]	Calcification OR [95% CI]
Model 1					
Markers of innate immunity					
Granulocytes	1.99 [1.26;3.14]	0.09 [0.05;0.13]	1.27 [0.86;1.86]	0.90 [0.65;1.26]	1.37 [0.90;2.10]
Platelets	1.46 [0.83;2.58]	0.04 [-0.01;0.09]	0.63 [0.39;1.00]	0.88 [0.58;1.33]	0.92 [0.53;1.60]
Monocytes	0.98 [0.71;1.35]	0.03 [0.00;0.06]	1.17 [0.88;1.54]	1.15 [0.90;1.46]	1.32 [0.97;1.78]
Marker of adaptive immunity					
Lymphocytes	0.72 [0.41;1.25]	-0.10 [-0.15;-0.05]	0.56 [0.35;0.89]	0.77 [0.51;1.17]	0.70 [0.40;1.24]
Balance innate & adaptive immunity					
GLR	1.42 [1.07;1.89]	0.06 [0.03;0.08]	1.27 [1.00;1.62]	1.02 [0.83;1.26]	1.23 [0.94;1.62]
PLR	1.37 [0.95;2.00]	0.06 [0.03;0.10]	1.05 [0.77;1.44]	1.06 [0.80;1.40]	1.13 [0.77;1.65]
SII	1.35 [1.07;1.70]	0.05 [0.03;0.07]	1.08 [0.89;1.32]	0.99 [0.84;1.18]	1.14 [0.91;1.43]
MLR	1.07 [0.81;1.41]	0.05 [0.02;0.07]	1.31 [1.03;1.67]	1.19 [0.96;1.46]	1.34 [1.02;1.75]
Model 2					
Markers of innate immunity					
Granulocytes	1.44 [0.88;2.34]	0.06 [0.02;0.10]	0.99 [0.66;1.49]	1.06 [0.74;1.51]	1.11 [0.69;1.77]
Platelets	1.48 [0.83;2.63]	0.04 [-0.01;0.09]	0.62 [0.39;1.00]	0.87 [0.57;1.32]	0.90 [0.51;1.60]
Monocytes	0.85 [0.62;1.18]	0.02 [-0.01;0.05]	1.08 [0.82;1.43]	1.21 [0.94;1.54]	1.25 [0.90;1.71]
Marker of adaptive immunity					
Lymphocytes	0.80 [0.45;1.42]	-0.09 [-0.14;-0.04]	0.60 [0.37;0.97]	0.71 [0.47;1.09]	0.78 [0.43;1.38]
Balance innate & adaptive immunity					
GLR	1.22 [0.90;1.63]	0.05 [0.02;0.07]	1.15 [0.89;1.47]	1.12 [0.90;1.39]	1.11 [0.83;1.48]
PLR	1.31 [0.89;1.92]	0.06 [0.02;0.09]	1.02 [0.74;1.40]	1.09 [0.82;1.44]	1.07 [0.72;1.58]
SII	1.22 [0.96;1.55]	0.04 [0.02;0.06]	1.01 [0.82;1.23]	1.05 [0.88;1.25]	1.05 [0.83;1.33]
MLR	0.94 [0.70;1.25]	0.04 [0.01;0.06]	1.21 [0.95;1.55]	1.26 [1.02;1.56]	1.26 [0.95;1.68]

OR = odds ratio; 95% CI = 95% confidence interval; GLR = granulocyte-lymphocyte ratio; PLR = platelet-lymphocyte ratio; SII = systemic immune-inflammation index; MLR = monocyte-lymphocyte ratio. Maximum plaque thickness and all immunity markers are Ln transformed.

Model 1: adjusted for age, sex and maximum plaque thickness *

Model 2: adjusted for age, sex, maximum plaque thickness *, systolic blood pressure, diastolic blood pressure, use of antihypertensive medication, total cholesterol, HDL, use of lipid lowering medication, diabetes mellitus, and current smoking

** except for maximum plaque thickness as dependent variable*

Table S2. *P* interaction values for stratified analyses

	Stenosis > 30%	Maximum plaque thickness	Intraplaque hemorrhage	Lipid-rich necrotic core	Calcification
P interaction values for age					
Granulocytes	0.36	0.02	0.63	0.03	0.14
Platelets	0.55	0.003	0.33	0.96	0.52
Monocytes	0.42	0.72	0.9	0.29	0.48
Lymphocytes	0.40	0.05	0.78	0.21	0.93
GLR	0.33	0.01	0.99	0.04	0.37
PLR	0.37	0.35	0.35	0.38	0.61
SII	0.30	0.46	0.73	0.09	0.28
MLR	0.22	0.24	0.71	0.95	0.59
P interaction values for sex					
Granulocytes	0.72	0.76	0.72	0.74	0.74
Platelets	0.68	0.50	0.79	0.58	0.22
Monocytes	0.42	0.56	0.55	0.50	0.49
Lymphocytes	0.52	0.14	0.79	0.52	0.28
GLR	0.92	0.54	0.75	0.87	0.48
PLR	0.49	0.59	0.73	0.95	0.90
SII	0.80	0.82	0.70	0.91	0.92
MLR	0.32	0.21	0.50	0.36	0.93
P interaction values for history of CVD					
Granulocytes	0.05	0.21	0.87	0.86	0.23
Platelets	0.48	0.40	0.66	0.96	0.36
Monocytes	0.23	0.31	0.53	0.14	0.27
Lymphocytes	0.65	0.22	0.93	0.18	0.72
GLR	0.33	0.14	0.87	0.54	0.35
PLR	0.45	0.17	0.73	0.38	0.38
SII	0.60	0.13	0.74	0.64	0.24
MLR	0.39	0.82	0.54	0.56	0.40
P interaction values for use of NSAIDs					
Granulocytes	0.17	0.33	0.84	0.97	0.78
Platelets	0.99	0.24	0.59	0.40	0.14
Monocytes	0.36	0.03	0.009	0.40	0.33
Lymphocytes	0.08	0.28	0.45	0.27	0.54
GLR	0.08	0.96	0.59	0.56	0.64
PLR	0.21	0.96	0.87	0.86	0.14
SII	0.14	0.59	0.84	0.90	0.30
MLR	0.91	0.21	0.06	0.20	0.59

Shown are *P* interaction values between participant characteristics and blood cell counts per plaque characteristic. IPH = intraplaque hemorrhage; LRNC = lipid-rich necrotic core; GLR = granulocyte-lymphocyte ratio; PLR = platelet-lymphocyte ratio; SII = systemic immune-inflammation index; MLR = monocyte-lymphocyte ratio. Interaction terms were considered significant when *P*-interaction

Chapter 4
Brain pathology

4.1 Amyloid-beta, total-tau and neurofilament light chain and the risk of stroke

ABSTRACT

Objective To unravel whether Alzheimer's disease-related pathology or neurodegeneration play a role in stroke etiology, we determined the effect of plasma levels amyloid β (Aβ), total-tau and neurofilament light chain (NfL) on risk of stroke and its subtypes.

Methods Between 2002 and 2005, we measured plasma Aβ40, Aβ42, total-tau, and NfL in 4,893 participants from the population-based Rotterdam Study. Associations between these markers with prior stroke were established using multivariable logistic regression. Within stroke-free participants at baseline, we used Cox proportinonal-hazards models to determine the association with incident stroke for the entire cohort, per stroke subtype, and by median age, sex, *Apolipoprotein E* (*APOE*) ε4 carriership, and education.

Results At baseline, 232 (4.7%) participants had prior stroke. Participants with higher levels of log2 NfL were more likely to have had a prior stroke (odds ratio per standard deviation (SD) increase 1.44 (95% CI: 1.23–1.68). After a mean follow-up of 10.8±3.3 years, 379 participants suffered a first-ever stroke. Log2 total-tau at baseline showed a non-linear association with risk of any stroke and ischemic stroke. Log2 NfL was associated with an increased risk of any stroke (HR per SD increase 1.27, 95% CI: 1.12–1.44), ischemic stroke, and hemorrhagic stroke (HR 1.64, 95% CI: 1.17–2.28). Log2 Aβ40, Aβ42, and Aβ42/40 ratio levels were not associated with stroke risk.

Conclusions Participants with higher total-tau and NfL at baseline had a higher risk of stroke and several stroke subtypes. These findings support the role of accumulating brain pathology in the etiology of stroke.

INTRODUCTION

Stroke and dementia are frequent neurologic diseases in the elderly, often co-occurring in the same patient.[1,2] Indeed, the introduction of the term vascular cognitive impairment in recent decades demonstrates the importance of cerebrovascular pathology in the development of cognitive impairment.[3] Conversely, several studies have shown that memory decline[4], cognitive impairment[5] and even dementia may develop before clinical manifestation of vascular pathology, and even increase the risk of stroke.[6-9] It is therefore plausible that - besides vascular pathology linking stroke and dementia - other manifestations of brain pathology, e.g. amyloid plaques and neurofibrillary tangles, could also impact the risk of stroke.[10]

Amyloid plaques consist mainly of amyloid β (Aβ) peptides and neurofibrillary tangles of tau protein, and characterize Alzheimer's disease (AD) pathologically.[11] A link between these markers of AD-related brain pathology and vascular disease is most clearly demonstrated in the case of cerebral amyloid angiopathy (CAA), where deposition of amyloid β in the vascular wall leads to loss of integrity, leading to hemorrhagic stroke.[12] Elevated levels of these biomarkers have also been found in patients with ischemic stroke[13] and have been shown to predict post-stroke cognitive impairment.[14] In addition to these established markers, neurofilament light chain protein (NfL), a major component of the axonal cytoskeleton similar to tau, has more recently emerged as a biomarker reflecting axonal damage in a wide variety of neurological disorders.[10] Few studies have also linked elevated tau and NfL levels with stroke and small vessel disease.[15-17] However, whether the levels of Aβ, total-tau, and NfL are related to an increased risk of stroke in the general population remains unclear.

In this study, we determined the association of plasma Aβ, total-tau, and NfL levels with prior and incident stroke and its main subtypes in a prospective population-based study.D

METHODS

Study setting and population

This study is based on the Rotterdam Study, a large prospective population-based cohort in the Netherlands designed to study occurrence and determinants of age-related diseases in the general population.[18] The cohort was initiated in 1990 (RS-I) and was expanded in 2000 (RS-II) and 2006 (RS-III), with a total of 14,926 participants aged 45 years or older. Examination rounds with home interviews and extensive physical examinations are repeated every four years. Between 2002 and 2005, 5,069 participants from RS-I and RS-II underwent venipuncture at the research center and had sufficient plasma stores available for analysing biomarkers. We excluded 170 participants with all missing or invalid test results for plasma levels of biomarkers (for more details see 'Measurements of plasma concentrations of Aβ,

total-tau, and NfL') and six participants without informed consent to access medical records during follow-up, leaving 4,893 participants for analysis.

Measurements of plasma concentrations of Aβ, total-tau, and NfL

Blood from participants was sampled in ethylenediamine tetra-acetic-treated (EDTA) containers, aliquoted and frozen at -80^0 Celsius according to standard procedures. Measurements were performed in two separate batches at Quanterix (Lexington, MA, USA) on a single molecule array (Simoa) HD-1 analyzer platform.[19] The Simoa Human Neurology 3-Plex A assay (N3PA) was used to measure concentrations of Aβ40, Aβ42 and total-tau. NfL was measured by using the Simoa NF-light® advantage kit.[20] Samples were tested in duplicate. Two quality control samples were run on each plate for each analyte. When duplicates or single measurements were missing, or if the concentration coefficient of variation exceeded 20%, or control samples were out of range, data was excluded from analysis (n = 170).

Stroke assessment

Stroke was defined according to the World Health Organization (WHO) criteria as a syndrome of rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin.[21] History of stroke was assessed during baseline interview and verified by reviewing medical records obtained from general practitioners. After enrolment, participants were continuously monitored for incident stroke through automated linkage of the study database with files from general practitioners and nursing home physicians. Files from nursing home physicians and general practitioners of participants who moved out of the district were also checked on a regular basis. Additional information was obtained from hospital records. The information collected on potential strokes was reviewed and classified by research physicians and verified by experienced stroke neurologists in a consensus panel. Strokes were further classified as ischemic or hemorrhagic based on neuroimaging reports. If neuroimaging was not conducted or reported, a stroke was classified as unspecified. This classification corresponds with ICD-10 codes I61, I63 and I64. Participants who had a history of stroke at the time of enrolment in the Rotterdam Study or had an incident stroke before the baseline (date of venipuncture) of this current study, were categorized as those with prior stroke. Participants who were stroke-free at baseline could contribute a maximum of 14 years to follow-up, that is from baseline (date of venipuncture) until first-ever stroke, death, lost to follow-up or last health status update when they were known to be free of stroke, whichever came first, until January 2016. Follow-up was virtually complete (99.0%).

Assessment of participant characteristics

Participants were interviewed at home every four years with standardized questionnaires on health status, medication use and medical history. They additionally underwent physi-

cal examinations at the dedicated research center. Educational level was classified as low (primary, unfinished secondary and lower vocational), intermediate (intermediate vocational or higher general education) or high (higher vocational education or university). Smoking behavior was classified as current, former or never. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m^2). Blood pressure was measured at the right upper arm using a sphygmomanometer during two consecutive readings and the average of the two readings was used for further analysis. Blood samples were drawn to assess levels of cholesterol, high-density lipoprotein (HDL), glucose, creatinine, and *Apolipoprotein E* (*APOE*) genotype. In 111 participants with missing *APOE* genotype from this blood sampling, genotype was determined by genetic imputation (Illumina 610K and 660K chip; imputation with Haplotype Reference Consortium reference panel [v1.0] with Minimac 3). The estimated glomerular filtration rate (eGFR) was calculated with the CKD-EPI formula using creatinine level, age, sex and ethnicity.[22] Because creatinine was only assessed in a sample of our study participants between 2002 and 2005 (n = 1,967), for participants in whom creatinine was not assessed at baseline, we used, if available, the creatinine level of the previous research visit (n = 2,829). Diabetes mellitus type 2 was defined as a fasting glucose of ≥7.0 mmol/L, a non-fasting or post-load serum glucose of ≥11.1 mmol/L, or use of blood glucose-lowering medication. Dementia, atrial fibrillation, and coronary heart disease were assessed using baseline interviews that were verified by medical records and continuous monitoring of medical records. More detailed definitions and procedures have been described previously.[23-25]

Statistical analysis

We used five-fold multiple imputation by chained equations for missing data on patient characteristics based on all covariates and outcomes. The percentage of missing values ranged from 0.1% to 9.6%. Plasma concentrations (pg/ml) on Aβ40, Aβ42, total-tau, and NfL were log2 transformed to approach a normal distribution and standardized to facilitate comparison across different biomarkers. The Aβ42/40 ratio was calculated by dividing plasma levels of Aβ42 by plasma levels of Aβ40. We used logistic regressions to assess the cross-sectional association of log2 levels of Aβ40, Aβ42, Aβ42/40 ratio, total-tau, and NfL with prior stroke. We also performed analyses by the median number of years of prior stroke (four years or longer before baseline versus less than four years before baseline). Models were adjusted for age, sex, and batch (Model I) and additionally for educational level, smoking, BMI, systolic and diastolic blood pressure, blood pressure-lowering medication use, total cholesterol, HDL, lipid-lowering medication use, eGFR, diabetes mellitus type 2, coronary heart disease, and atrial fibrillation (Model II). Next, using Cox proportional-hazards models we estimated hazard ratios for risk of incident stroke per standard deviation (SD) increase of log2 levels of Aβ40, Aβ42, Aβ42/40 ratio, total-tau, and NfL. Both model I and II were used and the proportional hazard assumption was met. We repeated the analyses

by stroke subtype and after excluding participants with dementia at baseline. We tested for possible non-linear associations using the ANOVA test to compare the fit of a model with and without added splines for each biomarker. Besides the continuous analyses, we estimated the adjusted hazard ratios of incident stroke per quartile of each biomarker or ratio, with the first quartile as a reference group. We also constructed Kaplan-Meier curves for stroke-free survival probability per year follow-up for each of the biomarker quartile groups. Finally, we stratified by median age, sex, *APOE* genotype, and educational level to determine whether there was any effect modification. *APOE-ε4* carriership was defined as ε3/ε4 or ε4/ε4 genotype versus ε2/ε3, ε2/ε2, or ε3/ε3 genotype. Participants with ε2/ε4 were excluded from the stratified analysis (n = 172). To avoid overfitting in the stratified analyses, we calculated a propensity score for Aβ40, Aβ42, total-tau, and NfL based on all covariates of model II except age and sex.[26] This propensity score was added as a covariate to the model along with age and sex. All analyses were performed using R 3.6.3 software. Alpha level (type 1 error) was set at 0.05.

Standard protocol approvals, registrations, and patient consents

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study Personal Registration Data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands National Trial Register and into the WHO International Clinical Trials Registry Platform under shared catalogue number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians.

Data availability Statement

The corresponding author has full access to all the data in the study and has final responsibility for the decision to submit for publication. Rotterdam Study data can be made available to interested researchers upon request. Requests can be directed to data manager Frank J.A. van Rooij (f.vanrooij@erasmusmc.nl). Because of restrictions based on privacy regulations and informed consent of the participants, data cannot be made freely available in a public repository.

RESULTS

Baseline characteristics of the participants included in the analysis are presented in Table 1. The mean ± SD age of the study population was 72.1 ± 7.5 years and 2,803 (57.3%)

participants were women. Mean baseline plasma levels of Aβ40, Aβ42, total-tau and NfL were 265.9 ± 55.3, 10.5 ± 2.9, 2.6 ± 2.2, and 16.1 ± 12.7 pg/ml, respectively.

Table 1. Baseline characteristics of study population (n = 4,893)

Characteristic	
Age (years)	72.1 ± 7.5
Women	2,803 (57.3%)
Body mass index (kg/m^2)	27.6 ± 4.1
Smoking status	
Current	708 (14.5%)
Former	2,718 (55.5%)
Never	1,467 (30.0%)
Systolic blood pressure (mm Hg)	149 ± 21
Diastolic blood pressure (mm Hg)	80 ± 11
Blood pressure-lowering medication use	2,222 (45.4%)
Total cholesterol (mmol/L)	5.6 ± 1.0
High-density lipoprotein (mmol/L)	1.5 ± 0.4
Lipid-lowering medication use	1,117 (22.8%)
eGFR (mL/min/1.73 m²)	73.5 ± 14.5
Educational level	
Low	2,720 (55.6%)
Intermediate	1,485 (30.3%)
High	688 (14.1%)
Apolipoprotein E ε4 carriership*	1,161 (23.7%)
Diabetes mellitus type 2	714 (14.6%)
Coronary heart disease	489 (10.0%)
Atrial fibrillation	312 (6.4%)
Amyloid β40 (pg/ml)	265.9 ± 55.3
Amyloid β42 (pg/ml)	10.5 ± 2.9
Amyloid β42/40 ratio	0.04 ± 0.01
Total-tau (pg/ml)	2.6 ± 2.2
Neurofilament light chain (pg/ml)	16.1 ± 12.7

Data presented as frequency (percentage) for categorical values and mean ± standard deviation for continuous variables.

**Apolipoprotein E* ε4 genotype was missing in 157 participants. Participants with ε2/ε4 genotype (n = 132) were not considered as *Apolipoprotein E* ε4 carriers and were excluded from the stratified analyses.

In total, 232 (4.7%) participants had prior stroke at baseline. Participants with higher levels of log2 Aβ40, Aβ42, Aβ42/40 ratio, total-tau, and NfL were more likely to have had a prior stroke (Table 2). After additional adjustments in model II, only higher levels of log2 NfL were significantly associated with a higher odds ratio for prior stroke (OR per SD increase 1.44, 95% CI: 1.23; 1.68). The association of Aβ42 and Aβ42/40 ratio with prior stroke was stronger in participants with earlier stroke (four or more years before baseline) compares with participants with more recent prior stroke (Table 3). This difference in association was not found in the other biomarkers.

Table 2. Association of plasma amyloid β40, amyloid β42, total-tau, and neurofilament light chain with prior stroke.

		Model I	Model II
	n/N	**Odds ratio** (95% CI)	**Odds ratio** (95% CI)
Amyloid β40	214/4,668	1.21 (1.04; 1.40)	1.03 (0.88; 1.20)
Amyloid β42	195/4,510	1.26 (1.07; 1.47)	1.07 (0.90; 1.28)
Amyloid β42/40 ratio	191/4,382	1.22 (1.03; 1.46)	1.07 (0.90; 1.28)
Total-tau	215/4,716	1.25 (1.09; 1.43)	1.13 (0.98; 1.30)
Neurofilament light chain	230/4,830	1.49 (1.29; 1.71)	1.44 (1.23; 1.68)

Estimates indicate odds ratio for prior stroke per standard deviation increase in log2 biomarker.
Model I: Adjusted for age, sex and batch.
Model II: Additionally adjusted for educational level, smoking, BMI, systolic and diastolic blood pressure, blood pressure-lowering medication use, total cholesterol, high-density lipoprotein, lipid-lowering medication use, eGFR, diabetes mellitus type 2, coronary heart disease, and atrial fibrillation.
Abbreviations:, CI; confidence interval, n; number of participants with prior stroke, N; number of participants in analysis.

Table 3. Association of plasma amyloid β40, amyloid β42, total-tau, and neurofilament light chain with prior stroke, comparing more recent strokes to older strokes.

	Stroke <4 years		Stroke ≥4 years	
	n/N	**Odds ratio** (95% CI)	**n/N**	**Odds ratio** (95% CI)
Amyloid β40	78/4,532	1.22 (0.95; 1.55)	62/4,516	1.32 (1.00; 1.73)
Amyloid β42	76/4,391	0.93 (0.73; 1.17)	54/4,369	1.27 (0.92; 1.74)
Amyloid β42/40 ratio	72/4,263	0.93 (0.74; 1.18)	54/4,245	1.22 (0.89; 1.68)
Total-tau	81/4,582	1.18 (0.97; 1.45)	61/4,562	1.29 (1.05; 1.60)
Neurofilament light chain	89/4,689	1.63 (1.31; 2.03)	64/4,664	1.44 (1.11; 1.86)

Estimates indicate odds ratio for prior stroke per standard deviation increase in log2 biomarker.
Models are adjusted for age, sex, and propensity score.
Abbreviations:, CI; confidence interval, n; number of participants with prior stroke, N; number of participants in analysis.

Table 4. Association of plasma amyloid β40, amyloid β42, total-tau, and neurofilament light chain with risk of stroke and stroke subtypes.

	All strokes		Ischemic stroke		Hemorrhagic stroke		Unspecified stroke	
per log2 SD increase	**n/N**	**HR** (95% CI)	**n/N**	**HR (95% CI)**	**n/N**	**HR** (95% CI)	**n/N**	**HR** (95% CI)
Amyloid β40	367/4,454	0.96 (0.85; 1.08)	266/4,454	0.96 (0.83; 1.10)	45/4,454	1.12 (0.80; 1.57)	56/4,454	1.10 (0.82; 1.47)
Amyloid β42	373/4,579	0.94 (0.83; 1.06)	270/4,579	0.91 (0.79; 1.05)	46/4,579	0.98 (0.70; 1.39)	57/4,579	0.81 (0.58; 1.14)
Amyloid β42 / β40 ratio	367/4,454	0.96 (0.85; 1.08)	266/4,454	0.92 (0.80; 1.06)	45/4,454	0.90 (0.65; 1.25)	56/4,454	0.71 (0.52; 0.97)
Total-tau	370/4,501	1.08 (0.97; 1.20)	268/4,501	1.20 (1.06; 1.35)	46/4,501	0.97 (0.72; 1.31)	56/4,501	0.96 (0.73; 1.27)
Neurofilament light chain	373/4,600	1.27 (1.12; 1.44)	271/4,600	1.24 (1.07; 1.43)	46/4,600	1.64 (1.17; 2.28)	56/4,600	1.18 (0.87; 1.60)

Estimates indicate hazard ratio for risk of stroke subtype per standard deviation increase in log2 biomarker.
Model for incident all strokes: adjusted for age, sex, batch, educational level, smoking, BMI, systolic and diastolic blood pressure, blood pressure-lowering medication use, total cholesterol, HDL, lipid-lowering medication use, eGFR, diabetes mellitus type 2, coronary heart disease, and atrial fibrillation.
Models for incident ischemic, hemorrhagic and unspecified stroke: adjusted for age, sex, and propensity score.
Abbreviations: CI; confidence interval, HR; hazard ratio, n; number of participants with incident stroke, N; number of participants in analysis, SD; standard deviation.

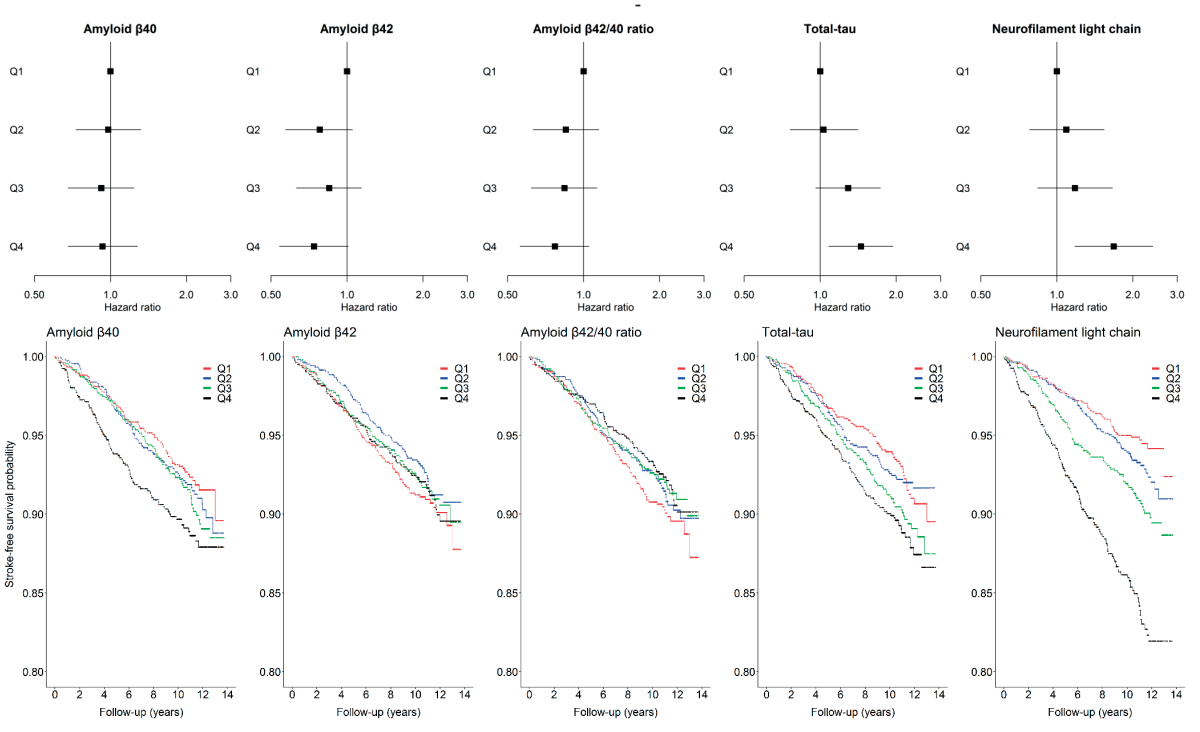

Figure 1. Association of plasma amyloid β40, amyloid β42, total-tau, and neurofilament light chain with risk of stroke. Forest plots showing hazard ratios for incident stroke and 95% CI per quartile of plasma levels (pg/ml) of total-tau, amyloid β40, amyloid β42, amyloid β42/40 ratio, and neurofilament light chain, with the lowest quartile (Q1) as reference group. The models were adjusted for age, sex, batch, educational level, smoking, BMI, systolic and diastolic blood pressure, blood pressure-lowering medication use, total cholesterol, HDL, lipid-lowering medication use, eGFR, diabetes mellitus type 2, coronary heart disease, and atrial fibrillation. Below Kaplan-Meier curves depicting the stroke-free survival probability during follow-up in years for quartiles of plasma levels of total-tau, amyloid β40, amyloid β42, amyloid β42/40 ratio, and neurofilament light chain (lowest group, Q1: red, Q2 blue, Q3: green, and Q4: black).

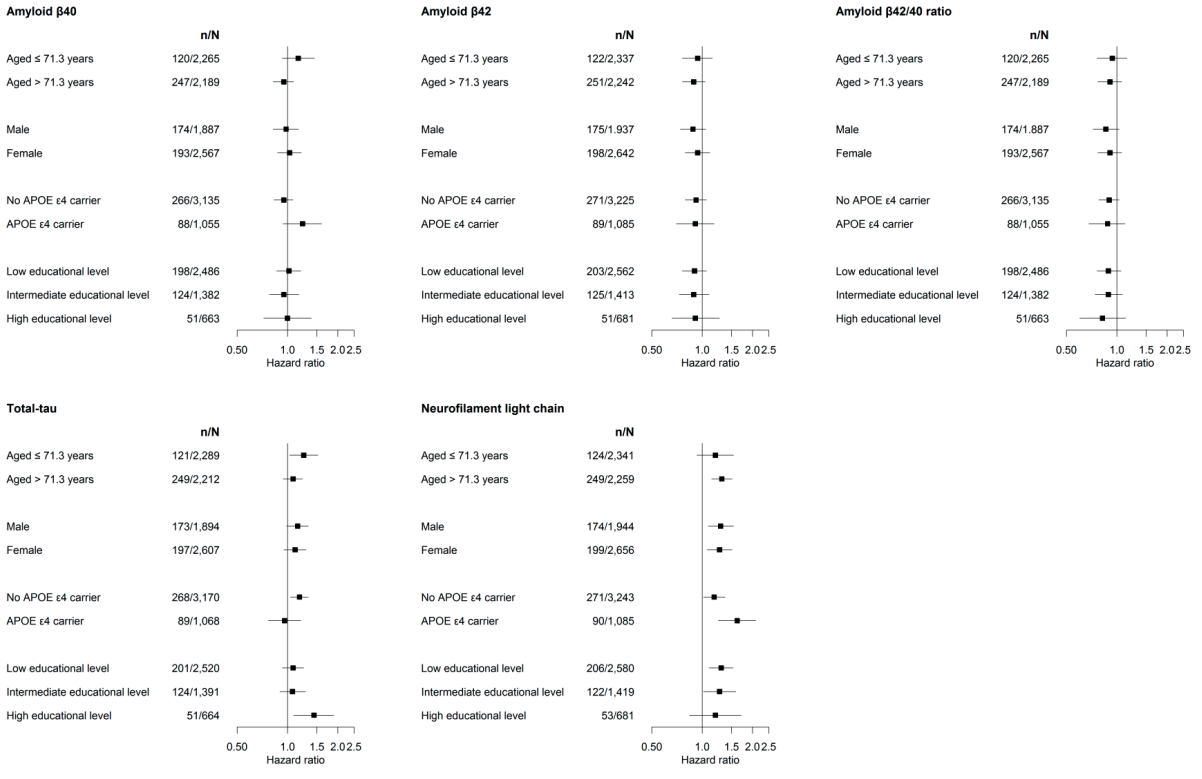

Figure 2. Stratified analyses for the association between plasma amyloid β40, amyloid β42, amyloid β42/40 ratio, total-tau, and neurofilament light chain with incident stroke. Forest plots showing hazard ratios for incident stroke and 95% CI per standard deviation increase in log2 plasma levels (pg/ml) of total-tau, amyloid β40, amyloid β42, amyloid β42/40 ratio, and neurofilament light chain after stratification by median age, sex, *APOE* ε4 carriership, and educational level. The models were adjusted for age, sex (if applicable), and propensity score.

After a median follow-up of 10.8 ± 3.3 years, 379 participants suffered a first-ever stroke after baseline. Of these strokes, 274 (72.3%) were classified as ischemic, 47 (12.4%) as hemorrhagic, and 58 (15.3%) as unspecified stroke. Log2 Aβ40, Aβ42, and Aβ42/40 ratio were not significantly associated with incident stroke or its subtypes (Table 4). Total-tau showed a non-linear association with the risk of incident stroke with the ANOVA test revealing a better fit using a quadratic term for total-tau. Analyses per quartile and the Kaplan-Meier plots also demonstrated a non-linear association between log2 total-tau and incident stroke (Figure 1). An increased stroke risk was found in participants in the third (HR 1.29, 95% CI: 0.96; 1.73) and fourth (HR 1.45, 95% CI: 1.08; 1.94) quartile of total-tau level, compared to those in the lowest quartile. An increase in log2 NfL was associated with the risk of any stroke (HR per SD increase: 1.27, 95% CI: 1.12; 1.44) and all stroke subtypes, in particular with incident hemorrhagic stroke (HR per SD increase: 1.64, 95% CI: 1.17; 2.28). Repeating the analyses after exclusion of participants with prior dementia (n = 37) did not change the results (data not shown).

Stratified analyses showed that the association between levels of log2 Aβ40 and NfL with incident stroke was in particular evident in *APOE-ε4* carriers (Figure 2) In participants below the median age of 71.3 years, the association between log2 Aβ40 and total-tau with incident stroke was stronger, although this difference was not seen for the other biomarkers. Furthermore, the association of levels of log2 total-tau and incident stroke was more evident in participants with the highest educational level in contrast to the association of log2 NfL and incident stroke, which was present across all educational levels.

DISCUSSION

In this study, participants with higher levels of log2 NfL were more likely to have had a prior stroke, and participants with higher levels of total-tau and NfL at baseline were associated with a higher risk of incident stroke. These findings were contrary to Aβ40, Aβ42 and the Aβ42/40 ratio: only higher levels of Aβ40 were related to higher stroke risk in *APOE-ε4* carriers. The association between NfL and incident stroke was strongest in *APOE-ε4* carriers and for incident hemorrhagic stroke.

Previous studies have shown altered levels of Aβ, total-tau, and NfL after stroke,[15,27,28] speculating that post-stroke impaired perivascular space integrity, inflammation, hypoxia, and blood-brain barrier breakdown can lead to accelerated deposition within brain parenchyma or vessels. To our knowledge, there are no previous studies determining the association between Aβ and incident stroke. The lack of association between Aβ and the Aβ42/40 ratio with any stroke and especially hemorrhagic stroke in our study is unexpected considering that cerebral amyloid angiopathy is an important cause of primary lobar intracerebral hemorrhage in older adults.[29] We did find that a higher plasma Aβ40 relates to higher

stroke risk in *APOE-ε4* carriers, although not significantly. This may relate to a previous study linking *APOE-ε4* carriership with higher overall CAA scores in brain samples from pathologically confirmed AD cases[30], supporting a potential link between Aβ and stroke in our data. Unfortunately we did not have enough data to study stroke subtypes when stratifying for *APOE-ε4* carriership to assess if this association is driven by hemorrhagic strokes. Furthermore, we previously found associations between lower and not higher plasma Aβ with a higher dementia risk in the same study population.[31] Lower plasma levels of Aβ might reflect an increased amyloid brain deposition. Indeed, previous reports using the Pittsburgh compound B (PiB) positron emission tomography (PET) scan support the notion that lower plasma Aβ reflects the aggregation of Aβ in the brain.[32-35] Aβ in the brain interstitial fluid can be cleared via perivascular drainage pathways, or deposited as neuritic plaques in the brain parenchyma or as CAA along vessel walls. Factors that favor vascular Aβ deposition over parenchymal deposition include termination of Aβ at amino acid position 41.[36] The relation between plasma Aβ with stroke risk within *APOE-ε4* carriers in our study more probably reflects CAA along vessel walls instead of Aβ deposition as neuritic plaques for two reasons: 1) We found that not lower but higher plasma Aβ increases stroke risk and 2) specifically Aβ40 instead of Aβ42 was associated with stroke risk in our study. Further studies involving other measures of Aβ including neuroimaging are warranted.

In contrast to Aβ, previous studies have looked at total-tau and NfL in relation to incident stroke. A study including 101 stroke cases showed a similar relation between total-tau and incident all-stroke compared to our study, especially in those with total-tau levels in the highest quantile.[16] Another study in diabetic adults assessing the relation between NfL and incident stroke found an increased stroke risk for individuals in the second to fifth quintiles of NfL levels compared to those in the lowest quintile, with HRs of 3.91 (1.45-10.53), 4.05 (1.52-10.79), 5.63 (2.16-14.66), and 9.75 (3.84-27.71).[17] Interestingly, we found that total-tau was mainly associated with the risk of ischemic stroke and NfL was associated with ischemic and especially hemorrhagic stroke. A potential explanation for our findings is the presence of subclinical pre-existing vascular pathology caused by repeated subclinical insults and injuries due to the atherosclerosis process before the clinical onset of stroke.[2] It is possible that these damages already cause NfL (and perhaps total-tau) to start leaking.[37] We also know that these subclinical events are strong harbingers of incident stroke[38], making the link between total-tau and NfL with incident stroke likely. Another explanation is that since both total-tau and NfL are axonal cytoskeletal proteins involved in axonal transport[39,40], these proteins could itself be causally involved in stroke etiology. Indeed, in experimental studies on stroke, cells present in the ischemic brain area contain decreased levels of acetylated α-tubulin, and pharmacological inhibition of the deacetylation successfully improves the functional recovery in rats.[41,42] Further experimental studies are needed to study the potential role of cytoskeletal proteins and axonal transport deficits in the etiology of stroke.

This study is not without limitations. First, we were unable to compare our measurements in plasma with those in cerebrospinal fluid (CSF) of the same participants, as CSF is not systematically collected in the population-based Rotterdam Study. However, plasma and CSF levels of these biomarkers are strongly correlated.[43-45] Second, our subtype analyses were limited by a small number of incident hemorrhagic stroke events. Third, our study participants are predominantly Caucasians, limiting the external generalizability of our study.

In conclusion, we found that individuals in the general population with higher levels of total-tau and NfL had a higher risk of stroke whereas baseline levels of Aβ40 were only related to higher risk of stroke in *APOE-ε4* carriers. Our findings support the role of accumulating brain pathology in the etiology of stroke.

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4.2 Immunity and amyloid-beta, total-tau and neurofilament light chain

ABSTRACT

Introduction

We investigated how components of immunity relate to biomarkers of Alzheimer's disease (AD) in plasma and explored the influence of AD genetic risk factors in the population-based Rotterdam Study.

Methods

In 7,397 persons, we calculated the granulocyte-to-lymphocyte ratio[GLR], platelet-to-lymphocyte ratio[PLR], and systemic immune-inflammation index[SII]. In 3,615 of these persons, plasma amyloid-beta (Aβ)-42 and Aβ-40 were measured. Next, we constructed an overall genetic risk score[GRS] based on genome-wide significant variants, both including and excluding *APOE*-ε4.

Results

All innate immunity phenotypes were related to higher Aβ, most strongly with a doubling in GLR leading to a 1.9% higher Aβ-42 (95% confidence interval [95%CI] 0.4;3.3%) and 3.2% higher Aβ-40 [95%CI 2.0;4.3%]. Higher AD GRS including *APOE*-ε4 was associated with higher immunity markers.

Discussion

Higher levels of immunity markers were associated with higher Aβ in plasma. Participants with a higher genetic predisposition to AD had higher immunity markers, where these effects were mainly driven by *APOE*-ε4.DIntroduction

INTRODUCTION

Alzheimer's disease (AD) is characterized pathologically by accumulation of amyloid-beta (Aβ) as amyloid plaques and phosphorylated tau as neurofibrillary tangles [1]. This accumulation gave rise to the amyloid hypothesis, posing that Aβ activates a cascade of pathologic changes [2]. As an extension to the amyloid hypothesis, the antimicrobial protection model was recently proposed suggesting that Aβ oligomerization is not intrinsically pathological, but emerges as an innate immune response [3]. Genetic variants identified through genome-wide association studies (GWAS) support this role for immunity [4]. It has also been found that higher activation of the innate immune system and lower activation of the adaptive immune system leads to higher dementia risk [5]. Yet, the link between immunity and AD-related brain pathology is largely unknown.

In the early 1990s, Apolipoprotein E (ApoE) was found to be a component of amyloid plaques [6, 7]. However, how ApoE's function as a lipid-carrier is in itself related to AD, either in relation to amyloid-β or other factors, is not entirely clear. Lipids serve more than pure nutritional purposes; they also play essential roles in immune regulation [8]. Innate immunity in turn affects amyloid-β and tau pathology buildup. Insoluble tau isolated from postmortem AD brain is shown to be taken up by microglia in vitro and in vivo [9]. This taking in of tau may participate in tau spreading by microglia subsequently releasing some form of tau [10].

To study further how AD relates to the immune response, we may use serum levels of various blood cells which reflect a systemic immune response [11] and relate them with biomarkers of AD pathology and progression. Serum levels of granulocytes and platelets are known biomarkers of the innate immunity, whereas lymphocyte levels may yield information on the adaptive immunity [12]. Combining these measurements into ratios is thought to even better reflect the relative balance between innate and adaptive immunity, obtaining the granulocyte-to-lymphocyte ratio (GLR), platelet-to-lymphocyte ratio (PLR) and systemic immune-inflammation index (SII) as phenotypical markers of immunity [13]. Whereas for the diagnosis of AD, biomarkers are grouped into those of β amyloid deposition, pathologic tau, and neurodegeneration [ATN] according to the recent classification by Jack et al [14], yielding plasma Aβ, total-tau and neurofilament light chain (NfL) as biomarkers of AD-related brain pathology, respectively.

In an earlier study investigating the role of various biological pathways based on a genetic risk score, we found an important role for the *immune response* pathway in early AD pathology [15]. Relating phenotypical immunity and AD-related markers to each other and additionally to genetic predisposition to AD may help to understand the role of immunity biomarkers in AD pathology and their relation with AD genetic risk, and ultimately identify therapeutic strategies targeting upstream events of altered immune response in amyloidosis and neurodegeneration. We previously found that higher levels of the GLR, PLR and SII

over time reflecting higher innate immunity increase dementia risk [5]. Since we also previously found that low Aβ-42 and high NfL plasma levels were associated with risk of AD [16], we hypothesized that higher innate immunity affects the serum levels of Aβ-42 to be lower and NfL to be higher. We tested this hypothesis by investigating whether serum GLR, PLR and SII were associated with Aβ, total-tau and NfL and how genetic predisposition to AD affected these markers.

METHODS

Study population

The present study is embedded within the Rotterdam Study, a prospective population-based cohort study in Rotterdam, the Netherlands. The Rotterdam Study started in 1989 with 7,983 persons (response of 78%) aged ≥55 years and residing in the district Ommoord, a suburb of Rotterdam. This first subcohort (RS-I) was extended with a second subcohort (RS-II) in 2000, consisting of 3,011 persons (response of 67%) and with a third subcohort (RS-III) in 2006, composed of 3,932 persons aged ≥45 years (response of 65%). The design of the Rotterdam Study has been described in detail previously [17]. In brief, participants were examined at study entry and follow-up visits every three to five years. They were interviewed at home by a trained research nurse, followed by two visits at the research facility for additional interviewing and laboratory assessments. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus Medical Center and by the board of the Netherlands Ministry of Health, Welfare, and Sports. Written informed consent was obtained from all participants.

Laboratory tests for granulocytes, platelets, and lymphocytes were introduced from 2002 onwards, corresponding with the following assessment rounds in the Rotterdam Study (baseline for this study): fourth round of RS-I (RS-I-4), second round of RS-II (RS-II-2), and first round of RS-III (RS-III-1). Plasma biomarkers of AD-related brain pathology were only assessed in RS-I-4 and RS-II-2. Blood was drawn from 11,496 participants for genotyping. Of these participants, 7,413 underwent granulocytes, platelets, and lymphocytes assessments, while 3,627 participants underwent plasma Aβ, total-tau and NfL assessment. We excluded participants with missing *APOE* genotype and participants with incomplete assessments of serum markers, leaving (n=7,397) participants for analysis with complete blood assessment for immunity markers and 3,615 participants with complete plasma Aβ, total-tau and NfL assessment (**Figure 1**).

Genotyping

DNA genotyping was performed at the internal genotyping facility of the Erasmus Medical Center, Rotterdam. All samples were genotyped with the 550K, 550K duo or 610K Illumina

arrays. Genotyping quality control criteria include call rate <95%, Hardy-Weinberg equilibrium of $P < 1.0 \times 10^{-6}$, and minor allele frequency <1%. Moreover, study samples with excess autosomal heterozygosity, call rate <97.5%, ethnic outliers, and duplicate or family relationships were excluded during quality control analysis. Genetic variants were imputed from the Haplotype Reference Consortium reference panel (version 1.0) [18], using the Michigan imputation server [19]. The server uses SHAPEIT2 (v2.r790) [20] to phase the genotype data and performs imputation with Minimac 3 software [21]. For this study, we used genetic variants that had imputation quality (R^2) > 0.5. *APOE* genotype was determined separately by PCR on coded DNA samples in the baseline cohort and with a bi-allelic Tacqman assay in the extensions of the Rotterdam Study [22].

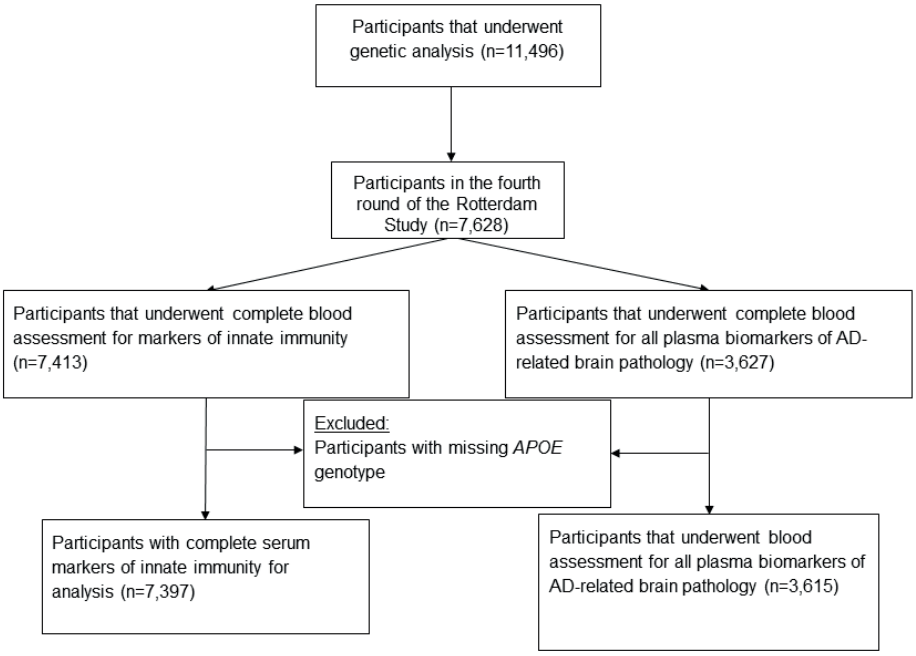

Figure 1. Flow Diagram

Assessment of blood cell counts and their derived ratios

Full blood count measurements were performed using the COULTER® Ac·T diff2™ Hematology Analyzer (Beckman Coulter, San Diego, California, USA) directly after blood sample drawn. Laboratory measurements included absolute granulocyte, platelet, and lymphocyte counts in 10^9 per litre. The GLR and PLR were calculated as the ratio of granulocyte count to lymphocyte count, and as the ratio of platelet count to lymphocyte count, respectively. The SII was defined as platelet count times the GLR [13]. We use the GLR as proxy measure for

the commonly used neutrophil-to-lymphocyte ratio, as granulocytes are the most abundant subtype of neutrophils.

Assessment of plasma Aβ, total-tau and NfL

Blood was sampled in EDTA treated containers and centrifuged. Subsequently plasma was aliquoted and frozen at -80^{0}C according to standard procedures. Measurements were done in two separate batches. The first batch included in total 2,000 samples, obtained from a random selection of 1,000 participants from sub-cohort RS-I-4 and 1,000 from RS-II-2. The second batch included in total 3,094 samples from the remaining participants.

All measurements were performed at Quanterix (Lexington, MA, USA) on a single molecule array (Simoa) HD-1 analyzer platform [23]. Samples were tested in duplicate. Two quality control (QC) samples were run on each plate for each analyte. NfL was measured with the NF-light advantage kit [24]. The Simoa Human Neurology 3-Plex A assay (N3PA) was used for measuring the concentration of total-tau, Aβ-42 and Aβ-40. When duplicates or single measurements were missing (the majority of missing samples were due to system failures (n=279), and few because of insufficient volume (n=47)) or in the case the concentration coefficient of variation (CV) exceeded 20% (14 to 87 samples) or control samples were out of range (none), participant's data were excluded from the analyses [16].

Genetic risk scores

We computed genetic risk scores, by selecting late-onset AD-associated single-nucleotide polymorphisms (SNPs) reaching genome-wide significance ($P < 5.0 \times 10^{-8}$). Among common variants, we considered only variants identified by the International Genomics of Alzheimer's Project (IGAP) meta-analyses [4]. In addition, we included four rare variants which can be classified under immune response based on their functional role [25, 26], leading to a total of 28 independent genome-wide significant AD-associated variants. A weighted genetic risk score was constructed using the effect sizes (log of odds ratio) of the genome-wide significant variants from the IGAP meta-analysis as weights and their respective allele dosages from imputed genotype data of our study cohorts. A genetic risk score was constructed as the sum of the products of SNP dosages and their corresponding weights. We constructed genetic risk scores in three ways: (1) combining all 28 selected variants, (2) excluding the *APOE*-ε4 variant to identify the joint independent effect of all other genome-wide significant SNPs and (3) clustering the variants into the immune response pathway. We classified AD associated SNPs into immune system pathway based on information on current investigations and reviews [4, 27-29]. Of the 28 SNPs, 9 could be clustered into the immune response pathway-based genetic risk score; **Table S1**. All genetic risk scores were standardized to allow direct comparison of results.

Covariates

Potential confounding factors were chosen on the basis of previous literature [13, 30]. All covariates were measured at the same rounds as the assessments of AD-related brain pathology and immunity markers. Smoking habits were categorized as current vs former and never smoking. Body mass index (BMI) was calculated as weight in kilograms per height in meters squared. Blood pressure was measured twice at the right brachial artery with the participant in sitting position, out of which the mean was used. Diabetes mellitus was defined as use of antidiabetic medication, fasting serum glucose level ≥7.1 mmol/L (≥127.9 mg/dL), or random non-fasting serum glucose level ≥11.1 mmol/L (≥200.0 mg/dL) [31]. Additional information from the serum samples was collected on high sensitivity C-reactive protein (hs-CRP) and creatinine in a subsample (N=1,342).

Statistical analysis

As the GLR, PLR and SII had a skewed distribution, analysis was based on the natural logarithmic (Ln) transformation of their values. For the same reason, all plasma biomarkers of AD-related brain pathology, except Aβ-40 which had a normal distribution, were Ln transformed. We first determined the association between the GLR, PLR and SII with plasma biomarkers of AD-related brain pathology using linear regression. For this analysis, we conducted two different transformations: 1) Because we transformed both exposure and outcome: $\log Yi = \alpha + \beta \log Xi + \varepsilon$, we reported mean differences in percentages with 95% confidence intervals (CIs) obtained by exponentiating Ln(2) times the estimated coefficients from the linear regression model to facilitate interpretation of results: These mean differences correspond to the percentage change in plasma Aβ, total-tau or NfL for a doubling of the GLR, PLR and SII. For this analysis, Aβ-40 was also Ln transformed. 2) We standardized all blood markers to allow direct comparison of the results. We adjusted all models for age, sex and study cohort (model I). In addition, the following covariates were added to a second model (model II):, smoking, diabetes, BMI and systolic blood pressure and *APOE*-ε4 carriership. Since plasma Aβ, total-tau and NfL were measured in two batches, we additionally corrected for batch effects in models. We performed additional adjustment for platelet count when analysing GLR in model II. We assessed effect measure modification by *APOE* genotype by stratifying for participants having the ε2/ε2 or ε2/ε3, ε3/ε3 and ε3/ε4 or ε4/ε4 genotype. We formally tested interaction between *APOE* genotype and ratios of blood cell counts on the multiplicative scale by adding interaction terms to model II. In addition, we assessed effect measure modification by activity of the innate immune system by stratifying for median granulocyte count (as pure innate immunity marker rather than studying the effect of the balance between innate and adaptive immunity) when assessing the associations between genetic risk scores and plasma biomarkers of AD-related brain pathology as outcome. We formally tested interaction between genetic risk scores and granulocyte counts on the multiplicative scale by adding interaction terms to the model. As a sensitivity analysis,

we also adjusted for creatinine and hs-CRP in a subsample of participants in which these markers were measured. Because hs-CRP is also an important inflammatory biomarker, we also assessed the association between Ln(hs-CRP) as exposure and the standardized plasma biomarkers of AD-related brain pathology as outcome using linear regression. We adjusted all models for age, sex, cohort, smoking, diabetes, BMI, systolic blood pressure, *APOE*-ε4 carriership, batch effects, platelet count and creatinine.

We then determined associations between all three genetic risk scores per standard deviation (SD) increase as exposure with the standardized immunity markers (Ln(GLR), Ln(PLR) and Ln(SII)) and with the standardized plasma biomarkers of AD-related brain pathology (Ln(Aβ-42, Aβ-40, Ln(Aβ-42/40 ratio), Ln(total-tau) and Ln(NfL)) as separate outcomes, using linear regression. These analyses were all adjusted for age, sex and study cohort.

Furthermore, we investigated the association between *APOE*-allele carriership as exposure with serum markers of immunity and plasma biomarkers of AD-related brain pathology as outcomes. For this analysis, we defined *APOE*-allele carriership as either carrying ε2 (ε2/ε2 or ε2/ε3 genotype), being homozygous for ε3 (ε3/ε3 genotype) or carrying ε4 (ε3/ε4 or ε4/ε4 genotype). People with the ε2/ε4 genotype were excluded from the analyses and ε3 homozygosity was considered the reference group. Analyses were adjusted for age, sex and study cohort. We calculated mean levels of hs-CRP and creatinine levels within the different *APOE* genotypes.

Missing covariate data (maximum 0.7%) were imputed using 5-fold Multiple Imputation by Chained Equations based on determinant, outcome, and included covariates. All analyses were performed using RStudio version 1.0.153 (R version 3.6.1, RStudio, Inc., Boston, MA). We corrected for multiple testing using Bonferroni adjustment for all analyses using the total population (i.e. not stratified or no subgroup analyses): For the associations between serum markers of immunity and plasma biomarkers of AD-related brain pathology, results are considered statistically significant if *P* below 0.05/(6×5)=0.002; for the associations of genetic risk scores reflecting AD including and excluding *APOE*-ε4 and immune response with plasma biomarkers of AD-related brain pathology and serum markers of immunity, results were considered statistically significant if *P* below 0.05/(9×5)=0.001 and for the associations of *APOE* genotypes with plasma biomarkers of AD-related brain pathology and serum markers of immunity, results are considered statistically significant if *P* below 0.05/16=0.003. For these analyses, a suggestive association was considered at the level alpha=0.05. All other analyses were considered statistically significant at the level alpha=0.05.

RESULTS

Characteristics of study participants are displayed in **Table 1**. The mean age of participants with serum markers of innate immunity was 66.1 (±10.4) years of which 4,208 (57%) were

Table 1. Characteristics of study population.

Characteristic	Sample with serum markers of immunity (N = 7,397)	Sample with plasma biomarkers of AD-related brain pathology (N = 3,615)
Women	4,208 (56.9%)	2,059 (56.8%)
Age, years	66.1 ±10.4	71.9 ±7.3
Study cohort		
Cohort 1	2,656 (35.9%)	2,159 (59.7%)
Cohort 2	1,704 (23.0%)	1,456 (40.3%)
Cohort 3	3,037 (41.1%)	-
Current smokers	1,427 (19.4%)	536 (14.9%)
Diabetes mellitus type 2	433 (5.9%)	214 (5.9%)
Body mass index, kg/m^2	27.6 ±4.3	27.6 ±4.1
Systolic blood pressure, mmHg	142.8 ±21.9	149.2 ±21.0
APOE genotype		
ε2/ε2	46 (0.6%)	31 (0.9%)
ε2/ε3	952 (12.9%)	490 (13.6%)
ε2/ε4	202 (2.7%)	103 (2.8%)
ε3/ε3	4293 (58.0%)	2119 (58.6%)
ε3/ε4	1741 (23.5%)	810 (22.4%)
ε4/ε4	163 (2.2%)	62 (1.7%)
Granulocyte count, × 10^3/microL	4.0 ±1.4	4.0 ±1.3
Platelet count, × 10^3/microL	268.2 ±67.2	256.4 ±64.4
Lymphocyte count, × 10^3/microL	2.3 ±1.2	2.2 ±1.3
Granulocyte-to-lymphocyte ratio	1.9 ±0.9	2.0 ±0.9
Platelet-to-lymphocyte ratio	128.2 ±47.3	130.0 ±49.8
Systemic immune-inflammation index	517.3 ±280.1	522.0 ±290.9
Amyloid-beta 42, pg/mL	-	10.6 ±3.0
Amyloid-beta 40, pg/mL	-	265.6 ±54.6
Amyloid-beta 42/40 ratio	-	0.04 ±0.009
Total-tau, pg/mL	-	2.6 ±2.5
Neurofilament light chain, pg/mL	-	15.7 ±11.6

Abbreviations: N = number of participants included in study. Data presented as mean (standard deviation) for continuous variables and number (percentages) for categorical variables. Data here are unimputed. Number of missing values for the immunity cohort: 39 (0.5%) for smoking, 19 (0.3%) for diabetes, 20 (0.3%) for BMI, 37 (0.5%) for systolic blood pressure, 0 for *APOE* carriership. Number of missing values for the sample undergoing serum AD measurements are: 25 (0.7%) for smoking, 8 (0.2%) for diabetes, 0 for BMI, 12 (0.3%) for systolic blood pressure and 0 for *APOE* genotype.

women and the mean age of the sample undergoing serum AD marker measurements was 71.9 (±7.3) years of which 2,059 (57%) were women.

We found that a doubling of SII was associated with a 1.2% higher serum Aβ-42, albeit not statistically significant (95% confidence interval (95% CI) -0.005% to 2.3%, *P*=0.051) and a 1.8% higher serum Aβ-40 (95% CI 0.9 to 2.7%, *P*=<0.001). Estimates were even higher for a doubling of GLR, leading to a 1.9% higher serum Aβ-42 (95% CI 0.4 to 3.3%, *P*=0.010) and 3.2% higher serum Aβ-40 (95% CI 2.0 to 4.3%, *P*=<0.001). The Aβ-42/40 ratio was lower with a doubling in GLR only (-1.2% [-2.3 to -0.1%], *P*=0.028). Only a doubling in PLR was significantly associated with lower total-tau (-3.6% [-5.5% to -1.7%], *P*=<0.001). NfL was higher with all innate immunity phenotypes, most strongly with a doubling in GLR (4.7% [2.5 to 7.1%], *P*=<0.001); **Table 2** and **Table S2**. Stratification did not reveal statistically significant differences across strata of *APOE* carriership, with the effects in the *APOE* ε3/ε4 or ε4/ε4 stratum being only non-significantly stronger than in the other strata, especially for NfL; **Figure 2** and **Table S2**. In addition, the associations between all genetic risk scores with lower Aβ-42/40 ratio became lower in participants with granulocyte counts higher than the median (mean difference = -0.18, 95% = CI -0.23; -0.13, *P*=<0.001 for the overall AD genetic risk score, mean difference = -0.06, 95% CI = -0.11; -0.02, *P*=0.007 for the overall AD genetic risk score excluding *APOE* and mean difference = -0.05, 95% CI = -0.10; -0.004, *P*=0.035 for the immune response pathway-based genetic risk score, yet the interaction remained non-significant; **Table S3**). When additionally adjusting for hs-CRP and creatinine (in a subsample: N=1,341), the associations between GLR and SII with higher Aβ-42 and NfL disappeared, the associations between immunity markers with higher Aβ-40 were borderline significant and the associations with lower Aβ-42/40 ratio and lower total-tau persisted; **Table S4**. Higher hs-CRP was significantly associated with a higher Aβ-40 and higher total-tau; **Table S5**.

Furthermore, we found that a standard deviation (SD) increase in the overall AD genetic risk score including *APOE*-ε4 was associated with higher GLR (mean difference in Ln(GLR) [SD] = 0.024, 95% CI = 0.002; 0.046, *P*=0.032) and significantly with lower Aβ-42 and Aβ-42/40 ratio (mean difference in Aβ-42 [SD] = -0.129, 95% CI = -0.162; -0.095, *P*=<0.001 and Ln(Aβ-42/40) [SD] = -0.157, 95% CI = -0.190; -0.124, *P*=<0.001, respectively). These associations were mainly driven by the *APOE*-ε4 variant; **Figure 3**. The genetic risk score reflecting the immune response showed a suggestive association with lower Aβ-42/40 ratio (mean difference = -0.038, 95% CI = -0.070; -0.006, *P*=0.020), but not with the serum markers of innate immunity; **Figure 3** and **Table S6**. We found that *APOE*-ε2 carriers displayed lower serum markers of innate immunity levels compared to ε3/ε3, while these markers were elevated in *APOE*-ε4; **Figure 4** and **Table S7**. Mean levels of hs-CRP and creatinine levels within the different *APOE* genotypes are shown in **Table S8**.D

Table 2. Associations between serum markers of immunity and plasma biomarkers of AD-related brain pathology.

Per doubling in serum markers of immunity	Percentage change in Aβ, total-tau or NFL, 95% CI									
	Aβ-42	P	Aβ-40	P	Aβ-42/40 ratio	P	Tau	P	NFL	P
Model I										
GLR*	1.6 (0.2; 3.1)	0.025	3.2 (2.1; 4.3)	<0.001	-1.5 (-2.6; -0.4)	0.008	0.1 (-1.8; 2.0)	<0.001	4.9 (2.5; 7.2)	<0.001
PLR	-0.7 (-2.2; 0.8)	0.351	-0.2 (-1.4; 0.9)	0.688	-0.5 (-1.6; 0.7)	<0.001	-4.4 (-6.3; -2.5)	<0.001	2.9 (0.5; 5.3)	0.018
SII	1.0 (-0.1; 2.2)	0.078	1.8 (0.9; 2.7)	<0.001	-0.8 (-1.7; 0.1)	0.093	-0.4 (-1.9; 1.1)	0.590	3.3 (1.5; 5.2)	<0.001
Model II										
GLR*	1.9 (0.4; 3.3)	0.010	3.2 (2.0; 4.3)	<0.001	-1.2 (-2.3; -0.1)	0.028	-0.1 (-1.9; 1.8)	0.927	4.7 (2.5; 7.1)	<0.001
PLR	-0.8 (-2.2; 0.7)	0.314	-0.2 (-1.3; 1.0)	0.790	-0.6 (-1.8; 0.6)	0.309	-3.6 (-5.5; -1.7)	<0.001	1.5 (-0.8; 3.9)	0.205
SII	1.2 (0.00; 2.3)	0.051	1.8 (0.9; 2.7)	<0.001	-0.6 (-1.5; 0.3)	0.190	-0.5 (-2.0; 1.0)	0.539	2.9 (1.1; 4.8)	0.002

Abbreviations: CI = confidence interval, Ln = natural logarithmic transformation, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = amyloid-beta 42, Aβ-40 = amyloid-beta 40, Aβ-42/40 ratio = amyloid-beta 42-to-40 ratio, Tau = total-tau, NFL = neurofilament light chain. Model I adjusted for age, sex, study cohort and batch effects. Model II adjusted for age, sex, study cohort, smoking, diabetes, BMI, platelets, systolic blood pressure, *APOE*-ε4 and batch effects. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity. *Additional adjustment for platelet count. Results are considered statistically significant if *P* below 0.05/(6×5)=0.002.DSupporting information

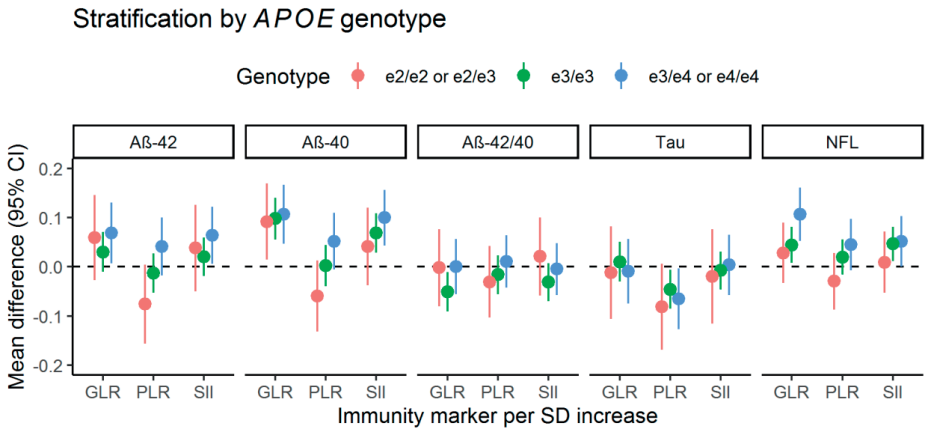

Figure 2. Associations between serum markers of immunity and plasma biomarkers of AD-related brain pathology.

Abbreviations: CI = confidence interval, SD = standard deviation, Ln = natural logarithmic transformation, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = amyloid-beta 42, Aβ-40 = amyloid-beta 40, Aβ-42/40 ratio = amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain, e2/e2 or e2/e3 = apolipoprotein Eε2/ε2 genotype, e3/e3 = apolipoprotein Eε3/ε3 genotype, e3/e4 or e4/e4 = apolipoprotein Eε3/ε4 or ε4/ε4 genotype. Model adjusted for age, sex, study cohort, smoking, diabetes, BMI, platelets, systolic blood pressure, *APOE*-ε4 and batch effects. Additional adjustment for platelet count when analysing Ln(GLR). All phenotypical markers were Ln(transformed) except for Aβ-40. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity.

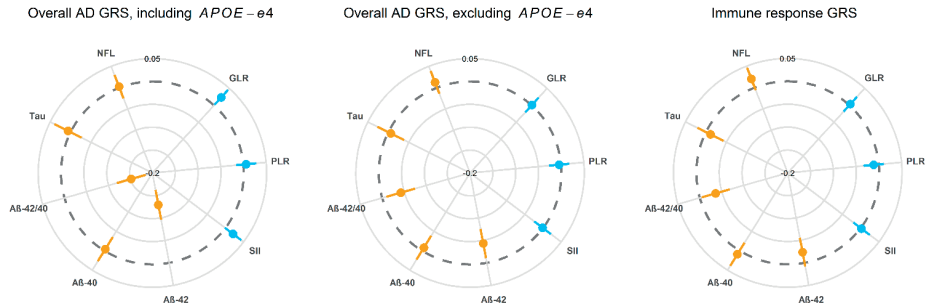

Figure 3. Associations of genetic risk scores reflecting AD including and excluding *APOE*-ε4 and immune response with plasma biomarkers of AD-related brain pathology and serum markers of immunity.

Abbreviations: Ln = natural logarithmic transformation, AD = Alzheimer's disease, GRS = genetic risk score, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = Amyloid-beta 42, Aβ-40 = Amyloid-beta 40, Aβ-42/40 = Amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain. Model adjusted for age, sex and

study cohort. GRS are per SD increase. All phenotypical markers were Ln(transformed) except for Aβ-40. Gray dotted circle indicates the null with associations becoming increasingly negative towards the centre of the circle. Orange indicates the plasma biomarkers of AD-related brain pathology while blue indicates the immunity phenotypes. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity.

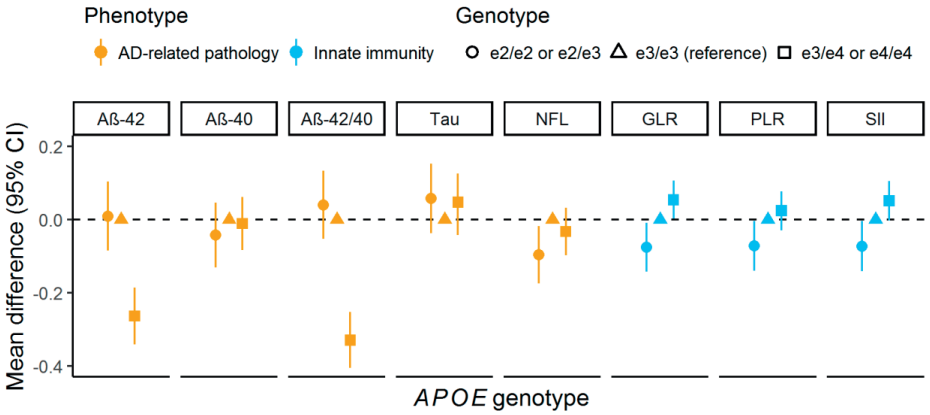

Figure 4. Associations of *APOE* genotypes with plasma biomarkers of AD-related brain pathology and serum markers of immunity.

Abbreviations: CI = confidence interval, SD = standard deviation, GRS = genetic risk score, AD = Alzheimer's disease, Ln = natural log transformation, GLR = granulocyte-to-lymphocyte ratio, SII = systemic immune inflammation index, Aβ-42 = Amyloid-beta 42, Aβ-40 = Amyloid-beta 40, Aβ-42/40 = Amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain, *APOE* = Apolipoprotein E, e2/e2 or e2/e3 = apolipoprotein Eε2/ε2 genotype, e3/e3 = apolipoprotein Eε3/ε3 genotype, e3/e4 or e4/e4 = apolipoprotein Eε3/ε4 or ε4/ε4 genotype. Adjusted for age, sex and study cohort. All phenotypical markers were Ln(transformed) except for Aβ-40. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity.

DISCUSSION

In this study, we found associations between higher levels of GLR, PLR and SII reflecting higher innate immunity, with higher Aβ42 and 40, lower Aβ-42/40 ratio, lower total-tau and higher NfL in plasma. In *APOE*-ε4 carriers, these associations were even stronger. The overall genetic risk score including *APOE*-ε4 was associated with higher GLR and SII and with lower Aβ-42 and Aβ-42/40 ratio. These effects were mainly driven by the *APOE*-ε4 variant. The genetic risk score reflecting the immune response was associated with lower Aβ-42/40 ratio, but not with the serum immunity markers. Furthermore, we found that *APOE*-ε2 carriers displayed lower serum markers of innate immunity compared to *APOE*-ε3/ε3, while these markers were elevated in *APOE*-ε4 carriers.

Interestingly, we found that higher innate immunity was associated with higher serum Aβ-42, but with even higher Aβ-40. The 40-residue peptide represents the most abundant Aβ isoform in the brain [32], while the 42-residue shows a significant increase with certain forms of AD. Our findings are concordant with the notion that Aβ functions as an anti-microbial peptide (AMP), because the physiochemical and biological properties previously reported for Aβ are similar to those of AMPs. In addition, experiments have shown that Aβ is active against at least eight common and clinically relevant microorganisms [33]. Activity was isoform-specific for six organisms with Aβ-42 showing greater potency compared to Aβ40 [33]. Taken all evidence together, we propose that *APOE-ε4* carriers display stronger innate immune responses, and thus produce more/excess Aβ in response to pathogens. Of the two isoforms, Aβ42 will aggregate in the brain (possibly due to its larger size) while Aβ40 will not aggregate (or to a lesser extent) and as a consequence will be higher in serum. Further study is needed to confirm this hypothesis.

Furthermore, we found that both higher serum markers of innate immunity and the genetic risk score reflecting the immune response associate with the lower serum Aβ-42/40 ratio. *APOE*-ε4 carriership, which is the major genetic risk factor for AD, also displays lower serum Aβ-42/40 ratio as well as lower Aβ-42. In line with this, a meta-analysis of prospective cohort studies has shown that lower serum Aβ-40, even lower Aβ-42 and consequently lower Aβ-42/40 ratio lead to higher AD risk.[34] Previous reports of associations of low plasma Aβ-42/40 ratio with increased amyloid brain uptake, as measured by Pittsburgh compound B (PiB) positron emission tomography (PET) scan, support the notion that lower plasma Aβ reflect the aggregation of Aβ in the brain.[35-38] In this context, our finding that *APOE*-ε4 carriers have higher activity of innate immunity compared to ε3/ε3, while we see the opposite in *APOE*-ε2 carriers suggests that *APOE*-ε4 might have an overactive innate immune response. Our results support experimental studies showing the capacity of *APOE* to modulate inflammation. Indeed, in healthy humans challenged with intravenous lipopolysacharide (LPS) infusion, ε4 carriers demonstrated significantly higher elevation of body temperature and plasma tumor necrosis factor levels than ε4 non-carriers. [39] In this same study, when whole blood isolates from human subjects were stimulated ex-*vivo* with Toll-like receptor ligands, increased production of a wide panel of cytokines and chemokines was observed in blood from ε4+ donors compared with ε4– donors. A higher immune response associated with the ε4 allele is also observed in human *APOE*-targeted replacement mice and in cultured microglia and/or macrophages upon LPS stimulation.[39-41] How *APOE* achieves this is not yet well-understood.[8] Evidence shows that lipid rafts play an essential role in immune activation by serving as platforms for signaling complexes. [42] *APOE*-ε4 is reported to be less effective than *APOE*-ε3 in inducing cholesterol efflux from macrophages[43], which leads to cholesterol accumulation on cell membranes.[39, 43] This mechanism has been proposed to explain the higher immune reactivity associated with

APOE-ε4[8], but further studies are needed to explore this or potential other mechanisms further.

In addition to the role of immune response pathways in Aβ, our data show that higher innate immunity related to lower plasma total-tau. Others assessed the effect of microglial activation on tau pathogenesis, where they found that microglial activation is shown to precede tau pathology in a tauopathy mouse model (Yoshiyama et al. 2007) and administering an immunosuppressant drug FK506 from an early age drastically reduces tau pathology [44]. According to previous studies, higher plasma tau is associated with AD dementia [16, 45], although correlations were weak [45] and non-linear (J-shape) [16], making the role of immunity in tau elusive. Future studies are implicated relating the same crude markers of the immune system in relation to phospho tau.

The associations between immunity markers with higher Aβ-42 and NfL disappeared when additionally adjusting for hs-CRP and creatinine. There could be two explanations: 1) This analysis was performed within a subsample of the total population, impeding comparison of the results in the total population and decreasing the power of the analysis. 2) We found that higher hs-CRP was also associated with higher NfL, albeit not statistically significant, suggesting that hs-CRP could be acting as a mediator.

Our result that the immune GRS was not related to the immunity biomarker levels is surprising, especially since several immune genes are key players for innate immunity, including *TREM2* [46]. TREM2 overexpression is thought to enhance microglial phagocytotic capacity, but transcription analyses show mixed microglial activation patterns with suppression of certain disease-associated microglia (DAM) genes, but further activation patterns of other DAM genes [47], making the role of *TREM2* in AD unclear. Further studies are needed to identify the precise gene signatures of microglia that mediate pathology-and neurodegeneration-associated sterile inflammation.

Our study has several limitations. First, ours is a cross-sectional study, limiting our ability to draw causal inferences. Longitudinal cohort studies are required to confirm our findings. Second, we were limited to studying crude markers of the immune system in this population-based setting. These types of measures have been associated with other outcomes like cancer-related, pancreatitis, and many other responses [48]. These ratios could be proxies for different systemic inflammatory responses that affect mobilization of bone marrow-derived cells and egress of leukocytes into tissue [49]. It could also be related to immunosenescence and this might explain the association with NfL because it too, increases with age [50]. Third, we were similarly limited to crude markers of AD-related brain pathology which are not diagnostic of AD, especially since the ATN-classification includes AD biomarkers as measured by PET, structural MRI or CSF and we do not have phospho tau measured. However, plasma and CSF levels of these biomarkers are strongly correlated [51-53]. Also NfL is a global biomarker and not specific for AD. Fourth, we were unable to categorise the age groups into early and late onset AD respectively, due to the low number of early

onset AD patients within our population-based cohort. It would be helpful to categorise the biomarkers according to early and late onset AD in future studies, because Aβ has a better correlation in early onset AD. Early onset AD differs from late onset AD not only with respect to genetic predisposition and pathology but also in relation to the clinical outcome and the natural course. Only 11% of early AD patients has familial mutations with *APP*, *PSEN1* and *PSEN2*. For example, patients with early AD have a greater burden in precuneus and parietal lobes and to a lesser extent in the frontal lobes [54]. Fifth, we did not offer replication data. We hope that our study provides a stimulus for other cohort studies to replicate our findings. Lastly, our study contains predominantly Caucasians, which limits its external generalizability. Further study in other ethnicities is needed to identify potential ethnic differences.

In summary, in this population-based study we showed that higher innate immunity, as reflected by higher levels of serum GLR, PLR and SII, were associated with higher plasma Aβ42, 40, and NfL, and with lower Aβ-42/40 and total-tau. Furthermore, *APOE*-ε2 carriers displayed lower serum markers of innate immunity levels compared to ε3/ε3, while these markers were elevated in *APOE*-ε4. Knowledge on the potential pro-inflammatory role of *APOE*-ε4 should encourage future studies to find ways to scale down innate immune responses in *APOE*-ε4 carriers to limit AD-related brain pathology to prevent AD.

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SUPPORTING INFORMATION

Table S1. Clustering of genome-wide significant variants into the immune response pathway.

Pathway	Gene	Rare or common	Assigned SNP	Kunkle et al. [4]	Dourlen et al. [22]	Jones et al. [23]	Guerreiro et al. [24]
Immune Response	CLU	Common	rs9331896	Yes	Yes	Yes	Yes
	CR1	Common	rs4844610	Yes	Yes	Yes	Yes
	INPP5D	Common	rs10933431	Yes	Yes	Yes	Yes
	EPHA1	Common	rs10808026	-	-	-	Yes
	MS4A2	Common	rs7933202	-	Yes	-	Yes
	MEF2C	Common	rs190982	Yes	-	-	Yes
	ABI3	Rare	rs616338	-	Yes	-	-
	PLCG2	Rare	rs72824905	Yes	Yes	Yes	-
	TREM2	Rare	rs75932628	-	Yes	-	Yes
	TREM2	Rare	rs143332484	-	Yes	-	-

Table S2. Associations between serum markers of immunity and plasma biomarkers of AD-related brain pathology stratified for *APOE* genotype.

Per SD increase in serum markers of immunity	SD change in Aβ, total-tau or NFL, 95% CI									
	Ln(Aβ-42)	P	Aβ-40	P	Ln(Aβ-42/40)	P	Ln(Tau)	P	Ln(NFL)	P
APOE ε2/ε2 or ε2/ε3										
Ln(GLR)*	0.06 (-0.03; 0.15)	0.177	0.09 (0.01; 0.17)	0.021	0.00 (-0.08; 0.08)	0.964	-0.01 (-0.11; 0.08)	0.801	0.03 (-0.03; 0.09)	0.367
Ln(PLR)	-0.08 (-0.16; 0.00)	0.064	-0.06 (-0.13; 0.01)	0.106	-0.03 (-0.10; 0.04)	0.406	-0.08 (-0.17; 0.01)	0.069	-0.03 (-0.09; 0.03)	0.312
Ln(SII)	0.04 (-0.05; 0.13)	0.394	0.04 (-0.04; 0.12)	0.307	0.02 (-0.06; 0.10)	0.607	-0.02 (-0.12; 0.08)	0.687	0.01 (-0.05; 0.07)	0.773
APOE ε3/ε3										
Ln(GLR)*	0.03 (-0.01; 0.07)	0.147	0.10 (0.06; 0.14)	<0.001	-0.05 (-0.09; -0.01)	0.013	0.01 (-0.03; 0.05)	0.627	0.04 (0.01; 0.08)	0.018
Ln(PLR)	-0.01 (-0.05; 0.03)	0.527	0.00 (-0.04; 0.04)	0.911	-0.02 (-0.06; 0.02)	0.428	-0.05 (-0.09; -0.01)	0.024	0.02 (-0.02; 0.06)	0.283
Ln(SII)	0.02 (-0.02; 0.06)	0.301	0.07 (0.03; 0.11)	0.001	-0.03 (-0.07; 0.01)	0.109	-0.01 (-0.05; 0.03)	0.695	0.05 (0.01; 0.08)	0.008
APOE ε3/ε4 or ε4/ε4										
Ln(GLR)*	0.07 (0.01; 0.13)	0.030	0.11 (0.05; 0.17)	0.001	0.00 (-0.06; 0.06)	0.994	-0.01 (-0.07; 0.06)	0.781	0.11 (0.05; 0.16)	<0.001
Ln(PLR)	0.04 (-0.02; 0.10)	0.174	0.05 (-0.01; 0.11)	0.078	0.01 (-0.04; 0.06)	0.697	-0.07 (-0.13; 0.003)	0.040	0.04 (-0.01; 0.10)	0.094
Ln(SII)	0.06 (0.01; 0.12)	0.031	0.10 (0.04; 0.16)	0.001	0.00 (-0.06; 0.05)	0.859	0.004 (-0.06; 0.07)	0.901	0.05 (0.00001; 0.10	0.050
APOE genotype	**P for interaction**									
Ln(GLR)*	0.364		0.994		0.198		0.298		0.169	
Ln(PLR)	0.696		0.418		0.990		0.801		0.515	
Ln(SII)	0.518		0.624		0.690		0.930		0.931	

Abbreviations: SD = standard deviation, CI = confidence interval, Ln = natural logarithmic transformation, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = amyloid-beta 42, Aβ-40 = amyloid-beta 40, Aβ-42/40 ratio = amyloid-beta 42-to-40 ratio, Tau = total-tau, NFL = neurofilament light chain. Model adjusted for age, sex, study cohort, smoking, diabetes, BMI, platelets, systolic blood pressure and batch effects. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity. *Additional adjustment for platelet count.

Table S3. Associations of genetic risk scores reflecting AD including and excluding *APOE*-ε4 and immune response with plasma biomarkers of AD-related brain pathology and serum markers of immunity stratified for median granulocyte counts.

Per SD increase in genetic risk scores	SD change in Aβ, total-tau or NFL, 95% CI									
	Ln(Aβ-42)	P	Aβ-40	P	Ln(Aβ-42/40)	P	Ln(Tau)	P	Ln(NFL)	P
Granulocyte counts lower than the median										
Overall AD GRS, including APOE-ε4	-0.10 (-0.15; -0.06)	<0.001	0.00 (-0.04; 0.04)	0.942	-0.13 (-0.18; -0.09)	<0.001	0.00 (-0.05; 0.05)	0.972	0.02 (-0.02; 0.06)	0.285
Overall AD GRS, excluding APOE-ε4	-0.04 (-0.09; 0.00)	0.057	-0.03 (-0.07; 0.01)	0.204	-0.03 (-0.07; 0.02)	0.237	-0.01 (-0.06; 0.04)	0.682	0.00 (-0.04; 0.04)	0.886
Immune response GRS	-0.03 (-0.08; 0.01)	0.129	-0.01 (-0.05; 0.03)	0.672	-0.03 (-0.07; 0.01)	0.156	-0.04 (-0.09; 0.00)	0.079	0.02 (-0.02; 0.06)	0.308
Granulocyte counts higher than the median										
Overall AD GRS, including APOE-ε4	-0.15 (-0.20; -0.11)	<0.001	-0.02 (-0.06; 0.03)	0.523	-0.18 (-0.23; -0.13)	<0.001	0.01 (-0.04; 0.06)	0.692	-0.02 (-0.06; 0.02)	0.321
Overall AD GRS, excluding APOE-ε4	-0.05 (-0.10; 0.00)	0.047	0.01 (-0.04; 0.05)	0.773	-0.06 (-0.11; -0.02)	0.007	0.00 (-0.05; 0.04)	0.958	0.02 (-0.02; 0.06)	0.369
Immune response GRS	-0.02 (-0.07; 0.02)	0.318	0.02 (-0.02; 0.07)	0.344	-0.05 (-0.10; -0.00)	0.035	0.01 (-0.03; 0.06)	0.585	0.02 (-0.02; 0.06)	0.312
	P for interaction									
Overall AD GRS, including APOE-ε4	0.996		0.512		0.292		0.526		0.387	
Overall AD GRS, excluding APOE-ε4	0.591		0.654		0.512		0.488		0.387	
Immune response GRS	0.794		0.953		0.657		0.640		0.615	

Abbreviations: SD = standard deviation, Ln = natural logarithmic transformation, AD = Alzheimer's disease, GRS = genetic risk score, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = Amyloid-beta 42, Aβ-40 = Amyloid-beta 40, Aβ-42/40 = Amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain. Model adjusted for age, sex and study cohort. GRS are per SD increase. All phenotypical markers were Ln(transformed) except for Aβ-40. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity.

Table S4. Associations between serum markers of immunity and plasma biomarkers of AD-related brain pathology with additional adjustments.

	SD increase in Aβ, total-tau or NFL, 95% CI									
Adjustments for creatinine and hs-CRP, N=1341	**Ln(Aβ-42)**	**P**	**Aβ-40**	**P**	**Ln(Aβ-42/40)**	**P**	**Ln(Tau)**	**P**	**Ln(NFL)**	**P**
Ln(GLR)*	0.003 (-0.049; 0.055)	0.922	0.045 (-0.001; 0.091)	0.054	-0.039 (-0.096; 0.019)	0.187	-0.055 (-0.106; -0.004)	0.036	-0.031 (-0.073; 0.010)	0.135
Ln(PLR)	-0.033 (-0.080; 0.014)	0.166	0.017 (-0.024; 0.058)	0.415	-0.056 (-0.106; -0.003)	0.038	-0.063 (-0.109; -0.017)	0.007	-0.010 (-0.048; 0.027)	0.583
Ln(SII)	0.002 (-0.046; 0.051)	0.923	0.040 (-0.003; 0.083)	0.070	-0.030 (-0.084; 0.024)	0.275	-0.050 (-0.099; -0.002)	0.041	-0.018 (-0.056; 0.021)	0.371

Abbreviations: SD= standard deviation, hs-CRP = high-sensitive CRP, CI = confidence interval, Ln = natural log transformation, GLR = granulocyte-to-lymphocyte ratio, SII = systemic immune inflammation index, Aß-42 = Amyloid-beta 42, Aß-40 = Amyloid-beta 40, Aß-42/40 = Amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain. Model II: Adjusted for smoking, diabetes, BMI, platelets, systolic blood pressure, apolipoprotein E4ε carriership and batch effects. *Additional adjustment for platelet count.

Table S5. Associations between serum markers of immunity and plasma biomarkers of AD-related brain pathology.

Per log increase of hs-CRP N= 1341	SD increase in Aβ, total-tau or NFL, 95% CI									
	Ln(Aβ-42)	**P**	**Aβ-40**	**P**	**Ln(Aβ-42/40)**	**P**	**Ln(Tau)**	**P**	**Ln(NFL)**	**P**
Ln(CRP)	0.02 (-0.03; 0.06)	0.481	0.06 (0.02; 0.11)	0.002	-0.03 (-0.08; 0.02)	0.226	0.07 (0.03; 0.12)	0.002	0.03 (-0.01; 0.07)	0.124

Abbreviations: SD = standard deviation, hs-CRP = high-sensitive CRP, CI = confidence interval, Ln = natural logarithmic transformation, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = amyloid-beta 42, Aβ-40 = amyloid-beta 40, Aβ-42/40 ratio = amyloid-beta 42-to-40 ratio, Tau = total-tau, NFL = neurofilament light chain. Model adjusted for age, sex, study cohort, smoking, diabetes, BMI, platelets, systolic blood pressure, *APOE*-ε4, batch effects and creatinine.D

Table S6. Associations of genetic risk scores reflecting AD including and excluding *APOE*-ε4 and immune response with plasma biomarkers of AD-related brain pathology and serum markers of immunity.

Per SD increase in genetic risk scores	SD change in Aβ, total-tau or NFL, 95% CI									
	Ln(Aβ-42)	P	Aβ-40	P	Ln(Aβ-42/40)	P	Ln(Tau)	P	Ln(NFL)	P
Overall AD GRS, including APOE-ε4	-0.13 (-0.16; -0.10)	<0.001	0.00 (-0.04; 0.03)	0.779	-0.16 (-0.19; -0.12)	<0.001	0.01 (-0.03; 0.04)	0.742	0.00 (-0.03; 0.03)	0.973
Overall AD GRS, excluding APOE-ε4	-0.04 (-0.08; -0.01)	0.007	-0.01 (-0.04; 0.02)	0.591	-0.05 (-0.08; -0.01)	0.006	-0.01 (-0.04; 0.03)	0.670	0.01 (-0.02; 0.04)	0.424
Immune response GRS	-0.02 (-0.06; 0.01)	0.138	0.01 (-0.02; 0.04)	0.542	-0.04 (-0.07; -0.01)	0.020	-0.01 (-0.04; 0.02)	0.456	0.02 (-0.01; 0.05)	0.151
Per SD increase in genetic risk scores	**SD change in GLR, PLR and SII**									
	Ln(GLR)	**P**	**Ln(PLR)**	**P**	**Ln(SII)**	**P**				
Overall AD GRS, including APOE-ε4	0.02 (0.00; 0.05)	0.032	0.01 (-0.02; 0.03)	0.579	0.02 (0.00; 0.04)	0.062				
Overall AD GRS, excluding APOE-ε4	0.00 (-0.02; 0.02)	0.921	0.00 (-0.03; 0.02)	0.715	0.00 (-0.02; 0.02)	0.936				
Immune response GRS	0.00 (-0.02; 0.03)	0.760	-0.01 (-0.03; 0.01)	0.343	0.00 (-0.02; 0.03)	0.833				

Abbreviations: SD = standard deviation, Ln = natural logarithmic transformation, AD = Alzheimer's disease, GRS = genetic risk score, GLR = granulocyte-to-lymphocyte ratio, PLR = platelet-to-lymphocyte ratio, SII = systemic immune-inflammation index, Aβ-42 = Amyloid-beta 42, Aβ-40 = Amyloid-beta 40, Aβ-42/40 = Amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain. Model adjusted for age, sex and study cohort. GRS are per SD increase. All phenotypical markers were Ln(transformed) except for Aβ-40. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity. Results are considered statistically significant if *P* below 0.05/(9×5)=0.001.

Table S7. Associations of *APOE* genotypes with plasma biomarkers of AD-related brain pathology and serum markers of immunity.

APOE genotypes	SD change in Aβ, total-tau or NFL, 95% CI									
	Ln(Aβ-42)	**P**	**Aβ-40**	**P**	**Ln(Aβ-42/40)**	**P**	**Ln(Tau)**	**P**	**Ln(NFL)**	**P**
APOE ε2/ε2 or ε2/ε3	0.01 (-0.08; 0.10)	0.845	-0.04 (-0.13; 0.05)	0.343	0.04 (-0.05; 0.13)	0.402	0.06 (-0.04; 0.15)	0.231	-0.10 (-0.17; -0.02)	0.016
APOE ε3/ε3 (reference)	0	-	0	-	0	-	0	-	0	-
APOE ε3/ε4 or ε4/ε4	-0.26 (-0.34; -0.19)	<0.001	-0.01 (-0.08; 0.06)	0.766	-0.33 (-0.41; -0.25)	<0.001	0.05 (-0.03; 0.12)	0.237	-0.03 (-0.10; 0.03)	0.315
APOE genotypes	**SD change in GLR, PLR and SII**									
	Ln(GLR)	**P**	**Ln**(PLR)	**P**	**Ln**(SII)	**P**				
APOE ε2/ε2 or ε2/ε3	-0.08 (-0.14; -0.01)	0.025	-0.07 (-0.14; 0.00)	0.037	-0.07 (-0.14; 0.00)	0.038				
APOE ε3/ε3 (reference)	0	-	0	-	0	-				
APOE ε3/ε4 or ε4/ε4	0.05 (0.00; 0.11)	0.041	0.02 (-0.03; 0.08)	0.379	0.05 (0.00; 0.10)	0.064				

Abbreviations: CI = confidence interval, SD = standard deviation, GRS = genetic risk score, AD = Alzheimer's disease, Ln = natural log transformation, GLR = granulocyte-to-lymphocyte ratio, SII = systemic immune inflammation index, Aβ-42 = Amyloid-beta 42, Aβ-40 = Amyloid-beta 40, Aβ-42/40 = Amyloid-beta 42-to-40 ratio, NFL = neurofilament light chain, *APOE* = Apolipoprotein E, e2/e2 or e2/e3 = apolipoprotein Eε2/ε2 genotype, e3/e3 = apolipoprotein Eε3/ε3 genotype, e3/e4 or e4/e4 = apolipoprotein Eε3/ε4 or ε4/ε4 genotype. Adjusted for age, sex and study cohort. All phenotypical markers were Ln(transformed) except for Aβ-40. The GLR, PLR and SII reflect the balance between innate and adaptive immunity, with higher markers indicating a dysbalance towards higher innate immunity. Results are considered statistically significant if *P* below 0.05/16=0.003.

Table S8. Descriptive statistics of high-sensitive CRP and creatinine levels within *APOE* genotypes.

APOE genotype	Mean hs-CRP level	Mean creatinine level
ε2/ε2 or ε2/ε3 (N=203)	3.5	82.1
ε3/ε3 (N=799)	3.4	84.3
ε3/ε4 or ε4/ε4 (N=304)	2.3	84.3

Abbreviations: hs-CRP = high-sensitive CRP, *APOE* = Apolipoprotein E, e2/e2 or e2/e3 = apolipoprotein Eε2/ε2 genotype, e3/e3 = apolipoprotein Eε3/ε3 genotype, e3/e4 or e4/e4 = apolipoprotein Eε3/ε4 or ε4/ε4 genotype.

4.3 Telomere length and the risk of dementia

ABSTRACT

There is a wide interest in biomarkers that capture the burden of detrimental factors as these accumulate with the passage of time, i.e., increasing age. Telomere length has received considerable attention as such a marker, because it is easily quantified and it may aid in disentangling the aetiology of dementia or serve as predictive marker. We determined the association of telomere length with risk of Alzheimer disease and all-cause dementia in a population-based setting. Within the Rotterdam Study, we performed quantitative PCR to measure mean leukocyte telomere length in blood. We determined the association of telomere length with risk of Alzheimer's disease until 2016, using Cox regression models. Of 1,961 participants (mean age 71.4±9.3 years, 57.1% women) with a median follow-up of 8.3 years, 237 individuals were diagnosed with Alzheimer's. We found a U-shaped association between telomere length and risk of Alzheimer's: compared to the middle tertile the adjusted hazard ratio was 1.59 (95% confidence interval (CI), 1.13-2.23) for the lowest tertile and 1.47 (1.03-2.10) for the highest tertile. Results were similarly U-shaped but slightly attenuated for all-cause dementia. In conclusion, shorter and longer telomere length are both associated with an increased risk of Alzheimer's disease in the general population.

Keywords: Dementia, Alzheimer disease, telomere, population-based, prospective cohort study

INTRODUCTION

Dementia is a devastating disease affecting millions of people worldwide. While advances over the past decades have unravelled several pathological mechanisms underlying dementia, chronological age remains the most important predictor for dementia onset (Collaborators, 2018). There is, therefore, a wide interest in biomarkers that capture the burden of detrimental factors as these accumulate with the passage of time, i.e., increasing age. Such markers, that are preferably easily quantified, may aid in disentangling the aetiology of dementia or ultimately serve as predictive markers. Against this background, telomere length has received considerable attention (Blackburn *et al.*, 2015).

Telomeres are repetitive base pair sequences at the end of chromosomes and facilitate complete chromosome replication (Martinez and Blasco, 2015). Since the replication machinery is unable to copy the ends of DNA, telomeres will shorten with each cell division. A change in telomere length resulting in shortened telomeres induces a DNA damage response that leads to a growth arrest during which cells attempt to repair the damage and if DNA damage is irreparable, replicative senescence or cell death is triggered (Martinez and Blasco, 2018). Very short telomeres have relatively low cancer risk (due to cell senescence blocking cell division), but could be associated with increased DNA damage (Min *et al.*, 2017). Given the tight link with cell death, short telomere length has been linked to poorer survival (Fasching, 2018; Wang *et al.*, 2018) and other age-related diseases, including dementia as has been shown in a recent meta-analysis (Forero *et al.*, 2016). Conversely, there is evidence that over-elongated telomeres represent pathological cell function with decreased DNA repair and increased cancer risk (Zhang *et al.*, 2017), which has also been shown in human embryonic stem cells (Rivera *et al.*, 2017), while "normally" long telomeres are protective, including against DNA damage. With respect to Alzheimer's disease, there is also evidence suggesting an increased risk of mild cognitive impairment related to both short and long telomeres (Roberts *et al.*, 2014). Additionally, hippocampal cells of Alzheimer's disease brains are shown to have longer telomeres compared to control samples (Thomas *et al.*, 2008). It is, thus, conceivable that not only shorter but also longer telomere length may be detrimental for dementia risk. However, the temporal relation between longer telomere length and dementia has so far not been investigated. Therefore, the aim of this study is to investigate the association between blood cell telomere length and risk of Alzheimer's disease and to explore non-linearity within this association.

METHODS

Design and study population

This study was embedded within the Rotterdam Study, a prospective population-based cohort study among middle-aged and elderly individuals in the Netherlands. Three cohorts were established in 1990, 2000 and 2006, respectively. From each cohort, participants were invited every 4-5 years to undergo follow-up examinations. We refer the reader to the Rotterdam Study methods paper for further information (Ikram *et al.*, 2017). For this study, a total of 2,140 participants were randomly selected from the first (1990-2004, N=7983) and third cohorts (2006-2008, N=3932) for telomere length measurement. From these participants, 1,961 were dementia-free at baseline visit and had complete telomere length data available at baseline (**Figure 1**).

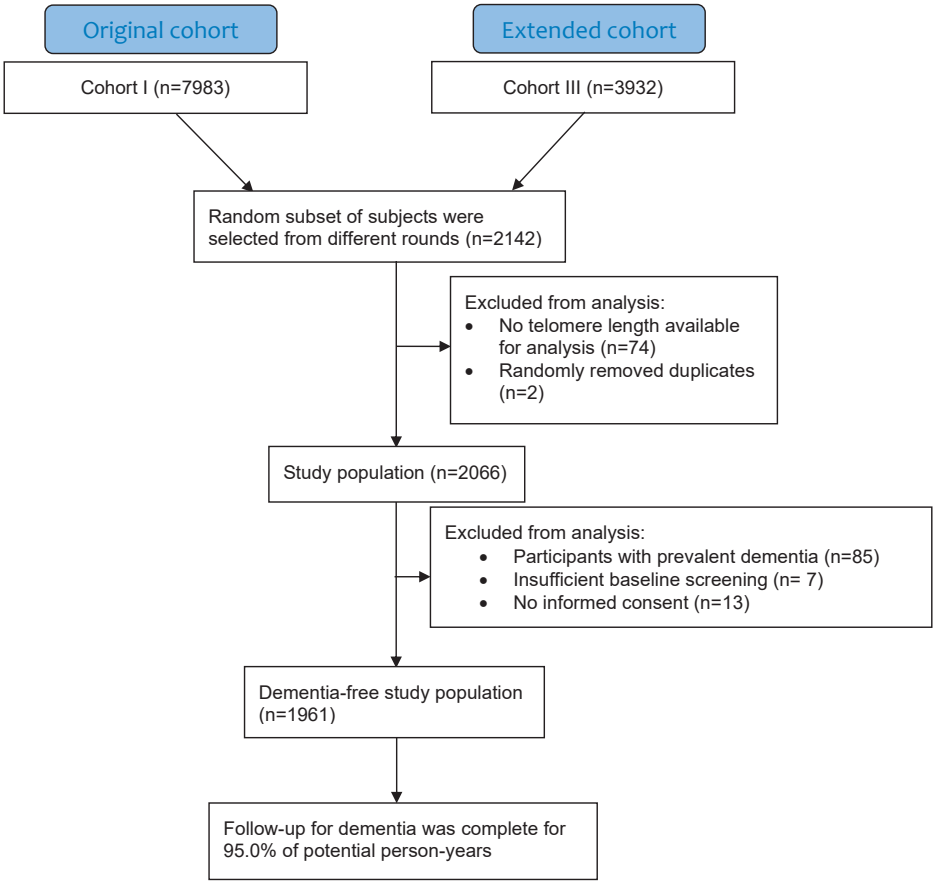

Figure 1. Flow-chart of T/S ratio study population for dementia.

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study). All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

Assessment of telomere length

Blood for all samples was collected in EDTA tubes and stored at -20°C until DNA isolation. DNA isolation was done with a method based on salting out, with different lysis buffers for the different cohorts. The OD260/280 and OD260/230 for both isolation methods did not differ significantly between the two cohorts. The average OD260/280 ratio was 1.90 with a standard deviation of 0.10 and the average OD260/230 ratio was 1.61 with a standard deviation of 0.46 in a random sample of 168.Telomere length was measured using a qPCR assay based on the method described by Cawthon (Cawthon, 2002) with minor modifications. The SDS software of the 7900HT qPCR machine is not able to analyse the qPCR data produced at two different temperature points. Therefore we chose the singleplex assay for this study. For each sample the telomere and 36B4 assay were run in separate wells but in the same 384 wells PCR plate. Each reaction contained 5 ng DNA, 1 uM of each of the telomere primers (tel1b-forward: GGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT, tel2b-reverse: GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT) or 250 nM of the 34B4 primers (36B4u-forward: CAGCAAGTGGGAAGGTGTAATCC, 36B4d-reverse: CCCATTCTATCATCAACGGGTACAA) and 1x Quantifast SYBR green PCR Mastermix (Qiagen). The reactions for both assays were performed in duplicate for each sample in a 7900HT machine (Applied Biosystems). Ct values and PCR efficiencies were calculated per plate using the MINER algorithm (Codd *et al.*, 2010). Duplicate Ct values that had a Coefficient of Variance (CV) of more than 1% were excluded from further analysis. Using the average Ct value per sample and the average PCR efficiency per plate the samples were quantified using the formula Q=1/(1+PCR eff)^Ct. The relative telomere length was calculated by dividing the Q of the telomere assay by the Q of the 34B4 assay. To validate the assay 96 random samples were run twice and the CV of that experiment was 4.5%.Telomere length was measured in 37 batches.

Ascertainment of incident dementia

Baseline and follow-up ascertainment methods for dementia have previously been described in detail (de Bruijn *et al.*, 2015). In brief, participants were screened for dementia at baseline and subsequent center visits. In addition, the entire cohort was continuously under surveillance for dementia through electronic linkage of the study database with medical records. A consensus panel led by a consultant neurologist established the final diagnosis according to standard criteria for dementia (DSM-III-R) and Alzheimer's disease (NINCDS–ADRDA).

Within this period, participants were censored at date of dementia diagnosis, death, loss to follow-up, or 1st of January 2016, whichever came first.

APOE genotyping

APOE genotype was determined using polymerase chain reaction on coded DNA samples in the baseline cohort (Slooter *et al.*, 1998) and with a bi-allelic Tacqman assay (rs7412 and rs429358) in the extensions of the Rotterdam Study (Ikram *et al.*, 2017). *APOE*-ε4 carrier status was defined carriers versus non-carriers of the ε4 allele.

Other covariates

Potential confounding factors were chosen on the basis of previous literature (de Bruijn *et al.*, 2015; Nilsonne *et al.*, 2015). All covariates were measured at baseline. Educational attainment was categorized as low (primary education or lower vocational education), intermediate (secondary education or intermediate vocational education), and high educational level (higher vocational education or university). Smoking habits were categorized as current, former and never smoking. BMI was calculated as weight in kilograms per height in meters squared. Blood pressure was measured twice at the right brachial artery with the participant in sitting position, out of which the mean was used. Hypertension was defined as a resting blood pressure exceeding 140/90 mmHg or the use of blood pressure lowering medication. Hypercholesterolemia was defined as a total cholesterol of ≥6.2 mmol/L or the use of serum lipid-reducing agents. White blood cell counts were assessed with a Coulter AcT diff2 Hematology Analyzer at the research centre in Ommoord. At 14 February 2005 a new device of the same brand and type was installed. Prevalent stroke was assessed at baseline during interview and we verified these data with medical records. After study entry, we continuously monitored participants after enrollment for incident stroke through linkage of the study database with files from general practitioners. Files of nursing home physician's and files from general practitioners of participants who moved out of the district were also checked. We obtained additional information from hospital records. When potential strokes were found, they were reviewed by research physicians and verified by an experienced neurologist.

Statistical analysis

Missing covariate data (maximum 2.2%) were imputed using 5-fold multiple imputation based on determinant, outcome, and included covariates. In all models, we adjusted for age continuously, sex and center visit since data was randomly selected from 4 center visits from 2 subcohorts. We additionally adjusted for educational attainment, *APOE*-ε4 carrier status, smoking, body mass index (BMI), hypertension, hypercholesterolemia and white blood cell count in a second model, all at time of blood draw. We first explored linearity of telomere length with Alzheimer's disease and dementia risk by entering T/S ratio as restricted cubic spline with 3 knots in Cox regression models. In order to further quantify potential

non-linearity, T/S ratio was subsequently divided into tertiles, defined as T/S ratio of 0.31-0.87, 0.87-1.02 and 1.02-1.79, respectively. To provide a parsimonious description of the telomere-dementia relationship and facilitate communication of the results, we assessed the association between telomere length in tertiles, comparing lowest and highest tertiles that represent the shortest and longest telomere lengths, respectively, to the middle tertile, with risk of Alzheimer's disease and dementia. We repeated the analysis for Alzheimer's disease, by adjusting not only for age at blood draw, but also for age at dementia diagnosis or date of last follow-up as an attempt to assess survival bias. We further verified that age was robustly controlled for by comparing results across models using five different ways of age-adjustment. These included no adjustment for age, entering age and age^2 in the model, entering age as cubic splines, repeating the analyses with age rather than follow-up time as the time scale and finally regressing the effect of age out of T/S ratio before creating T/S ratio tertiles. We performed an additional adjustment for plate number to rule out batch effects. The proportional hazards assumption was assessed using Schoenfeld residuals. We next repeated the analyses for AD, but excluding all participants with prior clinical stroke at baseline and censoring for incident clinical stroke during follow-up using Cox models.

We also assessed interaction by age, sex, education or *APOE*-ε4 carrier status by stratification and testing for multiplicative interaction by including the product of the interacting factor and T/S ratio continuously as a restricted cubic spline (4 knots) to take into account the non-linear relationship between T/S ratio and Alzheimer's disease. We stratified for age by 3 groups, namely 45-65 years, 65-85 years and 85-105 years.

All analyses were performed using RStudio version 0.99.903 (R version 3.4.3(Team, 2017), RStudio, Inc., Boston, MA). We used the following R packages for our analyses: mice, survival, rms and scales.

RESULTS

Baseline characteristics of the study population are presented in **Table 1**. The mean age of the participants was 71.4±9.3 years with 57.1% women. During a median follow-up of 7.3 (interquartile range 4.9-11.7) years (26,025 person-years), 305 individuals were diagnosed with dementia of whom 237 were Alzheimer's disease.

Table 1. Baseline characteristics of the dementia-free study population.

Characteristics	**Dementia-free cohort** (N = 1961)
Women	1120 (57.1%)
Age, years	71.4 ±9.3
Cohort	
First	1543 (78.7%)
Third	418 (21.3%)
Education	
Primary education	366 (18.9%)
Lower/intermediate general education	806 (41.5%)
Intermediate vocational education	535 (27.6%)
Higher vocational education	233 (12.0%)
Apolipoprotein E4ε carriership	
Non-carrier (ε2/ε2 , ε2/ε3 or ε3/ε3)	1411 (72.0%)
Carrier (ε4/ε4, ε3/ε4 or ε2/ε4)	550 (28.0%)
Smoking	
Current	405 (21.1%)
Former	921 (48.0%)
Never	592 (30.9%)
BMI, kg/m^2	27.2 ±4.1
Hypertension	700 (36.5%)
Hypercholesterolemia	949 (48.8%)
White blood cell count	6.9 ±2.3
Telomere length, T/S ratio	0.95 ±0.18

Abbreviations: N = number of participants included in study. Data presented as mean (standard deviation) for continuous variables and number (percentages) for categorical variables. T/S ratio is relative telomere to single copy gene ratio. Data represent original data without imputed values. Number of missing values are 43 (2.2%) for smoking, 21 (1.1%) for education, 27 (1.4%) for hypertension, 17 (0.9%) for hypercholesterolemia, 29 (1.5%) for body mass index and 157 (8.0%) for white blood cell count. Data for apolipoprotein E4ε carriership was complete.

There was evidence of non-linearity in the association between telomere length and Alzheimer's disease; **Figure S1**. Compared to the middle tertile of telomere length, we found an adjusted hazard ratio (aHR) of 1.59; 95% confidence interval (CI), 1.13-2.23 for lowest tertile of telomere length and 1.47; 95%CI, 1.03-2.10 for highest tertile of telomere length for Alzheimer's disease, with similar but lower estimates for dementia (**Table 2)**. When additionally adjusting for age at dementia diagnosis or date of last follow-up, we found an aHR of 1.52; 95%CI, 1.07-2.18 for lowest tertile of telomere length and 1.75; 95%CI, 1.21-2.54 for highest tertile of telomere length for Alzheimer's disease, compared to the middle tertile. Other approaches of age-adjustment yielded similar results (**Table 3**). There were no batch effects. The proportional hazards assumption was met for all models. Results were slightly attenuated but remained U-shaped after excluding participants with prior clinical stroke at baseline and censoring for incident clinical stroke: compared to middle tertile aHR: 1.44, 1.02-2.05 for lowest tertile of telomere length and aHR 1.30, 95% CI 0.90-1.89 for highest tertile of telomere length.

Table 2. Telomere length and the risk of Alzheimer's disease and all-cause dementia.

	n/N	Alzheimer's disease		n/N	All-Cause dementia	
		Model I HR, 95% CI	Model II HR, 95% CI		Model I HR, 95% CI	Model II HR, 95% CI
Telomere length						
Tertile 1 (T/S ratio 0.31-0.87)	106/654	1.54, 1.10 – 2.15	1.59, 1.13 – 2.23	129/654	1.25, 0.94 – 1.66	1.27, 0.96 – 1.70
Tertile 2 (T/S ratio 0.87-1.02)	54/654	1 (reference)	1 (reference)	80/654	1 (reference)	1 (reference)
Tertile 3 (T/S ratio 1.02-1.79)	77/653	1.41, 0.99 – 2.00	1.47, 1.03 – 2.10	96/653	1.19, 0.88 – 1.60	1.25, 0.92 – 1.69

Abbreviations: n = number of cases; N = number of persons at risk; HR = hazard ratio; CI = confidence interval; T/S ratio = relative telomere to single copy gene ratio. Cox regression model I: Adjusted for age, sex and visit. Cox regression model II: Adjusted for age, sex, visit, education, *APOE*-ε4 carrier status, smoking, BMI, hypertension, hypercholesterolemia and white blood cell count.

In the stratified analyses, the association between shorter and longer telomeres and Alzheimer's disease was stronger in *APOE*-ε4 carriers compared to non-carriers (**Figure 2**) with the multiplicative interaction term yielding p=0.087. Stratification for age, sex and education did not provide different risk estimates (P-values for interaction: p = 0.554, p = 0.600 and p = 0.123, respectively).

Table 3. Association between telomere length and Alzheimer's disease using different modes of age adjustment.

Adjustment	**Alzheimer's disease**					
		No age adjustment	**Age + age^2**	**Cubic splines for age†**	**Age as time-scale**	**Regressing out the effect of age of T/S ratio***
	n/N	HR, 95% CI	HR, 95% CI	HR, 95% CI	HR, 95% CI	HR, 95% CI
Telomere length						
Tertile 1 (shortest)	106/654	2.08, 1.49-2.91	1.64, 1.17-2.30	1.64, 1.17-2.30	1.63, 1.16-2.29	1.33, 0.96-1.86
Tertile 2 (middle)	54/654	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Tertile 3 (longest)	77/653	1.25, 0.88-1.77	1.45, 1.01-2.06	1.45, 1.02-2.06	1.44, 1.01-2.06	1.22, 0.87-1.69

Abbreviations: n = number of cases; N = number of persons at risk; HR = hazard ratio; CI = confidence interval; T/S ratio = relative telomere to single copy gene ratio. †Cubic splines with 3 knots, similar results for higher degree polynomials.

Models adjusted for age, sex, visit, education, *APOE*-ε4 carrier status, smoking, BMI, hypertension, hypercholesterolemia and white blood cell count.*Regressing the effect of age out of T/S ratio before creating T/S ratio tertiles; no additional adjustment for age in the model; similar results for additionally regressing out the effect of sex. *Regressing the effect of age out of T/S ratio before creating T/S ratio tertiles; similar results for additionally regressing out the effect of sex.

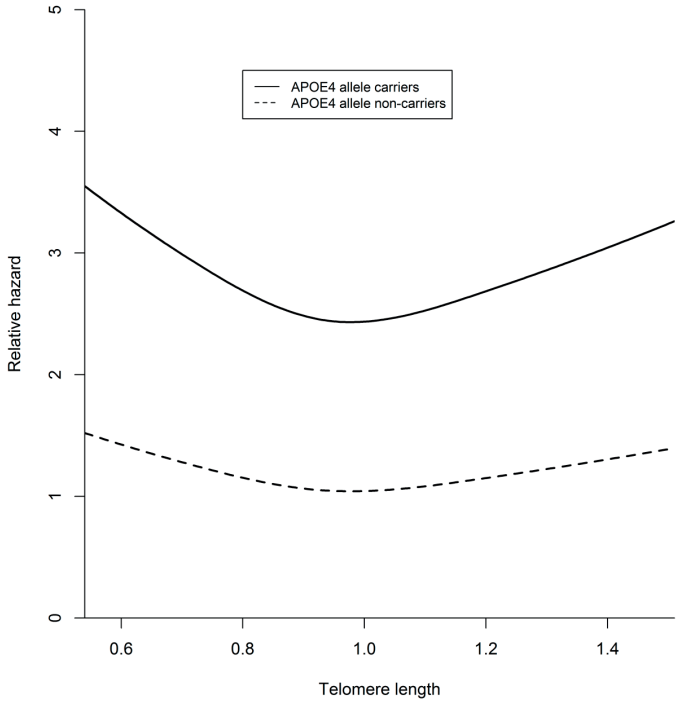

Figure 2. The association between telomere length and the risk of Alzheimer's disease stratified for APOE4 allele carriers and non-carriers.
A visual representation of telomere length and the risk of Alzheimer's disease (AD) stratified by APOEε4 carriership with restricted cubic splines in Cox model II, adjusted for age, sex, study visit, education, APOEε4 carrier status, smoking, BMI, hypertension, hypercholesterolemia and white blood cell count. Number of AD cases in APOEε4 carrier group: 102 with 550 persons at risk; number of cases in the APOEε4 non-carrier group: 135 with 1411 persons at risk.

DISCUSSION

In this study, shorter as well as longer telomere length were significantly associated with increased risk of dementia, specifically Alzheimer's disease.

Thus far, focus in the dementia field has been on shorter telomere length with studies finding observationally as well as genetically associations between shorter telomere length and increased risk of AD (Honig *et al.*, 2012; Zhan *et al.*, 2015; Guo and Yu, 2019; Scheller Madrid *et al.*, 2019). Moreover, a recent meta-analysis which included 13 primary studies demonstrated significantly shorter telomere length in 860 AD patients compared to 2,022 controls (Forero *et al.*, 2016). Interestingly, we found that in addition to shorter telomere length also longer telomere length was associated with an increased dementia risk. This

finding is in line with previous studies (Wikgren *et al.*, 2012a; Wikgren *et al.*, 2012b), particularly the study by Roberts and others (Roberts *et al.*, 2014) showing that short and long telomeres increase risk of amnestic mild cognitive impairment. Yet, we emphasize that further replication is necessary to confirm the robustness of our findings with dementia.

An explanation for our observations with longer telomere length might be survival bias, where presumably participants with the longest telomere length live long enough to develop dementia. This is however less likely since associations remained after additional adjustment for age at dementia diagnosis or end of follow-up. Alternatively, it is plausible that a disturbed equilibrium of telomere length towards either direction is harmful. Indeed, over-elongated telomeres become fragile and accumulate DNA damage, as has been shown by an experimental study in human embryonic stem cells, and very tight control of telomere length homeostasis is regulated by not only telomerase-dependent elongation, but also by telomere trimming events (Rivera *et al.*, 2017). Another study has shown that telomerase RNA component (TERC) knockout mice with AD, which present with telomere shortening, slows down the progression of Aβ pathology, showing a more protective effect of short telomere length (Rolyan *et al.*, 2011), thereby supporting a detrimental effect of longer telomere length. The potential biological link between over-elongated telomeres and higher AD risk remains to be further elucidated. In contrast with longer telomere length, more is known about the potential biological link between shorter telomeres and increased AD risk. Neurons lacking telomerase reverse transcriptase (TERT) in knockout mice and thus have shorter telomeres, display an increased production of oxidative species and an increase in cellular oxidative damage in response to tau, demonstrating a harmful effect of shorter telomeres (Spilsbury *et al.*, 2015). Therefore, telomerase may play different roles in the tau and amyloid pathology via multiple mechanisms (Liu *et al.*, 2018). In addition, microglial cellular senescence may also be an important mechanism in the development of AD, which is suggested to be exacerbated by the presence of amyloid (Flanary, 2005; Flanary *et al.*, 2007). Further studies are warranted to differentiate between a true biological association and survival bias. If a true biological association exists between longer telomere length and risk of dementia, telomerase therapies, which have been successful in mice (Jaskelioff *et al.*, 2011; Bernardes de Jesus *et al.*, 2012), would need further fine-tuning to prevent over-elongation of telomere length.

Interestingly, we found stronger effects for Alzheimer's disease compared to all-cause dementia. This might be reflective of the fact that Alzheimer's disease is a more homogeneous entity than all-cause dementia, which includes a plethora of etiologies.

Certain limitations must be taken into account. First, we measured telomere length in leukocytes in blood. This may not be representative of telomere length in the brain, in particular glial cells. However, a previous study has shown a direct correlation between telomeres measured from peripheral blood and cerebellum (Lukens *et al.*, 2009), suggesting blood cell telomere length to be a valid measurement. Another limitation of measuring telo-

mere length in leukocytes is that lymphocytes are the only peripheral blood cells expressing telomerase activity and therefore are able to maintain telomere length despite proliferation, while granulocytes lack the ability for further cell division (Akbar and Vukmanovic-Stejic, 2007). Therefore, it is possible that the older individuals without dementia have a greater proportion of lymphocytes (fewer granulocytes) (van der Willik *et al.*, 2019), and thus longer telomere length. Unfortunately, we were not able to adjust for the percentage of lymphocytes to overcome this issue, since this data was not available for all center visits. Another issue is that leukocytes divide peripherally in response to various stresses (e.g., infection) and the telomere length may therefore change unexpectedly. Also, research centers may vary in reliability in measuring leukocyte telomere length. Second, we measure mean telomere length, while cell senescence is suggested to be related to and modulated by the shortest telomere length per cell (Hemann *et al.*, 2001). Third, the external validity of the results may be limited due to the single centre nature of this study. Finally, despite using different approaches yielding similar results, the effect of age may not have been taken into account entirely. Nevertheless, given the link of older age with shorter telomere length and older age with Alzheimer's disease, controlling for age would if anything strengthen the association between longer telomere length and risk of Alzheimer's disease.

In conclusion, shorter and longer telomere length are associated with an increased risk of Alzheimer's disease in a sample of elders in The Netherlands. Further studies are warranted to confirm our findings, in particular studies with imaging or fluid biomarkers available for dementia diagnosis, and to unravel any underlying biological pathway from possible methodological bias.

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SUPPORTING INFORMATION

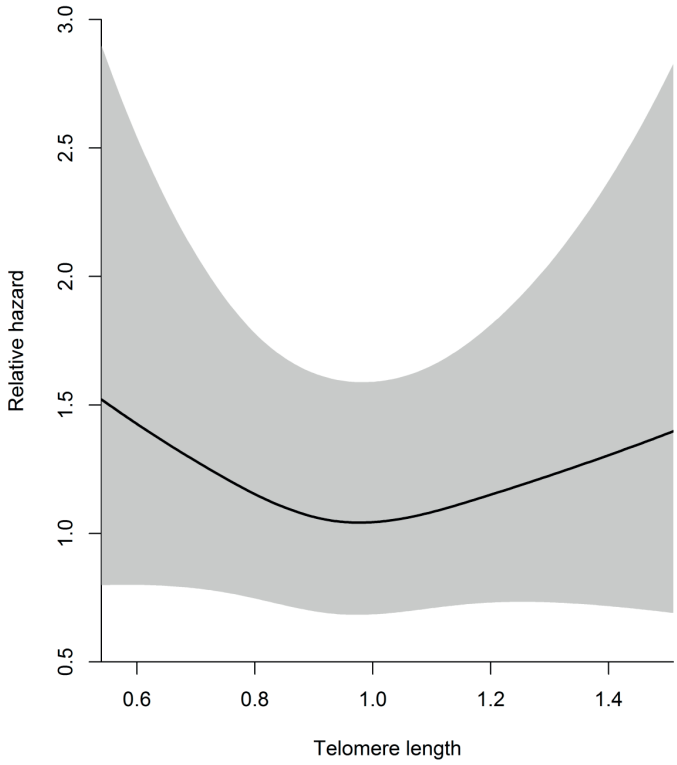

Figure S1. The association between telomere length and the risk of Alzheimer's disease.
A visual representation of telomere length and the risk of Alzheimer's disease (AD) with restricted cubic splines in Cox model II, adjusted for age, sex, study visit, education, APOEε4 carrier status, smoking, BMI, hypertension, hypercholesterolemia and white blood cell count. The U-shaped association between telomere length and Alzheimer's disease risk is clearly visible, showing elevated risk for both shorter and longer telomere length. The black line indicates the hazard ratio and gray area indicates the 95% confidence interval.

Chapter 5
Brain hemodynamics

5.1 Global brain perfusion and the risk of transient ischemic attack and ischemic stroke

ABSTRACT

Background – The role of subtle disturbances of brain perfusion in the risk of TIA or ischemic stroke remains unknown. We examined the association between global brain perfusion and risk of TIA and ischemic stroke in the general population.

Methods and Results - Between 2005-2015, 5289 stroke-free participants (mean age 64.3 years, 55.6% women) from the Rotterdam Study underwent phase-contrast brain magnetic resonance imaging at baseline to assess global brain perfusion. These participants were followed for incident TIA or ischemic stroke until January 1st, 2016. We investigated associations between global brain perfusion (mL blood flow/100mL brain/minute) and risk of TIA and ischemic stroke using Cox regression models with adjustment for age, sex, and cardiovascular risk factors. Additionally, we investigated whether associations were modified by retinal vessel calibers, small and large vessel disease, blood pressure and heart rate. During a median follow-up of 7.2 years (36,103 person-years), 137 participants suffered a TIA and another 108 an ischemic stroke. We found that lower global brain perfusion was associated with a higher risk of TIA but not with the risk of ischemic stroke (adjusted hazard ratio (aHR), 95% confidence interval (CI), per standard deviation decrease of global brain perfusion: 1.29, 1.07–1.55 for TIA and aHR of 1.06, 0.87–1.30 for ischemic stroke). Across strata of wider arteriolar retinal calibers, lower brain perfusion was more prominently associated with TIA, but not with ischemic stroke.

Conclusions - In a community-dwelling population, impaired global brain perfusion increased the risk of TIA but not of ischemic stroke.

INTRODUCTION

Stroke is caused by insufficient perfusion to specific regions of the brain, leading to neuronal dysfunction and ultimately cell death[1]. If the disruption is of short duration, it may instead lead to a transient ischemic attack (TIA) [2]. Maintenance of sufficient perfusion of the brain is achieved primarily by arterioles, which either dilate or contract[3]. In the presence of cardiovascular risk factors and arteriolosclerosis this regulating ability of arterioles is affected and can lead to disruptions in brain perfusion. In this light, cerebral hypoperfusion has been suggested as a potential link between vascular damage and TIA and stroke and is a potential target for preventive interventions [4, 5]. However, the temporal relationship between cerebral hypoperfusion and the risk of TIA or stroke remains undefined. Global brain perfusion is a hemodynamic factor, which reflects more subtle and chronic changes in brain perfusion[6]. Until recently, studies assessing global brain perfusion have only been performed in people with previous stroke or in patients with asymptomatic or symptomatic high-grade carotid stenosis, in which lower global brain perfusion increased the risk of stroke[7]. Yet, it is unknown whether in the absence of high-grade carotid stenosis global brain perfusion is related to the occurrence of TIA and stroke. Moreover, it is unclear whether any impact of poor global brain perfusion on stroke risk is augmented by additional pathology along the brain vasculature, either as large vessel or small vessel disease. Identifying individuals who are at increased hemodynamic risk of stroke can be of major importance for preventive purposes. Against this background, we investigated the association of global brain perfusion at baseline with the risk of TIA and ischemic stroke in a large sample of community-dwelling elderly with an average follow-up of 7 years. We hypothesized that lower global brain perfusion is a risk factor for incident TIA and ischemic stroke and that this association is modified by markers of small and large vessel disease.

METHODS

Study population

This study is embedded within the Rotterdam Study, a large population-based cohort study in the Netherlands that started in 1990. The study is aimed at investigating determinants of various chronic diseases among elderly people[8]. The original study population consisted of all residents of Ommoord, a district in Rotterdam, aged 55 years and older. In 2000, the cohort was expanded with another 3,011 participants with the same inclusion criteria and in 2006, a further expansion of the cohort was initiated in which 3,932 participants were included, aged 45-54 years. Follow-up examinations at the research center are performed every 3 to 5 years. From 2005 onwards, brain MRI was implemented in the core study protocol and all participants were invited to undergo brain MRI[9].

The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/network/primary/en/) under shared catalogue number NTR6831. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians. Requests to access the dataset from qualified researchers trained in human subject confidentiality protocols may be sent to Dept. of Epidemiology, Erasmus MC University Medical Center at f.vanrooij@erasmusmc.nl.

Population for analysis

Of 5,644 persons undergoing a first MRI scan, no reliable measure of cerebral blood flow could be obtained in 5 (0.09%) persons. After excluding participants with prevalent TIA or stroke (n = 320) and 30 participants with missing follow-up (n = 30) data, 5,289 participants were included for analysis. Follow-up for TIA and stroke was complete for 93.7% of potential person-years (Figure 1).

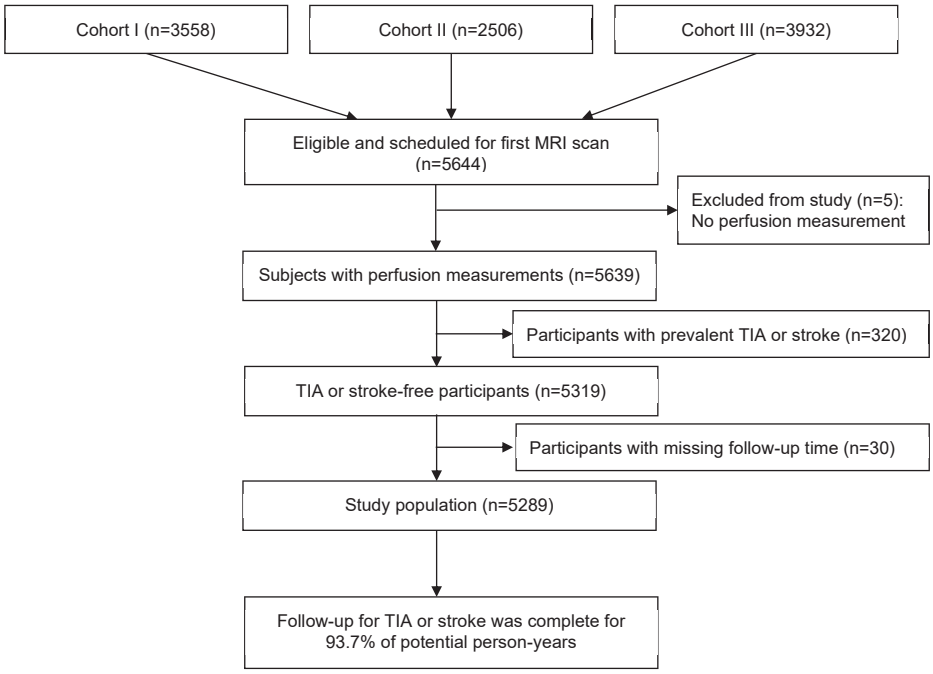

Figure 1. Flow-chart of study population.

Assessment of global brain perfusion

All participants were scanned at baseline on a 1.5-Tesla MRI scanner (General Electric Healthcare, Milwaukee, WI, USA) with a dedicated eight-channel head coil. The full MRI protocol has been described in detail before[9]. Specifically, for the flow measurements, we performed a 2D phase-contrast sequence[10, 11]. A 2D thick slab projection phase contrast angiographic localizer (60 mm thick, velocity encoding (VENC) = 60 cm/sec) is positioned sagittally in order to determine the location of the carotid and basilar arteries. Next, a thin slice perpendicular to all three vessels at the level of the precavernous internal carotid artery is positioned (VENC = 120 cm/s, slice thickness 5 mm, NEX = 8). From the resulting images, we calculated cerebral blood flow using interactive data language-based custom software (Cinetool version 4, General Electric Healthcare, Milwaukee, WI, USA). Two independent, experienced technicians drew circular to elliptical regions of interest (ROIs) manually around both carotids and the basilar artery at the level of the clinoid segment on the phase-contrast images, encompassing the entire lumen of the vessel (inter-rater correlations > 0.94 for all vessels)[12]. For improved visualization of vessel boundaries, the contrast between the arteries of interest and the background were inverted. In each ROI, the value of mean signal intensity reflected the flow velocity in the vessel (cm/sec). By multiplying the average velocity with the cross-sectional area of the vessel, flow (in mL/sec) was calculated. To obtain global cerebral blood flow (tCBF) in mL/min, flow rates for the carotid arteries and the basilar artery were summed and multiplied by 60 secs/min. We calculated global brain perfusion (in mL/min blood per 100 mL brain) by dividing tCBF (mL/min) by each individual's brain volume (mL) and multiplying the obtained result by 100 mL brain volume. Brain volume was calculated by adding up gray and white matter volumes, converted to millilitres.

Assessment of retinal vessel calibers

Retinal microvascular calibers are considered markers of small vascular pathology, but also markers of vascular autoregulation[13, 14], which we therefore stratified for. We assessed retinal microvascular calibers (arteriolar and venular calibers (in µm)) using fundus photographs and the Retinal Vessel Measurement System (Retinal Analysis, Optimate, WI; Department of Ophthalmology and Visual Science, University of Wisconsin-Madison)[15] as previously described[16, 17]. Retinal microvascular calibers were assessed in a subset of 3219 individuals.

Assessment of small vessel and large vessel disease

For the lacunar infarcts and WMHs measurements on MRI scans, gray matter volume (GM), white matter volume (WM) and cerebrospinal fluid were quantified using an automated tissue segmentation method, based on a k-nearest-neighbour brain tissue classifier algorithm[18]. This was extended with a custom-developed WMH (white matter hyperintensity) segmentation[19]. Segmentation results were visually inspected and if needed corrected manually. All scans were furthermore rated by trained research physicians, blinded to clinical data, for the

presence of lacunar infarcts (defined as focal lesions ≥3 and <15 mm in size with the same signal characteristics as cerebrospinal fluid on all sequences, and, if located supratentorially, with a hyperintense rim on the fluid attenuated inversion recovery sequence).

Markers of large vessel disease (intima-media thickness and carotid plaques) were measured as follows: Doppler ultrasound was used to measure carotid stenosis (≥50% stenosis). To measure carotid intima-media thickness and carotid plaques, ultrasonography of the common carotid artery, carotid bifurcation, and left and right internal carotid artery was performed with a 7.5-MHz linear-array transducer (ATL Ultra-Mark IV)[20].

Stroke and TIA assessment

The definition of stroke was based on the WHO criteria[21], describing a syndrome of rapidly developing symptoms of focal or global cerebral dysfunction lasting 24 hours or longer or leading to death, with apparent vascular cause. Prevalent stroke was assessed at baseline during interview and we verified these data with medical records. After study entry, we continuously monitored participants after enrollment for incident stroke through linkage of the study database with files from general practitioners. Files of nursing home physician's and files from general practitioners of participants who moved out of the district were also checked. We obtained additional information from hospital records. When potential strokes were found, they were reviewed by research physicians and verified by an experienced neurologist. We then categorized strokes into ischemic or haemorrhagic on the basis of neuro-imaging reports. If neuroimaging was missing and we could not differentiate the type of stroke according to the symptoms, the stroke was classified as unspecified. Subarachnoid haemorrhages due to ruptured aneurysms were not considered stroke events. For this study, we were mainly interested in ischemic stroke as outcome. Follow-up for incident stroke was complete until January 1st 2016.

A similar work-up was performed to determine prevalent and incident TIA[22]. *Transient neurological attacks (TNA)* were defined as attacks of sudden neurological symptoms that completely resolved within 24 hours, with no clear evidence for the diagnosis of migraine, epilepsy, Meniere disease, hyperventilation, cardiac syncope, hypoglycemia, or orthostatic hypotension[23]. If only focal brain symptoms (hemiparesis, hemihypesthesia, dysphasia, dysarthria, amaurosis fugax, hemianopia, hemiataxia, diplopia, or vertigo) were reported, the event was classified as a *focal TNA*. If only nonfocal brain symptoms (decreased consciousness, unconsciousness, confusion, amnesia, unsteadiness, nonrotatory dizziness, positive visual phenomena, cardiac or vegetative signs, paresthesias, bilateral weakness, and unwell feelings) were reported, the event was classified as *nonfocal TNA*. If both focal and nonfocal symptoms were reported for the same attack, a *mixed TNA* was diagnosed. For this study, we only used the focal and mixed TNAs as outcome for TIA.

Other measurements in the Rotterdam Study

Information on cardiovascular risk factors was obtained by interview, physical examination and blood sampling[8]. We assessed history of smoking (current, former, never), and use of antihypertensive, lipid-lowering and anti-thrombotic medication at baseline by interview. Body mass index was calculated as weight (kg)/length (m^2). Diastolic and systolic blood pressures were measured twice on the right arm with a random-zero sphygmomanometer; for analyses the mean of these readings was used. Heart rate was measured during sitting blood pressure measurement. Total cholesterol and high-density lipoprotein (HDL) cholesterol were measured by an automated enzymatic procedure. Diabetes mellitus type 2 was defined as the use of blood glucose-lowering medication at baseline or having a fasting serum glucose level of ≥126 mg/dL or ≥7.0 mmol/l[24].

Statistical analysis

First, we investigated the association of global brain perfusion (per standard deviation decrease, and in tertiles) with the risk of TIA and ischemic stroke separately and combined (whichever came first) using Cox proportional hazards models. In model 1, we adjusted for age, sex and cohort. In model 2 we additionally adjusted for smoking status, systolic and diastolic blood pressure, blood pressure- and lipid lowering-medication, antithrombotic medication use, diabetes mellitus, BMI, carotid stenosis, total cholesterol, HDL and MRI-defined lacunar infarcts. We censored for incident TIAs during follow-up in the analyses on ischemic stroke and for incident stroke in the analyses on TIA. We repeated the same analyses while not censoring for incident TIAs during follow-up in the analyses on stroke to compare what the effect is of brain perfusion when having a TIA before a stroke. For all Cox models, we tested the proportional hazard assumption using Schoenfeld residuals.

Second, we investigated potential effect modification by retinal vessel calibers, small and large vessel disease and finally blood pressure and heart rate on the association of brain perfusion with TIA and ischemic stroke. As markers of small vessel disease, we considered lacunar infarcts and WMH. While lacunar infarcts were dichotomized into yes versus no, we stratified WMH at their median (3.0 mL) and retinal calibers into tertiles. Regarding the association of blood pressure, lacunar infarcts and WMH with global brain perfusion we would like to point to the work of our colleagues in this same sample of community-dwelling people[25, 26]. As markers of large vessel disease, we considered carotid intima media thickness and carotid plaques. While we stratified carotid intima media thickness using a cut-off of 1 mm, carotid plaques were dichotomized into presence and absence. As cardiovascular determinants we chose systolic and diastolic blood pressure and heart rate since these modify the diameter of blood vessels. Due to potential overfitting of the models, we only adjusted these analyses for age, sex and study cohort. In addition to these stratified analyses, we also tested for interaction on the multiplicative scale by adding interaction terms to the further adjusted regression models (model 2).

Third, since we defined TIA and ischemic stroke as clinical diagnoses, we assessed the effect of brain infarction on MRI by performing a sensitivity analysis in which we excluded participants with the presence of cortical infarcts on brain MRI. Finally, we excluded people with a carotid stenosis at baseline to preclude the effect of carotid stenosis on the association between global brain perfusion and TIA or ischemic stroke.

Missing data on covariables (maximum 10.3%) were imputed using 5-fold multiple imputation based on determinant, outcome and covariables with 20 iterations for each imputation. Distribution of covariables was similar in the imputed versus non-imputed dataset.

Analyses were done using RStudio version 1.0.153 (2009-2017 RStudio, Inc).

RESULTS

Of the 5289 stroke-free participants at baseline, mean age was 64.3 years, (standard deviation 10.6) with 55.6% women and a mean global brain perfusion of 56.2 ml/min per 100 mL brain (standard deviation 9.7; Table 1). During a median follow-up of 7.2 years (36,103 person-years), 137 suffered a TIA (mean global brain perfusion 52.7) and 137 a stroke (mean global brain perfusion 53.3), whichever came first (incidence rate for both events: 3.8 per 1000 person-years). Within the stroke group, 108 participants developed ischemic stroke (mean global brain perfusion 53.4), 20 participants developed haemorrhagic stroke and 9 participants developed a stroke which was not further specified. Among participants who developed TIA, 93 participants had only focal symptoms and 44 participants had a mix of focal and non-focal symptoms. The majority developed a single TIA, while 22 participants had multiple attacks.

We found that global brain perfusion at baseline associated with a higher risk of TIA (adjusted hazard ratio (aHR), 95% confidence interval (CI), per standard deviation decrease: 1.30; 1.07–1.57) and not of ischemic stroke (aHR, 1.06; 95% CI, 0.87–1.30); Table 2. Effect estimates were higher for the risk of ischemic stroke when not censoring for previous TIA (aHR 1.09; 95% CI, 0.90 – 1.33). Results for the association for lower global brain perfusion with risk of TIA or any stroke combined are shown in Table 3. The proportional hazard assumption was met for all models.

After stratifying the analyses on retinal vessel diameter, lower global brain perfusion was associated more prominently with TIA across strata of wider arteriolar and wider venular retinal vessel calibers compared to strata of smaller retinal vessel calibers (aHR, 2.04, 95% CI, 1.18–3.50 for arterioles and aHR, 2.30, 95% CI, 1.26–4.17 for venules in the widest strata). For ischemic stroke, there were no differences between strata of retinal vessels (aHR, 1.15, 95% CI, 0.64 – 2.06 for arterioles and aHR, 1.07, 95% CI, 1.72–0.79 for venules in the widest strata); Figure 2. However, within strata of higher diastolic blood pressure, participants had an 51% increased risk of developing an ischemic stroke per SD decrease of global brain perfusion, with the interaction term being borderline significant (p=0.056),

whereas higher diastolic blood pressure did not modify the risk of TIA (p=0.444); Figure 2. For other markers of small and large vessel disease, no significant differences were found across strata for the risk of TIA or ischemic stroke (data not shown).

Table 1. Baseline characteristics of the study population

	Total cohort (N = 5289)
Women	2941 (55.6)
Age, years	64.3 (10.6)
Smoking	
Current	1003 (21.1)
Former	2293 (48.3)
Never	1447 (30.5)
Systolic blood pressure, mmHg	139.4 (21.3)
Diastolic blood pressure, mmHg	82.6 (11.0)
Use of blood-pressure lowering medication	1787 (34.1)
Serum total cholesterol, mmol/l	5.6 (1.1)
Serum high-density lipoprotein cholesterol, mmol/l	1.5 (0.4)
Use of lipid-lowering medication	1227 (23.4)
Diabetes mellitus type 2	506 (9.8)
Body mass index, kg/m^2	27.5 (4.2)
Carotid stenosis	141 (2.7)
Use of antithrombotic medication	878 (16.7)
Presence of lacunar infarcts	325 (6.2)
Global brain perfusion, ml/min per 100 mL brain	56.2 (9.7)

Abbreviations: N = number of participants included in study.
Data presented as mean (standard deviation) for continuous variables and number (percentages) for categorical variables. Data represent original data without imputed values.
Number of missing values are 546 (10.3%) for smoking, 38 (0.7%) for systolic blood pressure, 38 (0.7%) for diastolic blood pressure, 42 (0.8%) for use of blood-pressure lowering medication, 89 (1.7%) for serum total cholesterol, 89 (1.7%) for serum high-density lipoprotein cholesterol, 42 (0.8%) for use of lipid-lowering medication, 114 (2.2%) for diabetes mellitus type 2, 32 (0.6%) for body mass index, 89 (1.7%) for carotid stenosis, 42 (0.8%) for use of antithrombotic medication and 12 (0.2%) for the presence of lacunar infarcts.

We found no evidence for a change in effect of brain perfusion on TIA or ischemic stroke after exclusion of all persons with cortical infarcts (n=66) on the MRI scan (aHR, 1.31, 95% CI, 1.08 – 1.58 for TIA and aHR, 1.07, 95% CI, 0.87–1.31 for ischemic stroke). Finally, after excluding people with carotid stenosis (n=190), also no difference was found in effect of brain perfusion on TIA or ischemic stroke (aHR, 1.24, 95% CI, 1.02 – 1.51 for TIA and aHR, 1.06, 95% CI, 0.86 – 1.31 for ischemic stroke).

Table 2. Global brain perfusion and the risk of TIA and ischemic stroke.

	TIA			Ischemic stroke		
	n/N	**Model I**	**Model II**	**n/N**	**Model I**	**Model II**
		HR, 95% CI	**HR, 95% CI**		**HR, 95% CI**	**HR, 95% CI**
Global brain perfusion (per SD decrease)	137/5289	1.34, 1.10 – 1.62	1.30, 1.07 – 1.57	108/5289	1.09, 0.89 – 1.34	1.06, 0.87 – 1.30
Tertile 1 (19 - 52)	59/1716	1.83, 1.14 – 2.92	1.78, 1.11 – 2.85	49/1716	1.33, 0.81 – 2.18	1.29, 0.78 – 2.12
Tertile 2 (52 - 59)	51/1762	1.84, 1.15 – 2.95	1.74, 1.08 – 2.80	33/1762	1.28, 0.77 – 2.13	1.31, 0.79 – 2.18
Tertile 3 (59 - 154)	27/1811	1 (reference)	1 (reference)	26/1811	1 (reference)	1 (reference)

Global brain perfusion tertiles presented as lowest, middle, highest (mL/min per 100 mL).

Abbreviations: n = number of cases; N = number of persons at risk; HR = hazard ratio; CI = confidence interval.

Cox regression model I: Adjusted for sex, age and study cohort.

Cox regression model II: As model I, additionally adjusted for systolic blood pressure, diastolic blood pressure, blood pressure lowering medication, serum total cholesterol, serum high-density lipoprotein cholesterol, lipid-lowering medication, smoking, diabetes mellitus type 2, body mass index, carotid stenosis, antithrombotic medication use, and silent brain infarcts (lacunar infarcts).

Table 3. Global brain perfusion and the risk of TIA or ischemic stroke combined.

	TIA or ischemic stroke		
	n/N	Model I	Model II
		HR, 95% CI	HR, 95% CI
Global brain perfusion (per SD decrease)	246/5289	1.22, 1.06 – 1.40	1.18, 1.03 – 1.36
Tertile 1 (19 - 52)	108/1716	1.55, 1.10 – 2.17	1.49, 1.06 – 2.09
Tertile 2 (52 - 59)	84/1762	1.46, 1.03 – 2.05	1.45, 1.03 – 2.05
Tertile 3 (59 - 154)	54/1811	1 (reference)	1 (reference)

Total brain perfusion tertiles presented as lowest, middle, highest (mL/min per 100 mL).
Abbreviations: n = number of cases; N = number of persons at risk; HR = hazard ratio; CI = confidence interval.
Cox regression model I: Adjusted for sex, age and study cohort.
Cox regression model II: As model I, additionally adjusted for systolic blood pressure, diastolic blood pressure, blood pressure lowering medication, serum total cholesterol, serum high-density lipoprotein cholesterol, lipid-lowering medication, smoking, diabetes mellitus type 2, body mass index, carotid stenosis, anti-thrombotic medication use, and silent brain infarcts (lacunar infarcts).

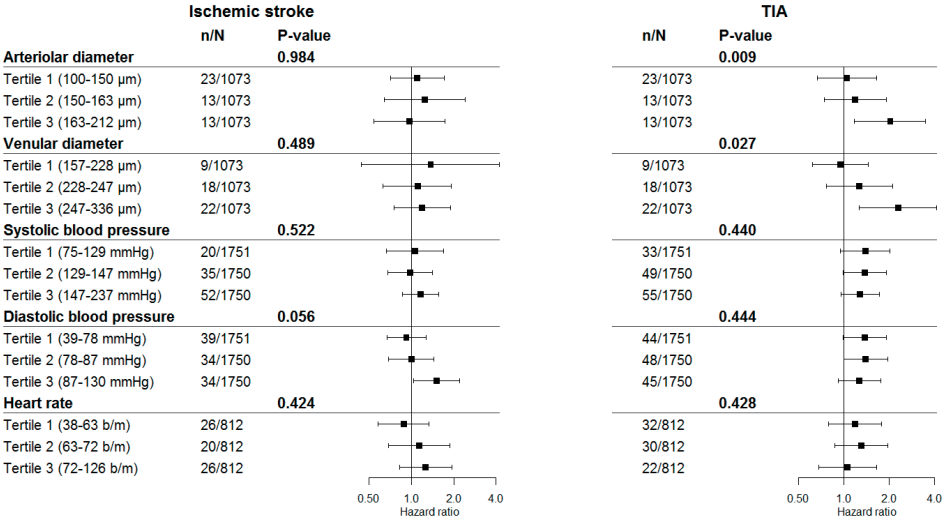

Figure 2. Assessment of effect measure modification between global brain perfusion per SD decrease and various cardiovascular determinants on the risk of TIA and ischemic stroke.
Assessing effect measure modification by stratification and interaction terms between global brain perfusion per SD decrease and markers of microvascular disease (arteriolar and venular retinal diameter), and cardiovascular determinants (systolic and diastolic blood pressure and heart rate). P-value indicates p-value for interaction. Abbreviations: µm = micrometer; mmHg = millimetre of mercury; b/m = beats per minute; n = number of cases; N = number of persons at risk, which may differ per stratified analyses depending on the availability of data within the study population. Cox regression model I: Adjusted for sex, age, study cohort and when assessing retinal vessels, the other retinal vessel was included in the model.

DISCUSSION

In this population-based study, we observed that lower global brain perfusion was associated with a higher risk of TIA but not of ischemic stroke.

Previous studies have assessed the association between global brain perfusion and the risk of TIA or stroke among patient-based samples[27, 28]. A meta-analysis showed that lower brain perfusion is associated with increased risk of ischemic events in patients with symptomatic or asymptomatic carotid stenosis or occlusion[7]. In this meta-analysis, the odds ratio (OR) for stroke was 3.87 (95% CI 1.99-7.48) and for TIA and stroke combined 4.65 (95% CI 2.65-8.14). Interestingly, we found no association of lower brain perfusion with a higher risk of stroke, even when censoring for previous TIA, which is in contrast with these earlier clinical studies. An important explanation for this may be the differences in study population. All of the prior findings were based on patients with carotid artery stenosis, whereas our study population comprised of asymptomatic community-dwelling elderly. A study population comprising of patients with carotid stenosis will have more comorbidity, thereby increasing the risk of developing a TIA or stroke.

Surprisingly, we found that the effect of lower global brain perfusion was more pronounced for TIA than ischemic stroke. This implies that lower global brain perfusion is more likely a hemodynamic risk factor for focal transient ischemia than permanent ischemia. It is known that a reduction in global cerebral blood flow may give focal worsening of an existing neurological deficit in the brain, i.e. after TIA or stroke[29]. However, in people with lower global brain perfusion without history of TIA or stroke, probably the combination of a short disruption of global brain perfusion with impaired autoregulatory vasodilation leads to a TIA, while the presence of cardiovascular risk factors are necessary to cause a longer drop in global brain perfusion sufficient to lead to an ischemic stroke. An explanation could be that within an individual, perfusion in the cerebral arteries and larger arterioles fluctuates with the cardiac cycle[30]. Given a lower mean brain perfusion, these fluctuations resulting in peaks and troughs, may during a trough drop below the critical ischemia point causing a mild and short ischemia in the brain and consequently a TIA, but not a stroke due to a fast recovery from this trough in this brain perfusion cycle[31]. Such a harmful drop in brain perfusion will especially occur when cerebral autoregulation is poor for example by impaired myogenic mechanisms[32]. Indeed, in the analyses in which we stratified by retinal vessel diameters, we found evidence that in participants with wider retinal calibers, the association between global brain perfusion and TIA was stronger, but did not modify the association with ischemic stroke (Figure 2). Myogenic autoregulation is widely distributed over vascular beds including the retina and brain[33]. Autoregulation of the retinal vessels may thus reflect autoregulation in the small brain vessels. If arterioles are already fully vasodilated, they may not be able to widen any further in response to a drop in perfusion. Thus, our findings of more pronounced effects in persons with widened retinal vessels suggest exhausted vasodila-

tory response to be an important contributing factor for TIA in the general population. For ischemic stroke to occur, however, it is likely that more is needed than a drop in global brain perfusion, namely the presence of cardiovascular risk factors. This is supported by our finding that participants with higher diastolic blood pressure show an increased risk for developing ischemic stroke per decrease of global brain perfusion. Another possibility is that a longer drop in global brain perfusion is needed to develop a stroke, for which a lower global brain perfusion in the general population is not a risk factor alone. Although TIA and ischemic stroke share many risk factors, specifically with regard to the role of global brain perfusion our study also suggests the existence of specific etiological differences. Further studies are needed to determine to which factors the lack of effect of global brain perfusion on ischemic stroke in the general population are attributable, for instance collateral circulation in the brain.

Limitations of our study include the following. First, we used single global brain perfusion measurements in relation to the risk of TIA or stroke. We could not perform analyses on repeated measurements of brain perfusion, due to small numbers at this moment. Hence, we could not assess fluctuations in perfusion or display changes of perfusion over a longer period of time. In addition, more advanced imaging techniques such as arterial spin labelling tend to have lower and slightly less variable absolute perfusion measures due to post processing with a single value deconvolution model, compared to phase contrast imaging measures of perfusion, where overestimation of brain perfusion can particularly occur in aged brains due to usage of a max slope model[34]. But such a systematic deviation would not influence obtained relative risks and a larger variability would only lead to dilution of effect estimates. It is important to acknowledge that using our phase-contrast method we obtain a measure of blood flow in major brain arteries which is assumed to represent homogeneous flow in the entire brain under normal conditions. This directly highlights that potentially interesting inhomogeneities of perfusion among different brain regions cannot be taken into account. There may be compensation on other brain regions and these hypo- and hyperperfusion regions may average out in the carotid and basilar arterial flow. Second, the vast majority of our population is of European ancestry, limiting generalizability to other ethnicities. More specifically, it is known that the impact of small vessel and large vessel disease on stroke is different between ethnic groups, where in Caucasians stroke is more often associated with large vessel disease (carotid stenosis) compared to small vessel disease (lacunar infarcts) in Africans and Asians[35]. Further study is needed to confirm that these findings indeed apply to other ethnicities and geographical regions.

Strengths of our study are the population-based setting and the thorough assessment and follow-up for stroke and TIA. Another strength is the amount of stroke and TIA cases, which allowed us to investigate the association with brain perfusion for these outcomes separately. Finally, our cohort includes both women and men, allowing us to investigate a heterogenic

population, although we found no effect modification of the association between brain perfusion and risk of ischemic stroke or TIA by sex in our study (data not shown).

In conclusion, impaired global brain perfusion increases the risk of TIA but not of ischemic stroke in this community-dwelling population. These findings support a role of global brain perfusion as a hemodynamic risk factor for developing a TIA. Further studies are warranted to unravel mechanisms in relation to compensatory mechanisms such as autoregulatory vasodilation and collateral circulation in the brain. Also more detailed regional changes that cannot be detected by phase-contrast imaging are important to take into consideration in order to assess the potential of global brain perfusion as a target for prevention of vascular events.

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5.2 Thyroid function and brain hemodynamics

ABSTRACT

Background: Thyroid dysfunction and decreased brain perfusion have been associated with risk for Alzheimer's disease and stroke. Whether thyroid dysfunction is related to altered brain circulation in the general population remains unknown. We determined the association of thyroid hormones with four different markers of brain circulation within community-dwelling elderly.

Methods: A total of 5142 participants (mean age, 63.8 years; 55.4% women) from three subcohorts of the population-based Rotterdam Study, starting in 1989, 2000 and 2006, underwent venapuncture to measure serum thyroid-stimulating hormone (TSH), free thyroxine (FT4) and thyroid peroxidase antibodies (TPO). Between 2005 and 2015, all participants underwent phase-contrast brain magnetic resonance imaging to assess global brain perfusion (mL of blood flow/100 mL of brain/min). Arteriolar and venular retinal calibers were assessed in 3105 participants as markers of microcirculation. Finally, 901 participants underwent transcranial Doppler to assess cerebrovascular CO_2 reactivity. We investigated associations of TSH, FT4 and TPO with brain circulation measures using (non-)linear regression models with adjustment for age, sex, smoking, alcohol and diet.

Results: Higher and lower FT4 levels (in pmol/L) were associated with lower global brain perfusion. The difference was up to -2.44 mL (95% confidence interval (95%CI)=-4.31;-0.56) when comparing persons with a higher FT4 (25 pmol/L) with middle FT4 levels (FT4=15 pmol/L; *P* non-linearity=0.002). Similarly, higher and lower levels of FT4 were associated with arteriolar retinal vessels (mean difference up to -2.46 µm, 95%CI -4.98; 0.05 for lower FT4). Furthermore, arteriolar retinal vessel diameters were narrower in participants with higher TSH and TPO levels (mean difference -0.93 µm, 95%CI -1.66;-0.21 for TSH and -1.93 µm, 95%CI -3.49;-0.36 for TPO). There was no association between thyroid hormones and venular retinal vessels nor cerebrovascular CO_2 reactivity.

Conclusions: Our data show that global brain perfusion was lower in middle-aged and elderly with lower as well as higher FT4 levels. Furthermore, arteriolar retinal vessel diameters were narrower in elderly with higher TSH and TPO levels, in the general population. These results suggest that thyroid dysfunction may play a role in brain diseases such as stroke or dementia through a suboptimal brain circulation that is potentially amendable.

Keywords: Thyroid, cerebral blood flow, brain perfusion, general population, cohort study, free thyroxine

INTRODUCTION

Thyroid dysfunction is common in the general population, yet underdiagnosed, and associated with an increased risk of stroke and dementia.[1-3] Despite available evidence for these associations, the mechanism through which thyroid dysfunction causes dementia or stroke remains unclear. Reduced perfusion of the brain is implicated in dementia[4] as well as crucial in the pathophysiology of acute transient ischemic attack (TIA) and stroke.[5] There is growing evidence that persons with thyroid dysfunction have an altered brain circulation, which in turn could lead to an increased risk of cerebrovascular and neurodegenerative disease.[6] For example, several previous studies have shown that in patients with Alzheimer's disease, higher serum levels of thyroid-stimulating hormone (TSH) were correlated with lower brain perfusion in the temporal regions.[12-14] In addition to hypothyroidism where reduced perfusion of the brain occur mainly through a lower cardiac output[9], relative hypoperfusion could also occur with hyperthyroidism proposedly through increased metabolism in the brain which causes a faster utilization of oxygen.[15] When the vasodilatory response is exhausted, the brain becomes hypoperfused. Thus far, the relation between thyroid dysfunction and circulation of the brain has only been shown in specific patient populations, such as in patients with mood disorders and thyrotoxicosis.[6-11] Yet, whether thyroid status, even within the normal range, is related to an altered brain circulation in the general population remains unknown. These changes might be important for the pathogenesis of stroke and dementia and these diseases may thus be amendable by altering thyroid function status (e.g. treating thyroid dysfunction).Against this background, we determined the association between TSH, free thyroxine (FT4) and thyroid peroxidase antibodies (TPO) with different measures of brain circulation available in the population-based Rotterdam Study, including global brain perfusion as assessed by MRI, and alterations in retinal vessel diameter, as a measure of brain microcirculation. In addition, we assessed the effect of thyroid hormones on cerebrovascular CO_2 reactivity (CVR) as assessed by transcranial Doppler, all in the population-based Rotterdam Study.

METHODS

Study population

This study was performed within the Rotterdam Study, a large population-based cohort. The rationale, design and aims have been described elsewhere.[16] The Rotterdam Study was initiated in 1989 in Rotterdam, The Netherlands, with 7983 participants aged 55 years or older. The Rotterdam study was extended in 2000 with a second cohort of 3011 participants. A third cohort of 3932 participants, this time 45 years or older, was added in 2006. For the current study, 5142 participants from these three independent Rotterdam Study cohorts

underwent laboratory measurements to assess TSH, FT4 and TPO (thyroid function was measured once per participant): 1043 participants from the first cohort (1996 - 1999), 1274 participants from the second cohort (2000 - 2001) and 2825 participants from the third cohort (2006 - 2008). Between 2005 and 2015, all 5142 participants underwent 2D phase-contrast brain magnetic resonance imaging to assess global brain perfusion. Of these participants, 3105 underwent arteriolar and venular retinal calibers assessment between 2004 and 2008 during the second visit of the second cohort and first visit of the third cohort. Finally, a smaller sample of 901 participants underwent cerebrovascular CO_2 reactivity measurements between 1997 and 2001 during the third visit of the first cohort and the first visit of the second cohort; **Figure 1**.

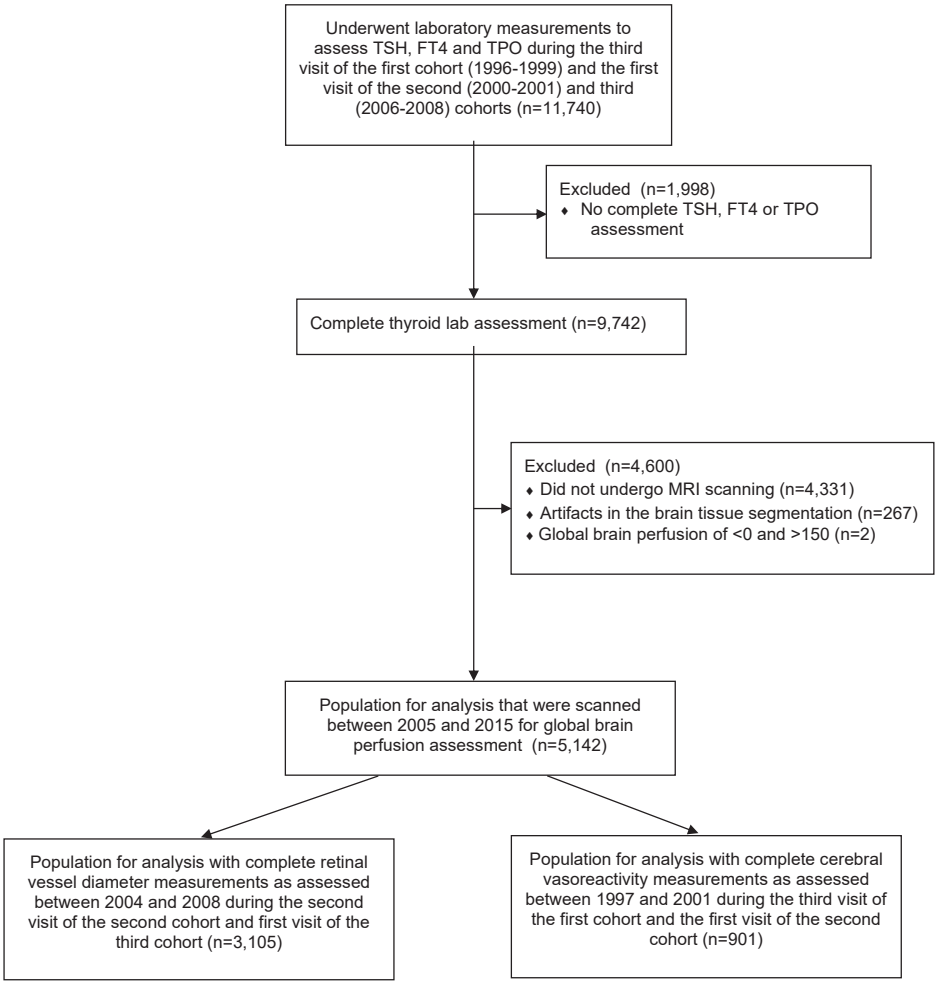

Figure 1. Flow Diagram

The Rotterdam Study has been approved by the Medical Ethics Committee of Erasmus University with the registration number: MEC 02.1015 and by the Ministry of Health,Welfare and Sport of the Netherlands (Population Screening Act WBO, license number 1071272-159521-PG), implementing the Population Study Act Rotterdam Study. The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; www.who.int/ictrp/network/primary/en/) under shared catalogue number NTR6831. In accordance with the Declaration of Helsinki, all participants in this analysis provided written informed consent to participate in the study and to have their information obtained from treating physicians.

Assessment of thyroid function

TSH, FT4 and TPO were measured on serum samples stored at -80°C (The electrochemiluminescence immunoassay "ECLIA", Roche). The normal range values were defined for TSH as 0.4-4.0 mIU/L and for FT4 as 0.86-1.94 ng/dL according to guidelines and previous research.[17] TPO levels greater than 35 kU/mL were considered as positive according to manufacturer recommendations.

Assessment of global brain perfusion

All participants were scanned between 2005 and 2015 on a 1.5-Tesla MRI scanner.[4, 22] Specifically for the flow measurements, we performed a 2D phase-contrast sequence as described before[22], a sequence that allows for measuring blood flow velocities by phase. Blood flow was calculated using interactive data language-based custom software (Cinetool version 4, General Electric Healthcare, Milwaukee, WI, USA) from the 2D phase-contrast sequence images. The calculation of cerebral blood flow (CBF, in mL/min) was made by summing the flow rates for the carotid arteries and the basilar artery (mL/secs) and multiplying this by 60 secs/min[23]. Global brain perfusion (mL/min blood per 100 mL brain) was computed as the division of CBF by each individual's brain volume (mL) and by multiplying the result by 100.[4]

Retinal vessel measurements

Between 2004 and 2008, simultaneous stereoscopic fundus color photographs centered on the optic disc were taken (pharmacological mydriasis, 20°; Topcon Optical Co., Tokyo, Japan; digitized with a high-resolution scanner model LS-4000; Nikon Corp., Tokyo, Japan).[24] For each participant, the image of 1 eye with the best quality was studied with the Retinal Vessel Measurement System (Retinal Analysis, Optimate, WI; Department of Ophthalmology and Visual Science, University of Wisconsin Madison).[24] One summary value was calculated for the arteriolar diameters (in μm) and one for the venular diameters of the blood column for each participant.[25, 26] Because of different magnification in case of refractive changes due to

corneal curvature, lens and axial length differences, we adjusted this summary vessel measure with Littmann's formula to approximate absolute measures.[26, 27]

Transcranial Doppler (TCD) assessment

TCD monitoring (Multi-Dop X-4; DWL, Sipplingen, Germany) was performed between 1997 and 2001 to measure the CBF velocity in the middle cerebral artery on both sides as described before.[28] End diastolic, peak systolic, and mean CBF velocities (cm/sec) were recorded automatically[29]. End-tidal CO_2 pressure (kPa) was recorded continuously with a CO_2 analyzer (Multinex; Datascope, Hoevelaken, the Netherlands).[28] CVR was assessed by continuous measurement of flow velocity in the middle cerebral artery, when participants breathed room air followed by 5% carbon dioxide inspiration through an anaesthetic mask for 2 minutes.[28] We defined CVR as the percentage increase in flow velocity during inspiration of 5% CO_2, divided by the absolute increase in end-tidal CO_2 in the same period.[28] We used the mean of right and left circulatory parameters for the analyses. In case of one-sided window absence, the contralateral parameters were used for analyses.[28]

Covariates

Smoking was assessed with a questionnaire and categorized as never, past and current smoking. Alcohol consumption was measured with a questionnaire assessing the number of glasses of alcohol per day. Diet was self-reported using the food-frequency questionnaire, and a diet quality score was calculated as previously described.[30] Measures of height and weight were collected at the research center and BMI was defined as weight (kg)/height $(m)^2$. Blood pressure was measured twice using a sphygmomanometer and averaged. Blood pressure lowering medication was assessed through a home interview and pharmacy records. Diabetes mellitus was defined as use of antidiabetic medication, fasting serum glucose level ≥7.1 mmol/L (≥127.9 mg/dL), or random non-fasting serum glucose level ≥11.1 mmol/L (≥200.0 mg/dL). Physical activity was assessed using the validated LASA physical activity questionnaire and Zutphen physical activity questionnaire.[31, 32] Creatinine was measured using blood samples with an enzymatic assay method..

Statistical analyses

We cross-sectionally studied the association of thyroid hormones (i.e. TSH, FT4 and TPO) with measures of brain circulation, including global brain perfusion, arteriolar and venular retinal diameters and CVR. Covariables were selected based on the biological confounding plausibility[33]; **Figure S1**. The first model was adjusted by sex, age and cohort. For the analyses involving global brain perfusion, we also adjusted for time between thyroid assessment and MRI scan. We did not adjust for time difference when we explored the association between thyroid function and retinal vessels because the time difference was very small (median time difference: 0.03 months). Similarly, the association between thyroid function and CVR was

not adjusted for time difference because these measures were collected during the same research visits. The second model was additionally adjusted for smoking, alcohol consumption and diet quality score. In the third model, we additionally adjusted for covariables that could act as confounders but also as mediators, namely BMI, blood pressure lowering medication, diabetes mellitus, physical activity and glomerular filtration rate. In a fourth model, we additionally adjusted for systolic and diastolic blood pressure as potential mediators. TSH was studied using natural logarithmic transformation due to its right skewed distribution. TPO was studied continuously as well as dichotomous based on the cut-off (35 kU/mL). The possibility of a nonlinear association between the exposures (TSH, FT4 and TPO), the covariables and our outcomes were tested by using restricted cubic splines with 3 knots. We further quantified nonlinear associations between thyroid hormones and measures of brain circulation by plotting these associations and visually assessing the thyroid hormone value that was associated with the highest point for brain circulation and comparing that with other thyroid hormone values and their corresponding brain circulation outcomes.

We performed several sensitivity analyses. First, we restricted our analyses to euthyroid participants. Euthyroidism was defined based on TSH values between 0.4-4.0 mIU/L, and without thyroid medication and anti-thyroid medication drugs (e.g. Methimazole). Second, due to the long time between blood measurement and time of MRI scan in some participants, we performed our analyses in participants who had their thyroid and MRI assessment during the same visit scheme. We did the same for retinal vessel diameter as outcome. Since the thyroid hormones and CVR were measured in the same visit, we did not perform these extra analyses for CVR. Third, we studied our association between thyroid function and global brain perfusion separately for those without cortical infarcts, without carotid stenosis (>50% in the left or right carotid artery) and additionally adjusting for the other thyroid hormones (e.g. we included TSH in the analyses of FT4 and vice versa).

We explored effect modification by sex, age, smoking, time between thyroid measurement and MRI scan and finally each other brain circulation measure by including the product of the interacting factor and thyroid hormones and planned a stratified analysis if the *P*-value for interaction was < 0.1.

Multiple imputation by chained equations was performed for covariates with missing data. Statistical analyses were performed using R statistical software (mice, rms packages, R project, Institute for Statistics and Mathematics, R Core Team, version 3.2.2).

RESULTS

We included a total of 5142 subjects with their characteristics presented in **Table 1**. The mean age was 63.8 years and 55.4% were women. Median TSH was 2.0 mIU/L (interquartile range (IQR) 1.4, 2.8), mean FT4 was 15.6 pmol/L with a standard deviation (SD) of

2.2 and 684 participants (13.3%) were TPO positive. Median global brain perfusion was 55.3 mL of blood flowing per 100 mL of brain/min (IQR 49.7, 61.8). The mean arteriolar diameter was 156.7 µm (SD: 15.8), venular diameter 238.2 µm (SD: 22.6) and the median CVR was 39.2%/kPa with an IQR of 25.0, 56.0 (**Table 1**). The median time difference between thyroid function and MRI data collection was of 2.7 months (IQR 0.7, 116.0) and the median time difference between thyroid function and retinal vessels data collection was of 0.03 months (IQR 0.00, 1.3). There was no time difference between thyroid function and CVR data collection because these measures were assessed in the same research visit.

Table 1. Study population characteristics.

Characteristic	**N = 5,142**
Women	2,849 (55.4%)
Age, years	63.8 ±10.2
Smoking	
Current	1,059 (20.6%)
Former or never	4,083 (79.4%)
Alcohol, glasses per day	1.2 ±2.0
Diet, sum score adherence dietary guidelines (0-14)	6.9 ±1.9
Diabetes mellitus type 2	261 (5.1%)
Body mass index, kg/m^2	27.5 ±4.1
Systolic blood pressure, mmHg	138.0 ±20.1
Diastolic blood pressure, mmHg	81.3 ±10.7
Blood pressure lowering medication	1585 (31.2%)
Total physical activity, MET·h·week, standardized	0.10 ±1.0
Glomerular filtration rate, mL/min/1.73 m^2	83.9 ±14.4
Free thyroxine, pmol/L	15.6 ±2.2
Thyroid-stimulating hormone, mIU/L	2.0 (1.4-2.8)
Thyroid peroxidase antibodies positivity (cutoff, 35 kU/mL)	684 (13.3%)
Outcomes	
Global brain perfusion, mL/min/100 mL of brain, median (IQR)	55.3 (49.7 – 61. 8)
Arteriolar diameter, µm (N = 3105)	156.7 ±15.8
Venular diameter, µm (N = 3105)	238.2 ±22.6
CO2 vasoreactivity, %/kPa (N = 901)**, median** (IQR)	39.2 (25.0-56.0)

Abbreviations: N indicates number of participants included in study, IQR indicates interquartile range. Data presented as mean ±standard deviation for continuous variables and number (percentages) for categorical variables, unless otherwise specified. Number of missing values: 43 (0.8%) for smoking, 144 (2.8%) for alcohol, 1,129 (22.0%) for diet, 12 (0.2%) for diabetes mellitus type 2, 5 for body mass index (0.1%), 64 for systolic and diastolic blood pressure (0.8%), 69 for blood pressure lowering medication (0.9%), 829 for total physical activity (16.1%), 81 for glomerular filtration rate (1.6%).

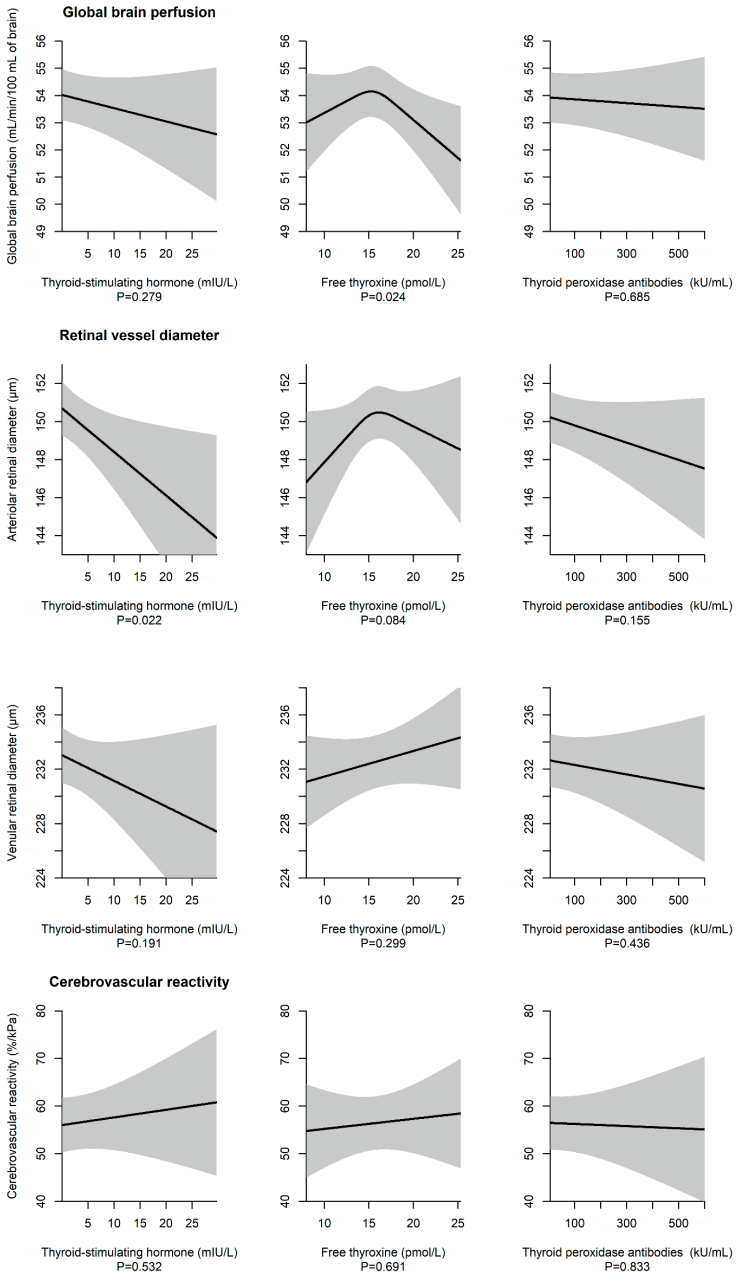

Figure 2. A visual representation of the associations between thyroid markers and brain perfusion measures. Models are corrected for age, sex, study cohort, smoking, alcohol and diet. For global brain perfusion, we additionally adjusted for time between thyroid measurement and MRI scan. The associations of free thyroxine and global brain perfusion and arteriolar retinal vessel diameter were performed using restricted cubic splines (3 knots). P denotes *P*-value of association.

Thyroid function and global brain perfusion

There was no statistically significant association between TSH and global brain perfusion (mean difference in mL perfusion per natural log increase in TSH = -0.22, 95% confidence interval (CI) -0.55; 0.10; **Figure 2 and Table S1).** On the other hand, we found that higher and lower FT4 levels (in pmol/L) were associated with lower global brain perfusion (inverted U-shaped) between FT4 and global brain perfusion (*P*-value for non-linearity = 0.002, *P*-value for association = 0.024; **Figure 2**), with on average 0.80 mL, 95% CI -1.95; 0.35 less brain perfusion in persons with a lower FT4 serum level of 10 pmol/L than in persons with a middle FT4 of 15 pmol/L and a difference of -2.44 mL, 95% CI -4.31; -0.56 when comparing persons with a higher FT4 of 25 pmol/L to FT4 of 15 pmol/L; **Table S2**. Finally, there was no statistically significant association between TPO positivity and global brain perfusion (mean difference in mL perfusion in TPO positives vs negatives = -0.25, 95% CI -1.00; 0.48).

Thyroid function and retinal vessels

We found on average 0.92 µm narrower arteriolar retinal vessels, 95% CI -1.64; -0.20 per natural log increase in TSH; **Figure 2** and **Table S1**. This association did not change materially after additional adjustment by systolic and diastolic blood pressure (mean difference = -0.79, 95% CI -1.48; -0.09, **Table S1**). There was an inverted U-shaped association between FT4 and arteriolar retinal vessels (*P*<0.0001 for non-linearity, *P*-value for association = 0.084; **Figure 2**). Participants positive for TPO antibodies had on average 1.78 µm narrower arterioles, 95% CI -3.33; -0.22, compared to persons negative for TPO antibodies. This association was attenuated after adjustment by systolic and diastolic blood pressure, and no longer statistically significant (mean difference = -1.35, 95% CI -2.85; 0.14, **Table S1**). There was no association between thyroid function and venular retinal vessels: mean difference in µm venular vessel diameter per natural log increase in TSH = -0.91 µm, 95% CI -1.96; 0.13, mean difference in µm vessel diameter per pmol/L increase in FT4 = 0.18 µm, 95% CI -0.16; 0.53 and mean difference in µm diameter in TPO positives vs negatives = -1.26, 95% CI -3.53; 1.01.

Thyroid function and cerebrovascular CO_2 reactivity

We did not find any association between thyroid function and CVR: mean difference in CVR per natural log increase in TSH was 0.03, 95% CI -0.03; 0.10, mean difference per pmol/L increase in FT4 -0.001, 95% CI -0.024; 0.023 and mean difference for TPO positive vs negative -0.02, 95% CI -0.17; 0.12.

Sensitivity and additional analyses

After we restricted our analyses to euthyroid participants, the non-significant association remained the same: TSH as well as TPO positive vs negative and global brain perfusion (mean

difference in mL per natural log increase in TSH = -0.16, 95% CI -0.51; 0.66 and mean difference in TPO positive vs negative: -0.41, 95% CI -1.38; 0.55). The inverse U-shaped association between FT4 and global brain perfusion persisted; **Figure S2**. The associations between TSH and TPO positive vs negative and arteriolar retinal vessels were slightly attenuated. When restricting the analyses to participants that had their thyroid and MRI assessment as well as their retinal vessel assessment at the same time, the inverted U-shaped associations between FT4 and global brain perfusion and arteriolar retinal vessel diameter remained; **Figure S3**. Furthermore, when excluding participants with cortical infarcts and carotid stenosis results were similar. Additional adjustment for the 2 other thyroid measures when assessing the relation between thyroid status and brain circulation did not alter results significantly. Finally, when exploring effect modification for sex, age, smoking and time between thyroid measurement and MRI scan, the p-value for all interaction terms was >0.1.

DISCUSSION

In this study, we found that lower as well as higher FT4 levels were associated with lower global brain perfusion in middle-aged and elderly within the general population. Furthermore, higher TSH and TPO levels associated with narrower arteriolar retinal vessel diameters. These associations remained in euthyroid participants and were robust for sensitivity analyses.

Previous studies investigating the association of thyroid status with brain circulation have been conflicting, where some studies find no association,[12, 13, 34] others report an association with low[9, 11, 35-38] and one study reports an association with high[8] thyroid function. However, most of these studies were performed in specific patient populations and none investigated non-linearity.

The observation of an inverted U-shaped association between FT4 and global brain perfusion is new compared to previous studies. As mentioned, one study found associations between higher FT4 and increased brain perfusion[8], but there are two important differences with our study: 1) short-term effects of hyperthyroidism were assessed, while we investigated the full range of thyroid function and 2) the previous study investigated regional brain perfusion while in our study we investigated global brain perfusion in the general population. Although a higher FT4 indeed increases cardiac output, studies have only shown that acutely increasing cardiac output increases brain perfusion[39], while a non-acutely altered cardiac output was not associated with brain perfusion.[40] One explanation is that vasoconstriction of other vascular beds shunts the flow from the periphery to the brain, resulting in a lesser extent of brain perfusion reduction than what would be expected based on both the cardiac output and the peripheral blood flow.[40] Another explanation is that the extent to which brain perfusion is changed is determined by the integrated effect of all brain perfusion-regulating

mechanisms, including other powerful mechanisms unrelated to cardiac output[39], such as cerebral perfusion pressure via cerebral autoregulation[19] and cerebral metabolic activity via neurovascular coupling.[41]

It is plausible that a chronic exposure to slightly elevated FT4 could be detrimental for brain circulation for several reasons. First, higher FT4 could lead to tachycardia and subsequently atrial fibrillation, even within the normal range of FT4.[42] Other potential mechanisms are elevated blood pressure[43], atherosclerosis[44] and increased coagulation[45]. Also, both hyperthyroidism and hypothyroidism can lead to heart failure[46], which has been linked to reduced CBF.[39] Indeed, additional adjustment for potential confounders that could also act as mediators in our third model attenuated our results. However, formal mediation analyses should be conducted to evaluate and quantify the role of these variables. Regarding lower thyroid function, further adjustment for blood pressure even removed the significant association between TPO positivity and narrower arteriolar retinal vessels, supporting elevated blood pressure as a mechanism between thyroid status and brain circulation.

The fact that the inversed U-shaped association between FT4 is not only present for global brain perfusion, but also for arteriolar retinal vessel diameter in our study adds robustness to our findings, since arteriolar retinal vessel diameter is another proxy for brain circulation. In fact, the caliber of the resistance vessels regulates brain perfusion, i.e. myogenic autoregulation[19] and this phenomenon is widely distributed over vascular beds, including the retina and brain.[47] Autoregulation of the retinal vessels may thus reflect autoregulation in the small brain vessels.

Interestingly, we did not find an association between thyroid status and CVR. Arterial blood CO_2 reactivity is only one of the four known physiologic processes that regulates the caliber of the cerebral resistance vessels.[21] It could be that thyroid hormones act through one or more of the other regulatory processes. The processes for a high-normal FT4 lowering brain perfusion are more likely to be the two other mechanisms that remain, which are arterial blood pressure and cerebral perfusion pressure via cerebral autoregulation[19] and cerebral metabolic activity via neurovascular coupling.[41] Which of these processes is affected by either higher or lower thyroid function remains to be elucidated.

Our findings are important, considering the clinical consequences of having a suboptimal brain perfusion. A previous study also measuring global brain perfusion by phase-contrast MRI in the Rotterdam Study found that lower brain perfusion was associated with higher risk of dementia.[4] A similar study showed that lower brain perfusion was associated with a higher risk of TIA.[5] Translating these findings to our study reveals that a person from a middle-aged and elderly population with an FT4 of 25 compared to 15, who has a decreased brain perfusion of 2.44 mL/100 mL/min, would have a higher risk of 7.5% for developing either dementia or a TIA (over a 7 year period).[4, 5]

It is important to acknowledge the limitations of our study. First, due to the cross-sectional nature of the study we cannot exclude reverse causation. Second, by using our

phase-contrast method, we obtain a measure of brain perfusion in major brain arteries which is assumed to represent homogeneous flow in the entire brain under normal conditions. This directly highlights that potentially interesting inhomogeneities of blood flow among different brain regions cannot be taken into account. Third, the time between thyroid and MRI (median 2.66 months) was long for some participants potentially introducing survival bias, although sensitivity analyses with participants having these measurements at the same time revealed similar results. Fourth, we did not have triiodothyronine (T3) measurements in our cohort, which could especially be interesting in this context due to the heart's vulnerability to reductions in biologically active T3[46], which could affect brain perfusion. Fifth, the vast majority of our population is of European ancestry, limiting generalizability to other ethnicities.

In summary, our data show that global brain perfusion was lower in middle-aged and elderly with lower as well as higher FT4 levels. Furthermore, arteriolar retinal vessel diameters were narrower in elderly with higher TSH and TPO levels, in the general population. These results suggest that thyroid dysfunction could lead to brain diseases such as stroke or dementia through a suboptimal brain circulation that is potentially amendable. These data need to be confirmed in prospective cohort studies and trials to assess whether changing thyroid function actually changes brain perfusion and whether this leads to a decrease in incidence of relevant clinical outcomes, such as dementia and TIA.

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SUPPORTING INFORMATION

Table S1. Linear associations between thyroid markers and measures of brain perfusion.

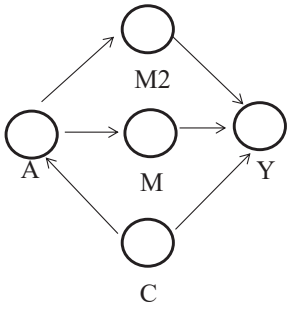
	Global brain perfusion (mL/min/100 mL of brain)*, N = 5142		
Model	**Ln(TSH)**		**TPO positivity**
	Mean difference, 95% CI		**Mean difference, 95% CI**
I	-0.255, -0.584; 0.074		-0.242, -0.989; 0.506
II	-0.222, -0.550; 0.107		-0.259, -1.006; 0.487
III	-0.197, -0.526; 0.132		-0.194, -0.938; 0.551
IV	-0.189, -0.519; 0.141		-0.190, -0.934; 0.554
	Arteriolar retinal vessel diameter (µm), N = 3105		
Model	**Ln(TSH)**		**TPO positivity**
	Mean difference, 95% CI		**Mean difference, 95% CI**
I	-1.090, -1.814; -0.366		-1.910, -3.480; -0.340
II	-0.931, -1.652; -0.296		-1.932, -3.493; -0.371
III	-0.923, -1.644; -0.202		-1.775, -3.329; -0.222
IV	-0.794, -1.489; -0.099		-1.354,-2.853; 0.144
	Venular retinal vessel diameter (µm), N = 3105		
Model	**Ln(TSH)**	**FT4**	**TPO positivity**
	Mean difference, 95% CI	**Mean difference, 95% CI**	**Mean difference, 95% CI**
I	-1.260, -2.329; 0.190	0.254, -0.103; 0.611	-1.333, -3.654; 0.988
II	-0.911, -1.962; 0.139	0.186, -0.165; 0.537	-1.260, -3.533; 1.013
III	-0.759, -1.811; 0.294	0.257, -0.095; 0.608	-1.324, -3.593; 0.944
IV	-0.744, -1.797; 0.308	0.282, -0.069; 0.633	-1.217, -3.484; 1.049
	Ln(Cerebrovascular reactivity, %/kPa), N = 901		
Model	**Ln(TSH)**	**FT4**	**TPO positivity**
	Mean difference, 95% CI	**Mean difference, 95% CI**	**Mean difference, 95% CI**
I	0.040, -0.029; 0.108	0.001, -0.023; 0.024	-0.025, -0.174; 0.123
II	0.038, -0.030; 0.107	-0.001, -0.024; 0.023	-0.028, -0.176; 0.121
III	0.042, -0.027; 0.111	0.001, -0.022; 0.025	-0.032, -0.181; 0.117
IV	0.038, -0.032; 0.107	0.004, -0.019; 0.028	-0.054, -0.204; 0.097

Abbreviations: N = number of participants, CI = confidence interval, FT4 = free thyroxine, TSH = thyroid-stimulating hormone, TPO = thyroid peroxidase antibodies (cutoff, 35 kU/mL), Ln=natural logarithm. Model I: corrected for age, sex, study cohort. *Additionally adjusted for time between thyroid measurement and MRI scan. Model II: additionally corrected for age, sex, study cohort, smoking, alcohol and diet. Model III: additionally corrected for BMI, diabetes mellitus, blood pressure lowering medication, physical activity and glomerular filtration rate. Model IV: additionally corrected for systolic and diastolic blood pressure. The analyses between FT4 and global brain perfusion and arteriolar retinal vessels are not shown due to their non-linear associations.

Table S2. The non-linear associations between free thyroxine and global brain perfusion and arteriolar retinal vessel diameter.

Thyroid function	**Global brain perfusion** (mL/min/100 mL of brain)**, N=5142**			
	Model I Mean difference, 95% CI	**Model II** Mean difference, 95% CI	**Model III** Mean difference, 95% CI	**Model IV** Mean difference, 95% CI
FT4				
FT4=10	-0.84, -1.99; 0.32	-0.80, -1.95; 0.35	-0.67, -1.82; 0.47	-0.63, -1.78; 0.52
FT4=15	1 (reference)	1 (reference)	1 (reference)	1 (reference)
FT4=20	-0.95; -1.77; -0.13	-1.04; -1.86; -0.22	-1.02, -1.84; -0.20	-1.00,-1.82; -0.19
FT4=25	-2.24; -4.12; -0.37	-2.44; -4.31; -0.56	-2.37, -4.24; -0.50	-2.33, -4.20;-0.45
	Arteriolar retinal vessel diameter (µm), N=3105			
FT4=10	-2.56, -5.08; -0.03	-2.46, -4.98; 0.05	-2.06 ,-4.56; 0.44	-2.02; -4.43; 0.40
FT4=15	1 (reference)	1 (reference)	1 (reference)	1 (reference)
FT4=20	-0.28, -1.98; 1.42	-0.58, -2.28; 1.11	-0.58, -2.26; 1.10	0.29, -1.34; 1.91
FT4=25	-1.11, -5.00; 2.79	-1.75, -5.63; 2.13	-1.67, -5.53; 2.18	0.24, -3.49; 3.96

Abbreviations: n = number of cases; N = number of persons at risk; CI = confidence interval; FT4 = free thyroxine. FT4 is modelled as restricted cubic spline with 3 knots. Cox regression model I: Adjusted for age, sex and visit. Cox regression model II: Additionally adjusted for smoking, alcohol and diet. Cox regression model III: Additionally adjusted for BMI (as restricted cubic spline with 3 knots), blood pressure lowering medication , diabetes mellitus, physical activity and glomerular filtration rate. Cox regression model IV: Additionally adjusted for systolic and diastolic blood pressure.



We performed our analyses using four different models:
Model 1: basic model, adjusting for sex, age and cohort
Model 2 variables that represent only confounders
Model 3: variables that can act as mediators and confounders at the same time
Model 4: variables that act as a cardiovascular mediator in our relation
A: Exposure
C: Confounder
Y: Outcome
M: Mediators
M2: Variables that act as mediators and confounders

Figure S1. Directed acyclic graph.

A: Thyroid function.

Y: Brain perfusion.

M: Systolic and diastolic blood pressure as potential mediators.

C: Smoking, alcohol, diet, age, sex, cohort.

M2: BMI, diabetes mellitus type 2, physical activity, glomerular filtration rate and blood pressure lowering medication.

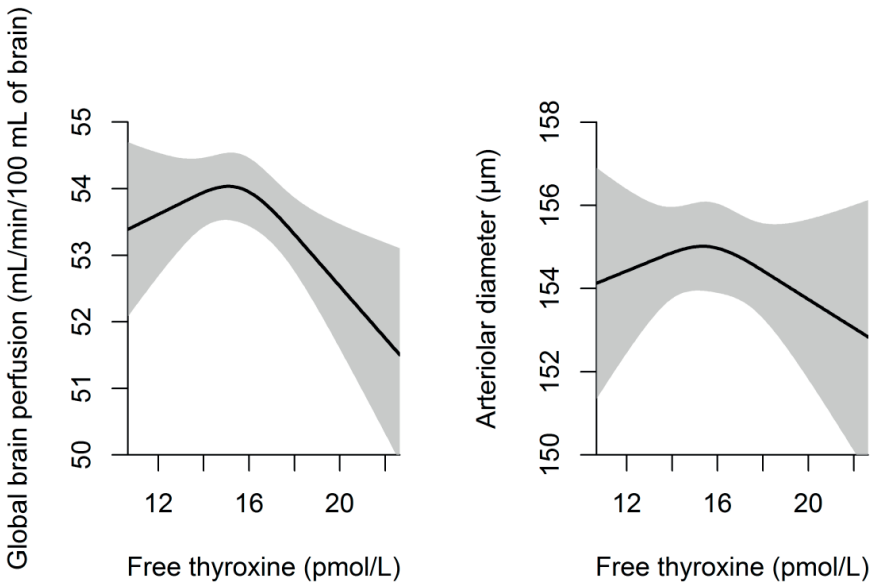


Figure S2. The association between free thyroxine and global brain perfusion and arteriolar diameter in euthyroid participants.

P-value for the association between FT4 and global brain perfusion: P=0.034.
P-value for the association between FT4 and arteriolar diameter: P= 0.257.
Euthyroid participants were defined as having TSH values ≥ 0.4 and ≤ 4.0 and without thyroxine medication use. A visual representation of free thyroxine and global brain perfusion with restricted cubic splines, adjusted for age, sex, study visit, time between blood measurement and MRI scan, smoking, alcohol and diet. The U-shaped association between free thyroxine and both global brain perfusion and arteriolar diameter is clearly visible, showing lower global brain perfusion and smaller arteriolar diameter for both lower and higher free thyroxine. The black line indicates the mean difference and gray area indicates the 95% confidence interval.

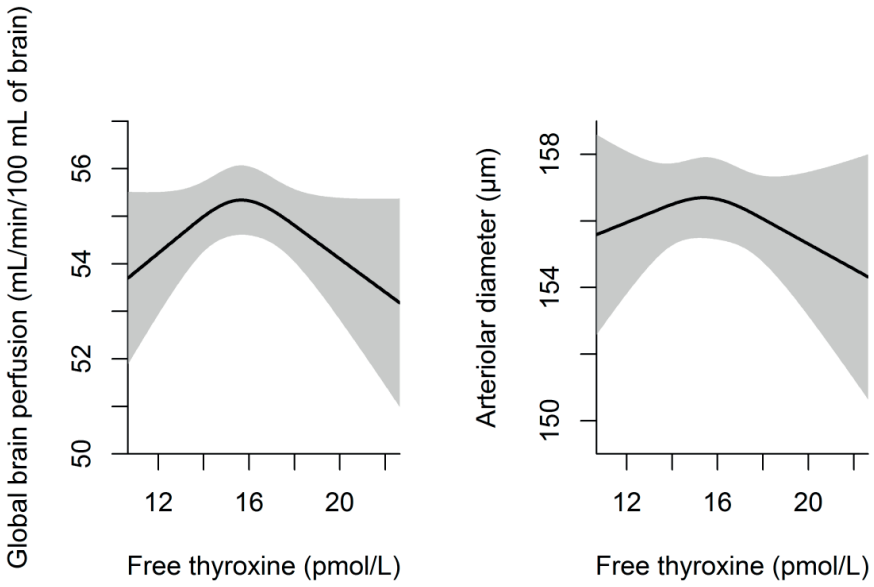


Figure S3. The association between free thyroxine and global brain perfusion and arteriolar diameter only in participants that received thyroid measurements and thyroid assessments at the same time (third cohort of Rotterdam Study).

P-value for the association between FT4 and global brain perfusion: P=0.036.
P-value for the association between FT4 and arteriolar diameter: P=0.170.
A visual representation of free thyroxine and global cerebral blood flow with restricted cubic splines, adjusted for age, sex, study visit, time between blood measurement and MRI scan, smoking, alcohol and diet. The U-shaped association between free thyroxine and both global cerebral blood flow and arteriolar diameter is clearly visible, showing lower global brain perfusion and smaller arteriolar diameter for both lower and higher free thyroxine. The black line indicates the mean difference and gray area indicates the 95% confidence interval.

Chapter 6
General Discussion

FINDINGS IN PERSPECTIVE AND METHODOLOGICAL CONSIDERATIONS

Neurodegenerative and vascular pathologies interact, which is clinically shown in the frequent co-occurrence of dementia and stroke. Therefore, widespread prevention measures of both dementia and stroke are becoming an inevitable necessity.[1,2] While stroke is an acute disorder with the key clinical feature of sudden onset of a focal neurological deficit, Alzheimer's disease (AD) is a progressive disorder, with blood-based biomarkers becoming increasingly important for the diagnosis and prognosis of AD. In the past, the only validated biomarkers for AD were amyloid and neurofibrillary tangles.[3] I aimed to uncover new promising biomarkers for AD in **chapters 2** and **4** using a population-based approach, with a focus on the role of immunity and inflammation.

I studied the balance between innate versus adaptive immunity in relation to the risk of dementia using blood cell counts in **chapter 2.1** and found that biomarkers reflecting this balance were associated with an increased dementia risk. I also found in **chapter 4.2** that these immune biomarker levels differ in *APOE* haplotype, which remains the risk factor with the largest effect for developing sporadic AD.

IMMUNITY AND DEMENTIA

To support the role of immunity in AD, I tried to triangulate our findings of **chapter 2.1** by performing a two-sample Mendelian Randomization (MR) Study (**chapter 2.2**). This study suggests however that lifelong elevated circulating immune biomarkers are not associated with AD risk or hippocampal volume. So is immunity then really playing a role in AD? Let us have a closer look at the findings in **chapter 2.1**. There I find that especially an increase of platelets over time leads to an increased risk of all-cause dementia, even when censoring for stroke. These estimates are higher for Alzheimer's disease and even higher for vascular dementia as outcomes. Therefore, I expected to find an association between platelets and Alzheimer's disease risk using MR. There are several potential methodological explanations as why I did not find this association with MR as outlined in **chapter 2.2**. However, it could also be that an increase in platelets solely is not causally related to AD, and that a larger immunity disruption is needed to cause AD, such as an additional disruption in the adaptive immune system. Indeed, I find in **chapter 2.2** that CD4 cell counts, important players of the adaptive immune system, show the strongest suggestive association with AD. This notion is supported by the findings in **chapter 2.1** where I show that a doubling in PLR, reflecting an imbalance in platelets and lymphocytes, is also significantly associated with AD and that a doubling of lymphocytes displays a protective effect for AD especially within *APOE* allele 4 carriers.

Several mechanisms could explain these associations. First, the classical vision on the innate immune system that it is merely the immediate mediator of host resistance and inflammation is switching into the notion that it also holds a memory similar to the adaptive immune system.[4] Although primarily a protective response by increasing resistance to reinfection, under predisposing conditions innate memory may endorse human diseases characterized by excessive inflammation.[5] Moreover, emerging evidence suggests that microglia are able to sense inflammatory signaling molecules originating outside the brain.[4,6] Thus, circulating innate immune myeloid cells could participate in the long-lasting dysregulated AD immune response by acting either directly when recruited to brain, or indirectly through the release of soluble mediators.[4,7] Second, regarding adaptive immunity, a study found that genetic ablation of peripheral immune cell populations significantly accelerates amyloid pathogenesis, worsens neuroinflammation, and alters microglial activation state.[8] They show that loss of IgG-producing B cells impairs microglial phagocytosis, thereby exacerbating amyloid deposition. Replacement of IgGs via direct injection or bone marrow transplantation reversed these effects and reduced Aβ pathology. Together, these results support the importance of the adaptive immune system and its interactions with microglia in the pathogenesis of AD.

But what triggers this imbalance in innate and adaptive immunity? A proposed new model suggests that infections of the brain, and infection elsewhere in the body that activates the immune system in the brain could be a potential trigger.[9] Particularly agents capable of producing an ineradicable infection, like herpesviruses, theoretically could trigger chronic neuroinflammation. In **chapter 2.4** I therefore linked herpes simplex virus type 1 (HSV-1) to AD, but found only an association between HSV-1 and risk of dementia within younger individuals. Several other microbes also have been linked to AD, including *Helicobacter pylori (H. pylori)*, which we also investigated **(chapter 2.3**). Similar to HSV-1, I found no association between *H. pylori* and AD risk in the total population, however risk estimates were higher in older participants, possibly due to exposure to more virulent strains in earlier years. Despite the null-findings considering infection and AD within the Rotterdam Study population, it is still plausible that infection leads to the formation of β-amyloid. For some investigators have suggested the "antimicrobial protection hypothesis", arguing that β-amyloid is capable of functioning as a natural antimicrobial peptide that is effective against not only viruses but also bacteria and fungi.[10] However, these smaller, soluble forms (oligomers) of β-amyloid are not only potential antimicrobials, they are also still neurotoxic. These neurotoxic effects typically become apparent only after age 60 years, potentially explaining its conservation throughout evolution for its beneficial effects earlier in life, at times where life expectancy was shorter and infectious diseases were important hazards.[9]

To assess causality of immune mechanisms with AD, several study designs were considered in **chapter 2**, with each having its own strengths and limitations. In **chapters 2.3** and **2.4**, I used the prospective cohort design using a single measurement for the determinant.

Limitations include reverse causation and residual confounding. In contrast to using a Cox regression model, the studies described in **chapters 2.1** and **3.1** again used the prospective cohort design but now using repeated measurements for the determinant using joint models. This statistical model is in particular useful here since the internal covariates (blood cell counts) are intermittently measured (biomarker levels are only known for the specific time points the participant visited the study center), and because they also contain measurement error, their complete path up to any time point t is not fully observed.[11] An important strength of the joint model is thus that we can successfully reconstruct the complete longitudinal history by postulating a suitable mixed-effects model to describe subject-specific time evolutions. The mixed model then accounts for the measurement error problem by postulating that the observed level of the blood cell count measurements equals the true level of the blood cell count measurements plus a random error term. However, since the survival function S depends on the whole history of the true marker levels (M), it is important to obtain a good estimate of M for an accurate estimation of S.[12] Thus, careful examination of data is important since effects could be for instance non-linear, as in **chapters 5.2** and **4.3**. Finally, we performed an MR study (**chapter 2.2**), a design in which genetic variants are used as 'instrumental variables' for a putative causal factor, theoretically minimizing the possible influence of confounding or reverse causality. If we seek to assess whether there is a causal effect of trait A (e.g. immune cells) on outcome B (AD), but not to provide an estimate of a causal effect parameter, then only the three instrumental variable assumptions listed in **Figure 1** are required. To estimate a causal effect parameter, further assumptions are required, namely 1) linearity of the risk factor-outcome association, and 2) the stable unit treatment value assumption (the value of the outcome for each individual depends on the value of the risk factor, and not on the mechanism by which the risk factor was intervened on). MR studies are often compared to randomized controlled trials (RCTs), but the causal effect estimate from an MR study should not be interpreted literally as the expected outcome of an intervention on the risk factor of interest. In almost all cases, we can only make inferences on the direction of the effect from an MR study rather than estimating an effect size.[13]

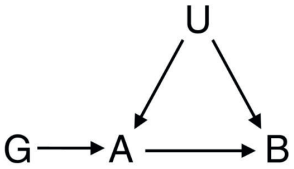


Figure 1. Diagram illustrating causal relationships between genetic variants G, putative causal trait (risk factor) A, putative effect trait (outcome) B, and confounders U necessary for instrumental variable assumptions to be satisfied.[13] The three instrumental variable assumptions are: 1) The set of genetic variants is associated with risk factor A; 2) Each genetic variant is independent of confounders U of the association between A and B; 3) If the risk factors were kept constant, intervention on the genetic variants would not have an effect on the outcome.

IMMUNITY AND STROKE

Besides the role of immunity in dementia, immunity disruption also seems to play a role in stroke development (**chapter 3**). Using a similar approach as in **chapter 2.1**, I studied how immune biomarkers relate to ASCVD risk since stroke shares many risk factors with other cardiovascular diseases although their relative importance varies (**chapter 3.1**).[14] In contrast to **chapter 2.1**, where an increase of platelets leads to the highest dementia risk, I found in **chapter 3.1** that granulocytes are the most important immune biomarkers for ASCVD risk, although platelets were still borderline significantly associated with a higher stroke risk, especially ischemic stroke. These findings suggest that immunity is indeed an overlapping mechanism between dementia and stroke, and that specifically platelets could form the bridge between the two diseases.[15] Not only do platelets play a role in vessel occlusion leading to stroke, they are also important players in inflammation.[16] Moreover, they express the amyloid precursor protein (APP) and display the complete enzymatic machinery to process APP proteins into β-amyloid as mentioned earlier.[17]

In addition to the role of platelets, in **chapter 3.1** I furthermore show that subclinical atherosclerosis as measured by carotid arterial calcifications potentially mediates the role of granulocytes in stroke risk. This suggests immunity-driven atherosclerosis as another important common mechanism between dementia and stroke.[18] Indeed, allelic variants in common genes including *APOE* predispose to both atherosclerosis and dementia.[18] Moreover, I found that innate immunity was linked to larger plaques, whilst the adaptive immunity (lymphocytes) related to smaller plaques and a lower frequency of intraplaque hemorrhage (**chapter 3.2**). I found similar protective effects of lymphocytes on all-cause dementia risk in **chapter 2.1**. Taken together, these results suggest that an imbalance in innate and adaptive immunity does not only play a role in dementia risk, but also in stroke risk through increased vulnerability to carotid atherosclerosis rupture.

Several methodological issues warrant consideration regarding the findings in this chapter. First, the mediation analysis presented in **chapter 3.1** presents large effect sizes, but they are not statistically significant. The assessed association between immunity and calcification is cross-sectional, is prone to measurement error and subject to reverse causality. I similarly used a cross-sectional design in **chapter 3.2**, highlighting that causality cannot be inferred solely based on these results. Second, multiple testing can be an issue because the more inferences are made, the more likely erroneous inferences are to occur. Several statistical techniques have been developed to deal with this issue, which generally require a stricter significance threshold for individual comparisons.[19] An example is the usage of the false discovery rate (FDR) as I have done in **chapter 2.2** when applying MR on multiple exposures or the usage of a Bonferroni correction as I have done in **chapter 4.2**.[19] However, in **chapters 3.1** and **3.2** I similarly perform multiple tests, where I associate multiple immune cells and their derived ratios with multiple outcomes, but I did not adjust for multiple

testing. This is because reducing the type I error for null associations also increases the type II error for those associations that are not null. Therefore, some researchers prefer not making adjustments for multiple comparisons because it will lead to fewer errors of interpretation when the data under evaluation are not random numbers but actual observations on nature, thereby avoiding the possibility of missing important findings.[20]

IMMUNITY: THE CONNECTION BETWEEN DEMENTIA AND STROKE

In **chapter 4.1** I have investigated how plasma β-amyloid and NfL relate to the risk of stroke within the Rotterdam Study and I found that higher levels of β-amyloid 40 were related to higher stroke risk in *APOE* ε4 carriers and within younger participants. Furthermore, higher levels of plasma NfL at baseline were associated with a higher risk of incident stroke in the total population. Interestingly, in **chapter 4.2**, I found that β-amyloid 40 and NfL were elevated in participants with higher immune biomarker levels, especially within *APOE* allele 4 carriers, suggesting that immunity disruption may be the trigger of β-amyloid 40 deposition in the vessel wall. In contrast to β-amyloid, blood NfL concentrations are non-specific, reflecting only underlying neuronal or axonal damage. This further underscores an early role of the immune system in neuronal damage, potentially inducing dementia and stroke.

An important methodological consideration is the validity of immune biomarker measurements in this thesis. The question is whether blood cell counts truly reflect the balance between innate versus adaptive immunity. Indeed, the immune system is extremely complex, and consists of many more components than blood cells. For instance, glial cells, more specifically microglia, are important players as immune cells of the central nervous system. However, there is not a paradigm for studying microglia within the Rotterdam Study. Therefore, I provide suggestions as to which biomarkers could additionally be measured to have a better reflection of the balance between innate and adaptive immunity in the **Future Research** section of the Discussion.

Another important consideration is the relative relevance of the findings in this thesis. For instance, in **chapter 4.2** the innate immunity phenotypes were related to higher Aβ, most strongly with a doubling in GLR leading to a 1.9% higher Aβ42 (95% confidence interval [95% CI] 0.4 to 3.3%) and 3.2% higher Aβ40 (95% CI 2.0 to 4.3%). These estimates, although statistically significant, are not very high. A valid question is therefore to what extent the immune system contributes to the onset of AD, or stroke, compared to other risk factors. The failures of several dementia prevention trials illustrates that identifying strong risk factors as potential targets for intervention is a field-wide problem. These trials have mainly focussed on reducing Aβ. Assuming the innate immune system and consequently the production of Aβ as a physiological process, it could be dangerous to inhibit this system to

prevent or treat AD. Regulating the more downstream effects of Aβ, however, could be more feasible considering the mere detrimental consequences of tau pathogenesis leading to tau-mediated neurodegeneration.[21] Thus, although effect sizes are generally small in this thesis, they do provide directions for further research, coming closer to successful interventions. At the end of this **Findings in perspective and methodological considerations** section, I therefore propose an updated model for AD pathogenesis, adding immunity to the other risk factors.

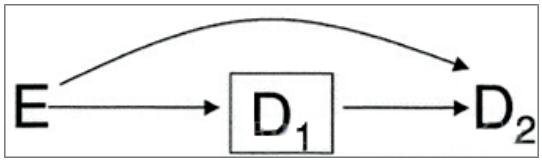
BRAIN PATHOLOGY

Another changing biomarker is tau, which is also assessed using blood-based assays within the Rotterdam Study. Total-tau has been previously assessed in association with dementia risk within the Rotterdam Study, where a J-shaped association was found. Similarly, in **chapter 4.1** we show a J-shaped association between this same biomarker and stroke risk within the Rotterdam Study. Interestingly, I found within **chapter 4.2** only an association between the platelet-to-lymphocyte ratio (PLR) and lower plasma total-tau levels within the same study population. This suggests a less clear role of the immune system in relation to tau, and suggests that tau changes are probably a consequence of β-amyloid abnormalities rather than being directly affected by the immune system. Since the PLR is only associated with higher β-amyloid and NfL within *APOE* allele 4 carriers, whereas these biomarkers are not altered in participants homozygous for *APOE* allele 3 and even lower within *APOE* allele 2 carriers, it could be that a lower plasma total-tau indeed reflects tau abnormalities within the brain when PLR is elevated. The reason that I only find a relation between the PLR and plasma total-tau, and not between the other immune biomarkers, could be because platelets are the primary source of β-amyloid in human blood (~90%)[22], and this secreted peptide is similar to those found in the senile plaques of Alzheimer's patients.[23]

Cognitive impairment is another important biomarker in the progression towards AD. Cognitive functions are mediated by specialized areas of neocortex distributed across the cerebral hemisphere in an orderly arrangement.[24] The cortex of each cerebral hemisphere is a continuous sheet of gray matter, which can be damaged by toxic peptides such as β-amyloid or by loss of neuronal integrity by the phosphorylation of tau. These aging-related processes can lead to cognitive impairment. Telomere length is an important biomarker for such aging-related processes, because telomeres shorten not only with each cell division, but also with aging and oxidative stress, both important factors in cognitive function.[25,26] As such, it has been found that community resident elders with longer telomere length exhibited less decline in global cognitive functioning over 7 years relative to elders with short or medium telomere length.[25] In contrast, another study has found that longer telomeres related to worse cognitive outcomes as CSF amyloid-β and tau increases.[27] In line with these contradictory

findings, I found in **chapter 4.3** that telomere length has a U-shaped association with AD risk, especially within *APOE* allele 4 carriers. However, telomere length seems to be related differently to stroke risk. I performed a preliminary analysis within the Rotterdam Study showing that telomere length is linearly associated with stroke, such that lower telomere length increases stroke risk. It could thus be that shorter telomere length reflects vascular pathology, while longer telomere length reflects more the AD-related brain pathology, the latter either through the upregulation of telomerase[27], or survival bias since shorter telomere length also increases risk of all-cause mortality (**Figure 2**).

a.



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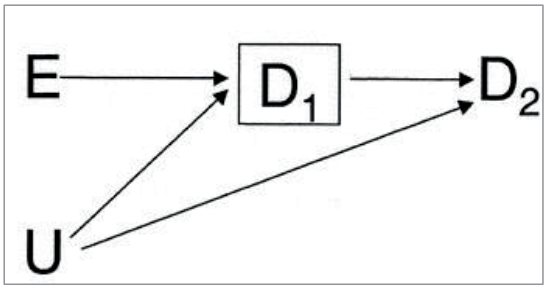


Figure 2a. Effect of telomere length (*E*) on survival (D_1) and dementia (D_2). Suppose the effect of exposure (longer telomere length) on D_1 is protective. Then the hazard at time 2 is the probability of dementia at time 2 among those who survived past time 1, i.e. D_1=0 (square around D_1 indicates this conditioning). The lack of an arrow from *E* to D_2 indicates that, although the exposure *E* has a direct protective effect (decreases the risk of death) at time 1, it has no direct effect on dementia at time 2. Exposure *E* would only have a protective effect on dementia at time 2 when there would be a direct arrow from E to D_2 as now drawn. **Figure 2b.** In the presence of an unmeasured shared cause of death and dementia, then the hazard ratio at time 2 is a biased estimate of the direct effect of exposure on dementia at time 2 from *E* to D_2 through D_1 and *U*. Modified from *Hernán, Miguel A.; Hernández-Díaz, Sonia; Robins, James M. Epidemiology15(5):615-625, September 2004.*

An important methodological issue needs to be taken into consideration, which is selection bias. This form of bias can be an issue in cohort studies, where informative censoring often takes place.[28] Informative censoring occurs when participants are lost to follow-up due to reasons related to the study. **Figure 2b** shows a scenario that is possible in cohort studies, where an unmeasured cause of death and dementia can be present. When I would only include survivors in the analyses, this would lead to a biased estimate for the telomere length – dementia relationship. However, when I would also include the non-survivors or

'censored' participants in the analyses by using their drop-out or mortality event as a study end-point (i.e. not conditioning on D_1), then the issue of informed censoring would be solved by closing the non-causal path from E to D_2 through D_1 and U as shown in **Figure 2**. I have correctly done so in **chapter 4.3**.

BRAIN HEMODYNAMICS

Changes in brain vasculature are one of the earliest changing biomarkers leading to AD progression. Indeed, a multifactorial data-driven analysis using the Alzheimer's Disease Neuroimaging Initiative (ADNI) data suggested that vascular dysregulation is an early step that leads to late-onset AD.[29] These data are supported by a study within the Rotterdam cohort showing that low global brain perfusion precedes cognitive impairment.[30] Since a large proportion of dementias can be prevented by preventing stroke[1], it is important to maximize our understanding of risk factors for stroke, moving beyond atherosclerosis and thromboembolism. While a reduced global brain perfusion is a risk factor for transient ischemic attack (TIA) in the general population (**chapter 5.1**), results in **chapter 5.1** also suggest that a reduced brain perfusion is only a risk factor for ischemic stroke within individuals with elevated diastolic blood pressure. These findings can be translated to a causal pie (**Figure 3**), where a reduction in global brain perfusion is sufficient to cause a TIA in middle- and older aged individuals, while an extra slice (i.e. component cause) is needed for the occurrence of a stroke.

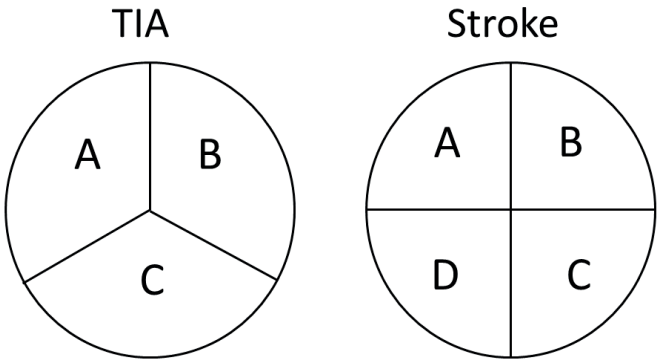


Figure 3. A TIA and stroke consist of a variety of overlapping component causes. Component causes A-C add up to sufficient cause for TIA and component causes A-D add up to sufficient cause for stroke. In this example component A may consist of a variety of cardiovascular risk factors, and component B of genetic risk. The findings from our study suggest that the addition of component cause C, in this case lowered brain perfusion, completes the causal pie for a TIA (i.e. leads to a TIA) whereas another component cause (i.e. D) is still required to lead to a stroke.

Moreover, since a reduced global brain perfusion seems to be a shared risk factor of dementia and stroke, it is of utmost importance to identify individuals that are at increased hemodynamic risk for developing these brain diseases. In **chapter 5.2** I showed that thyroid function acts as a promising determinant to intervene on brain perfusion.

The still hypothetical nature of brain perfusion mechanisms in AD and stroke highlights the impact of several methodological issues that slow the understanding of brain hemodynamic deficits in these brain diseases. First and foremost, the assessment of global brain perfusion using phase-contrast imaging within this population-based setting has limitations in comparison to other methodologies such as arterial spin labelling[31] or radioactive water perfusion measurements using PET.[32] There may be meaningful inhomogeneities of perfusion among brain regions that is not reflected by the average perfusion of the carotid and basilar arteries as measured by phase-contrast imaging. The lack of association between global brain perfusion and ischemic stroke thus remains to be confirmed. Second, I used retinal vessels as proxy for the brain microvasculature. If this can be extrapolated to the entire brain microvasculature remains an open question in the field, but over the years, it has been thought that these structures provide a direct measure for the vascular and neuronal status of the brain.[33] Reduced blood flow following Poiseuille's equation can be produced by altering vessel diameter, reducing viscosity and altering vessel length. I found an inverted U-shaped association between free thyroxine and arteriolar retinal vessel diameter, similar to global brain perfusion in **chapter 5.2**. Therefore, in the context of brain perfusion, I used retinal vessel diameter in this chapter as additional parameter of brain hemodynamics, although the effect is only within the retina and static, whereas with true autoregulation vessel diameter is naturally dynamic. Third, although most ischaemic stroke is due to embolism, there are also other major causes of stroke such as small vessel disease or other arterial diseases.[14] Unfortunately I was not able to further study ischemic stroke subtypes by stroke etiology due to the heterogeneous assessment of etiology within hospitals and due to a lack of power of stroke etiological subtypes.

Taking all these findings including their methodological considerations together, I propose a hypothetical updated Jack model of AD biomarkers to include the role of the immune system in addition to the more established roles of brain vasculature, amyloid PET, CSF β-amyloid and tau in AD (**Figure 4**). This model proposes that immunity is one of the changing biomarkers that leads to AD progression, but its exact position remains to be clarified. In the following sections, I will discuss how future research could shed more light on the position of immunity and inflammation within AD progression and stroke.

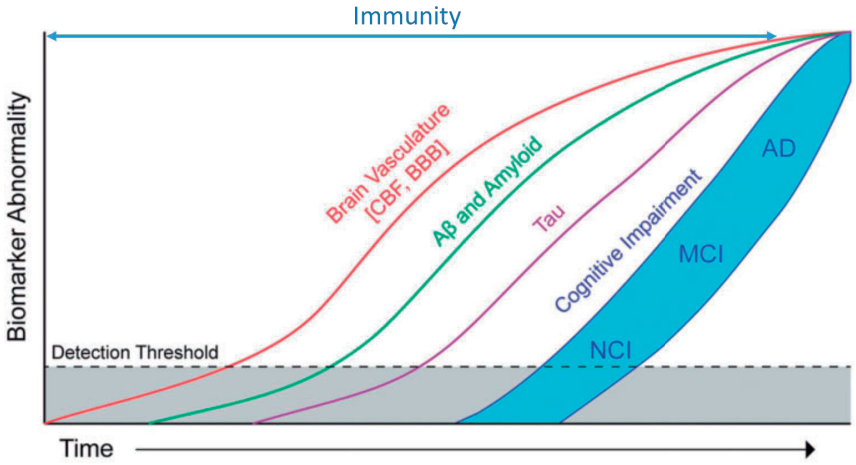
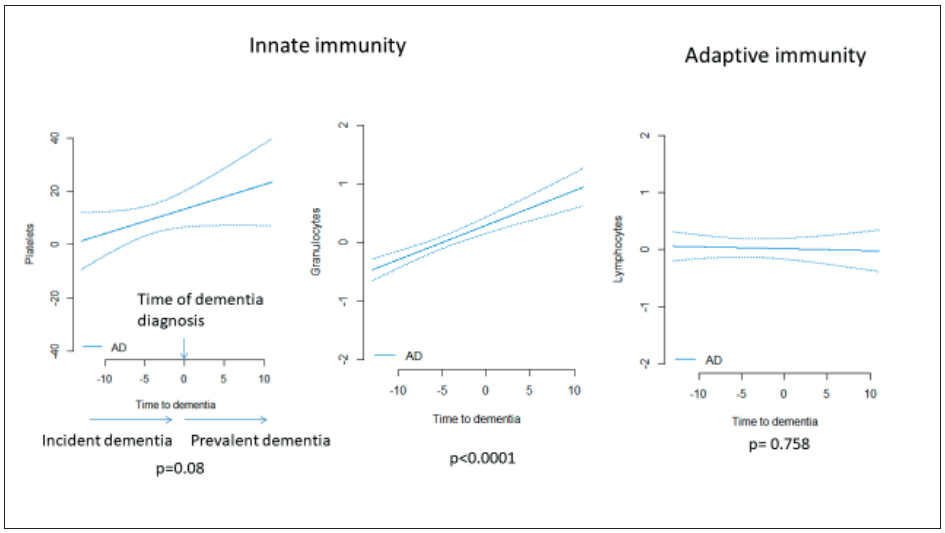


Figure 4. Hypothetical updated Jack model of Alzheimer's disease biomarkers to include the role of immunity. Hypothetical model of AD biomarker changes illustrating that immune changes may contribute to the initial stages of AD pathophysiological progression from NCI to MCI to AD. Abbreviations: AD, Alzheimer's disease; MCI, mild cognitive impairment; NCI, no cognitive impairment. *Modified from Hachinski V, Einhaupl K, Ganten D, et al. Special topic section: linkages among cerebrovascular, cardiovascular, and cognitive disorders: Preventing dementia by preventing stroke: The Berlin Manifesto. Int J Stroke 2019: 1747493019871915.*

FUTURE RESEARCH

To increase our knowledge on the mechanisms underlying dementia and stroke, the most obvious approach is to first reveal the order in which the different components of brain pathology occur, and in particular when inflammation and immunity changes occur. **A statistical technique that can be used for this purpose is an event-based model** (EBM), which is a class of interpretable disease progression models that estimate the timeline of neuropathologic change during AD progression.[34,35] A specific EBM technique, namely discriminative event based model (DEBM), allows inferring the order in which biomarkers for a disease become abnormal based on cross-sectional data, taking into account disease heterogeneity to a larger extent as compared to existing EBM methods.[35] But in order to use this model, it is important to test the assumptions of the DEBM. I propose a method for doing so in **Box** 1. I tested the assumption of a constant increase or decrease of markers over time by constructing a linear mixed model with random slope and intercept and observing if the slopes of the prevalent and incident AD subjects go up or remain constant (**Box 1**). As an example, I show that the assumptions are only met for innate immunity markers. Nevertheless, DEBM provides a useful tool for estimating ordering of biomarker changes provided that the assumptions are met. For this, using more specific adaptive immunity markers that show a constant increase or decrease over time is crucial.



Box 1. Testing the model assumptions of a discriminative event based model.

Conflicting data on the ordering of a disruption in the innate and adaptive immune system in amyloid metabolism have been reported, and remains limited to experimental studies.[36] I aimed to use a novel method for estimating central ordering of events, using the discriminative event-based model applied within the Rotterdam Study.[35] As markers of the innate immunity I used platelet counts and granulocyte counts and for the adaptive immunity we used lymphocyte counts. In order to use this model, the assumption of a constant increase or decrease of immune markers over time must be met. I tested this assumption by building a linear mixed model with non-AD subjects only to predict the effect of age and sex for granulocyte counts (1). Next, I found the residuals in demented subjects by running a model where I substracted the predicted granulocyte counts from model 1 from the raw granulocyte counts. (2). As a last step I would fit the residuals in a linear mixed model with time to dementia as the exposure variable (3). Results for innate and adaptive immunity markers are shown below, showing that the assumptions are only met for innate immunity markers.

Within this thesis, I have mainly used easily obtainable measures of immunity, which allow for repeated measurements in large samples. However, these measures are fairly gross and simplify the complexity of the immune system. This is especially the case for the adaptive immune system, for which I used lymphocyte count as proxy measure. But lymphocytes include natural killer cells, T cells, and B cells, and thus form a heterogeneous group with distinct functions.[37] Of these lymphocyte subsets, T cells seem the most promising target for AD as well as atherosclerosis so far.[38,39] T cells are involved in cell-mediated immunity, with T helper cells producing cytokines that direct the immune response, while cytotoxic T cells produce toxic granules.[40] A first step thus would be **to measure lymphocyte subsets, with a special focus on T cells and further T cell subtyping.** More specifically, in this thesis I suggest in **chapter 4.2** that in particular CD4 T cells are worthwhile exploring further. A recent study showed that CD4 T cells play an important role in microglia maturation which

in turn affects neuronal synapse maturation[41], and another study showed that plaques from symptomatic patients (recent stroke or transient ischemic attack) were characterized by a distinct subset of $CD4^+$ T cells.[39] The old-fashioned notion that the brain is cut off from the peripheral immune system by the blood-brain-barrier (immune privileged) is being reconsidered to the idea that there is truly immune surveillance of the brain.[42] There is promising evidence that temporarily reducing immunosuppressive mechanisms helps release effector T cell activity[43], and at the same time, maintains a sufficient number of anti-inflammatory cells in the periphery.[44] This allows trafficking of inflammation-resolving cells to the sites of brain pathology, in which they play an essential role.[45]

As mentioned earlier, also the measures used for the innate immune system could be expanded. For instance, translocator protein 18 kDa is a marker of activated glial cell response and could be measured using positron emission tomography in a population-based setting.[46-49] **Measuring markers for glial cells that could be detected by imaging therefore may serve as a solution for measuring innate immunity in the CNS.**

In addition to targeting T cells and glial cells, the antimicrobial hypothesis of AD poses that long-lasting infections lead to β-amyloid accumulation, so another approach is to target infections or pathogens to prevent chronic neuroinflammation. Another rationale for this is that there has been a decline in dementia incidence across Western high-income countries.[50] This decline could partly be explained by favorable trends in vascular risk factors. Concomitantly, there has been a decline of mortality by infectious diseases during the 20th century, which could be attributed to improved socio-economic indicators, or use of antibiotics.[51] There has been one randomized controlled trial to study the effect of antibiotics on the progressive decline of cognitive function in patients with AD. Combination antimicrobial therapy with doxycycline and rifampicin significantly reduced cognitive worsening after 6 months but not after 12 months.[52] Future researchers could **investigate the hypothesis that certain antibiotics have a protective effect on the development of dementia**.

To further reveal the order in which the different components of brain pathology occur, and in particular the position of inflammation and immunity changes, is by studying the relation between brain circulation and the immune system. Within the Rotterdam Study, there are ongoing efforts to add arterial spin labelling as a noninvasive measurement of regional cerebral blood flow (CBF).[53] The possibility of **taking these regional variations in CBF into account will allow for studying CBF physiology in relation to immunity and inflammation** within a population-based setting. In addition, it facilitates disentangling the effect of regional CBF on brain outcomes and the determinants that cause regional CBF changes.[54]

Eventually, the goal is to prevent dementia and stroke by understanding pathophysiology and determining clinical relevance of newly discovered pathways, such as the role of immunity and inflammation. This requires a **close collaboration between dementia and**

stroke researchers, epidemiologists, neuroscientists, infection & immunity experts and geneticists. This is crucial for facilitating translational research.

CONCLUSION

In this thesis, I have shown that immunity and inflammation play a crucial role in dementia and stroke. Furthermore, I describe how future research can build upon this work. The prevention of dementia and stroke can be boosted by enhancing our understanding of the order in which different components of brain pathology and hemodynamic changes occur. In particular, the position of immunity and inflammation changes within this cascade warrants further research.

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Chapter 7
Summary & Samenvatting

SUMMARY

The etiological similarity between dementia and stroke was already suggested in Alois Alzheimer's first major article on dementia, describing that those cases of atrophy without recognizable infarcts were due to microscopic infarcts. The similarity between dementia and stroke has been further confirmed by the evidence of substantial drops of dementia in high-income countries where stroke incidence and mortality has declined substantially. But what the exact underlying mechanism is of these two diseases is yet unknown. In this thesis, I examine immunity and inflammation as potential common mechanisms connecting dementia and stroke.

In the first part of this thesis, I demonstrate the role of an imbalance in the immune system towards innate immunity in the pathogenesis of dementia by showing that an increase of components of innate immunity increase dementia risk and an increase of components of adaptive immunity is protective for dementia risk in the general population (**chapter 2.1**). In **chapter 2.2** I did not find evidence to support associations of circulating biomarkers of immunity and inflammation with Alzheimer's disease (AD) and hippocampal volume, but CD4 count, an important player of adaptive immunity, showed the strongest suggestive association with AD. In **chapters 2.3** and **2.4** I found no evidence of *Helicobacter pylori* or herpes simplex type 1 infections to be associated with dementia risk in the general population.

Focussing next on the role of immunity and inflammation in stroke, I show in **chapter 3.1** that an increased granulocyte count reflecting innate immunity was associated with a higher risk of atherosclerotic cardiovascular disease (ASCVD) in the general population. Moreover, higher levels of granulocytes were associated with larger volumes of arterial calcification, with a mediation analysis suggesting that arterial calcifications may explain a proportion of the link between granulocytes and ASCVD. A causal relationship is supported by effects of granulocytes and other immune markers on plaque thickness and plaque components in **chapter 3.2**, suggesting that an imbalance in innate and adaptive immunity may play a role in the vulnerability of the carotid atherosclerotic plaque.

Chapter 4.1 further connects dementia and stroke by finding that participants with higher total-tau and neurofilament light chain at baseline as markers of AD-related brain pathology had a higher risk of stroke. In addition, higher levels of plasma amyloid-β (Aβ) 40 were associated with an increased stroke risk in *APOE-ε4* carriers only. Supporting the role of accumulating brain pathology from an immunity and inflammation perspective in the etiology of dementia and stroke, I found in **chapter 4.2** that higher levels of immunity markers were associated with higher Aβ in plasma. I further show that participants with a higher genetic predisposition to AD had higher immunity markers, where these effects were mainly driven by *APOE*-ε4. In contrast to the more traditional markers of AD-related brain pathology, I also studied telomere length as a promising marker of brain pathology in

chapter 4.3, where I found a U-shaped association between telomere length and risk of AD in the general population and even more strongly so in *APOE*-ε4 carriers.

In **chapter 5**, I shift focus to brain hemodynamics. I found in **chapter 5.1** that lower global brain perfusion was associated with a higher risk of transient ischemic attack, but not with the risk of ischemic stroke, in the general population. This association may be modified by wider arteriolar and venular retinal calibers for transient ischemic attack and elevated blood pressure for ischemic stroke. To identify a potentially amendable determinant to intervene on global brain perfusion, we studied thyroid function in **chapter 5.2** and we found that global brain perfusion was lower in middle-aged and elderly with lower as well as higher FT4 levels. Furthermore, arteriolar retinal vessel diameters were narrower in elderly with higher TSH and TPO levels.

To conclude, I discuss in **chapter 6** these main findings where I have shown that immunity and inflammation play a crucial role in dementia and stroke in light of published literature, and provide methodological considerations. Furthermore, I describe how future research can build upon this work by proposing that the prevention of dementia and stroke can be boosted by enhancing our understanding of the order in which different components of brain pathology and hemodynamic changes occur. Also the position of immunity and inflammatory changes within this cascade are important questions to be answered for future researchers.

SAMENVATTING

De etiologische gelijkenis tussen dementie en hersenberoertes kwam al naar voren in Alois Alzheimer's eerste grote artikel over dementie, waarin hij beschrijft dat patiënten met hersenatrofie zonder duidelijke infarcten waarschijnlijk microscopische infarcten hebben. De gelijkenis tussen dementie en hersenberoertes kwam verder naar voren bij de zichtbare afname van dementie in welvarende landen waar de incidentie van hersenberoertes en mortaliteit substantieel is afgenomen. Wat echter het exacte onderliggende mechanisme is van deze twee fascinerende aandoeningen is nog onduidelijk. In dit proefschrift beschouw ik het immuunsysteem en inflammatie als potentiële overlappende mechanismen die dementie en hersenberoertes kunnen verbinden.

In het eerste deel van dit proefschrift demonstreer ik de rol van een disbalans in het immuunsysteem richting het aangeboren immuunsysteem in de pathogenese van dementie door te laten zien dat een toename van componenten van het aangeboren immuunsysteem het risico op dementie verhogen terwijl een toename van componenten van het aangeleerde immuunsysteem beschermend is tegen het krijgen van dementie (**hoofdstuk 2.1**). In **hoofdstuk 2.2** heb ik geen bewijs gevonden die het verband tussen het immuunsysteem en Alzheimer ondersteunen, maar CD4 cellen, belangrijke spelers van het aangeleerde immuunsysteem, laten de sterkste suggestieve associatie zien met Alzheimer. Kijkend naar de rol van infectieziekten in het ontstaan van dementie, vond ik in **hoofdstukken 2.3** en **2.4** geen associatie tussen de maagbacterie *Helicobacter pylori* of het virus herpes simplex type 1 en dementie in de normale bevolking.

De focus verleggend naar de rol van het immuunsysteem en inflammatie in hersenberoertes, laat ik in **hoofdstuk 3.1** zien dat een toename van granulocyten, die het aangeboren immuunsysteem weerspiegelen, geassocieerd is met een hoger risico op het krijgen van cardiovasculaire aandoeningen. Via een mediatie analyse laat ik zien dat dit verband mogelijk voor een groot deel door de vorming van aderverkalking wordt veroorzaakt. Een causaal verband wordt ondersteund door te kijken naar de effecten van onder andere granulocyten op de dikte van aderverkalking en losse componenten van aderverkalking in **hoofdstuk 3.2**. Deze resultaten suggereren dat een disbalans in het aangeboren en aangeleerde immuunsysteem een rol zouden kunnen spelen in de kwetsbaarheid van aderverkalking om te scheuren.

Hoofdstuk 4.1 legt verder de verbinding tussen dementie en hersenberoertes door de bevinding dat mensen met meer total-tau en neurofilament light chain (biomarkers van Alzheimer-gerelateerde hersenpathologie) een hoger risico hebben op het ontwikkelen van hersenberoertes. Daarnaast zijn hogere spiegels van plasma amyloid-β (Aβ) 40 geassocieerd met een verhoogd risico op het krijgen van hersenberoertes in *APOE-ε4* dragers. Kijkend vanuit het perspectief van het immuunsysteem, heb ik verder in **hoofdstuk 4.2** gevonden dat hogere immuun- en inflammatiewaardes geassocieerd zijn met hogere Aβ in plasma. Verder hebben mensen met een verhoogd genetisch risico op het ontwikkelen van Alzheimer

ook verhoogde immuun- en inflammatiewaardes. Deze effecten worden vooral gedreven door *APOE*-ε4. In tegenstelling tot de meer traditionele markers van Alzheimer-gerelateerde breinpathologie heb ik ook telomeerlengte bestudeerd als een potentiële biomarker in **hoofdstuk 4.3**, waarbij ik heb gevonden dat er een U-vormige associatie is tussen telomeerlengte en het risico op het ontwikkelen van Alzheimer, waarbij deze associatie nog sterker is in *APOE*-ε4 dragers.

In **hoofdstuk 5** verleg ik de aandacht naar breinhemodynamica. Ik heb in **hoofdstuk 5.1** gevonden dat een lagere algehele breinperfusie geassocieerd is met een hoger risico op het krijgen van een transient ischemic attack, ofwel een TIA, maar dat het risico niet verhoogd is op het krijgen van een ischemische hersenberoerte. Deze associatie werd gemodificeerd door wijdere retinale arteriolen en venulen voor het krijgen van een TIA en door een hogere bloeddruk voor het krijgen van een ischemische hersenberoerte. Om een potentiële modificeerbare determinant te vinden om algehele breinperfusie te beïnvloeden, hebben we gekeken naar schildklierfunctie in **hoofdstuk 5.2** en heb ik gevonden dat de algehele breinperfusie lager was in ouderen met lagere alsook hogere FT4 spiegels. Verder waren de retinale arteriolen ook smaller in ouderen met hogere TSH en TPO spiegels.

Tot slot bediscussieer ik in **hoofdstuk 6** de bevindingen waarin ik laat zien dat het immuunsysteem en inflammatie een cruciale rol spelen in de ontwikkeling van dementie en hersenberoertes met inachtneming van de gepubliceerde literatuur en bespreek ik methodologische overwegingen en factoren. Ik beschrijf verder hoe toekomstig onderzoek kan bouwen op dit werk door voor te stellen dat de preventie van dementie en hersenberoertes een boost kan krijgen door te bestuderen wat de volgorde is van het veranderen van verschillende componenten van brein pathologie en hemodynamica. Wat de positie is van immuunsysteem- en inflammatieveranderingen in deze cascade is een cruciale vraag om door toekomstige onderzoekers beantwoord te worden.

Chapter 8
Appendices

PHD PORTFOLIO

Name PhD student: L. Fani **Erasmus MC Department: Epidemiology** **Research School: Netherlands Institute for Health Sciences** (NIHES)	**PhD period: February 2017 – July 2020** **Supervisors: Professors M.A. Ikram and M.K. Ikram**

1. PhD training	Year	ECTS
General courses		
Master of Science in Clinical Epidemiology (NIHES)	2017-2018	70
R course	2017	1.8
Scientific integrity (Erasmus MC)	2017	0.3
ESP62 Markers and Prediction Research (NIHES)	2019	0.7
Seminars and workshops		
Departmental journal club	2017-2020	2.0
Departmental seminars (Epidemiology & Neurology)	2017-2020	4.0
Chair of the seminar committee	2017-2019	1.0
8th Mix & Match meeting of Alzheimer Nederland	2017	0.5
2de gezamenlijke wetenschappelijke vergadering van de Neurovasculaire Werkgroep en het Neurovasculair Genootschap	2018	0.5
International conferences		
The 8th International Conference of The International Society of Vascular Behavioural and Cognitive Disorders (VASCOG)**. Amsterdam, NL**	2016	1.0
4th European Stroke Organisation Conference (ESOC)**. Gothenburg, Sweden**	2018	2.0
9th International Conference of The International Society of Vascular Behavioural and Cognitive Disorders. Hong Kong	2018	2.0
5th European Stroke Organisation Conference (ESOC)**. Milan, Italy**	2019	2.0
Virtual Alzheimer's Association International Conference (AAIC)	2020	1.0
Research visits		
CoSTREAM consortium meeting. Cambridge, UK	2017	1.0
Institute for Stroke and Dementia Research (ISD) **- LMU Klinikum, Ludwig-Maximilians-University in Munich, Germany**	2020	

2. Teaching	Year	ECTS
Teaching assistance		
SPSS and/or R computer practicals of the one-week Biostatistical Methods I	2019	0.5
ESP65 Computer Practical of Practice of Epidemiologic Analysis (NIHES)	2019	0.5

Project supervision		
Master's project: Vitamin D Status and Risk of Stroke	2019	1.5
Master's project: HSV1 and risk of dementia	2020	1.5
3. Other activities		
Peer-review	2018-2020	2.5
Scan appraisal for incidental findings in population imaging	2018-2020	2.0
Stroke data collection of the Rotterdam Study	2017-2020	4.0
Dementia screening with structured interview (CAMCOG/ CAMDEX)	2017-2020	1.5
Physician at ERGO centre performing exit interview	2017-2020	3.0

*1 ECTS (European Credit Transfer System) equals a workload of 28 hours

LIST OF PUBLICATIONS

Fani L, Ahmad S, Ikram MK, Ghanbari M, Ikram MA.: Immunity and amyloid beta, total tau and neurofilament light chain: Findings from a community-based cohort study. Alzheimers Dement. 2020 Nov 20.

Fani L, van der Willik KD, Bos D, Leening MJG, Koudstaal PJ, Rizopoulos D, Ruiter R, Stricker BHC, Kavousi M, Ikram MA, Ikram MK.: The Association of Innate and Adaptive Immunity, Subclinical Atherosclerosis, and Cardiovascular Disease in the Rotterdam Study: A Prospective Cohort Study. PLoS Med. 2020 May 7;17(5):e1003115.

Fani L, Hilal S, Sedaghat S, Broer L, Licher S, Arp PP, van Meurs JBJ, Ikram MK, Ikram MA.: Telomere Length and the Risk of Alzheimer's Disease: The Rotterdam Study. J Alzheimers Dis. 2020; 73(2): 707–714.

Fani L, Bos D, Mutlu U, Portegies MLP, Zonneveld HI, Koudstaal PJ, Vernooij MW, Ikram MA, Ikram MK.: Global Brain Perfusion and the Risk of Transient Ischemic Attack and Ischemic Stroke: The Rotterdam Study. J Am Heart Assoc. 2019 Apr 2; 8(7): e011565.

Fani L*, van der Willik KD*, Rizopoulos D, Licher S, Fest J, Schagen SB, Ikram MK, Ikram MA.: Balance between innate versus adaptive immune system and the risk of dementia: a population-based cohort study. J Neuroinflammation. 2019 Mar 30;16(1):68.

Fani L, Wolters FJ, Ikram MK, Bruno MJ, Hofman A, Koudstaal PJ, Murad SD, Ikram MA.: Helicobacter pylori and the risk of dementia: A population-based study. Alzheimers Dement. 2018 Oct;14(10):1377-1382.

Berghout BBP, Fani L, Ikram MK.: Response by Berghout et al to Letters Regarding Article, "Vitamin D Status and Risk of Stroke: The Rotterdam Study".: Stroke. 2019 Dec;50(12):e432.

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Mens MMJ, Heshmatollah A, Fani L, Ikram MA, Ikram MK, Ghanbari M. Circulatory MicroRNAs as Potential Biomarkers for Stroke Risk: The Rotterdam Study. Stroke. 2021 Feb 10:STROKEAHA120031543.

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van der Willik KD, Ghanbari M, Fani L, Compter A, Ruiter R, Stricker BHC, Schagen SB, Ikram MA. Higher Plasma Amyloid-β Levels Are Associated with a Higher Risk of Cancer: A Population-Based Prospective Cohort Study.: Cancer Epidemiol Biomarkers Prev. 2020 Oct;29(10):1993-2001.

Turner DT, Hu MX, Generaal E, Bos D, Ikram MK, Heshmatollah A, Fani L, Ikram MA, Penninx BWJH, Cuijpers. Physical Exercise Interventions Targeting Cognitive Functioning and the Cognitive Domains in Nondementia Samples: A Systematic Review of Meta-Analyses.: Geriatr Psychiatry Neurol. 2020 Apr 15:891988720915523.

Licher S, van der Willik KD, Vinke EJ, Yilmaz P, Fani L, Schagen SB, Ikram MA, Ikram MK.: Alzheimer's disease as a multistage process: an analysis from a population-based cohort study. Aging (Albany NY). 2019 Feb 27;11(4):1163-1176.

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Georgakis MK, de Lemos JA, Ayers C, Wang B, Björkbacka H, Pana TA, Thorand B, Sun C, Fani L, Malik R, Dupuis J, Engström G, Orho-Melander M, Melander O, Boekholdt SM, Zierer A, Elhadad MA, Koenig W, Herder C, Hoogeveen RC, Kavousi M, Ballantyne CM, Peters A, Myint PK, Nilsson J, Benjamin EJ, Dichgans M. Association of Circulating Monocyte Chemoattractant Protein-1 Levels With Cardiovascular Mortality: A Meta-analysis of Population-Based Studies. JAMA Cardiol. 2020 Nov 4.doi: 10.1001

Fani L, Bak S, Delhanty P, van Rossum EF, van den Akker EL.: The melanocortin-4 receptor as target for obesity treatment: a systematic review of emerging pharmacological therapeutic options. Int J Obes (Lond). 2014 Feb;38(2):163-9.

Fani L, de Jong TH, Dammers R, van Veelen ML.: Endoscopic third ventriculocisternostomy in hydrocephalic children under 2 years of age: appropriate or not? A single-center retrospective cohort study. Childs Nerv Syst. 2013 Mar;29(3):419-23.

ABOUT THE AUTHOR

Lana Fani was born on the 15th of June 1991 in Capelle aan den IJssel, the Netherlands. She grew up in a loving family of Syrian origin, in Rotterdam, and followed bilingual (English A1 higher level) education at the Wolfert van Borselen scholengroep. During her final years at high school she was introduced to the brain by following a Pre-University Class at the University of Leiden on Psychology, after which she started studying Medicine at the Erasmus University Rotterdam. She became an active member of the International Federation of Medical Students' Assocations (IFMSA) by joining the National Board of IFMSA-NL, while also working as a translator between doctors and patients for the Arabic (Middle Eastern) language at Erasmus MC. Her career in research started simultaneously early during her studies by working as a student-assistant at the department of Neurosurgery, which resulted in a first author publication in a peer-reviewed international medical journal. Her second first-author publication during her Bachelor followed soon after performing a systematic review at the department of Pediatric Endocrinology. Due to her increasing interest in research and the brain she followed the Neuroscience Research Master's program at Erasmus MC. After that, she completed her clinical rotations, and started as a PhD candidate at the Department of Epidemiology at Erasmus MC. During that period, she obtained a Research Master's degree in Clinical Epidemiology at the Netherlands Institute of Health Sciences. Eager to learn new research methods and to work in different environments, she performed part of the work of her thesis at the Ludwig-Maximilians-Universität in Munich (Germany) at the Institute for Stroke and Dementia Research, supported by a personal fellowship of the Dutch Alzheimer Foundation. During the completion of her thesis, she started working as an Internal Medicine Physician at Ikazia Hospital Rotterdam. Lana enjoys playing the piano in her free time. Her goal is to become a Neurologist, combining clinical and research activities in an academic setting.

EPILOGUE

The work in this thesis could not have been made possible without the guidance and support of many inspiring people.

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