Aged Arteries and B-vitamins

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*"Doch door wetenschap bereikt men veel,
doch slechts de liefde voert tot volmaaktheid"*

Rabindranath Tagore 1861-1941

CONTENTS

Chapter **1**

Introduction

1   INTRODUCTION

Cardiovascular disease is a very common condition, especially among elderly [1, 2]. Cardiovascular risk prediction via the Framingham risk score is the most commonly used risk prediction [3]. However, the Framingham risk score has only been validated until the age of 75 and the power of the classical risk factors have been shown to decline with advancing age [4, 5].

   A strong predictor of cardiovascular disease in older populations is arterial stiffness. Arterial stiffness is involved in the pathogenesis of cardiovascular disease, with pulse wave velocity being the major marker for cardiovascular risk [6-10]. Over the years, arterial stiffness have been shown to be an important pre-clinical stage of cardiovascular disease of interest for research, mainly based on preventive strategies. Arterial stiffness is associated with many lifestyle factors and disorders, among others smoking [11], vitamin D deficiency [12], physical functioning [13], renal failure [14], diabetes [15, 16] and constructive obstructive pulmonary disease (COPD) [17].

Arterial stiffness is a condition, which is caused by a disturbance in the interaction between stable and dynamic changes of the vessel wall [18]. These changes can be influenced by both hemodynamic forces, as well as by other external factors such as hormones. Also ageing itself leads to changes in the vascular tree that contribute to the arterial stiffening process [18]. The stability of the vascular wall depends on two proteins: collagen and elastin. When misbalance occurs due to for example inflammatory stimuli or hypertension, collagen is overproduced, which in turn leads to less elastin in the vascular wall, with vascular stiffness as a consequence [19]. Collagen and elastin are regulated by catabolic matrix metalloproteases (MMPs). Both vascular cells and inflammatory cells like macrophages produce MMPs. The balance between MMP-inhibitors and MMP-producers is important in controlling the remodeling of the vascular wall [20]. Another important pathway of the arterial stiffening process is through advanced glycation endproducts (AGEs) [21]. AGEs form irreversible cross-links in collagen. This collagen derived from AGEs is stiffer, not easy to break down and it therefore leads to an accumulation of this type of collagen with vascular stiffness as a consequence [22]. AGEs are also able to affect the endothelial function by stimulating stress and inflammatory responses, increasing radical oxidant formation, pro-inflammatory cytokines and vascular adhesion molecules [23]. This subsequently may lead to vascular stiffness via MMP signaling [24] or to endothelial dysfunction because the smooth muscle tone will increase,

flow-mediated dilation will decrease and atherosclerotic plaque formation will be enhanced [25, 26]. Next to these structural changes, endothelial cells and vascular smooth muscle cells also have a role in vascular stiffening. Vascular smooth muscle cells react on mechanostimuli, a.o. because of cell stretch [18]. Endothelial cells can suffer from disturbances in the nitric oxide expressions, which has also been associated with vascular stiffness [27, 28].

Arterial stiffness results in increased pulse pressure, which is the difference between the systolic and the diastolic pressure. This has its influence on the heart, in particular because the ventricle load increases. A stiffer arterial tree needs higher systolic pressures in order to have the same amount of net stroke volume. A stiff vascular wall thereby lowers the efficacy of the ejection fraction [29] and the perfusion of the coronary arteries [30]. In turn, the systolic hypertension caused by arterial stiffness, leads to a higher vascular tone, which further increases the amount of arterial stiffness by a disturbance in the collagen-elastin ratio and by stretching the vascular smooth muscle cells.

Arterial stiffness can be measured in several ways. The most commonly used method is assessing the pulse wave velocity (PWV) [31]. During each heart beat, a pulse wave travels from the heart down the arterial wall in advance of blood flow. The more rigid the wall of the artery, the faster the wave moves. The PWV is the time it takes for the pulse wave to travel from the heart to the branching point, often the iliacal bifurcation. When the wave hits the major branching points, these waves are reflected back so that they reverse direction and travel back to the point of origin. Normally, the reflected wave returns after the aortic valve is closed (**Figure 1.1**) [6]. This amplifies diastolic blood pressure and facilitates blood flow through the coronary arteries. Another measure of arterial stiffness is the augmentation index (AIx). The augmentation pressure is the contribution of the reflection wave to systolic arterial pressure. When the compliance of the arteries is reduced, the reflection wave will return earlier, so before the aortic valve has closed, causing a rise in systolic pressure. The augmentation index is the augmentation pressure divided by the pulse pressure and is therefore a indirect measurement of arterial stiffness (**Figure 1.2**) [6]. Also, the compliance and distensibility of an artery can be measured directly. The carotid distensibility is often used, because this artery is easy to approach by ultrasonography. With the distensibility of the carotid artery we measure the difference of expansion of the artery between the diastolic and the systolic phase. This is one of the
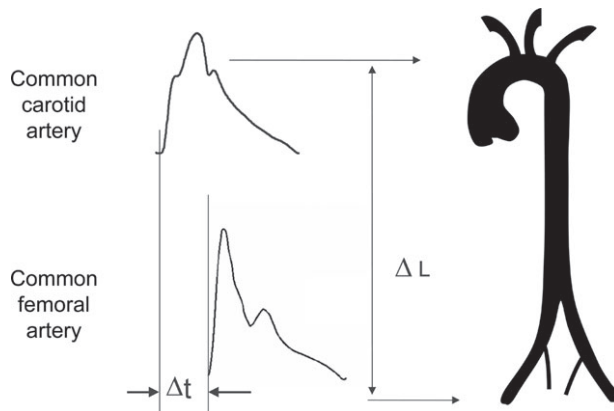
Figure 1.1. Measurement of carotid-femoral PWV.

From: Laurent et al. Eur Heart J 2006; 27: 2588-2605
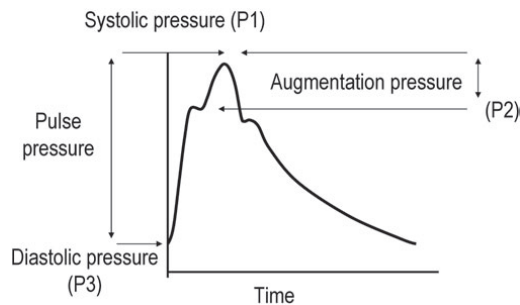
Figure 1.2 Augmentation Index.

The height of the late systolic peak (P1) above the inflection (P2) defines the augmentation pressure, and the ratio of augmentation pressure to pulse pressure defines the AIx (in %).

From: Laurent et al. Eur Heart J 2006; 27: 2588-2605.

parameters of arterial stiffening: the stiffer the vessels, the less expansion there will be [32, 33].

Another important cardiovascular risk indicator in elderly, next to arterial stiffness, is the amino-acid homocysteine. Elevated levels of homocysteine form an independent predictor for cardiovascular disease [34, 35], in particular in the oldest old [36]. In this age group, hyperhomocysteinemia has even been shown a better predictor for cardiovascular mortality than the traditional risk factors used in the Framingham risk score [36]. However, the underlying mechanism is not completely clear, especially since homocysteine-lowering intervention trials failed to demonstrate beneficial effects on cardiovascular

outcomes [37]. Therefore, it has been proposed that homocysteine may act as a biomarker, rather than a risk factor [38]. The question remains what the controversy can be between the prospective studies and the intervention studies. There is a discrepancy between studies which clearly demonstrate that homocysteine is a risk indicator and the randomized controlled trials, who failed to demonstrate a benefit of B-vitamin supplementation, while this would be expected based on pathophysiological grounds – the one carbon metabolism (**Figure 1.3**). There are several potential explanations. First of all, many trials were performed in folic-acid fortified and middle-aged populations. Next, the administration of B-vitamins did not always lead to the anticipated lowering of homocysteine. Also, the duration of the studies was relatively short, based on the pathophysiology of atherosclerotic disease. However, an alternative hypothesis is that the lack of effect may be explained by the fact that B-vitamin supplementation has dual, opposing effects. It has been hypothesized that B-vitamins on the one hand lower homocysteine, but on the other hand at the same time lead to increased inflammation status [38].

However, it is also possible that arterial stiffness is the actual link between homocysteine level and adverse cardiovascular events. Because arterial stiffness
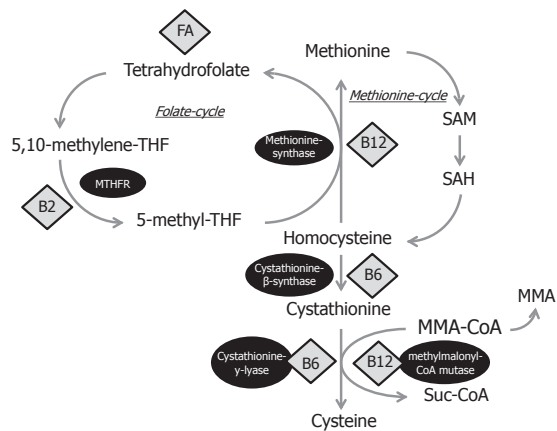
Figure 1.3. The one-carbon metabolism.

Vitamins, metabolites and enzymes which are involved in the one carbon metabolism. Abbreviations: THF: tetrahydrofolate; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; MTHFR: methylenetetrahydrofolate reductase; MMA: methylmalonic acid; MMA-CoA: methylmalonyl-CoA; Suc-CoA: succinyl-CoA; FA: folic acid; B12: vitamin B12; B2: vitamin B2; B6: vitamin B6.

From: van Dijk et al. Nutr Metab Cardiovasc Dis 2014, Jan; 31.

is a condition that is not easily or rapidly reversible, an association between homocysteine levels and arterial stiffness may explain the lack of beneficial effects of homocysteine-lowering therapy. There is already some evidence that homocysteine is indeed associated with arterial stiffness, however these association studies are equivocal, and were often done in younger populations with relatively low homocysteine levels [39-42]. Pathophysiologically, homocysteine leads to an increased thrombogenecity, increased oxidative stress and to an overactivation of inflammatory pathways (**Figure 1.4**).
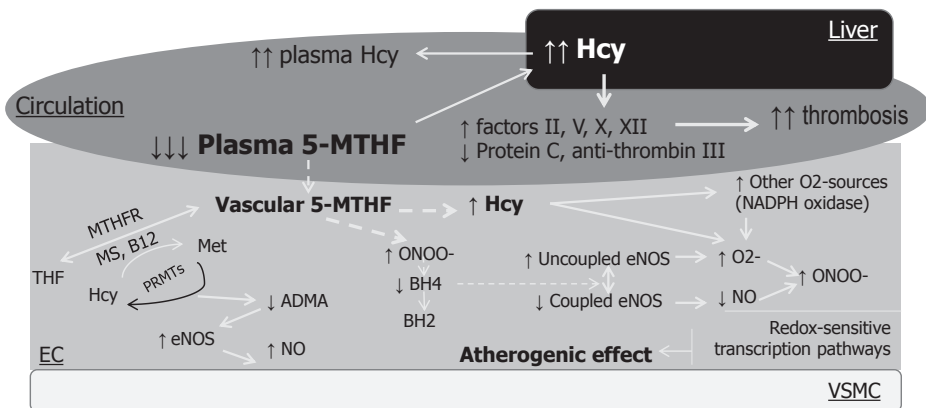
Figure 1.4. Pathophysiological effects of hyperhomocysteinemia.

This figure demonstrates the liver, the blood vessel, the endothelial cell and the vascular smooth muscle cell, which are all likely to be involved in the pathophysiological mechanism of homocysteine. First, high homocysteine levels in the liver increases the synthesis of pro-atherogenic factors and decreases the biosynthesis of anti-thrombotic factors, resulting into a net trombotic effect. Second, in the vascular endothelium, 5-MTHF increase intracellular homocysteine (Hcy) and a rise in O2 radicals. Also, 5-MTHF directly increase peroxynitrite (ONOO), leading to less oxidation of hydrobiopterin (BH), and therefore decreases endothelial nitric oxide synthase coupling (eNOS). Third, endogenous 5-MTHF is synthesized by methyl-tetrahydrofolate reductase (MTHFR). 5-MTHF is converted back to THF in parallel to the conversion of Hcy to methionine (Met) by methionine synthase (MS), which uses vitamin B12 as a co-factor. Due to high plasma Hcy levels, the conversion of methionine back to Hcy is less. Therefore the methylation of L-arginine (L-arg) to asymmetrical dimethylarginine (ADMA) is decreased, leading to an enhancement of eNOS. In the end, this all results in an reduction of vascular redox and endothelial function.

From: C. Antoniades et al. Eur Heart J 2009; 30: 6-15.

Because hyperhomocysteinemia is a very common condition among elderly, with a prevalence of 25-50% in European countries [43], and because hyperhomocysteinemia has been shown to be a strong cardiovascular risk predictor in this age group, we aimed to investigate the association between homocysteine and arterial stiffness within older individuals participating in

the B-PROOF trial [44]. The B-PROOF (B-vitamins for the PRevention Of Osteoporotic Fractures) trial is a large, multi-center placebo-controlled, double-blind, randomized controlled trial conducted in the Netherlands. Participants were included after the age of 65 and when they had mildly elevated homocysteine levels (12-50 μmol/L). Exclusion criteria were renal insufficiency (creatinine level >150 μmol/L) and the presence of a malignancy. The intervention included 500 μg vitamin B12 and 400 μg folic acid for a period of 2 years. Both the intervention and the placebo group received 15 μg (600 IE) vitamin D_3. In total, 2919 participants were included. The main outcome of this trial was osteoporotic fracture incidence. Also many secondary outcomes have been defined, such as cardiovascular disease incidence, arterial stiffness measures, cognitive functioning and physical performance [44].

The aim of this thesis is, because this large trial forms a unique opportunity, to not only investigate the association between homocysteine level and arterial stiffness, but also the effect of B-vitamin supplementation on arterial stiffness and other cardiovascular outcomes in an older population.

At first, we will focus on the methods for measuring arterial stiffness in our elderly population in Chapter 2. Furthermore, it would be of interest to explore other cardiovascular risk prediction markers in the elderly, especially in the pre-clinical phase as arterial stiffness represents. In Chapter 3 we explore the association between arterial stiffness and vitamin D, osteoporosis and physical function. In Chapter 4 the association between homocysteine and arterial stiffness are explored and we address the question whether B-vitamin supplementation has an influence on cardiovascular disease, arterial stiffness and endothelial function.

The results of this thesis will therefore be helpful in order to better understand the underlying mechanisms of arterial stiffness as being an important cardiovascular predictor in the elderly.

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Chapter **2**

Measurement of arterial stiffness
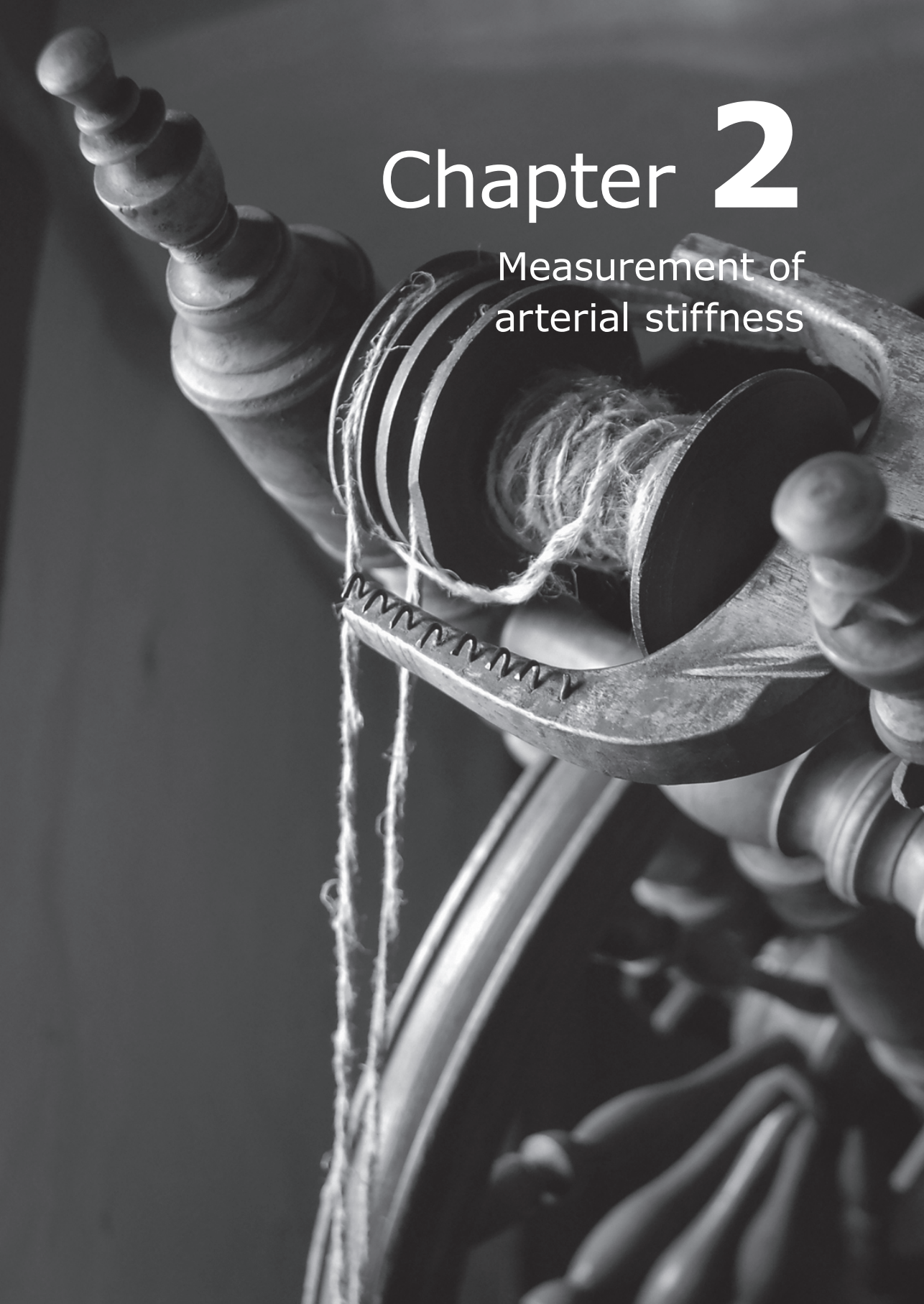
2.1

Oscillometry and applanation tonometry measurements in older individuals with elevated levels of arterial stiffness

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2.1.1   ABSTRACT

Objective

Indices of arterial stiffness and aortic pressure are usually assessed by applanation tonometry. The more recently introduced oscillometric device is simpler to use. Several studies have investigated the agreement between these two devices, but not in populations with elevated levels of arterial stiffness. Therefore, we evaluated the agreement in an elderly population with high risk of arterial stiffness.

Patients and methods

We included a subgroup of the B-PROOF study (n= 344, mean age 73 years, 60% men), whose aortic pulse wave velocity (aPWV), aortic augmentation index (AIx), aortic pulse pressure (PP), and aortic systolic blood pressure (SBP) were assessed both with applanation tonometry (SphygmoCor) and with oscillometry (Arteriograph). We investigated agreement between the two devices using Pearson correlations and Bland–Altman analysis. We carried out a stratified analysis in participants with more pronounced arterial stiffness (SphygmoCor aPWV > 12 m/s).

Results

The oscillometric method produced higher values of AIx, aortic PP, and aortic SBP (P <0.01) than applanation tonometry. aPWV values were lower (P < 0.01) and were not correlated (r= − 0.06, P = 0.92), whereas AIx measurements (r= 0.35 P <0.01), aortic PP (r =0.57 P < 0.01), and aortic SBP (r= 0.68 P < 0.01) measurements were correlated. Bland–Altman analysis showed insufficient agreement between the two devices, especially in those with elevated levels of arterial stiffness (aPWV > 12 m/s).

Conclusion

Particularly in the elderly with elevated levels of arterial stiffness, measurements of aPWV obtained with oscillometry and applanation tonometry show poor agreement. Also, AIx, aortic SBP, and aortic PP show clearly less than optimal agreement.

2.1.2 INTRODUCTION

Arterial stiffness has become a well-established marker for cardiovascular risk [1], and is measured routinely by pulse wave analysis, from which one can derive both pulse wave velocity (PWV) and augmentation index (AIx). The European Society of Hypertension and the European Society of Cardiology have even recommended pulse wave analysis for cardiovascular risk assessment [2].

Applanation tonometry is commonly used for pulse wave analysis to assess PWV and AIx. It uses the carotid–femoral transit time to provide an indirect measurement of the central artery system [3]. A disadvantage of applanation tonometry is that the accuracy of the measurements depends on the examiner's experience. Therefore, a computerized device that uses an oscillometric method to determine PWV and AIx has been developed. This method uses the changes in pulsatile pressure in the brachial artery as an indirect measurement for the pressure oscillations in the central artery system.

Several studies have recently compared these types of devices, but these studies were carried out only in younger adults and hypertensive patients, with relatively low PWV values [4–7]. Furthermore, the results of these studies were conflicting. None of the comparisons were performed in older individuals or individuals with elevated levels of arterial stiffness. Because it is still unclear whether the oscillometric method is comparable with applanation tonometry within a general elderly and arterial stiffness population, we compared the PWV and AIx and aortic systolic blood pressure (SBP) and aortic pulse pressure (PP) measurements obtained with applanation tonometry and oscillometry in an elderly population.

2.1.3 METHODS

Study population

This study was carried out within the framework of the B-vitamins for the Prevention Of Osteoporotic Fractures (B-PROOF) study. A detailed description of this randomized-controlled trial has been reported elsewhere [8]. Briefly, B-PROOF is a multicenter, randomized, placebocontrolled, double-blind trial investigating the effect of vitamin B supplementation on osteoporotic fractures in older patients.

B-PROOF has 2919 participants from the area in and around three Dutch cities: Rotterdam, Amsterdam, and Wageningen. The main inclusion criteria were age 65 years and older and a mildly elevated homocysteine level (12–50 µmol/l). Follow-up has been completed recently.

Clinical characteristics

During the baseline measurements, all participants filled in a questionnaire on their medical history, use of medication, use of nutritional supplements, current habits of coffee and alcohol intake, and smoking habits. Their height and weight were measured, and BMI was calculated as weight divided by height^2 (expressed as kg/m^2).

Cardiovascular disease was defined as a positive medical history for myocardial infarction, angina pectoris, heart failure, valvular disorders, arrhythmia, and aneurysms. The presence of hypertension, diabetes mellitus, hypercholesterolemia, and renal disease was also registered.

Vascular function tests

At the Erasmus Medical Center (Rotterdam) and the VU Medical Center (Amsterdam), a subsample of participants was invited to undergo vascular function tests (n=560). Participants with cardiac arrhythmia were excluded from these additional measurements. During the measurements, participants were placed in supine position on a flat examination couch in a quiet laboratory room for at least 5–10 min before the measurements, and the participants were not allowed to speak during the measurements. Use of alcohol or coffee during 12 h before the measurements was prohibited.

Applanation tonometry

Peripheral blood pressure used for applanation tonometry was measured once using a semiautomatic oscillometric device (Datascope Accurator Plus Device; Datascope Corp., Mahwah, New Jersey, USA) [9] after at least 5min of supine rest. All blood pressure measurements were performed on the right arm. Arterial tonometry was obtained once from the right radial, carotid, and femoral artery using the SphygmoCor device (SphygmoCor version 7.1; AtCor Medical, Sydney, New South Wales, Australia). With use of the radial arterial wave form, an estimate of the corresponding central aortic pulse wave was calculated using a validated generalized transfer function incorporated into the device. Using the integrated software, the central augmented pressure was calculated as the difference between the early and late systolic peaks of the estimated central pressure wave form. Central aortic AIx was

calculated as the augmented pressure expressed as a percentage of the PP. PWV was measured simultaneously with three-channel ECG recording and recording of the right carotid and femoral artery pulse waveforms, and was calculated as the delay between the femoral pulse wave and the carotid pulse wave, divided by the transit distance. Transit distances were assessed by body-surface measurement from the carotid artery to the femoral artery [3].

Oscillometric analysis

The participant was lying in a supine position for at least 10 min before the oscillometric measurement of the Arteriograph (Arteriograph; TensioMed, Budapest, Hungary) was obtained. The oscillometric measurement was performed once, directly after the SphygmoCor measurements, only at the Erasmus Medical Center. The conditions of the room and participant were similar. The blood pressure cuff was applied to the right upper arm and was inflated to a pressure of 35mmHg above the SBP on the brachial artery. The pressure in the underlying occluded artery is transmitted through the cuff to the pressure sensor. The first systolic peak corresponds to the ejection of the left ventricle, whereas the second peak is assumed to be the reflection of the first pressure wave from the periphery. The difference in time between the first and the second wave is the return time. The distance traveled is twice the distance between the aortic arch and the iliac bifurcation, and is measured with a tape measure as the distance between the sternal notch and the pubic symphysis. PWV was recorded as continuous data [10].

Statistical analysis

The comparison between applanation tonometry and oscillometry was tested by the Pearson correlation coefficient, Bland–Altman [11], and linear regression analysis. Only linear regression analysis had the possibility to account for possible confounders. We used a multivariate model that included all variables that caused a change in the point estimate of more than 10% or were considered clinically relevant. The covariates age and sex were considered as potential confounders. We also carried out a stratified analysis in a population with elevated levels of arterial stiffness, defined as an aortic pulse wave velocity (aPWV) measured with SphygmoCor more than 12 m/s, and we compared this with a population with less arterial stiffness (aPWV SphygmoCor ≤ 12 m/s). Another stratified analysis was carried out between populations aged 73 years or less and more than 73 years (mean age 73 years). Statistical analysis was carried out using SPSS 20.0 statistical soft-

ware package (SPPS Inc., Chicago, Illinois, USA). P-values of less than 0.05 were considered statistically significant.

2.1.4   RESULTS

In total, 344 participants had evaluable applanation tonometry and oscillometric measurements; 203 were men and 141 were women. Their mean age was 72.5±5.5 years, mean BMI was 26.5±3.4 kg/m2, and mean brachial blood pressure levels were 134.9/77.2mmHg measured using a semiautomatic datascope (**Table 1**).

Table 1. Participant characteristics, total population (n = 344)

Variable	Mean ± SD
Age (years and range)	72.5 ± 5.5 [65-95)
Sex (n, %)	
Male	203 (59.0%)
Brachial blood pressure (mmHg)[a]	
SBP	134.9 ± 16.9
DBP	77.2 ± 9.5
Plasma homocysteine (µmol/L)	14.3 [12.9 – 16.3]
MDRD (ml/min per 1.73m^2)	90.4 ± 34.0
BMI (kg/m^2)	26.5 ± 3.4
Medical history of	
Hypertension	131 (38.1%)
Diabetes mellitus	38 (11.0%)
Hypercholesterolemia	88 (26.7%)
Cardiac disease	74 (21.5%)
TIA / Stroke	26 (7.6%)
Smoking status	
Nonsmoker	126 (36.6%)
Former smoker	190 (55.2%)
Current smoker	28 (8.1%)

Values are presented as number and percentage or as mean ± SD, except for homocysteine [median (IQR)].
Abbreviations: DBP, diastolic blood pressure; MDRD, modification of diet in renal disease; SBP, systolic blood pressure; TIA, transient ischemic attack.
[a]Measured with a semiautomatic oscillometric device.

Peripheral blood pressure measurements obtained with a semiautomatic oscillometric device, used for applanation tonometry, were significantly correlated with the peripheral blood pressure measurements of the oscillometric method (SBP: r=0.76, P<0.01; diastolic blood pressure: r=0.65, P<0.01). The mean bias between the two blood pressure measurements is 5.6±12.7 mmHg, with 95% limits of agreement ranging from −19.2 to 30.4 mmHg. The peripheral SBP explains 4% of the variation in the PWV level and 80% in the variation with aortic SBP with applanation tonometry. With oscillometry, the peripheral SBP explains 11% of the PWV level and 89% of the aortic SBP level.

AIx ranged from 0 to 57% and aPWV measurements from 2.3 to 48.0 m/s with applanation tonometry. With oscillometry, AIx ranged from 1.8 to 74.4% and aPWV from 6.3 to 17.7 m/s. AIx and aPWV differed significantly between the two devices: oscillometry yielded higher values of the AIx (P<0.01) but lower values of aPWV (P<0.01) than applanation tonometry. When data were stratified on the basis of aPWV levels of more than 12 m/s, aPWV values only differed in participants with increased arterial stiffness (**Table 2**).

Table 2. Vascular characteristics (n = 344)

	Sphygmocor	Arteriograph	p-value
PWV ≤ 12 m/s			
AIx (%)	24.9 ± 10.2	37.4 ± 14.8	< 0.01
aPWV (m/s)	10.1 ± 2.0	9.9 ± 2.3	0.46
Aortic SBP (mmHg)	123.6 ± 16.7	133.0 ± 19.5	< 0.01
Aortic PP (mmhg)	46.2 ± 13.0	51.8 ± 13.5	< 0.01
PWV > 12 m/s			
AIx (%)	26.7 ± 9.8	37.8 ± 14.9	< 0.01
aPWV (m/s)	16.1 ± 4.1	10.3 ± 2.0	< 0.01
Aortic SBP (mmHg)	130.2 ± 18.9	145.5 ± 22.0	< 0.01
Aortic PP (mmhg)	51.2 ± 15.4	59.7 ± 16.3	< 0.01

Values are presented as mean±SD.
Abbreviations: AIx, augmentation index; aPWV, aortic pulse wave velocity; PP, pulse pressure; SBP, systolic blood pressure.

With applanation tonometry, aortic SBP ranged from 77 to 224 mmHg and the aortic PP from 21 to 144 mmHg, whereas with oscillometry, aortic SBP ranged from 98 to 198 mmHg and aortic PP from 29 to 102 mmHg; aortic

SBP and aortic PP were higher with the oscillometric method than with the respective values of applanation tonometry (P<0.01) (**Table 2**).

The AIx measurements between applanation tonometry and oscillometry were correlated, but rather weakly (r=0.35, P<0.01). The mean bias with Bland–Altman analysis was −12.6±14.8%, with 95% limits of agreement ranging from −41.6 to 16.4% (**Figure 2.1.1a**). aPWV assessed by applanation tonometry and oscillometry did not correlate at all (r= −0.06, P=0.92), and the level of agreement assessed by Bland–Altman analysis showed a
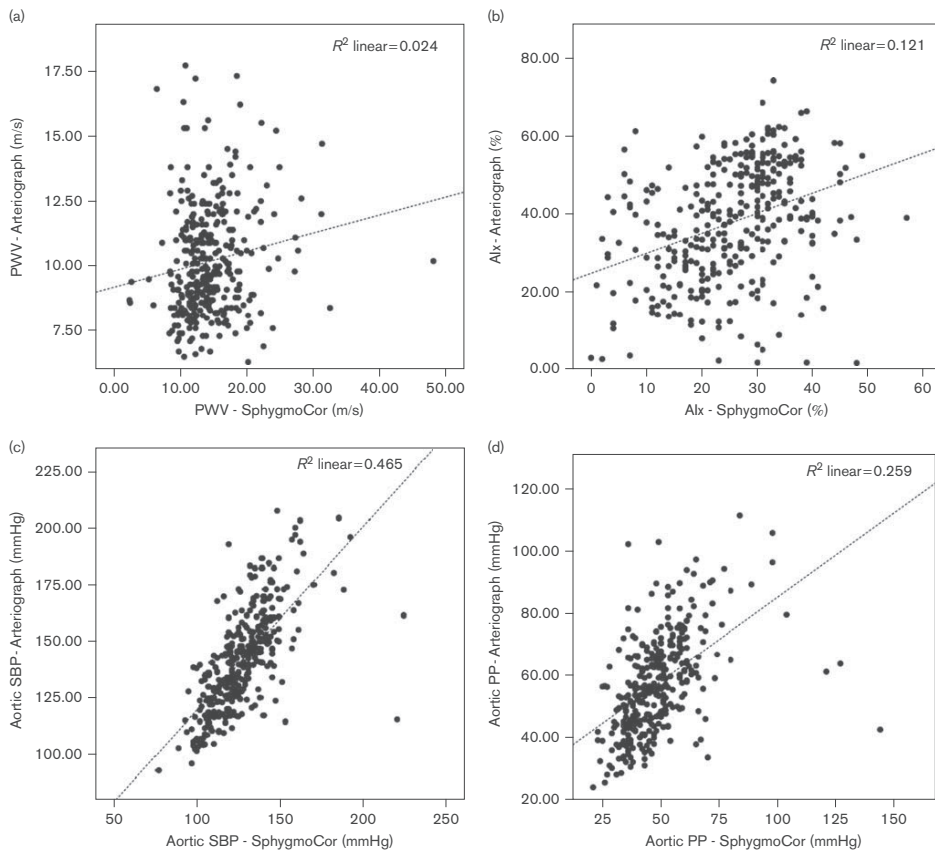
Figure 2.1.1.
Scatter plots of the (a) augmentation index (AIx) values measured with the Arteriograph versus the AIx values measured with the SphygmoCor, (b) the pulse wave velocity (PWV) values measured with the wArteriograph versus the PWV values measured with the SphygmoCor, (c) the aortic systolic blood pressure (SBP) values measured with the Arteriograph versus the aortic SBP values measured with the SphygmoCor, (d) the aortic pulse pressure (PP) values measured with the Arteriograph versus the aortic PP values measured with the SphygmoCor.

mean bias of 4.2±4.9 m/s, with 95% limits of agreement varying from −5.3 to 13.7m/s (**Figure 2.1.1b**).

The oscillometric and applanation tonometric aortic SBP measurements correlated moderately (r=0.68, P<0.01). Bland–Altman analysis showed a mean bias of -13.5±15.3 mmHg, with the 95% limits of agreement ranging from −43.5 to 16.5 mmHg (**Figure 2.1.1c**). For aortic PP, the correlation coefficient was moderate (r=0.57, P<0.01). The level of agreement had a mean bias of −8.8±15.2 mmHg, with 95% limit of agreement ranging from −38.6 to 20.9 mmHg (**Figure 2.1.1d**).
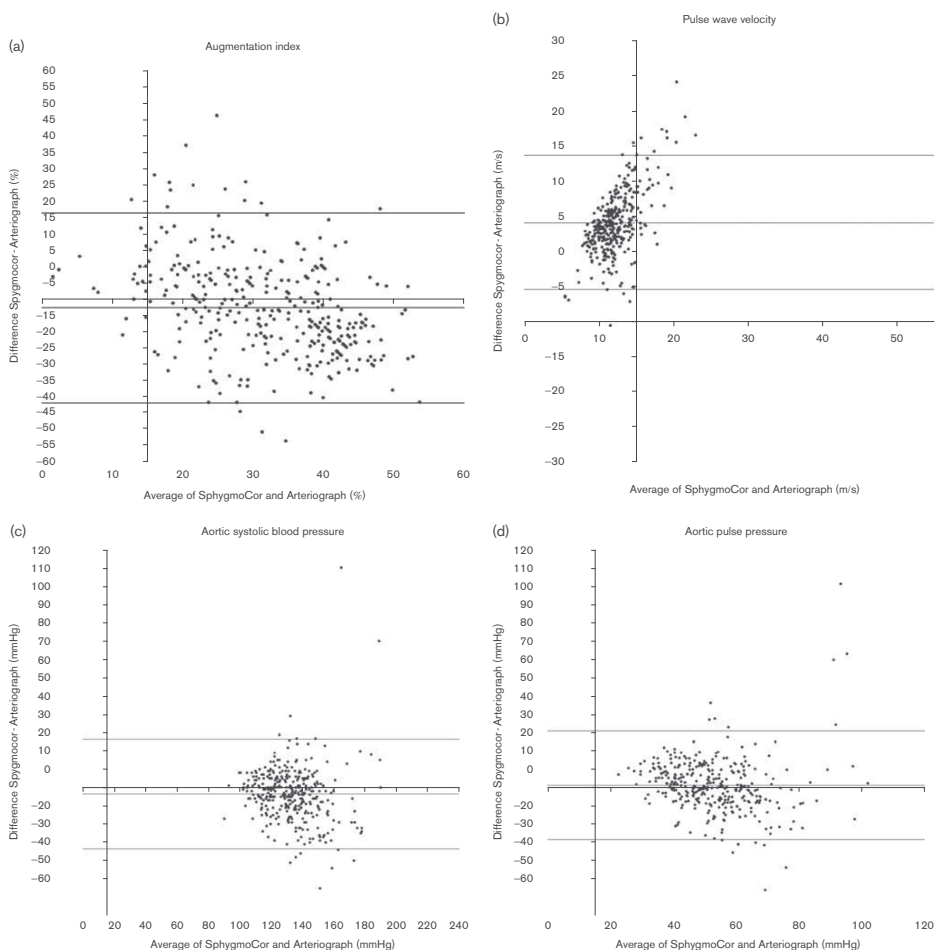
Figure 2.1.2.
Bland–Altman plots of (a) augmentation index, (b) pulse wave velocity, (c) aortic systolic blood pressure, and (d) aortic pulse pressure within the total population.

A stratified Bland–Altman analysis of aPWV applanation tonometry measurements more than 12 m/s (n=219) showed a mean bias of 6.1±4.5 m/s, with 95% limits of agreement ranging from −2.7 to 14.9 m/s (**Figure 2.1.2**). A mean bias of 0.29±2.8 m/s was found in a stratified analysis of aPWV applanation tonometry measurements 12 m/s or less (n=125), with 95% limits of agreement ranging from −5.3 to 5.9 m/s (**Figure 2.1.2**).

A stratified analysis of aPWV measurements between the age categories 73 years or less and more than 73 years showed no differences between the level of agreement. aPWV values in participants aged 73 years or less showed a mean bias of 3.1±4.5 m/s, with 95% limits of agreement ranging from −5.7 to 11.8 m/s, and for aPWV values of participants aged more than 73 years, a mean bias of 5.9±5.0 m/s was found, with 95% limits of agreement ranging from −3.8 to 15.7 m/s (data not shown).

2.1.5   DISCUSSION

In participants with normal arterial stiffness (PWV ≤ 12 m/s), we found a moderate agreement between applanation tonometry and oscillometry. However, the lack of agreement was considerable when there were elevated levels of arterial stiffness. The other arterial stiffness measurements AIx, aortic SBP, and aortic PP also differed between the devices.

Since oscillometry became available, several studies have compared this method with applanation tonometry [4–7]. A population-based research found a complex pattern of agreement in the peripheral and central AIx. Because the values of the AIx depend on age and diastolic blood pressure, agreement differed within subcategories [12]. In hypertensive populations (mean PWV 8–10 m/s), the devices did not agree sufficiently in their arterial stiffness measurements [5,6]. In patients with severe renal disease (mean PWV 11 m/s), arterial stiffness measurements with the oscillometric device did not correspond to those of the tonometric measurements [13]. However, none of these studies were carried out in the elderly with elevated levels of arterial stiffness, whereas accurate arterial stiffness measurements are particularly important in such a population, as this is important for enhanced cardiovascular risk prediction.

Both devices have been validated by comparison with invasive measurements of arterial stiffness [14–16]. Applanation tonometry is currently used as the standard for noninvasive arterial stiffness measurements and although oscillometry has also been validated invasively, in agreement with the studies mentioned above, our study found that also in an older population applanation tonometry and oscillometric measurements do not have comparable arterial stiffness measures. Therefore, some authors are skeptical about the data that oscillometry yields. To understand the difference in agreement, which appears to depend on the amount of arterial stiffness, determinants of aPWV values are important. We have found that age is not a determinant of aPWV levels measured with oscillometry, and also not when there are elevated levels of arterial stiffness. As a stratified analysis per age category did show a larger lack of agreement in the oldest age category, this fits with our hypothesis that increased arterial stiffness is not that accurately measured with oscillometry as with applanation tonometry. Furthermore, AIx is a significant determinant of aPWV levels measured with oscillometry, independent of the amount of arterial stiffness. As AIx is an arterial stiffness measurement, which also represents peripheral resistance [17], this could also be a clue that the oscillometric measurement is more sensitive to local brachial stiffness, as has been debated previously [10,18]. The conditions of measuring with a cuff result in the pulse wave traveling back and forth in the brachial artery, where it reflects distally on the occluded cuff and proximally on the reflection of the aortic junction. However, other authors suggest that the brachial artery does have higher PWV levels, but that these levels would be much less affected by age-related stiffness than the aorta and that this artery is often not involved in atherosclerotic changes.

Nevertheless, the above-mentioned results may be an explanation why the measurements obtained with the oscillometric method do agree more with applanation tonometry in a non-arterial stiffness population and that no agreement has been shown in a population with more prominent arterial stiffness. Yet, it is still unclear whether oscillometry and applanation tonometry measure similar vascular properties.

To the best of our knowledge, this study is the first to compare measurements of arterial stiffness obtained with both oscillometry and applanation tonometry in an elderly population with elevated levels of arterial stiffness. As arterial stiffness is an important cardiovascular risk predictor in older populations in whom arterial stiffness is more prominently present, accurate measurements of arterial stiffness are important. Despite aPWV being the

most robust marker for arterial stiffness, aPWV measurements have the largest lack of agreement. Because the technology of applanation tonometry is more complicated, the oscillometric method was developed for its ease of arterial stiffness assessment. Despite this easy-to-use technology, with our results, it may be questioned whether the lack of agreement of aPWV levels is too large for the oscillometric method to be used for clinical applications in an elderly population with elevated levels of arterial stiffness as this is an important marker for cardiovascular risk assessment.

Several limitations of this research should be mentioned. One is the selected group of participants. Our study was carried out in older individuals participating in the B-PROOF study, all of whom had increased homocysteine levels. As hyperhomocysteinemia is associated with arterial stiffness [19], our participants are more prone to have elevated arterial stiffness, as the mean value of aPWV of 14 m/s indicates. However, as we aimed to investigate the accuracy of measurements with oscillometry, especially in a population with elevated levels of arterial stiffness, our study population could also be considered as a strength. Our stratified analysis of elderly with more pronounced arterial stiffness endorses this importance. A second limitation of our study is that the analysis we used for comparison did not allow us to adjust for different transit distances used by applanation tonometry and oscillometry and it was more difficult to compare the methods. Applanation tonometry uses the carotid–femoral distance, and the oscillometric method uses the distance from the sternal notch to the pubic symphysis. Another limitation is that different blood pressure methods were used for the calculation of vascular function with the two devices. Peripheral blood pressure levels are needed for calculation of AIx and PWV. Applanation tonometry uses a separate automatic oscillometric device to measure peripheral blood pressure, and the oscillometric method measures peripheral blood pressure itself. This difference might have influenced the results. Nevertheless, the mechanisms of measuring blood pressure are similar. However, although the blood pressure measurements were obtained with different devices and not simultaneously, we found a statistically significant correlation between these measurements and for both devices the peripheral SBP explains the variability in PWV levels to the same extent. Furthermore, measurements with both devices were performed only once. Repeated measurements would have yielded a more accurate analysis comparing the devices. The fact that the known determinants of PWV values as mean arterial pressure and heart rate are not explaining much of the variance of the PWV levels in our popula-

tion is also worth mentioning. Therefore, one may question whether there is an accurate way of measuring arterial stiffness in such an older population at all. Also, we need to mention that it is possible that the oscillometric method will be appropriate for cardiovascular risk prediction, despite the fact that measurements are not in agreement with those of the applanation tonometry method. Because of our cross-sectional design, we cannot draw any conclusions on the predictive value of this device. However, in renal disease patients, oscillometry could not predict cardiovascular mortality [13]. It still needs to be established whether oscillometry has clinical value in cardiovascular risk assessment in the elderly and other patients with high risk of arterial stiffness.

In conclusion, in our study, we found that in elderly individuals with elevated levels of arterial stiffness, measurements with the oscillometric method do not agree with those of applanation tonometry. These findings should be taken into account when using the oscillometric device for cardiovascular risk assessment.

Acknowledgements

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Chapter **3**

Physical function, osteoporosis and arterial stiffness
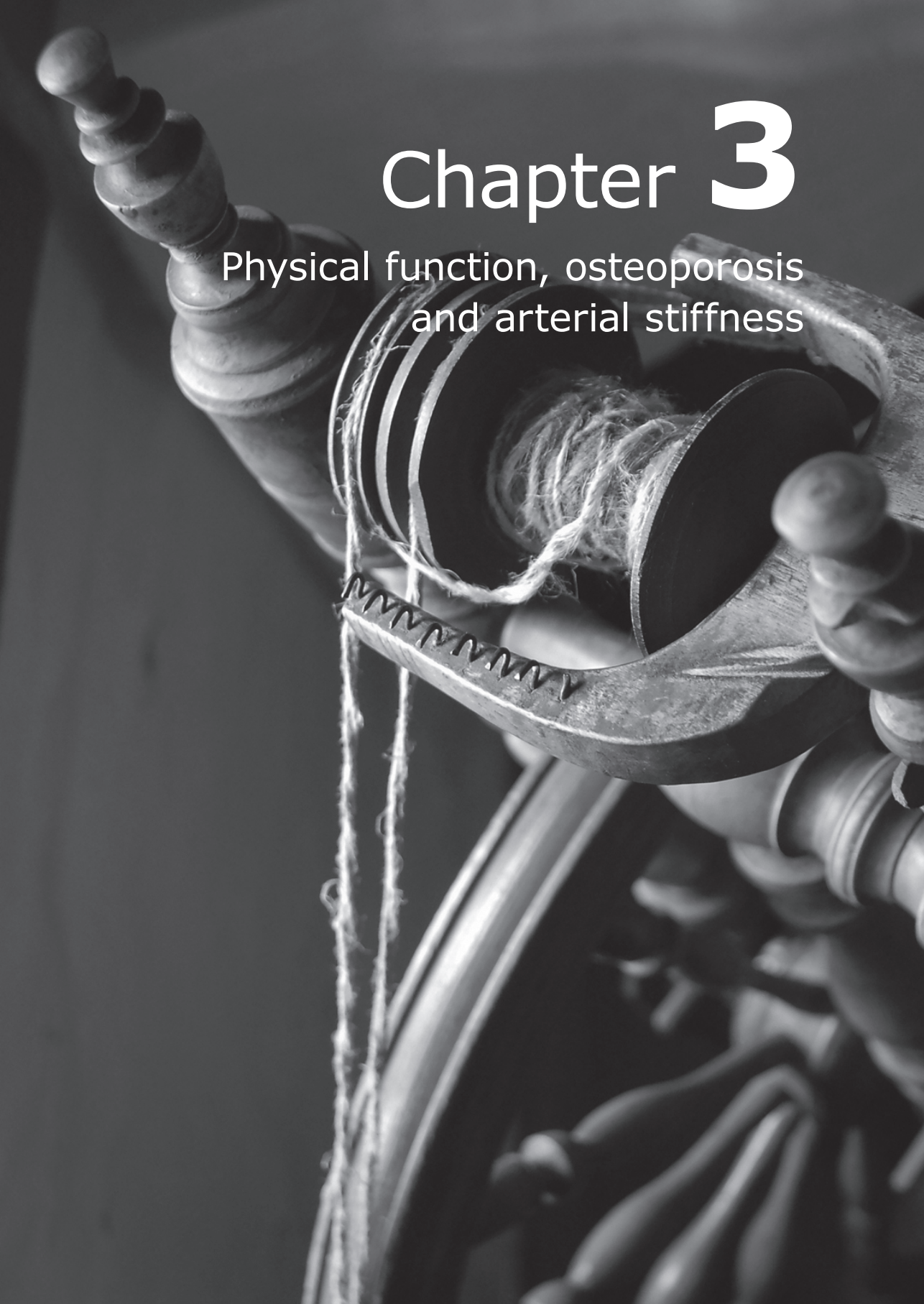
3.1

Physical fitness, activity and hand-grip strength are not associated with arterial stiffness in older individuals

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3.1.1 ABSTRACT

Background

Whereas evidence exists about the benefits of intensive exercise on cardiovascular outcomes in older adults, data are lacking regarding long-term effects of physical fitness and physical activity on cardiovascular health. Therefore, we aimed to investigate the longitudinal association of physical fitness, physical activity and muscle strength with arterial stiffness measures.

Design: Longitudinal with 2-yr follow-up.
Setting / Participants: A subgroup of the B-PROOF study (n=497, mean age 72.1 ± 5.4).

Measurements

The primary outcome was arterial stiffness after 2 years of follow-up as estimated by pulse wave velocity (PWV) measured with applanation tonometry. Furthermore, augmentation index and aortic pulse pressure were assessed. Physical activity was estimated using a validated questionnaire regarding daily activities. Physical fitness was measured with a physical performance score, resulting from a walking, chair-stand and balance test. Muscle strength was assessed with hand-grip strength using a handheld dynamometer.

Results

Our population was aged 72 ± 5.4 years and 57% was male. The median performance score was 9.0 [IQR 8.0-11.0], the mean physical activity was 744.4 (SD 539.4) kcal/day and the mean hand-grip strength was 33.1 (SD 10.2) kg. AIx differed between the baseline and follow-up measurement (26.2% (SD 10.1) vs. 28.1% (SD 9.9); $p < 0.01$), whereas PWV and aortic PP did not. In multivariable linear regression analysis, physical performance, physical activity and hand-grip strength at baseline were not associated with the amount of arterial stiffness after two years of follow-up.

Discussion

Physical fitness, activity and muscle strength were not associated with arterial stiffness. Possibly only intensive activity will modify arterial stiffness within an older population, especially since this is also the case in younger individuals.

3.1.2   INTRODUCTION

Physical fitness and exercise have been shown to reduce the risk of cardio-vascular disease to a relatively large extent in young and middle-aged popu-lations [1, 2]. A potential important pathophysiological pathway consists of an exercise-induced reduction of the inflammatory status. Research showed that short-term regular exercise reduces the atherogenic activity of blood mononuclear cells, with a decrease in the production of atherogenic cyto-kines and an increase in atheroprotective cytokines [3]. However evidence of the long-term benefit of physical fitness on cardiovascular outcomes in elderly is ambiguous as compared to younger populations [4].

Arterial stiffness is an interesting phenotype to target in this perspective, because it is a pre-clinical state of cardiovascular disease. Arterial stiffness is an abnormality of the vasculature, that has been shown to be an pre-clinical phase for cardiovascular disease at older age [6]. A variety of studies already showed that indeed short-term aerobic exercise (~12 weeks) improved arte-rial function in older persons [7-10]. It has however been hypothesized that the pathophysiology of vascular adaptation to exercise differs between short and long-term exercise [11]. Whereas short-term adaptations of the arteries are likely due to changes in the vascular smooth muscle tone or changes in the vascular endothelium vasodilator tone, long-term adaptations are more likely to be represented by changes in the elastin-collagen composition, both may lead to less arterial stiffness. However, because arterial stiffness is an abnormality that is not easily or rapidly reversible, it is plausible that exercise will be no longer beneficial when arteries have already stiffened as is often the case in older individuals.

Despite the promising results of short-term exercise in older adults, data are lacking regarding long-term effects of physical fitness and activity. Since the B-PROOF study included a general elderly population in which data are available regarding physical fitness and physical activity, we aim to investi-gate the association between these physical parameters and arterial stiffness longitudinally.

3.1.3 METHODS

Study population

The present study was conducted as a longitudinal analysis within the framework of the B-PROOF (B-vitamins for the Prevention Of Osteoporotic Fractures) study. A detailed description of this randomized controlled trial has been reported elsewhere [12]. In short, B-PROOF is a multi-center, randomized, placebo-controlled, double-blind trial including 2919 participants from three areas in the Netherlands. The main outcome is osteoporotic fractures. Secondary outcomes include a.o. physical function measures and cardiovascular outcomes [12]. The intervention comprises 500 µg vitamin B12 and 400 µ folic acid. Both the intervention and the placebo group received 15 µg vitamin D. Main inclusion criteria were age 65 years and older, and an elevated homocysteine level (12 – 50 µmol/l). Main exclusion criteria were renal insufficiency (creatinine level > 150 µmol/l) and presence of a malignancy (past 5 years). All participants gave written informed consent before the start of the study. The Wageningen Medical Ethics Committee approved the study protocol, and the Medical Ethics committees of Rotterdam and Amsterdam gave approval for local feasibility. The B-PROOF study has been registered with the Netherlands Trial Register (www.trialregister. nl) under identifier NTR 1333 since June 1, 2008 and with ClinicalTrials. gov under identifier NCT00696514 since June 9, 2008. In the current study participants were included in whom both physical parameters and arterial stiffness parameters were available.

Physical performance, activity and hand grip strength

Physical Performance

Physical fitness was measured with the physical performance score, which was assessed using three different tests: the walking test, i.e. the time needed to walk 3 meters, turn around and walk back as quickly as possible; the chair stands test, e.g. the time needed to rise from and sit down in a chair for five times without the use of arms, as quickly as possible; and the tandem stand, i.e. the ability to stand with one foot in front of the other for 10 seconds. Quartiles of the time needed to perform the walking test and chair stands were calculated [13]. The categories for walking were: score 0 (unable or able to hold < 4 s), score 1 (≥9 s), score 2 (7–8 s), score 3 (6 s) and score 4 (≤5 s); the categories for the chair stands were: score 0 (unable), score 1 (≥15 s), score 2 (12–14 s), score 3 (10–11 s) and score 4 (≤9 s). The tandem stand score was categorized as follows: score 0 (unable),

score 2 (able to hold position for 4–9 s), and score 4 (able to hold position for at least 10 s). A total physical performance score (range 0–12, with a score of 12 representing optimal physical performance) was calculated by summing up the scores of the three different tests. The physical performance test has been shown to be reliable and valid instrument to measure physical performance: A lower total score has been associated with an increased fall- and fracture risk, frailty and lower cognitive functioning [13-16].

Hand Grip strength
Muscle strength was assessed with hand-grip strength (kg), which was measured using a strain-gauged dynamometer (Takei, TKK 5401, Takei Scientific Instruments Co. Ltd., Japan, inter observer CV = 5%). Participants were asked to perform two maximum hand-grip trials with each hand in standing position with their arms along their body. Maximal hand-grip strength was defined as the average of the highest score of the left and right hand.

Physical Activity
Total physical activity (min/day) was measured with the validated LASA Physical Activity Questionnaire (LAPAQ) [17]. The activities in this questionnaire included walking, cycling, gardening, participation in sports and light and heavy household activities. Frequencies and duration of each activity during the last two weeks were assessed. Physical activity was subsequently calculated in kcal/day.

Arterial stiffness parameters
At the Erasmus MC (Rotterdam) and VU University Medical Center (Amsterdam), a subsample of participants of the B-PROOF study underwent vascular measurements (n = 567). Participants with cardiac arrhythmia were excluded from these additional measurements. During the measurements, participants were situated in supine position on a flat examination couch in a quiet laboratory room for at least 5-10 minutes prior to the measurements, and the participants were not allowed to speak during the measurements. Use of alcohol or coffee during 12 hours before the measurements was prohibited.

Blood pressure measurement
Peripheral blood pressure at the time of vascular function tests was measured once with a semi-automatic oscillometric device (DatascopeAccurator Plus device, Datascope Corp. New Jersey, USA) after at least five minutes of supine rest. All blood pressure measurements were conducted at the

right arm and measured in mmHg. The mean arterial pressure (MAP) was measured and the pulse pressure was calculated as the systolic minus the diastolic blood pressure.

Applanation tonometry
Arterial tonometry was obtained from the right radial, right carotid and right femoral artery using the Sphygmocor device (Sphygmocor version 7.1, AtCor Medical, Sydney, Australia). An estimate of the corresponding central aortic pulse wave was calculated, using a validated generalized transfer function incorporated in the device. With the integral software, the central augmented pressure was calculated as the difference between the early and late systolic peaks of the estimated central pressure wave form. Central aortic augmentation index (AIx) was calculated as the augmented pressure expressed as a percentage of the pulse pressure (intra CV = 3%, inter CV = 5%). Aortic pulse wave velocity (PWV) was measured with three channel ECG recording and simultaneously recording of the right carotid artery pulse wave form and subsequently the femoral artery pulse waveform. The PWV was calculated as the delay between the femoral pulse wave and the carotid pulse wave, divided by the transit distance (intra CV = 5 %, inter CV = 8%). Transit distance was assessed with body surface measurement from the carotid artery to the femoral artery [18].

Covariates

Alcohol intake, smoking habits and presence of cardiovascular risk factors: hypertension, hypercholesterolemia and diabetes mellitus were determined using a structured questionnaire. Questions regarding cardiovascular disease history, including angina pectoris, myocardial infarction, transient ischemic attack and/or stroke were included in this questionnaire.

Body mass index (BMI) was calculated as weight divided by squared height and expressed as kg/m^2.

Serum creatinine was measured with the enzymatic colorimetric Roche CREA plus assay (CV=2%). The estimated Glomerular Filtration Rate (eGFR) was defined as the Modification of Diet in Renal Disease (MDRD) which was calculated in $ml/min/1.73m^2$ with the formula: $186 * (serum\ creatinine\ (\mu mol/l)\ /\ 88.4)^{-1.154} * age\ (years)^{-0.203} * 0.742$ (for females) [19].

Serum CRP was measured with electrochemiluminescence immunoassay on a Roche Modular E170 (Roche, Almere, The Netherlands).

Statistical analysis

The associations between physical fitness, physical activity and muscle strength and arterial stiffness parameters were tested longitudinally. For these analyses, baseline measurements of the physical parameters were used and follow-up measures of arterial stiffness.

The distributions of all variables of interest were examined with histograms and Kolmogorov-Smirnov tests. If a variable was not normally distributed, a categorized variable was created. The linearity of associations of the prediction of arterial stiffness measures with physical performance, activity and hand grip strength was tested with curve estimation modeling. When linear relations had the best fit, Pearson correlation coefficients were calculated and multivariable linear regression analysis was done. If the relations were non-linear, cubic spline models were created with R version 3.0.0 [20].

Covariates which were considered as potential confounders were baseline measures of arterial stiffness parameters, age, gender, study center, treatment allocation of the participant in the BPROOF study, MAP, heart rate, smoking status, alcohol use, eGFR and BMI.

Furthermore, we tested whether gender or age modified the association between physical performance, activity, hand-grip strength and measures of arterial stiffness. If the interaction-term gender * physical performance, activity or hand-grip strength was significant ($p < 0.1$), a stratified analysis was done. This also accounted for the interaction terms with age.

In order to further explore the pathophysiological hypothesis that in participants with increased arterial stiffness, the association between physical parameters and arterial stiffness is different, we tested if this modified the association between physical performance, activity or hand-grip strength. If the interaction-term was significant ($p < 0.1$), a stratified the linear regression analysis was done for participants with and without increased arterial stiffness at baseline.

Statistical analyses were performed using the statistical software package of SPSS version 20.0 (SPSS Inc, Chicago, Illinois, USA). The α-level was set at 0.05.

3.1.4   RESULTS

In total, our population consisted of 497 participants for whom both arterial stiffness measurements and physical variables were available. The mean age of our population was 72 (SD 5.4) years and 57% was male. The characteris-

tics of the study population are depicted in **Table 1** and **Table 2**. The median performance score was 9.0 [IQR 8.0-11.0], the mean physical activity was 744.4 (SD 539.4) kcal/day and the mean hand-grip strength was 33.1 (SD 10.2) kg.

AIx differed between the baseline and follow-up measurement ($p < 0.01$), whereas PWV and aortic PP did not (**Table 2**).

Table 1. Population characteristics at baseline (n = 497)

Variable	Mean ± SD
Age (years and range)	72.1 ± 5.4
Sex (n, %) Male	 283 (57%)
BMI (kg/m^2)	27.0 ± 3.7

Treatment allocation B-PROOF trial (n, %)
  Intervention with B-vitamins
  Placebo
	
	260 (52.3%)
	237 (47.7%)

Smoking behavior
  Never
  Former
  Current
	
	183 (36.8%)
	272 (54.7%)
	42 (8.5%)

Alcohol consumption (n, %)
  Light
  Moderate
  Excessive
  Very excessive
	
	319 (64.2%)
	152 (30.6%)
	21 (4.2%)
	5 (1.0%)

SBP (mmHg)	137.1 ± 17.8
DBP (mmHg)	77.2 ± 9.4
Hypertension (n, %)	194 (39.4%)
Diabetes mellitus (n, %)	53 (10.7%)
Hypercholesterolemia (n, %)	138 (27.8%)
eGFR (ml/min per 1.73m^2)	91.8 ± 34.9
Plasma homocysteine (µmol/L)	14.2 [12.9 – 16.3]
CRP (mg/L)	1.0 [1.0 – 3.0]
Performance score (0-12)	9.0 [8.0 – 11.0]
Physical activity (kcal/day)	744.4 ± 539.4
Hand-grip strength (kg)	33.1 ± 10.2

Data are presented as mean ± SD, unless for categorical variables and variables with a non-normal distribution. Categorical variables are presented as number of cases and the percentage of cases and variables which were not normally distributed are presented as medians with IQR. Abbreviations: BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; CRP: C-reactive protein.

Table 2. Descriptives of the vascular parameters at both baseline and follow-up

	n	Baseline	Follow-up	p-value difference baseline – follow-up
PWV (m/s)	448	14.1 ± 4.3	14.2 ± 4.4	0.67
AIx (%)	483	26.2 ± 10.1	28.1± 9.9	< 0.01
Aortic PP (mmHg)	487	49.8 ± 15.0	48.6 ± 14.0	0.08

Data are presented as mean ± SD. Abbreviations: PWV: pulse wave velocity; AIx: augmentation index; PP: pulse pressure. P-values were based on the students' t-test.

In the multivariable linear regression analysis, neither physical performance, nor physical activity, nor hand-grip strength were associated with the amount of arterial stiffness after a follow-up period of 2 years (**Table 3**). Gender as well as age did not modify these models.

To test the hypothesis of arterial stiffness being irreversible, a stratified analysis was done comparing participants with and without increased arterial stiffness (PWV ≥ 12 m/s) at baseline. In both groups, physical performance, activity and hand-grip strength were not able to predict the amount of arterial stiffness after 2 years.

Table 3. Multivariate linear regression analysis of the association between physical parameters at baseline and arterial stiffness after 2yrs of follow-up

	PP	PA	HGS
PWV (m/s)	-0.04 [-0.20 ; 0.11]	$-2.92 \cdot 10^{-5}$ [-0.01 ; 0.01]	-0.03 [-0.10 ; 0.04]
AIx (%)	0.23 [-0.11 ; 0.57]	$-3.13 \cdot 10^{-4}$ [-0.01 ; 0.01]	0.08 [-0.06 ; 0.21]
Aortic PP (mmHg)	0.05 [-0.32 ; 0.43]	$-8.61 \cdot 10^{-4}$ [-0.01 ; 0.01]	-0.11 [-0.26 ; 0.05]

Data are presented as estimated beta with 95% confidence interval (CI) and were adjusted for baseline measure of arterial stiffness parameter, age, gender, treatment allocation, study center, MAP, heart rate, smoking status, alcohol use, eGFR, BMI. Abbreviations: PWV: pulse wave velocity; AIx: augmentation index; PP: pulse pressure; PP: physical performance score, PA: physical activity measured with LAPAQ; HGS: hand-grip strength.

3.1.5   DISCUSSION

In our study, physical performance, physical activity and hand-grip strength were not associated with arterial stiffness after a follow-up period of 2 years. Stratification for the presence of elevated arterial stiffness at baseline did not modify these results.

To the best of our knowledge, our study is the first to address the longitudinal relation between physical fitness, physical activity and muscle strength on vascular parameters among elderly individuals. Although many other studies have been done, these were mainly trials investigating the effect of exercise on vascular function, in general follow-up time was relatively short, with a mean duration of approximately 12 weeks [6-9]. In our study, we did not observe an association between physical fitness, physical activity and hand-grip strength with arterial stiffness longitudinally during a long-term follow-up of 2 years.

The lack of an association may be explained in several ways. First, there may be a difference between short-term and long-term adaptations of the arteries. On short-term, aerobic exercise has been shown to change endothelial function and inflammation, mediating the improvement of the arterial stiffness process [10]. It has also been shown that structural adaptations of the artery are possible, based on elastin and collagen changes, which are less likely to occur after a short period of time, but rather occur on the long-term [10]. Nevertheless, we did also not observe an association between physical parameters and arterial stiffness over a period of 2 years. Our finding might indicate that these structural changes are not present in older individuals. Potentially this has to do with the limited reversibility of the arterial stiffening process. If the latter holds true, the predictive value of the physical parameters would differ between participants with and without elevated arterial stiffness at baseline. Nevertheless, stratification did not show different results between both groups. Also in the participants without elevated arterial stiffness, physical fitness, physical activity and muscle strength were not associated with the amount of arterial stiffness after 2 years. Therefore we may conclude, that these mechanisms are probably of less importance in the arterial ageing process. Nevertheless, it is possible that in older persons only very intense physical exercise will lead to the adaptations mentioned above, as these vascular changes occur more within younger individuals during intensive activities.

Besides, we did not observed an association between hand-grip strength and arterial stiffness after a period of 2-years. Hand-grip strength is possibly only a reliable way for measuring muscle resistance in trained individuals, and not in an untrained older population. Especially since hand-grip strength is more often used as a marker of frailty in this age group and not as a measure of muscle strength [20, 21].

There are several limitations that need mentioning. First, our population consists of hyperhomocysteinemic elderly [11], which makes our findings not generalizable to the overall population. Nevertheless, almost 50% of all European elderly has increased homocysteine levels [22], making this condition very common. Furthermore, for the vascular measurements, participants were invited to come to the hospital for undergoing the tests and it cannot be ruled out that the more frail participants would be restrained for this appointment. Because our participants were slightly more active, had higher performance score and higher hand-grip strength compared to the total population, this is certainly a possibility. Third, all participants participated in a trial and all received low dose vitamin D during the study and were allocated in a B-vitamin treatment group or placebo [11]. Although the B-vitamin treatment did not influence the arterial stiffness parameters [23], a potential effect of vitamin D supplementation on arterial stiffness levels, in particular for AIx, cannot be excluded. However, a trial with vitamin D supplementation in postmenopausal women did not show beneficial effects on arterial stiffness [24]. Fourthly, physical activity was assessed with the LAPAQ, a self-report questionnaire. It is possible this self-report is not completely representative for the true activity load, especially for lighter activities [25]. Nevertheless, this questionnaire has been validated in an older population, showing to be valid and reliable for measuring physical activity [16], and we calculated the activity in kcal/day, therefore adding more value to heavier activities. Regarding the physical performance score, this score has been validated in a more frail population [12] compared to our relatively healthy study population. These instruments of measuring physical activity and fitness may therefore not be able to detect subtle differences among fitter elderly.

Conclusion

Physical fitness, physical activity and muscle strength were not associated with preclinical cardiovascular disease in the older B-PROOF population. Potentially only very intensive activity will be able to modify arterial stiffness rather than daily activity. Furthermore, with regard to the limited reversibility of arterial stiffness, the influence of physical activity would be minimal in older individuals. Nevertheless, more research is warranted to elucidate the long-term effects of daily and intensive physical activity on arterial stiffness in an elderly population, preferably by using an intervention trial. Only in this way knowledge regarding the long-term effects of physical activity on the prevention of cardiovascular disease in older persons can be obtained.

Acknowledgements

3.1.6   REFERENCES

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3.2

Arterial stiffness is not associated with bone measures in an older hyperhomocysteinemic population

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3.2.1   ABSTRACT

Introduction

Several studies have observed positive associations between bone disease and cardiovascular disease. A potential common pathway is hyperhomocysteinemia. However, up to now, data are lacking regarding hyperhomocysteinemic populations. Therefore we examined, both cross-sectionally and longitudinally, whether there is an association between bone parameters and arterial stiffness in a hyperhomocysteinemic population, and investigated the potential common role of homocysteine level on these associations.

Methods

Data of the B-PROOF study were used (n = 519). At both baseline and 2-year follow-up we determined bone measures: incident fractures and history of fractures, bone-mineral density (BMD) and quantitative ultrasound (QUS) measurement; arterial stiffness parameters were measured at baseline: pulse wave velocity (PWV), augmentation index (AIx) and aortic pulse pressure (PP) levels with applanation tonometry. Linear regression analysis was used to examine these associations and we tested for potential interaction of homocysteine level.

Results

Mean age was 72.3 years and 44.3% was female. Both cross-sectionally and longitudinally there was no association between arterial stiffness measures and BMD or QUS measurements, nor with incident fractures (n = 16) within the 2-3 years of follow-up. Homocysteine level did not modify the associations and neither did adjustment for homocysteine change the results.

Conclusion

Arterial stiffness was not associated with bone parameters and fractures, and homocysteine neither acted as a pleiotropic factor nor as a mediator. The potential association between bone and arterial stiffness is therefore not likely to be driven by hyperhomocysteinemia.

3.2.2   INTRODUCTION

Both cardiovascular disease and osteoporosis are common conditions in the older population. Research has demonstrated that diseases of vasculature and bone share several similar determinants, like for example advancing age, smoking and diabetes mellitus [1-5]. Recently, it has been shown that prevalence of cardiovascular disease is associated with new occurrence of hip fractures [6]. Furthermore, in several studies osteoporosis and bone mineral density have been shown to predict cardiovascular mortality, cardiovascular morbidity and atherosclerosis [7-10]. However, there are also a few studies that did not observe an association between BMD and cardiovascular mortality [11, 12]. Therefore, the question rises whether the relation between bone diseases and cardiovascular disease is causal, or may be driven by a common pathway.

One of the hypothesized common pathways is hyperhomocysteinemia [13-16]. Hyperhomocysteinemia is associated with both osteoporosis and with cardiovascular disease via dysregulation of the RANK-ligand/RANK axis and via inflammation [17-19]. Particularly in elderly, hyperhomocysteinaemia is associated with osteoporosis and hip fractures [16, 20]. Hyperhomocysteinaemia is also an important risk predictor for cardiovascular disease in this age group [21], predominantly via arterial stiffness, a preclinical cardiovascular condition [22, 23].

Up to now there are no reports addressing the association between bone and cardiovascular parameters in elderly or hyperhomocysteinemic populations. Nevertheless, different associations might be expected between bone and vascular parameters in individuals having a relatively low versus high homocysteine level. As mentioned above, high levels of homocysteine are associated with lower BMD levels, increased fracture risk and elevated arterial stiffness, and therefore, we expected a stronger relationship at higher homocysteine levels as compared to lower homocysteine levels. The first aim of this study was therefore to investigate whether arterial stiffness is associated with bone parameters and fractures in older persons with mildly elevated homocysteine levels (the B-PROOF study). Our second aim was to better explore the potential role of homocysteine in the etiology of osteoporosis and cardiovascular disease (**Figure 3.2.1**).
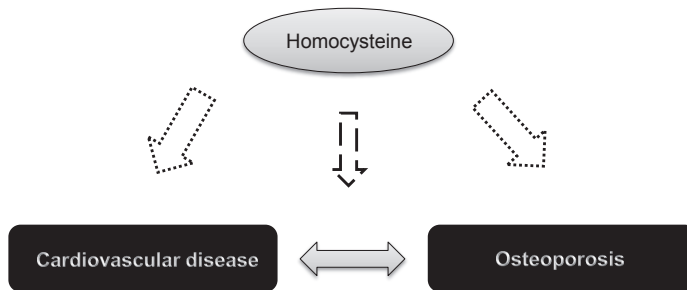
Figuur 3.2.1.

•••••••• Potential pleotropic effect

— — Potential mediating effect

3.2.3 METHODS

Study population

The present study was conducted within the framework of the B-PROOF (B-vitamins for the PRevention Of Osteoporotic Fractures) study. A detailed description of this randomized controlled trial has been reported elsewhere [24]. In short, B-PROOF is a multi-center, randomized, placebo controlled, double-blind trial including 2919 participants from three areas in the Netherlands. Main inclusion criteria were age 65 years and older, and an elevated homocysteine level (12 – 50 µmol/l). Main exclusion criteria were renal insufficiency (creatinine level > 150 µmol/l) and presence of a malignancy. All participants gave written informed consent before the start of the study. The Wageningen Medical Ethics Committee approved the study protocol, and the Medical Ethics committees of Rotterdam and Amsterdam gave approval for local feasibility. The present study was conducted in a randomly invited subsample of 519 participants in whom both vascular parameters and bone measures were available.

Laboratory measurements

Venous blood samples were obtained in the morning, when participants were in a fasted state, or had taken a restricted breakfast [24]. For total homocysteine analysis, a plasma EDTA tube was stored on ice immediately after blood drawing [24], and samples were processed within 4 hours in order to prevent a temperature- and time-dependent increase in plasma homocysteine [25]. Plasma homocysteine was measured using the Architect

i2000 RS analyzer (VU University Medical Center, intra assay CV=2%, inter assay CV=4%) and LC-MS/MS (Erasmus intra assay CV=5.5%, inter assay CV=1.3%). Outcomes of the centers did not differ significantly at cross-calibration. Serum creatinine was measured with the enzymatic colorimetric Roche CREA plus assay (CV=2%). The Modification of Diet in Renal Disease (MDRD) was calculated in ml/min/1.73m^2 with the formula: 186 * (serum creatinine (µmol/l) / 88.4)$^{-1.154}$ * age (years)$^{-0.203}$ * 0.742 (for females) [26].

Arterial stiffness

At the Erasmus MC (Rotterdam) and VU University Medical Center (Amster-dam), a subsample of participants underwent vascular measurements at the same day of BMD measurement. Participants with cardiac arrhythmia were excluded from these additional measurements. During the measurements, participants were situated in supine position on a flat examination couch in a quiet laboratory room for at least 5-10 minutes prior to the measurements, and the participants were not allowed to speak during the measurements. Use of alcohol or coffee during 12 hours before the measurements was pro-hibited.

Blood pressure measurement

Peripheral blood pressure at the time of vascular function tests was mea-sured once with a semi-automatic oscillometric device (Datascope Accurator Plus device, Datascope Corp. New Jersey, USA) after at least five minutes of supine rest. All blood pressure measurements were conducted at the right arm and were measured in mmHg. The mean arterial pressure was measured and the pulse pressure was calculated as the systolic minus the diastolic blood pressure.

Applanation tonometry

Arterial tonometry was obtained from the right radial, right carotid and right femoral artery using the Sphygmocor device (Sphygmocor version 7.1, AtCor Medical, Sydney, Australia). An estimate of the corresponding central aortic pulse wave was calculated, using a validated generalized transfer function incorporated in the device. With the integral software, the central augmented pressure was calculated as the difference between the early and late systolic peaks of the estimated central pressure waveform. Central aortic augmenta-tion index (AIx) was calculated as the augmented pressure expressed as a percentage of the pulse pressure (intra CV = 3%, inter CV = 5%). Aortic pulse wave velocity (aPWV) was measured with three channel ECG recording

and simultaneously recording of the right carotid artery pulse wave form and subsequently the femoral artery pulse waveform. The aPWV was calculated as the delay between the femoral pulse wave and the carotid pulse wave, divided by the transit distance (intra CV = 5 %, inter CV = 8%). Transit distance was assessed with body surface measurement from the carotid artery to the femoral artery [27].

Bone parameters (outcome variables)

Fractures

Fracture history was assessed at baseline using a structured questionnaire. During the 2-3 year follow-up, participants reported incident fractures on a study calendar every three months. Finally, at the end of follow-up participants were asked for the occurrence of fractures during the study period to confirm the data collected during follow-up. All reported fractures were verified with the participants' general practitioner or hospital.

Bone mineral density

At Erasmus MC (Rotterdam) and VU Medical Center (Amsterdam), participants were invited for bone mineral density (BMD) measurement. Dual-energy X-ray assessment (DEXA) was used to measure femoral neck and lumbar spine BMD (g/cm^2) under standard protocols within four weeks of the individual's start in the intervention. For all measurements, the Hologic QDR 4500 Delphi device (VUmc, (Hologic, USA)) or the GE Lunar Prodigy device (Erasmus MC, (GE Healthcare, USA)) were used. The two devices were cross-calibrated by measuring a European spine phantom (ESP) five times on both machines and all results were adjusted accordingly.

Bone quality – Quantitative ultrasound of the calcaneus

During the blood-drawing visit, a random subgroup of participants was invited to undergo Quantitative ultrasound (QUS) measurements of the calcaneus. These measurements were performed using a Hologic Sahara bone densitometer (Hologic, USA) (Erasmus MC, VUmc, WUR) or a CUBA Clinical system (VUmc). Subjects were excluded from QUS measurements if edema in the foot/ankle was visibly present, since this is known to affect the measurement [28]. Broadband ultrasound attenuation (BUA, dB/MHz) and speed of sound (SOS, m/s) were measured in duplicate in both the right and the left calcaneus. For each individual, averages of these four measurements were calculated. Measurements were excluded when violation of the

expected linear frequency-attenuation relation occurred, since this indicates invalid results.

Covariates

Height was measured in duplicate to the nearest 0.1 cm while standing erect and wearing no shoes, using a stadiometer [24]. Weight was measured with the participant wearing light garments without shoes and empty pockets to the nearest 0.5 kg using a calibrated weighing device (SECA 761) [24]. Body Mass Index (BMI) was calculated as weight divided by squared height and expressed as kg/m^2.

Alcohol intake and smoking habits were determined using a questionnaire [24]. Questions regarding cardiovascular disease risk factors including hypertension, hypercholesterolemia and diabetes mellitus were also included [24].

Statistical analysis

The distributions of all variables of interest were examined with histograms. The linearity of associations between arterial stiffness parameters and bone parameters was tested with curve estimation modeling. Because linear associations had the best fit, Pearson correlation coefficients were calculated and multivariable linear regression analysis was used. Potential confounders were age, gender, study center, smoking status, alcohol use, eGFR, BMI, diabetes, hypertension and hypercholesterolemia. These parameters were added to the final model when they were considered to be clinically relevant or contributed to a > 10% change of the point estimate.

We tested the association of arterial stiffness with bone parameters both cross-sectionally at baseline and longitudinally after 2 years of follow up. Arterial stiffness measures at baseline were used as independent variables and bone parameters, including fractures (at baseline and after 2 years of follow-up; fractures 2-3 years of follow-up), were the outcome variables. Regarding the longitudinal analysis, we considered it justified to include all B-PROOF participants who were also included in the cross-sectional analyses (the participants who received placebo as well as the participants who received the B-vitamin treatment), because the intervention with vitamin B12 and folic acid overall did not significantly prevent fractures and did not change the levels of BMD, BUA or arterial stiffness [29, 30], however in order to adjust for any potential residual confounding we did adjust for group assignment.

Within the cross-sectional multivariable linear regression analysis we tested whether adjustement for homocysteine affected the possible association between measures of arterial stiffness and bone parameters. Also for the longitudinal analysis we adjusted for homocysteine level.

Potential effect modification of homocysteine
We cross-sectionally tested whether plasma homocysteine modified the association between measures of arterial stiffness and bone parameters. If the interaction term homocysteine * PWV or another arterial stiffness measure was significant (p < 0.1), a stratified analysis was done. Strata were created based on homocysteine level < 18 μmol/L and homocysteine level ≥ 18 μmol/l.

We also tested whether the change in homocysteine level during the two years of follow-up modified the longitudinal analysis. If the interaction term homocysteine-change * PWV (or AIx/aortic PP) was significant (p < 0.1) a stratified analysis was done with participants with an increase in homocysteine level after 2yr follow-up, no change in homocysteine during 2 yrs and a decrease in homocysteine level after 2yr follow-up.

Statistical analyses were performed using the statistical software package of SPSS version 20.0 (SPSS Inc, Chicago, Illinois, USA). The α-level was set at 0.05.

3.2.4   RESULTS

Characteristics of the population are described in **Table 1**. The mean age was 72.3 years and 44.3% was female. The median number of fractures occurred in history was 0.0 [IQR 0-1], total number ranging from 0 to 7. One hundred ninety-nine participants had at least 1 fracture in history.

Within the cross-sectional analysis at baseline, PWV correlated negatively with FN-BMD (r = -0.09, p = 0.04), but not with LS-BMD, BUA or SOS. AIx correlated negatively with FN-BMD, LS-BMD, BUA and SOS levels (r=-0.24 p < 0.01; r=-0.21 p<0.01; r=-0.17 p<0.01; r=-0.12 p=0.04 respectively). Aortic PP correlated with FN-BMD (r=-0.16 p<0.01), but not with LS-BMD, BUA or SOS. The number of sustained fractures in history did not correlate with either of the vascular parameters.

Table 1. Population characteristics

	n	Mean ± SD
Age (years and range)	519	72.3 ± 5.4
Gender (n, %) Female	519	230 (44,3%)
Homocysteine (µmol/L)	519	14.1 [12.9 – 16.4]
Number of sustained fractures in history	519	0.6 ± 1.1 [range 0-7]
Cardiovascular risk factors		
Smoking behavior (n, %) Never Former Current	519	 186 (35.8%) 286 (55,1%) 47 (9,1%)
Alcohol use (n, %) Light Moderate Excessive Very excessive	519	 330 (63,6%) 159 (30,6%) 24 (4,6%) 6 (1,2%)
eGFR (ml/min per 1.73m^2)	519	90.5 ± 35.0
BMI (kg/m^2)	519	27.0 ± 3.7
Brachial blood pressure (mmHg) SBP DBP	519	 137.6 ± 17.9 77.3 ± 9.5
Hypertension (n, %)	519	210 (40,5%)
Diabetes mellitus (n, %)	519	57 (11,0%)
Hypercholesterolemia (n, %)	519	142 (27.4%)
Vascular parameters		
PWV (m/s)	479	14.2 ± 4.3
AIx (%)	509	26.3 ± 10.1
Aortic PP (mmHg)	510	50.0 ± 15.0

Data are presented as mean ± SD or as number (%) when categorical variables. Homocysteine is presented as median [IQR].

Abbreviations: eGFR: estimated glomerular filtration rate; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; PWV: pulse wave velocity; AIx: augmentation index; PP: pulse pressure.

Within the cross-sectional multivariable linear regression analysis, adjusted for age, gender and study center, none of the arterial stiffness measures were associated with the number of sustained fractures in history, FN-BMD, LS-BMD, BUA or SOS (**Table 3**). In particular the adjustment for age altered the associations to non-significant. Also in the longitudinal linear regression

Table 2. Bone characteristics at baseline and 2-yr follow-up

	Baseline	Follow-up	p-value
FN-BMD (g/cm^2)	0.87 ± 0.16	0.86 ± 0.16	< 0.01
LS-BMD (g/cm^2)	1.15 ± 0.24	1.16 ± 0.24	< 0.01
BUA (dB/mHz)	72.9 ± 16.3	72.5 ± 17.2	< 0.01
SOS (m/s)	1541.2 ± 29.4	1544.7 ± 32.8	0.47

Data are presented as mean ± SD. Abbreviations: FN-BMD: femoral-neck bone mineral density; LS-BMD: lumbal-spine bone mineral density; BUA: bone ultrasound attenuation; SOS: speed of sound.

analysis, we did not observe an association between the vascular parameters and bone parameters after a period of 2 years (**Table 4**). During the 2-year follow-up period, 16 participants suffered at least one fracture. We did not find an association between the vascular parameters and incident fractures (data not shown).

Additional adjustment for homocysteine (baseline level in the cross-sectional analysis and homocysteine change in the longitudinal analysis) did not significantly change the associations between arterial stiffness measures and bone parameters (**Table 3 and 4**).

Potential effect modification of homocysteine

In order to investigate the effect of homocysteine level on the association between bone and vascular parameters, we tested the interaction effect of homocysteine. The homocysteine * aortic PP interaction was significant in the association with FN-BMD (p = 0.02). However, in a stratified analysis for Hcy < 18 µmol/l and Hcy ≥ 18 µmol/l we did not observe any association between aortic PP and FN-BMD (data not shown). Regarding sustained fracture history, we observed a significant homocysteine * aortic PP interaction (p = 0.09) and a significant homocysteine * AIx interaction (p = 0.02) (data not shown). In the stratified analysis for Hcy < 18 µmol/l and Hcy ≥ 18 µmol/l, we observed a significant association between the number of sustained fractures in history and aortic PP in the group with homocysteine levels ≥ 18 µmol/l (beta -0.04 95%CI [-0.07 ; -0.01]). In other words, in the group with high homocysteine levels, lower number of fractures was associated with higher levels of aortic PP.

In the longitudinal analysis, the homocysteine change – aortic PP interaction was again significant (p = 0.01) for FN-BMD (at follow-up), but not for the

Table 3. Cross-sectional linear regression analysis between bone and vascular parameters at baseline

	Number of sustained fractures in history		FN-BMD (g/cm^2)		BUA (dB/mHz)	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
PWV (m/s)	0.007 [-0.018; 0.032]	0.006 [-0.019; 0.031]	4.63·10^{-4} [-0.003 ; 0.002]	-0.001 [-0.003 ; 0.002]	-0.078 [-0.458 ; -0.302]	-0.021 [-0.409 ; 0.367]
AIx (%)	0.003 [-0.007; 0.013]	0.002 [-0.008; 0.012]	-0.001 [-0.002 ; 2.70·10^{-4}]	-0.001 [-0.002 ; 1.6210^{-4}]	0.030 [-0.151 ; 0.211]	0.044 [-0.138 ; 0.226]
Aortic PP (mmHg)	-0.003 [-0.009; 0.004]	-0.004 [-0.012; 0.004]	-1.31·10^{-4} [-0.001 ; 0.001]	-4.14·10^{-4} [-0.001 ; 4.35·10^{-4}]	-0.026 [-0.076 ; 0.128]	0.001 [-0.123 ; 0.126]

	LS-BMD (g/cm^2)		SOS (m/s)	
	Model 1	Model 2	Model 1	Model 2
PWV (m/s)	0.002 [-0.003 ; 0.006]	0.002 [-0.001 ; 0.006]	-0.306 [-0.990 ; 0.378]	-0.238 [-0.931 ; 0.455]
AIx (%)	-0.001 [-0.003 ; 3.84·10^{-4}]	-0.002 [-0.003 ; 1.97·10^{-4}]	0.142 [-0.199 ; 0.483]	0.167 [-0.175 ; 0.509]
Aortic PP (mmHg)	-1.92·10^{-4} [-0.001 ; 0.001]	-4.64·10^{-4} [-0.002 ; 0.001]	0.075 [-0.117 ; 0.268]	0.043 [-0.191 ; 0.278]

Data are presented as beta's ± 95% CI. Model 1: adjusted for age, gender and study center. Model 2: adjusted for age, gender, study center, treatment, smoking status, alcohol use, eGFR, BMI, diabetes, cholesterol and hypertension. Abbreviations as in Table 1 and 2.

Table 4. Longitudinal linear regression analysis with bone measures after 2 yrs as outcome and vascular parameters at baseline as independent

	FN-BMD (g/cm^2)		LS-BMD (g/cm^2)	
	Model 1	Model 2	Model 1	Model 2
PWV (m/s)	$1.86 \cdot 10^{-5}$ [-0.001 ; 0.001]	$-3.14 \cdot 10^{-5}$ [-0.001 ; 0.001]	$3.72 \cdot 10^{-4}$ [-0.001 ; 0.001]	$4.64 \cdot 10^{-4}$ [-0.001 ; 0.002]
AIx (%)	$5.31 \cdot 10^{-5}$ [$-2.16 \cdot 10^{-4}$; $3.22 \cdot 10^{-4}$]	$8.32 \cdot 10^{-5}$ [$-1.90 \cdot 10^{-4}$; $3.56 \cdot 10^{-4}$]	$1.45 \cdot 10^{-4}$ [$-2.62 \cdot 10^{-4}$; 0.001]	$2.58 \cdot 10^{-4}$ [$-1.54 \cdot 10^{-4}$; 0.001]
Aortic PP (mmHg)	$8.99 \cdot 10^{-5}$ [$-2.63 \cdot 10^{-4}$; $0.83 \cdot 10^{-4}$]	$-1.06 \cdot 10^{-4}$ [$-3.12 \cdot 10^{-4}$; $1.00 \cdot 10^{-4}$]	$-1.70 \cdot 10^{-4}$ [$-4.33 \cdot 10^{-4}$; $0.93 \cdot 10^{-4}$]	$-2.32 \cdot 10^{-4}$ [-0.001 ; $0.79 \cdot 10^{-4}$]

	BUA (dB/mHz)		SOS (m/s)	
	Model 1	Model 2	Model 1	Model 2
PWV (m/s)	-0.239 [-0.464 ; -0.015]*	-0.213 [-0.443 ; 0.017]	-0.162 [-0.633 ; 0.309]	-0.222 [-0.704 ; 0.261]
AIx (%)	0.004 [-0.098 ; 0.106]	0.001 [-0.103 ; 0.105]	-0.061 [-0.282 ; 0.160]	-0.060 [-0.287 ; 0.166]
Aortic PP (mmHg)	-0.014 [-0.072 ; 0.044]	-0.004 [-0.075 ; 0.066]	0.009 [-0.117 ; 0.134]	0.027 [-0.126 ; 0.180]

Data are presented as beta's ± 95% CI. Model 1: adjusted for baseline measure, age, gender, study center and treatment. Model 2: adjusted for baseline measure of BMD/QUS, age, gender, study center, treatment, smoking status, alcohol use, eGFR, BMI, diabetes, cholesterol and hypertension. Abbreviations: as in Table 1 and 2.

other bone parameters (data not shown). Stratified analysis for homocysteine level <18 µmol/L and ≥18 µmol/L did also not demonstrate any significant association of the vascular parameters in the strata (data not shown). For the incident fractures during follow-up, we did not see any interactions with the homocysteine change.

3.2.5   DISCUSSION

Our study showed that in an elderly population with hyperhomocysteinemia, arterial stiffness is not associated with prevalent or incident fractures, BMD or quantitative ultrasound bone measures (QUS), neither cross-sectionally nor longitudinally. Although homocysteine level was significantly interacting in the association between aortic PP and FN-BMD and fracture history, stratified analysis based on homocysteine level did not confirm this interaction effect. Furthermore, adjustment for homocysteine did not change the results. Therefore it is unlikely that homocysteine is a pleiotropic factor or mediator in the relation between bone and vasculature.

To our knowledge, this is the first study investigating the possible association of measures of arterial stiffness markers in relation to bone parameters in hyperhomocysteinemic elderly. Contradictory to our study, most of the cross-sectional studies in literature used small sample sizes, often middle-aged women were included and most studies used calcaneus ultrasound for their BMD-measures instead of measuring the golden standard BMD with dual X-ray absorptiometry [31-38]. Furthermore, data regarding a hyperhomocysteinemic population are lacking and a major strength is that we used fractures, bone density and bone quality as outcome variables, thus a broad perspective regarding the association between the vasculature and bone. Other cross-sectional studies, which did show associations between bone and arterial stiffness, often demonstrated only weak associations of which the clinical relevance can be discussed [31-38]. The lack of an association between arterial stiffness and osteoporosis parameters both cross-sectionally and longitudinally in our study endorses recent studies regarding BMD and cardiovascular mortality, which also observed no association [11, 12]. Arterial stiffness is presumably not the underlying pathway, in the potential association between bone and cardiovascular disease, because we observed no relation between arterial stiffness and several bone parameters. Because arterial stiffness is an important pre-clinical phase of cardiovascular disease

and we did also not observed a longitudinal association, we might conclude that a (causal) association between bone and cardiovascular disease is not likely.

The major aim of this study was to investigate whether homocysteine levels underlie or influence the assumed associations between bone and vasculature. Hyperhomocysteinemia has been hypothesized to form an underlying condition, because as mentioned above it is associated with both osteoporosis and with cardiovascular disease via dysregulation of the RANK-ligand/RANK axis and via inflammation [17-19]. Increased homocysteine levels have been shown to down-regulate bone collagen-crosslinking and methylation [16] and it causes increased thrombogenecity, increased oxidative stress, impaired endothelial function, platelet aggregation and finally atherothrombosis [39-42]. Nevertheless, in our study, homocysteine did only modify the association between FN-BMD with aortic PP, but no interaction was seen with other outcome variables. Because this modification was only found in one parameter, it may well be a chance finding. Especially since stratification based on homocysteine level did not change the results. Although this also can imply the cut-off value of 18 µmol/l was not high enough to demonstrate an association in persons with high homocysteine levels, it does show that this potential pathway is not relevant for the majority of the population. Homocysteine adjustment did also not change the results, making it unlikely to be a potential pleiotropic factor. Therefore we can conclude that homocysteine is neither an mediator or pleiotropic factor in the association between arterial stiffness and bone parameters.

There are some limitations to our study. First, our study population consisted of hyperhomocysteinemic elderly only. Nevertheless, as explained above, this type of population is of particular interest in order to explore the role of homocysteine explaining the association between bone and vasculature and may therefore also be interpreted as a strength. Our results might therefore not be applicable for the general older population. However, hyperhomocysteinemia is very common among elderly, with a prevalence of 25-50% in European countries [43], thus a large part of the older population was included. Third, due to the trial design, all participants received low dose vitamin D during the study and were allocated in a B-vitamin treatment group or placebo [24]. Although the B-vitamin treatment did not influence the arterial stiffness and bone parameters [29, 30], a potential attenuating effect of vitamin D supplementation on the longitudinal analysis cannot be

excluded. An effect of vitamin D therapy on bone health has been studied extensively [44] and could therefore have masked the influence of arterial stiffness on the investigated bone parameters.

In conclusion, contrary to our expectations, arterial stiffness was not associated with bone measures in an older, hyperhomocysteinemic population. In the potential association between bone and cardiovascular status, homocysteine is probably not a common factor and may either be dependent on other mechanisms or may not be causal. More research is warranted in order to elucidate the exact mechanisms that explain relationships between the vasculature and bone.

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3.3

Non-linear associations between 25-OH vitamin D and indices of arterial stiffness and arteriosclerosis in an older population

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3.3.1   ABSTRACT

Background

Several studies have been pointing towards a non-linear relationship between serum 25(OH)D and cardiovascular disease. Next to vitamin D deficiency, also higher levels of 25(OH)D have been reported to be associated with increased cardiovascular risk. We aimed to investigate the nature of the relationship between serum 25(OH)D and measures of arterial stiffness and arteriosclerosis in an elderly population.

Design: cross-sectional.

Setting/subjects: a subgroup of the B-PROOF study was included to determine associations between serum 25(OH)D and arterial stiffness and atherosclerosis (n = 567, 57% male, age 72.6 ± 5.6 years, mean serum 25(OH)D 54.6 ± 24.1 nmol/l).

Methods

Carotid intima media thickness (IMT) was assessed using ultrasonography and pulse wave velocity (PWV) was determined with applanation tonometry. Associations were tested using multivariable restricted cubic spline functions and stratified linear regression analysis.

Results

The associations between serum 25(OH)D and carotid IMT or PWV were non-linear. Spline functions demonstrated a difference between 25(OH)D deficient and sufficient individuals. In serum 25(OH)D sufficient participants (≥50 nmol/l; n = 287), a positive association with IMT and serum 25(OH)D was present (β 1.24; 95%CI [0.002; 2.473]). PWV levels were slightly lower in vitamin D deficient individuals, but the association with 25(OH)D was not significant.

Conclusion

Our study demonstrates that associations of serum 25(OH)D and PWV and IMT in an elderly population are not linear. In particular from serum 25(OH)D levels of 50 nmol/l and up, there is a slight increase of IMT with increasing 25(OH)D levels.

3.3.2 INTRODUCTION

Cardiovascular disease is more common among individuals with low serum 25(OH)D levels compared with 25(OH)D sufficient individuals [1–6]. However, not only vitamin D deficiency has reported to be associated with increased cardiovascular risk but high serum 25(OH)D levels have been as well. Taken these findings together, evidence of the recent years has been pointing towards a non-linear association between serum 25(OH)D level and cardiovascular disease [7]. High serum 25(OH)D levels could affect the vascular wall, either indirectly or via a direct effect of serum 25(OH)D. These processes may also have a role in the arterial stiffening process [8, 9].

Up till now, only inverse linear associations between serum 25(OH)D level and arterial stiffness have been reported. Earlier studies have been performed in younger subjects and disease-specific populations, like diabetics [8–12]. Very recently, the Baltimore Longitudinal Study of Ageing confirmed such an inverse linear association in an older population [13]. An inverse association between serum 25(OH)D and arteriosclerosis has only been described in serum 25(OH)D deficient individuals [14–16]. Because both arterial stiffness and atherosclerosis are important pre-clinical stages of cardiovascular disease, we aimed to investigate the nature of the relationship (e.g. linear, monotone or other) between serum 25(OH)D levels and indices of arterial stiffness and arteriosclerosis in older individuals.

3.3.3 METHODS

Study participants

The present study was conducted as a cross-sectional baseline analysis within the framework of the vascular subgroup of the B-PROOF (B vitamins for the prevention of osteoporotic fractures) study (n = 567). A detailed description of this randomized controlled trial has been reported elsewhere [17].

In short, B-PROOF is a multi-centre, randomised, placebo controlled, double-blinded trial including 2919 participants from three areas in the Netherlands. Main inclusion criteria were age 65 years and older, and an elevated homocysteine level (12–50 µmol/l). Main exclusion criteria were renal insufficiency (creatinine level > 150 µmol/l) and presence of a malignancy. All participants gave written informed consent before the start of the study. The Wageningen Medical Ethics Committee approved the study protocol, and

the Medical Ethics committees of Rotterdam and Amsterdam gave approval for local feasibility. At the Erasmus Medical Center (Rotterdam) and VU University Medical Center (Amsterdam), a random subsample of participants underwent vascular measurements (n = 567).

Clinical characteristics

Height was measured in duplicate to the nearest 0.1 cm while standing erect and wearing no shoes, using a stadiometer [17]. Weight was measured with the participant wearing light garments without shoes and empty pockets to the nearest 0.5 kg using a calibrated weighing device (SECA 761) [17].

Body mass index (BMI) was calculated as weight divided by squared height and expressed as kg/m2.

Self-reported medication use, alcohol intake and smoking habits were determined using a questionnaire [17]. Alcohol intake was assessed using the Garret structure classifying alcohol use into four categories (very excessive, excessive, moderate and light) [18]. Questions regarding cardiovascular disease history, like angina pectoris, myocardial infarction, transient ischaemic attack and/or stroke were also included in this questionnaire [17]. Furthermore, cardiovascular risk factors such as hypertension, hypercholesterolemia and diabetes mellitus were assessed with this questionnaire [17].

Serum 25(OH) vitamin D

Morning venous blood samples were obtained when participants were in a fasted state, or after a restricted breakfast [17]. Samples were stored at −80°C until determination in 2012. In short, measurement of serum 25(OH)D occurred by releasing it from its binding protein(s) and by adding a denaturized internal standard IS: 25(OH)D3-d6. Samples were extracted and analysed by XLC–MS/MS (a Symbiosis online SPE system (Spark Holland, Emmen, the Netherlands) coupled to a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA, USA). The inter-assay coefficient of variation was 9% at a level of 10 ng/mL (=24.9 nmol/l) and 6% at a level of 25 ng/mL (=62.4 nmol/l). All analyses were performed in the Endocrine Laboratory of the VU University Medical Center. Vitamin D deficiency was defined as a serum 25(OH)D level < 50 nmol/L according to literature since 25 (OH)D levels <50 nmol/l have reported to be related to several clinical disorders such as osteoporosis [19].

Serum creatinine

Serum creatinine was measured with the enzymatic colorimetric Roche CREA plus assay (CV = 2%). The estimated glomerular filtration rate (eGFR) was estimated with the Modification of Diet in Renal Disease (MDRD) and was calculated in ml/min/1.73 m2 with the formula: 186 × (serum creatinine (µmol/l)/88.4)−1.154 × age (years)−0.203 × 0.742 (for females) [20].

Vascular measurements

Only a random subsample of the B-PROOF study underwent vascular measurements (n = 567). Participants with cardiac rhythmic disturbances were excluded from these additional measurements. During the measurements, participants were situated in supine position on a flat examination couch in a quiet laboratory room for at least 5–10 min prior to the measurements. They were not allowed to speak during the measurements. Use of alcohol or coffee during 12 h before the measurements was prohibited [21].

Blood pressure measurement
Peripheral blood pressure at the time of vascular function tests was measured once with a semi-automatic oscillometric device (Datascope Accurator Plus device, Datascope Corp., NJ, USA) after at least 5 min of supine rest. Blood pressure measurements were conducted at the right arm and measured in mmHg. The mean arterial pressure (MAP) was measured and pulse pressure was calculated as systolic minus diastolic blood pressure.

Applanation tonometry
Arterial tonometry was obtained from the right carotid and right femoral artery using the Sphygmocor device (Sphygmocor version 7.1, AtCor Medical, Sydney, Australia). Aortic pulse wave velocity (aPWV) was measured with a three-channel ECG recording and simultaneously recording of the right carotid artery pulse wave form and subsequently the femoral artery pulse waveform. The aPWV was calculated as the delay between the femoral pulse wave and the carotid pulse wave, divided by the transit distance (intra-CV = 5%, inter-CV = 8%). Transit distance was assessed with body surface measurement from the carotid artery to the femoral artery [21].

Increased arterial stiffness was defined as a PWV>12 m/s, as this level is associated with increased cardiovascular risk [22].

Carotid intima media thickness

For carotid B-mode ultrasonography, the L105 40 mm 7.5 MHz array transducer was used (Picus, Pie Medical Equipment, Maastricht, the Netherlands) on the right carotid artery. Intima media thickness (IMT) is evaluated as the distance luminal − intimal interference and the media − adventitial interface (Art.Lab, Esoate Europe, Maastricht, the Netherlands) at ~1 cm proximal from the carotid bifurcation. In order to obtain accurate measurements, the standard deviation of the mean values of six independent measurements had to be <10% of the mean (intra-CV = 8%, inter-CV = 15%).

Statistical analysis

We have included all participants who had data available regarding serum 25(OH)D and carotid IMT and PWV measures in order to do a complete case analysis. For missing covariates, the statistical program automatically uses the mean for missing cases.

First, normal distribution was tested in all continuous variables and subsequently curve estimation models were created in order to investigate whether the associations between serum 25(OH)D levels and arterial stiffness parameters and carotid IMT were linear. Curve estimation models were tested by analysis of variance of the linear and non-linear component of the association between serum 25(OH)D and variables of interest. Statistics were performed using the statistical package SPSS 20.0 (SPSS Inc., Chicago, IL, USA). In case the associations were not linear, cubic spline analysis was performed to investigate non-linear associations between serum 25(OH)D and arterial stiffness measures and carotid IMT using R version 3.0.0. Spline regression models are piecewise polynomial functions that join smoothly at points called knots. In contrast to categorical models that assume a constant association within categories, in spline models all data points are used, providing a better estimate of dose−effect relationships [23]. Spline models were visually tested with three to five knots. In the final analyses, we used three knots based on the size of our study sample and because this gave the best fit, measured with the likelihood ratio. We adjusted for covariates that were considered clinically relevant or gave a considerable change of the likelihood ratio.

After this, we performed a multivariable linear regression analysis using SPSS 20.0, stratified for serum 25(OH)D levels based on the depicted spline plots. We accounted for confounders that were considered clinically relevant or contributed to a >10% change of the point estimate. Potential confound-

ers were age, gender, study centre, BMI, eGFR, MAP, heart rate, smoking, alcohol use, CRP and physical performance.

Smoking was added to the model as a binary variable: yes or no and this was also done for alcohol use: yes (moderate—very excessive) and no use (light). Furthermore, we tested whether age, gender, cardiovascular disease history, diabetes, hypercholesterolemia and use of calcium and/or vitamin D supplementation use significantly interacted with the association between serum 25(OH)D and pre-clinical measures of cardiovascular disease. If a variable interacted, stratified analysis was performed. Concerning all analyses, two-sided P-values of <0.05 were considered statistically significant.

3.3.4   RESULTS

Characteristics of the study population (n = 567) are shown in **Table 1**. Mean age was 72.5 ± 5.6 years and gender was equally distributed. The mean serum 25(OH)D level in our population was 54.6 ± 24.1 nmol/l and 50.3% of the participants were vitamin D deficient (<50 nmol/l).

Curve estimation modelling showed that the relationship between carotid IMT and serum 25(OH)D was not linear, and that a cubic model had the best fit (**Table 2**). The association between serum 25(OH)D and PWV was also not linear, but rather quadratic or cubic because of the explained variability, although neither of the models fitted the data well (**Table 2**).

In **Figure 3.3.1**, the spline curves created for each variable are depicted. As demonstrated, high levels of serum 25(OH)D were associated with higher measures of carotid IMT. Furthermore, both low and high levels of serum 25(OH)D were associated with lower levels of PWV. Since the association between serum 25(OH)D and carotid IMT visually appeared to be linear above a level of serum 25(OH)D of 50 nmol/l, we performed a stratified linear regression analysis for vitamin D deficient versus vitamin D sufficient participants. This analysis showed a positive association between serum 25(OH)D and carotid IMT (β 1.24; 95% CI: [0.002; 2.473]) in participants with serum 25(OH)D levels ≥ 50 nmol/l. In vitamin D sufficient older persons, an increase of 1 nmol/L of serum 25(OH)D corresponded to an increase of 1.24 μm carotid IMT (**Table 3**). Age was a significant effect modifier in this association (p = 0.01), showing that the association was only present in participants under the age of 80 (β 1.51 95% CI: [0.25; 2.77]) (data not

Table 1. Clinical and hemodynamic characteristics total population (n = 567)

| Variable	Mean ± SD
Age (years)	72.5 ± 5.6
80+	61 (10.8%)
Gender (n, %)	
Males	315 (55.6%)
BMI (kg/m^2)	27.3 ± 3.7
Smoking behavior (n, %)	
Never	198 (34,9%)
Former	53 (9,3%)
Current	316 (55,7%)
Alcohol use (n, %)	
Light	358 (63,1%)
Moderate	179 (31,6%)
Excessive	25 (4,4%)
Very excessive	5 (0,9%)
Self-reported medical history of	
Cardiac disease	61 (10.8%)
TIA or stroke	45 (7.9%)
Diabetes	65 (11.5%)
Hypertension	211 (38.1%)
Hypercholesterolemia	151 (26.6%)
Self-reported use of	
Vitamin D supplementation	9 (1.6%)
Calcium supplementation	20 (3.5%)
Calcium + vitamin D supplementation	18 (3.2%)
Mean dosage of used vitamin D (IE)	39.1 ± 144.8
Serum vitamin D level (nmol/l)	54.6 ± 24.1
Vitamin D deficiency (< 50 nmol/l)	274 (48.3%)
Serum creatinine level (μmol/l)	83.2 ± 17.2
eGFR (ml/min per 1.73m^2)	91.3 ± 35.9
Blood pressure	
SBP (mmHg)	137.7 ± 18.1
DBP (mmHg)	77.3 ± 9.6
Hypertension during measurement*	339 (59.8 %)
PWV (m/s)	14.3 ± 4.5
PWV > 12 m/s	313 (55.2%)
Carotid IMT (μm)	717.0 ± 163.2

Data are presented as number and percentage or as mean ± SD.

Abbreviations: eGFR: estimated glomerular filtration rate; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: Pulse Pressure; PWV: aortic pulse wave velocity; IMT: intima media thickness.

*Hypertension defined as SBP > 140 and/or DBP > 90 mmHg.

Table 2. Curve estimation models for both carotid IMT and PWV

	R^2	p-value
Carotid IMT		
Linear	0.001	0.65
Logarithmic	$0.46 \cdot 10^{-4}$	0.89
Quadratic	0.028	0.004
Cubic	0.045	$0.40 \cdot 10^{-3}$
PWV		
Linear	$0.23 \cdot 10^{-3}$	0.73
Logarithmic	$0.66 \cdot 10^{-4}$	0.85
Quadratic	0.003	0.41
Cubic	0.009	0.20
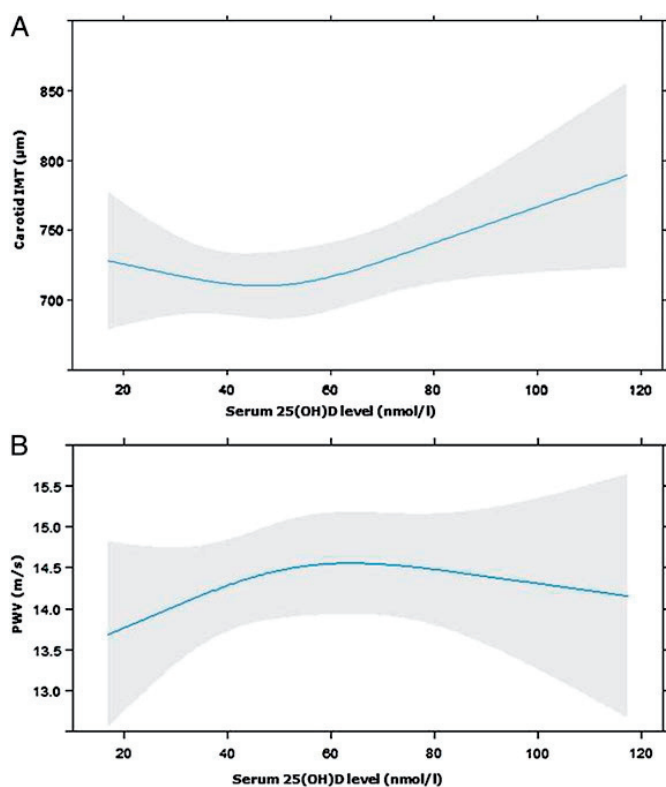
Figuur 3.3.1.

A. Carotid IMT. B. PWV. Carotid IMT data were adjusted for age, gender and eGFR. PWV data were adjusted for age, gender, study center, eGFR, MAP and heart rate. Abbreviations: as in table 1.

shown). In vitamin D insufficient participants, calcium use was a significant effect modifier (P = 0.02); however, stratified analysis did not demonstrate any differences between users and non-users. Gender, cardiovascular disease history, diabetes and hypercholesterolemia did not interact with the association between serum 25(OH)D and carotid IMT.

Table 3. Linear regression analysis of the associations between serum 25(OH)D level and IMT and PWV stratified per vitamin D category

Serum 25(OH)D < 50 nmol/l n = 274	
Carotid IMT	-0.79 [-3.217 ; 1.644]
PWV	0.04 [-0.020 ; 0.090]
Serum 25(OH)D ≥ 50 nmol/l n = 287	
Carotid IMT	1.24 [0.002 ; 2.473]*
PWV	$-0.31 \cdot 10^{-3}$ [-0.030 ; 0.029]

Values are presented as beta ± 95% CI and according to carotid IMT adjusted for age, gender and eGFR. Adjustments made for the analysis with PWV were age, gender, study center, eGFR, MAP and heart rate. * = p<0.05. Abbreviations: as in Table 1.

Furthermore, the direction of the association between serum 25(OH)D and PWV also changed at the level of 50 nmol/l, showing an inverse association between PWV levels and 25(OH)D below the concentration of serum 25(OH) D level of 50 nmol/l. In order to further investigate this association, we performed a stratified linear regression analysis for vitamin D deficient versus vitamin D sufficient individuals; however, we found no significant associations between serum 25(OH)D level and PWV for the separate groups (**Table 2**). Age, gender, cardiovascular disease history, diabetes, hypercholesterolemia or calcium use was not interacting within the association between serum 25(OH)D and PWV.

3.3.5   DISCUSSION

Our study shows that the association between serum 25 (OH)D and pre-clinical stages of cardiovascular disease in elderly subjects is non-linear. In particular, we have shown that high levels of serum 25(OH)D, starting from serum 25(OH)D levels of ≥50 nmol/l, were associated with higher values of carotid IMT in vitamin D sufficient individuals. Also, the association between serum 25(OH)D and PWV was non-linear, potentially monotone; however,

in contrast with the effect on IMT the effect size for PWV was small and not within clinically relevant ranges.

In contrast to other studies, we did not find linear associations between serum 25(OH)D and indices of arterial stiffness or arteriosclerosis [10, 12, 13]. Our study supports the abovementioned non-linear association with cardiovascular morbidity and mortality [7] because in particular for vitamin D sufficient individuals (serum 25(OH)D ≥ 50 nmol/l) an increase in carotid IMT per point increase of serum 25(OH)D level was present. Although this association was clear, one may argue about the clinical relevance because the carotid IMT only increased 1.24 μm per 1 nmol/l of serum 25(OH)D. Nevertheless, our finding gives an insight into potential mechanisms in which serum 25(OH)D might lead to increased cardiovascular risk.

Although it has been reported that vitamin D deficiency is associated with cardiovascular disease and with higher IMT levels [1–3, 14–16, 24], we were not able to confirm the latter in our study. The spline plot of carotid IMT did however show a significant positive association between serum 25(OH)D levels with carotid IMT in high-normal ranges of serum 25(OH)D. Several mechanisms may explain why high serum 25(OH)D levels are associated with an elevated carotid IMT. First, an increase of vitamin D levels will lead to an increased calcium absorption in the gastrointestinal tract, which in turn may result in higher circulating calcium concentrations. These higher calcium levels could contribute to vascular calcification, in particular in atherosclerotic plaques in the vessel wall. This may be further aggravated because high levels of vitamin D are known to up-regulate vitamin D receptors in vascular smooth muscle cells, reducing the activity of matrix metalloproteinases, which also contributes to calcium deposition in the vessel wall [25]. Furthermore, extrarenal activated macrophages express 1,α-hydroxylase, converting 25(OH)D into the hormonally active 1.25(OH)D form and activate pro-calcification in the vessel wall, which also contributes to arterial stiffness [8, 9].

In comparison with the recently published Baltimore study [13], we were not able to confirm a linear association between serum 25(OH)D and PWV. Visually, the association between 25(OH)D and PWV even appeared to be opposed compared with the association of carotid IMT with serum 25(OH)D. However, the differences in PWV levels between vitamin D sufficient and insufficient were very small and not likely to be clinically relevant. The small

difference in PWV levels may also explain why the serum 25(OH)D stratified analysis with continuous PWV measures did not demonstrate a significant association with serum 25(OH)D. Therefore, we may conclude that an association between serum 25(OH)D and PWV level is absent in our study, indicating that serum 25(OH)D within relatively large ranges does not directly affect the arterial stiffening process.

Calcium supplement use was significantly interacting in the association between serum 25(OH)D and carotid IMT in vitamin D deficient individuals. A meta-analysis demonstrated that calcium usage is potentially unsafe in terms of higher cardiovascular risk [26]; however, in our study we could not confirm calcium use being harmful for arteriosclerotic processes. The lack of interaction in vitamin D sufficient individuals potentially has to do with the lower number of calcium users within the vitamin D sufficient group compared with the group with low levels of serum 25(OH)D.

Our study has several limitations. First, this study has a cross-sectional design. Therefore, causality cannot be ascertained. In order to investigate the causal pathway of the association between serum 25(OH)D and indices of arterial stiffness and arteriosclerosis in older individuals, a prospective, preferably randomised controlled trial, comparing vitamin D supplementation with placebo, within this age group is needed. A second limitation is that our findings cannot be easily translated to the general population because our study population consists of mildly hyperhomocysteinemic elderly, which is an inclusion criteria of the B-PROOF study. Third, one might argue about the sample size of our study. Although a sample size calculation is not appropriate because of the non-linearity of the models, other studies investigating this relation had equal population sizes, making a power issue less likely. Furthermore, the number of participants was quite equal between the groups and can therefore not explain the lack of an association in participants with low serum 25(OH)D levels. Also, with regard to the vascular measurements, we followed the restrictions as provided by the manufacturer. Since detailed restrictions were not clearly included in guidelines at the time of the measurements as they are today, our measures may not be fully comparable with other, more recent studies. However, we tried to minimize the potential influence for example of smoking and physical exercise by considering these factors as potential covariates. The main strengths of our study are our accurately measured cardiovascular parameters and multiple measures of pre-clinical cardiovascular disease.

Conclusion

Overall, the association between serum 25(OH)D and parameters of arterial stiffness and arteriosclerosis in elderly was non-linear. Caution is therefore warranted when investigating this association. In particular, we demonstrated that vitamin D sufficient participants have higher carotid IMT levels compared with vitamin D deficient individuals. This finding suggests that also normal ranged vitamin D levels might potentially increase cardiovascular risk because of its association with pre-clinical cardiovascular disease.

Acknowledgements

3.3.6 REFERENCES

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Chapter **4**

Homocysteine, B-vitamins and arterial stiffness
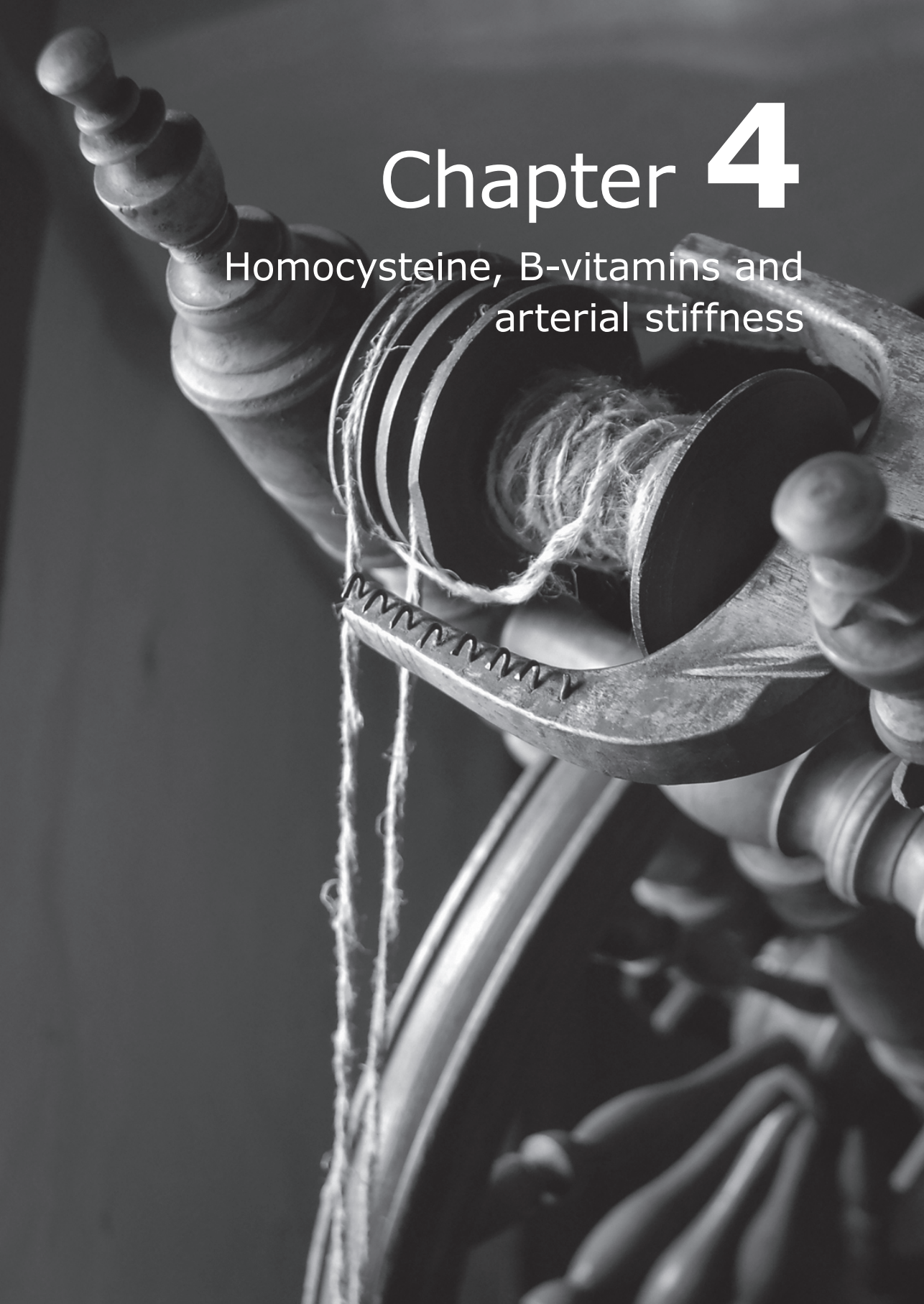
4.1

Homocysteine level is associated with aortic stiffness in elderly: cross-sectional results from the B-PROOF study

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4.1.1 ABSTRACT

Objective

Homocysteine has been shown to be a more accurate predictor of cardio-vascular mortality in very old persons than models based on classical risk factors. Arterial stiffening is a structural abnormality involved in the pathway of cardiovascular disease. We expect this underlying pathophysiology to be a possible explanation for the association between homocysteine and cardio-vascular risk, particularly in older populations.

Methods

Baseline cross-sectional data of the B-PROOF study were used to determine associations between homocysteine and outcomes of vascular function and structure. The cardiovascular subgroup of the B-PROOF study was included [n = 560, 58% men, age 72.6 ± 5.5 years, median homocysteine level 14.2 µmol/l (IQR 13.0–16.6)]. We assessed carotid distensibility coefficient, carotid compliance coefficient, aortic pulse wave velocity (aPWV), augmentation index (AIx) and aortic pulse pressure (aortic PP). Associations were tested using linear regression analysis and ANCOVA and were adjusted for possible confounders including age, sex, renal function, mean arterial pressure and heart rate.

Results

Ln-homocysteine was strongly associated with aPWV [β 0.005 95% confidence interval (0.001–0.009)]. Furthermore, this association was shown to be agedependent (P = 0.02) and it was most strong in the upper tertile of age (77–98 years). No significant associations with ln-homocysteine were observed for AIx, carotid distensibility coefficient and compliance coefficient and aortic PP. Sex stratification shows the association between ln-homocys-teine and aPWV is only significant in men.

Conclusion

In older persons, homocysteine is associated with aortic stiffness, predominantly in the oldest old. This suggests that the strong association between homocysteine and cardiovascular mortality in the elderly may be mediated by aortic stiffness.

4.1.2   INTRODUCTION

Hyperhomocysteinemia and cardiovascular disease are both highly prevalent among elderly persons. Elevated homocysteine has been shown to be a moderately strong and independent cardiovascular risk factor in healthy populations [1]. The association of homocysteine level with cardiovascular disease appears to be particularly strong in the very old and homocysteine level has even been shown to be a better predictor of cardiovascular mortality in this age group than models based on classical Framingham risk factors [2]. However, underlying mechanisms remain unclear, especially after homocysteine-lowering intervention trials reported a lack of short-term (3–5 years) benefit of B-vitamins in middle-aged populations [3].

Arterial stiffness, which is another strong predictor of cardiovascular disease especially in older populations [4,5], may be the link between hyperhomocysteinemia and adverse cardiovascular outcomes. Arterial stiffness is involved in the pathogenesis of cardiovascular disease and acts as a major marker for cardiovascular risk [4,6–9]. Particularly for aortic stiffness this association is consistent, with aortic pulse wave velocity (aPWV) emerging as the most potent predictor of cardiovascular events [7–9]. As arterial stiffness is a structural abnormality that is not easily reversible, an association between homocysteine and arterial stiffness might help explain why B-vitamins fail to exert beneficial effects on cardiovascular outcomes within the follow-up period of the intervention trials.

Evidence exists that homocysteine level may be associated with aortic stiffness in the general population; however, these studies were equivocal [10–13]. These studies were performed in relatively young populations and homocysteine levels were normal in a large proportion of studied patients. In order to gain more knowledge about the pathophysiological background of the association between homocysteine level and cardiovascular disease, specifically in older persons, we investigated whether homocysteine level is associated with arterial stiffness within the B-PROOF study [14]. The B-PROOF study consists of an elderly population (age 65–98 years) with elevated homocysteine levels (>12μmol/l). As mentioned, arterial stiffness and hyperhomocysteinemia are both common conditions and risk indicators for cardiovascular disease in this age group. Because the association between arterial stiffness and homocysteine level is not always present and not yet investigated in elderly, we will explore this association in older individuals.

4.1.3   METHODS

Study participants

The present study was conducted as a cross-sectional baseline analysis within the framework of the B-PROOF (B-vitamins for the Prevention of Osteoporotic Fractures) study. A detailed description of this randomized controlled trial has been reported elsewhere [14]. In short, B-PROOF is a multicenter, randomized, placebo-controlled, double-blind trial including 2919 participants from three areas in the Netherlands. Main inclusion criteria were age 65 years and older, and an elevated homocysteine level (12–50μmol/l). Fifty-one percent of the approached population could be included based on their homocysteine level. Main exclusion criteria were renal insufficiency (creatinine level >150μmol/l) and presence of a malignancy. All participants gave written informed consent before the start of the study. The Wageningen Medical Ethics Committee approved the study protocol, and the Medical Ethics committees of Rotterdam and Amsterdam gave approval for local feasibility.

At the Erasmus Medical Center (Rotterdam) and VU University Medical Center (Amsterdam), a subsample of participants underwent vascular measurements (n = 560). Participants with cardiac arrhythmia were excluded from these additional measurements. During the measurements, participants were situated in supine position on a flat examination couch in a quiet laboratory room for at least 5–10 min prior to the measurements, and the participants were not allowed to speak during the measurements. Use of alcohol or coffee during 12 h before the measurements was prohibited.

Clinical characteristics

Height was measured in duplicate to the nearest 0.1 cm using a stadiometer, with the participant standing erect and wearing no shoes [14]. Weight was measured using a calibrated weighing device (SECA 761), with the participant wearing light garments without shoes and empty pockets, to the nearest 0.5 kg [14]. BMI was calculated as weight divided by squared height and expressed as kg/m^2. Self-reported medical history, alcohol intake and smoking habits were determined using a questionnaire [14].

Homocysteine and renal function

Venous blood samples were obtained in the morning, when the participants were in a fasted state, or had taken a restricted breakfast [14]. For total homocysteine analysis, a plasma ethylenediaminetetraacetic acid tube was stored in ice immediately after blood drawing [14], and samples were

processed within 4 h in order to prevent a temperaturedependent and time-dependent increase in plasma homocysteine [15]. Plasma homocysteine was measured using the Architect i2000 RS analyzer (VU University Medical Center, intra-assay CV=2%, inter-assay CV=4%) and LC-MS/MS (Erasmus Medical Center, CV=3.1%). Outcomes of the centers did not differ significantly at cross-calibration. Serum creatinine was measured with the enzymatic colorimetric Roche CREA and assay (CV=2%). The estimated glomerular filtration rate (eGFR) was estimated with the Modification of Diet in Renal Disease (MDRD) and was calculated in ml/min per $1.73m^2$ with the formula: 186 * (serum creatinine (μmol/l)/88.4) − [1.154] * age (years) − [0.203] * 0.742 (for women) [7].

Carotid distensibility coefficient and compliance coefficient

The vessel wall movement–detector system has been described in detail previously by Hoeks et al [16]. This system consists of a wall track system and data-acquisition system (Art.Lab, Esoate Europe, Maastricht, the Netherlands). With the L105 40mm 7.5MHz transducer, with the M-mode, a M-mode line perpendicular to the right vessel was selected. Measurements were conducted during the cardiac cycles and in order to obtain accurate measurements, the SD of the mean values of six independent measurements was less than 10% of the mean (intra-CV=8%, inter-CV=10%). Distensibility coefficient was calculated using the following equation: distensibility coefficient = (2DD/D)/PP (10^{-3}/kPa) [17,18], where D is diameter during diastole and DD is the measured distensibility (systolic diameter – diastolic diameter) and PP is the aortic pulse pressure. The compliance coefficient was calculated as: compliance coefficient = (π*ΔD*D)/2PP (in mm^2/kPa) [19]. The aortic PP was derived from radial applanation and was used in both equations.

Applanation tonometry

Arterial tonometry was obtained from the right radial, right carotid and right femoral artery using the Sphygmocor device (Sphygmocor version 7.1, AtCor Medical, Sydney, Australia). An estimate of the corresponding central aortic pulse wave was calculated, using a validated generalized transfer function incorporated in the device. With the integral software, the central augmented pressure was calculated as the difference between the early and late systolic peaks of the estimated central pressure wave form.

Central aortic augmentation index (AIx) was calculated as the augmented pressure expressed as a percentage of the pulse pressure (intra-CV=3%, inter-CV=5%). Aortic pulse wave velocity (aPWV) was measured with

a three-channel ECG recording and simultaneously recording of the right carotid artery pulse wave form and subsequently the femoral artery pulse waveform. The aPWV was calculated as the delay between the femoral pulse wave and the carotid pulse wave, divided by the transit distance (intra-CV=5%, inter-CV=8%). Transit distance was assessed with body surface measurement from the carotid artery to the femoral artery [20].

Blood pressure measurement

Peripheral blood pressure at the time of vascular function tests was measured once with a semiautomatic oscillometric device (Datascope Accurator Plus device; Datascope Corp. New Jersey, USA) after at least 5 min of supine rest. All blood pressure measurements were conducted at the right arm. The mean arterial pressure was measured and the pulse pressure was calculated as the SBP minus the DBP.

Statistical analysis

The association between homocysteine level arterial stiffness was investigated using multiple steps. First, normal distribution of all variables was tested by histograms and Kolmogorov–Smirnov tests. Because of skewed distribution, we used both homocysteine level and ln-homocysteine level for analysis. Second, associations between homocysteine level and aortic pulse pressure (PP), carotid distensibility coefficient and compliance coefficient, aPWV and AIx were investigated with linear regression analysis. Third, analysis of covariance (ANCOVA) was performed to compare mean values of these variables per tertiles of homocysteine level. Potential confounders were evaluated in a stepwise model and included age, sex, creatinine, eGFR, mean arterial pressure (MAP), heart rate, smoking, medical history of cardiovascular disease, hypertension, hypercholesterolemia and diabetes mellitus. In the end, we ran a multivariable model containing all variables that caused a change in the point estimate (β coefficient) of more than 10%, or were considered clinically relevant.

Finally, we used age, sex, creatinine, MAP and heart rate in this multivariate model. We also performed a stratified analysis with sex, in order to investigate the effect of sex in the association between homocysteine and arterial stiffness.

We investigated tertiles of homocysteine levels and its association with aPWV per tertile of age with ANCOVA. Furthermore, we tested whether age was an interactionterm in the association between homocysteine and aPWV. Next, in order to investigate the correlation between all arterial stiffness

parameters, we used Pearson correlation and univariate linear regression. The aPWV is considered as the 'golden standard'. Statistical analysis was performed using the statistical software package of SPPS 17.0 (SPPS Inc., Chicago, Illinois, USA). P values of less than 0.05 were considered statistically significant.

4.1.4   RESULTS

Baseline characteristics of the participants (n = 560) are shown in **Table 1**. Mean age was 72.5 ± 5.6 years and sex was equally distributed. The median plasma homocysteine level was 14.2µmol/l. Mean brachial SBP and DBP were 137.7 ± 18.1 and 77.3 ± 9.6 mmHg, respectively (**Table 1**). Sex stratification showed a median plasma homocysteine level of 14.6 (13.1–16.8) µmol/l for men and 14.1 (12.9–16.3) µmol/l for women (p = 0.06).

In univariate analysis, ln-homocysteine was positively associated with aPWV (p < 0.01) (**Figure 1**), but not with AIx, distensibility coefficient, compliance coefficient and aortic PP. In the final model of the multivariate analysis, the

Table 1. Baseline characteristics of the B-PROOF vascular sub-population; n = 560

Variable	Mean ± SD
Age (years and range)	72.5 ± 5.6 [65-98]
Sex (n, %)	
Male	314 (56.1%)
BMI (kg/m^2)	26.5 ± 3.7
Plasma homocysteine (µmol/L)	14.2 (13.0 – 16.6)
Creatinine (µmol/L)	83.2 ± 17.0
eGFR (ml/min per 1.73m^2)	90.8 ± 38.3
Brachial blood pressure (mmHg)	
SBP	137.7 ± 18.1
DBP	77.3 ± 9.6
Aortic PP (mmHg)	50.1 ± 15.1
Carotid DC (10^{-3}/kPa)	14.5 ± 8.6
Carotid CC (mm^2/kPa)	618.9 ± 1106.5
AIx (%)	26.2 ± 10.0
aPWV (m/s)	14.3 ± 4.5

Values are presented as number and percentage or as mean_SD, except for homocysteine: median (IQR) and age: mean± SD and (range). AIx, augmentation index; aPWV, aortic pulse wave velocity; CC, compliance coefficient; DC, distensibility coefficient; mean ± SD; eGFR, estimated glomerular filtration rate; PP, pulse pressure.

positive association between ln-homocysteine and aPWV remained significant [β 0.005 95% CI (0.001–0.009)] (**Table 2**, **Figure 4.1.1**). None of the other arterial stiffness markers were significantly associated with ln-homocysteine.

Furthermore, there was an interaction effect of age on the association between ln-homocysteine and aPWV (p = 0.02), as demonstrated in **Figure 4.1.2.** This was confirmed by the stratified multivariate linear analysis of this association per tertile of age [tertile 1: β 0.004 95%CI (-0.002 to 0.010); tertile 2: β 0.007 95%CI (-0.004 to 0.014) and tertile 3: β 0.008 95% CI (0.001–0.016)]. The associations between ln-homocysteine and AIx, distensibility coefficient, compliance coefficient and aortic PP did not change with increasing age.
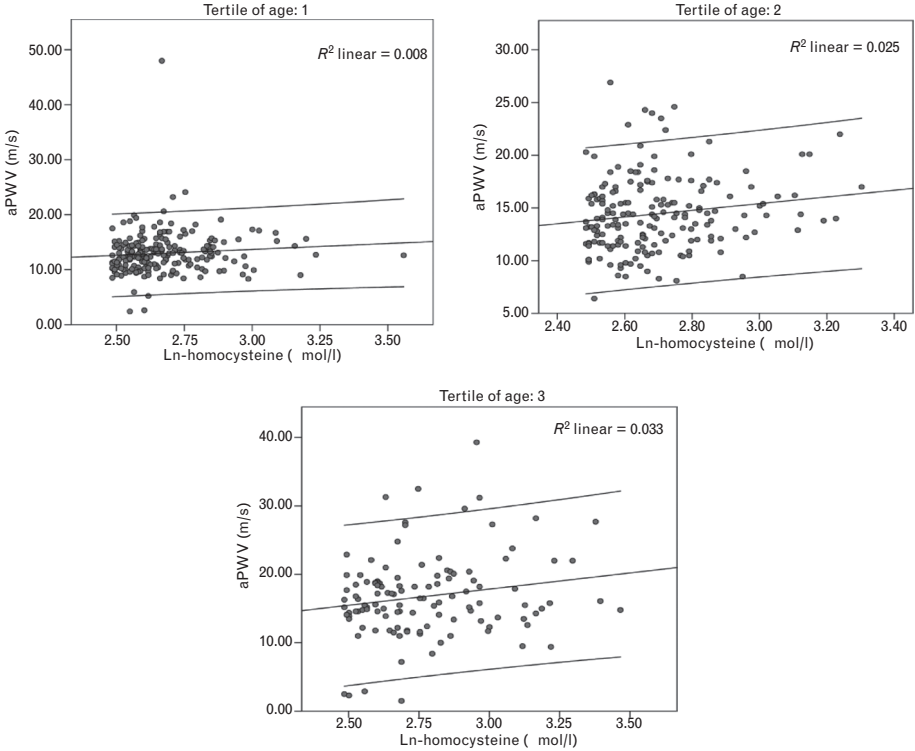
Figuur 4.1.1.

Scatterplots of aortic pulse wave velocity measurements stratified by tertile of age. Abbreviations as in Table 1.

Table 2. Multivariate linear regression analysis of ln-homocysteine to vascular parameters

Variable	Model 1 β (95%CI)	Model 2 β (95%CI)	R^2
Aortic PP (mmHg)	-0.00040 (-0.00100 to 0.00100)	-0.00100 (-0.00200 to 0.00030)	0.095
Carotid DC (10^{-3}/kPa)	-0.00060 (-0.00200 to 0.00200)	0.00100 (-0.00100 to 0.00300)	0.115
Carotid CC (mm^2/kPa)	-0.00010 (-0.00020 to 0.00002)	-0.00010 (-0.00030 to 0.00001)	0.119
AIx (%)	-0.00020 (-0.00200 to 0.00100)	-0.00100 (-0.00200 to 0.00100)	0.096
aPWV (m/s)	0.00800 (0.00500 to 0.01200)*	0.00500 (0.00100 to 0.00900)*	0.113

Values are presented as beta (95% CI) and R2. Final model: adjusted for age, sex, MAP, heart rate and creatinine.

* p-value less than 0.05. AIx, augmentation index; aPWV, aortic pulse wave velocity; CC, compliance coefficient; DC, distensibility coefficient; PP, Pulse Pressure.

Table 3. Stratified multivariate linear regression analysis for gender

	Men		Women	
	Model 1	Model 2	Model 1	Model 2
Variable	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)
Aortic PP (mmHg)	0.00005 (-0.001; 0.001)	-0.001 (-0.002 to 0.001)	-0.001 (-0.002 to 0.001)	-0.0003 (-0.003 to 0.002)
Carotid DC (10^{-3}/kPa)	-0.001 (-0.004 to 0.001)	-0.00002 (-0.002 to 0.002)	0.003 (-0.002 to 0.007)	0.002 (-0.002 to 0.007)
Carotid CC (mm^2/kPa)	-0.00001 (-0.00003 to 0.000004)	-0.00001 (-0.00003 to 0.000002)	-0.00003 (-0.0001 to 0.00009)	-0.00002 (-0.0001 to 0.0001)
AIx (%)	0.001 (-0.001 to 0.003)	-0.001 (-0.003 to 0.001)	-0.001 (-0.003 to 0.002)	-0.001 (-0.004 to 0.002)
aPWV (m/s)	0.010 (0.006 to 0.014)*	0.007 (0.002 to 0.011)*	0.005 (-0.0004 to 0.011)	0.003 (-0.003 to 0.009)

Values are presented as beta (95% CI). Model 1: unadjusted; Model 2: adjusted for age, sex, MAP, heart rate and creatinine. *P value less than 0.05. AIx, augmentation index; aPWV, aortic pulse wave velocity; CC, compliance coefficient; DC, distensibility coefficient; PP, pulse pressure.

A stratified analysis with sex showed the association between ln-homocysteine and aPWV is only present in men (**Figure 4.1.3**, **Table 3**).

In our population, AIx and aortic PP were positively, and distensibility coefficient was negatively correlated with aPWV. The respective correlation coefficients were r = 0.09 (p = 0.04), r = 0.20 (p<0.01), r = -0.16 (p = 0.01). Compliance coefficient and aPWV were not correlated. Although overall significant, the strength of the association between AIx and aPWV reduced
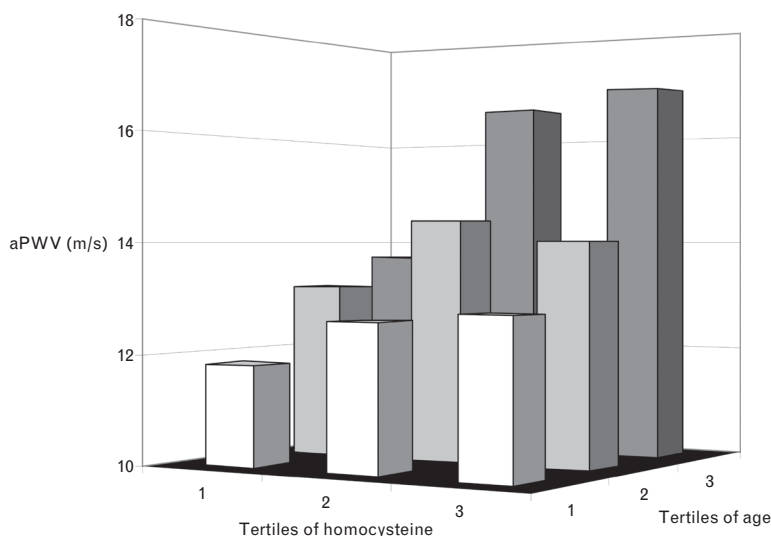
Figuur 4.1.2.

Interactive effects of age and plasma homocysteine on aortic pulse wave velocity. Values are presented as adjusted means. Model adjusted for sex, MAP, heart rate and creatinine. Overall P value for interaction for this model: P = 0.02. Tertiles of age: 1: 65–70 years; 2: 71–76 years; 3: 77–98 years. Tertiles of homocysteine: 1: 12.0–13.4mmol/l; 2: 13.5–15.6mmol/l; 3: 15.7–49.0mmol/l. Abbreviations as in Table 1.

with increasing age and did not reach significance in between the tertiles. Per tertile of age the associations between AIx and aPWV were, respectively, β 0.19 for the tertile 1 (p = 0.31); β 0.04 for the tertile 2 (p = 0.86); β 0.01 for the tertile 3 (p = 0.93). Such an age-dependent effect was also seen for the association between aortic PP and aPWV [tertile 1 β 0.94 (p<0.01); tertile 2 β 0.43 (p = 0.13); tertile 3 β 0.35 (p = 0.22)]. Age-dependency was not present for the association between distensibility coefficient or compliance coefficient and aPWV.

4.1.5   DISCUSSION

Our study in older participants with elevated homocysteine levels shows a positive association between plasma homocysteine level and aPWV, as a measure of aortic stiffness. This association was the strongest in the oldest old. Importantly, this association between homocysteine and aortic stiffness was independent of renal function and blood pressure. The association with
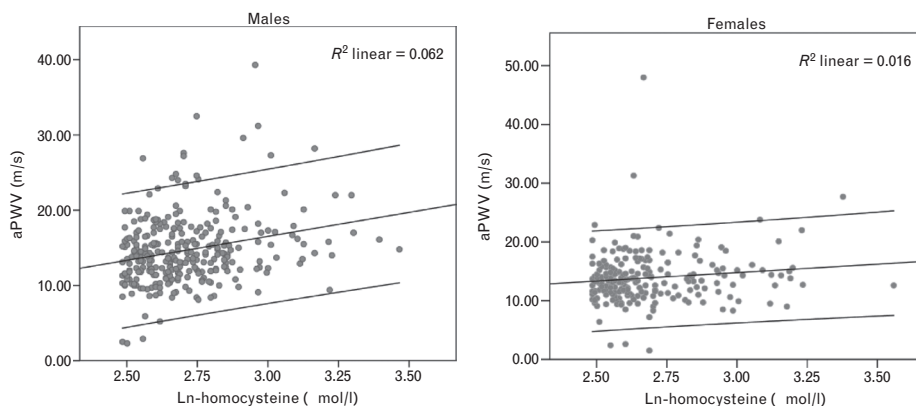
Figuur 4.1.3.
Stratified scatterplots of ln-homocysteine with sex. Abbreviations: as in Table 1.

homocysteine level was not present for other estimates of arterial stiffness. Stratification for sex shows this association is only significant in men.

To our knowledge, this is the first study showing that the positive association between homocysteine level and aortic stiffness holds true in a hyperhomocysteinemic population and is the strongest in the oldest old. The association between homocysteine level and aPWV has been investigated earlier in younger populations, however, these findings were conflicting [11–13]. One study, exclusively performed in men, did not report a relation between homocysteine level and PWV [13]. However, this is in contrast with the findings of the Framingham Heart Study, where an association between homocysteine and aortic stiffness was demonstrated in men, but not in women [11]. Furthermore, within a Czech study, homocysteine was strongly and independently associated with PWV in both men and women [12]. Nevertheless, all these studies have been performed in younger populations with mean ages between 40 and 60 years. Our study endorses that homocysteine level is independently and significantly associated with aortic stiffening in elderly and particularly in the oldest old.

There are several potential mechanisms, which may explain the relation between hyperhomocysteinemia and aortic stiffness. Main hypotheses are a direct effect of homocysteine via the combination of increased thrombogenicity, increased oxidative stress and overactivation of redox-sensitive inflammatory pathways, leading to impaired endothelial function and platelet aggregation,

and finally atherothrombosis [21–26,15]. During age, homocysteine is more important in the association with aPWV. This age-dependent effect might be explained by the fact that homocysteine is a more important cardiovascular risk indicator with advancing age, in comparison with traditional indictors. Nevertheless, future research will be necessary to investigate the underlying mechanisms.

The sex-effect of the association between ln-homocysteine and aortic stiffness might be due to the fact that there are more extreme aPWV measurements and higher homocysteine levels in males. In our study, higher homocysteine levels are more strongly related to higher aPWV levels, therefore, this sex-effect strengths our hypothesis. A reason we did not demonstrate this association in females might be there are less females in our population. Therefore, we may not definitively conclude there is no association between ln-homocysteine and aPWV in women.

In our study, we did not observe an association between homocysteine level and AIx. This may be explained by the curvelinear pattern of the change of AIx with age, where a plateau is reached after the age of 60 years [27] and because augmentation is also influenced by other factors than stiffness, including peripheral resistance and cardiac performance [28]. Nevertheless, even though our participants were older than 65 years, we did observe a small correlation between AIx and aPWV. However, in concordance with the curvelinear hypothesis, this correlation decreased with advancing age. Homocysteine level was not associated with distensibility coefficient and compliance coefficient in our study. The lack of an association between homocysteine level and distensibility coefficient and compliance coefficient in our study could possibly be explained by the fact that distensibility coefficient and compliance coefficient are also influenced by other factors, like systolic function. A second explanation might be that carotid distensibility coefficient and compliance coefficient are local measurements of the carotid artery, which is an elastic artery. With aPWV, more territories are reflected, providing information about mainly elastic, but also muscular arteries. As structural and functional properties of the arterial wall differ within the arterial tree, it is plausible the pathophysiology of homocysteine differs between elastic and muscular arteries. However, this is hypothetical and there may be other underlying mechanisms. Even though aortic PP is closely related to arterial stiffness [29], we found no association between homocysteine level and aortic PP in our study and no age-dependent effect. This might endorse the hypothesis

that aortic PP is a more indirect measurement of arterial stiffness than aPWV, as PP will also be influenced by other factors like cardiac performance.

A potential limitation of the current study is the cross-sectional design; therefore, we cannot be certain that the observed associations are truly causal. We will further study the role of aortic stiffness in the currently running randomized intervention trial (B-PROOF). A second limitation is that, the B-PROOF trial included only participants with hyperhomocysteinemia. It is conceivable that the associations with cardiovascular disease and aortic stiffening will be different when lower homocysteine levels are taken into account. However, the adjusted reference value of aPWV for individuals aged 70 years and over is 12.1 m/s [30]. In our population the mean aPWV is 14.3 m/s, which is clearly higher compared with a general, nonhyperhomocysteinemic population. Third, we cannot exclude the possibility that the association between homocysteine and aPWV is based on the play of chance, because other parameters of arterial stiffness are not associated with homocysteine. The relative small sample size of our population might explain why AIx, aortic PP, distensibility coefficient and compliance coefficient are not associated with homocysteine. However, aPWV is the most powerful measurement of arterial stiffness and might better reflect this condition compared with the other vascular parameters.

In conclusion, this study demonstrated a clear association between homocysteine level and aortic stiffness, particularly in males. Overall, the strength of the association increased with advancing age. Homocysteine level is an important risk indicator of cardiovascular mortality in elderly and our findings may indicate that aortic stiffness is a mediator in this process. Nevertheless, our hypothesis remains to be confirmed by the currently running B-PROOF intervention trial.

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4.2

B-vitamin levels and genetics of hyperhomocysteinemia are not associated with arterial stiffness

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4.2.1 ABSTRACT

Background and aims

Hyperhomocysteinemia is associated with arterial stiffness, but underlying pathophysiological mechanisms explaining this association are to be re-vealed. This study was aimed to explore two potential pathways concerning the one-carbon metabolism. A potential causal effect of homocysteine was explored using a genetic risk score reflecting an individual's risk of having a long-term elevated plasma homocysteine level and also associations with B-vitamin levels were investigated.

Methods and results

Baseline cross-sectional data of the B-PROOF study were used. In the cardiovascular subgroup (n=567, 56% male, age 72.6 ± 5.6 yrs) pulse wave velocity (PWV) was determined using applanation tonometry. Plasma concentrations of vitamin B12, folate, methylmalonic acid (MMA) and holo transcobalamin (holoTC) were assessed and the genetic risk score was based on 13 SNPs being associated with elevated plasma homocysteine. Associations were examined using multivariable linear regression analysis. B-vitamin levels were not associated with PWV. The genetic risk score was also not associated with PWV. However, the homocysteine – gene interaction was significant ($p < 0.001$) in the association of the genetic risk score and PWV. Participants with the lowest genetic risk of having long-term elevated homocysteine levels, but with higher measured homocysteine levels, had the highest PWV levels.

Conclusion

Homocysteine is unlikely to be causally related to arterial stiffness, because there was no association with genetic variants causing hyperhomocyste-inemia, whereas non-genetically determined hyperhomocysteinemia was associated with arterial stiffness. Moreover, the association between ho-mocysteine and arterial stiffness was not mediated by B-vitamins. Possibly, high plasma homocysteine levels reflect an unidentified factor, that causes increased arterial stiffness.

4.2.2   INTRODUCTION

Hyperhomocysteinemia has been shown to be an important cardiovascular risk indicator, especially in the oldest old [4]. Arterial stiffness is considered to be a pre-clinical state of cardiovascular disease and recently we have reported an association between plasma homocysteine level and arterial stiffness within an older, mild hyperhomocysteinemic population [20]. Whether this association is truly causal is not yet known, in particular since trials with B-vitamin supplementation aimed to reduce plasma homocysteine concentrations failed to demonstrate beneficial effects on cardiovascular outcomes [3].

A causal effect of homocysteine on the arterial stiffness process can be evaluated by exploring the relation between genetic determinants of elevated homocysteine levels and arterial stiffness. Genetic polymorphisms are an inherited phenotype, being constant over time, reflecting long-term elevated homocysteine levels. Furthermore, genotypes are in principle not modified by disease processes and are not affected by non-genetic confounding, and is referred to as the Mendelian randomization principle [16]. Recently, van Meurs et al described a genetic risk score of hyperhomocysteinemia (GRS Hcy), consisting of a combination of the most common single nucleotide polymorphisms (SNPs), which are associated with high homocysteine levels [22]. An association between this risk score and arterial stiffness measurements would suggest a pathophysiological link as explained above. Such a causal effect of homocysteine could be initiated for example via the combination of increased thrombogenecity, increased oxidative stress and over-activation of redox-sensitive inflammatory pathways [8, 12, 15, 26]. Alternatively, homocysteine may not be causally associated with arterial stiffness. A direct effect of vitamin B12 or folate on arterial stiffness is also possible, since these vitamins are essential in the one-carbon metabolism cycle and are strongly correlated with homocysteine. There are studies demonstrating an association of B-vitamins levels with blood pressure [11, 17] and atherosclerosis [5, 14], but to this date reports regarding arterial stiffness are lacking.

We aim to explore underlying mechanisms of different parts of the one-carbon metabolism cycle (**Figure 4.2.1**), that may underlie the association between plasma homocysteine and arterial stiffness. Therefore, we investigated the association between the genetic risk score of hyperhomocysteinemia and arterial stiffness and also the association between B-vitamin levels and arterial stiffness.
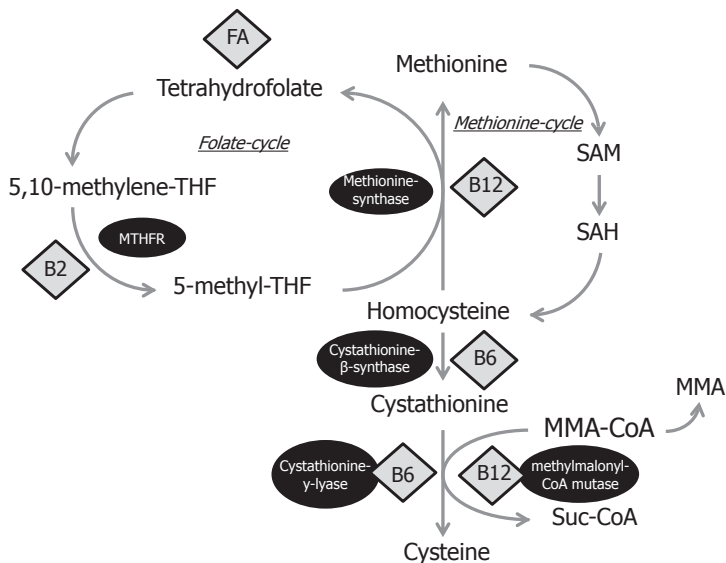
Figuur 4.2.1.

Vitamins, metabolites and enzymes which are involved in the one carbon metabolism. Abbreviations: THF: tetrahydrofolate; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; MTHFR: methylenetetrahydrofolate reductase; MMA: methylmalonic acid; MMA-CoA: methylmalonyl-CoA; Suc-CoA: succinyl-CoA; FA: folic acid; B12: vitamin B12; B2: vitamin B2; B6: vitamin B6.

4.2.3   METHODS

Study population

The present study was conducted as a cross-sectional baseline analysis within the framework of the B-PROOF (B-vitamins for the Prevention of Osteoporotic Fractures) study. A detailed description of this randomized controlled trial has been reported elsewhere [23]. In short, B-PROOF is a multi-center, randomized, placebo controlled, double-blind trial including 2919 participants from three areas in the Netherlands. Main inclusion criteria were age 65 years and older, and a mildly elevated homocysteine level (12 – 50 μmol/l). Fifty-one percent of the approached population could be included based on their homocysteine level. Main exclusion criteria were renal insufficiency (serum creatinine level > 150 μmol/l) and presence of a malignancy. All participants gave written informed consent before the start of the study. The Wageningen Medical Ethics Committee approved the study protocol, and the Medical Ethics committees of Rotterdam and Amsterdam gave approval for local feasibility.

At the Erasmus Medical Center (Rotterdam) and VU University Medical Center (Amsterdam), a subsample of participants underwent vascular measurements (n = 567). Participants with cardiac arrhythmia were excluded from these additional measurements. During the measurements, participants were situated in supine position on a flat examination couch in a quiet laboratory room for at least 5-10 minutes prior to the measurements, and the participants were not allowed to speak during the measurements. Use of alcohol or coffee during 12 hours before the measurements was prohibited.

Laboratory measurements

Venous blood samples were obtained in the morning, when the participants were in a fasted state, or had taken a restricted breakfast [23].

Measurements of folate and vitamin B12 status

Serum vitamin B12 and folate were measured using immunoelectrochemi-luminescence assay (Elecsys 2010, Roche GmbH, Mannheim, Germany) (CV vitamin B12 5.1% at 125 pmol/l and 2.9% at 753 pmol/l; CV folate: 5.9% at 5.7 nmol/l and 2.8% at 23.4 nmol/l) [21]. Serum holotranscobalamin (HoloTC) was determined by the AxSYM analyser (Abbott) (CV<8%) and serum methylmalomic acid (MMA) was measured by LC-MS/MS (CV<9%) [6]. MMA also reflects vitamin B12 status, next to serum vitamin B12 level and homocysteine level and is considered as the most representative indicator of metabolic vitamin B12 deficiency [1]. HoloTC is the fraction of vitamin B12 which is available for cells in the body, also referred to as active vitamin B12. HoloTC has a better diagnostic accuracy than vitamin B12 in order to detect vitamin B12 deficiency [6].

Homocysteine

For total homocysteine analysis, a plasma EDTA tube was stored in ice immediately after blood drawing [23], and samples were processed within 4 hours in order to prevent a temperature- and time-dependent increase in plasma homocysteine [13]. Plasma homocysteine was measured using the Architect i2000 RS analyzer (VU University Medical Center, intra assay CV=2%, inter assay CV=4%) and LC-MS/MS (Erasmus intra assay CV=5.5%, inter assay CV=1.3%). Outcomes of the centers did not differ significantly at cross-calibration.

Serum creatinine

Serum creatinine was measured with the enzymatic colorimetric Roche CREA plus assay (CV=2%). The estimated glomerular filtration rate (eGFR) was

calculated with the formula of Modification of Diet in Renal Disease in ml/min/1.73m^2: 186 * (serum creatinine (µmol/l) / 88.4) − 1.154 * age (years) − 0.203 * 0.742 (for females) [9].

Genotyping

DNA was isolated from buffycoats for genotyping. All participants were genotyped using the Illumina Omni-express array (Illumina Inc., San Diego, CA, USA) according to the manufacturers' protocol and quality standards. The dataset was imputed to the HapMap v22 CEU reference panel ($\approx$ 2.5 million single-nucleotide polymorphisms [SNPs]). Hidden Markov Model-based algorithms were used to infer unobserved genotypes probabilistically as implemented in either MACH [10]. Imputation quality control metrics included the ratio of observed/expected variance of the allele dosage $\leq$0.01.

Genetic risk score of Hyperhomocysteinaemia (GRS Hcy)

The GRS Hcy was generated as described by van Meurs et al [22]. The method outlined by Horne et al [7] was used, where the individual's GRS is equal to the sum of the expected number of risk alleles at each SNP weighted by their effect sizes on plasma homocysteine (beta-coefficients, obtained from the homocysteine meta-analysis). The meta-analysis identified SNPs of 13 independent loci exceeding the GWAS threshold (p < 5·10^{-8}). These included 6 previously unreported loci in or near the genes *MMACHC* (p=2.1x10^{-9}), *SLC17A3* (1.0x10^{-8}), *GTPB10* (1.7x10^{-8}), *CUBN* (p=7.5x10^{-10}), *HNF1A* (1.2x10^{-12}), and *FUT2* (6.6x10^{-9}). In addition, 7 loci previously reported were confirmed to be associated with homocysteine levels at or near the *MTHFR*, *MTR*, *CPS1*, *MUT, NOX4*, *DPEP1*, and *CBS* genes.

The MTHFR genotype was explored also separately, because of its strong individual association with hyperhomocysteinemia [24].

Clinical measures

Height was measured in duplicate to the nearest 0.1 cm using a stadiometer, with the participant standing erect and wearing no shoes [23]. Weight was measured using a calibrated weighing device (SECA 761), with the participant wearing light garments without shoes and empty pockets, to the nearest 0.5 kg [23]. Body Mass Index (BMI) was calculated as weight divided by squared height and expressed as kg/m^2. Self-reported medical history, alcohol intake and smoking habits were determined using a questionnaire [23].

Blood pressure measurement

Peripheral blood pressure at the time of vascular function tests was measured once with a semi-automatic oscillometric device (Datascope Accurator Plus device, Datascope Corp. New Jersey, USA) after at least five minutes of supine rest. Blood pressure measurements were conducted at the right arm and measured in mmHg.

Applanation tonometry

Arterial tonometry was obtained from the right radial, right carotid and right femoral artery using the Sphygmocor device (Sphygmocor version 7.1, AtCor Medical, Sydney, Australia). Aortic pulse wave velocity (aPWV) was measured with a three channel ECG recording and simultaneously recording of the right carotid artery pulse wave form and subsequently the femoral artery pulse waveform. The aPWV was calculated as the delay between the femoral pulse wave and the carotid pulse wave, divided by the transit distance (intra CV = 5 %, inter CV = 8%). Transit distance was assessed with body surface measurement from the carotid artery to the femoral artery [19].

Statistical analysis

Normality of distributions was examined with histograms and Kolmogorov-Smirnov tests. If a variable was not normally distributed, it was log-transformed. Associations between B-vitamins and arterial stiffness measurements were tested for linearity with curve estimation modeling. When linear relations had the best fit, we calculated Pearson correlation coefficients and subsequently tested the associations using multivariable linear regression analysis. Potential confounders were evaluated in a stepwise model and included age, gender, study center, eGFR, MAP, heart rate, smoking, alcohol consumption, presence of hypercholesterolemia and diabetes. Covariates were added to the final model as confounders if they caused a change of the point estimate (beta coefficient) of more than 10% or were considered clinically relevant. Furthermore, in a separate analysis, we tested whether homocysteine modified the association between B-vitamin levels and PWV measurements in order to further explore a potential direct effect of homocysteine. If the interaction term homocysteine – B-vitamins was significant (p < 0.05), a stratified analysis was done comparing participants with homocysteine concentrations under and above the median. Because age was a significantly interacting in the association between homocysteine and PWV [20] we also tested the interaction between age and B-vitamin concentrations within the multivariable linear regression analysis and performed a stratified analysis based on the mean age if this interaction term was significant.

Associations between GRS Hcy and PWV were also tested using multivariable linear regression analysis after confirmation of linearity. Next, ANCOVA was used to compare adjusted means of PWV values per quintile of GRS Hcy. Furthermore, the interaction between homocysteine level and GRS Hcy was examined in order to further explore causality. Stratification was performed based on homocysteine concentrations above and below the median if this interaction-term was significant ($p < 0.05$). Also we tested the interaction between age and GRS Hcy, because age was significantly interacting in the association homocysteine and PWV [20] and with this we could explore the survival effect of the SNPs. The same potential confounders mentioned above were used in a step-wise model.

Statistical analyses were performed using the statistical software package of SPSS version 20.0 (SPSS Inc, Chicago, Illinois, USA). P-values < 0.05 were considered to be statistically significant.

4.2.4   RESULTS

Characteristics of the study population (n = 567) are shown in **Table 1**. The mean age of our population was 72.5 ± 5.6 years and 55.6% was male. The median homocysteine concentration was 14.2 [13.0 – 16.4] µmol/l.

Plasma vitamin B12, folate, MMA and holoTC were all inversely correlated with plasma homocysteine concentrations (r = -0.29, $p < 0.001$; r = -0.30 $p < 0.001$; r = 0.29, $p < 0.001$; r = -0.36, $p < 0.001$ respectively). MMA and holoTC were correlated with PWV (r = 0.13, p = 0.002; r = -0.10, p = 0.03), whereas vitamin B12 and folate were not.

Multivariate linear regression analysis showed that folate, vitamin B12, MMA and holoTC were not associated with PWV (**Table 2**). In the associations between vitamin B12, folate, MMA and HoloTC with PWV, the interaction with homocysteine level was significant ($p < 0.001$, $p < 0.001$, p = 0.04, p = 0.004 respectively). However, after stratification for low and high levels of homocysteine we did not find any associations between B-vitamin levels and PWV.

Although the age – B-vitamin level interaction was significant within all associations of B-vitamins with PWV, age-stratified analysis did not show other associations between different age groups (data not shown).

Table 1. Population characteristics vascular subgroup B-PROOF (n = 567)

Variable	Mean ± SD
Age	72.5 ± 5.6 (range: 65 – 98)
Gender (n, %) Male	315 (55.6%)
BMI (kg/m^2)	27.0 ± 3.7
SBP (mmHg)	137.7 ± 18.1
DBP (mmHg)	77.3 ± 9.6
Pulse wave velocity (m/s)	14.3 ± 4.5
Medical history of (n, %) CHD Hypertension TIA / Stroke Diabetes mellitus Hypercholesterolemia	 61 (10.8%) 211 (37.2%) 45 (7.9%) 65 (11.5%) 151 (26.6%)
Laboratory values	
Homocysteine (µmol/L)	14.2 [13.0 – 16.4]
Vitamin B12 (pmol/l)	291.6 ± 122.5
Folate (nmol/l)	20.5 ± 7.2
MMA (µmol/L)	0.26 ± 0.20
HoloTC (pmol/L)	68.1 ± 24.5
eGFR (ml/min per 1.73m^2)	91.3 ± 35.9
Genotyping	
MTHFR genotype (n = 508) CC CT TT	 226 (44.5%) 205 (40.3%) 77 (15.2%)
GRS Hcy	0.90 ± 0.18

Values are presented as number and percentage or as mean ± SD or n (%), except for homocysteine: median (IQR) and age: mean ± SD and (range).

Abbreviations: BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; PWV: pulse wave velocity; CHD: coronary heart disease; TIA: transient ischemic attack; MMA: methylmalomic acid; holoTC: holotranscobalamin; eGFR: estimated glomerular filtration rate; MTHFR: Methylenetetrahydrofolate reductase, GRS Hcy: genetic risk score homocysteine.

Homocysteine levels were higher in participants with the MTHFR-TT genotype, compared with the MTHFR-CC participants and MTHFR-CT genotype, but these differences were not significant (p = 0.07 and p = 0.26 respectively). PWV levels between the MTHFR genotypes did not differ (p for trend = 0.08) (**Figure 2**).

Table 2. Linear regression analysis of the association between B-vitamin status and the genetic risk score of hyperhomocysteinemia with PWV

Variable	Model 1 β (95%CI)	Model 2 β (95%CI)
Vitamin B12 (pmol/l)	-0.003 [-0.006 ; 0.0001]	-0.002 [-0.005 ; 0.001]
Folate (nmol/l)	-0.022 [-0.079 ; 0.035]	-0.027 [-0.079 ; 0.026]
MMA (µmol/l)	-0.019 [-0.035 ; -0.002]*	-0.010 [-0.026 ; 0.005]
HoloTC (pmol/l)	3.307 [1.209 ; 5.405]	1.600 [-0.391 ; 3.592]
GRS Hcy	-2.09 [-4.34 ; 0.23]	-0.63 [-2.77 ; 1.52]

B-vitamin status: Model 1: crude; Model 2: adjusted for age, gender, study center, MAP, heart rate, eGFR.

GRS Hcy: Model 1: crude; Model 2: adjusted for age, gender and study center.

* $p < 0.05$.

Abbreviations: as in Table 1.

The GRS Hcy explained approximately 1% of the variation in plasma homocysteine (r = 0.10 ; p = 0.02) (**Figure 4.2.3**). No associations were observed between GRS Hcy and PWV levels (**Table 3**). Nevertheless, the lowest quintile of the GRS Hcy had significant higher PWV levels compared to the highest quintile (p = 0.03) (PWV 14.2 m/s in quintile 1 compared to PWV 13.9 m/s in quintile 5; data not shown).

Subsequently, we investigated the ln-homocysteine - GRS Hcy interaction for the association between GRS Hcy and PWV. The interaction term was added to the model and was highly significant (p = $0.54 \cdot 10^{-4}$). **Figure 4.2.4**
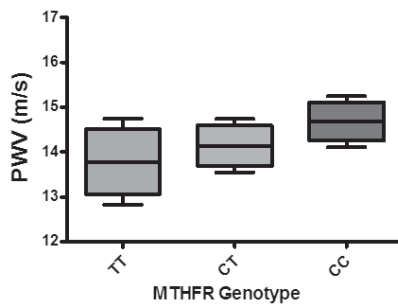
Figuur 4.2.2.

Values are depicted as Whiskers boxplots with estimated mean ± SE with 95% CI. Data are adjusted for age, gender, study center, MAP and heart rate.

Abbreviations: as in Table 1.
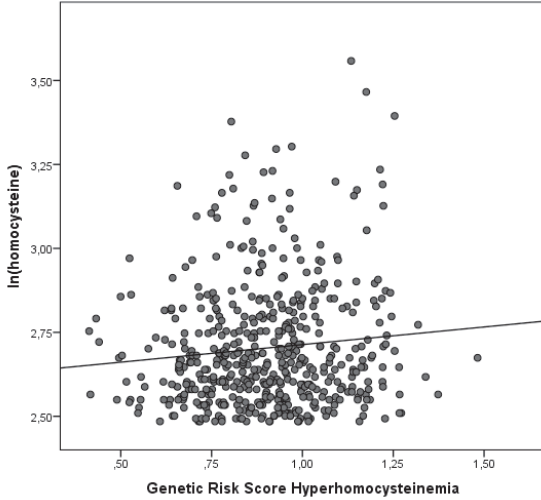
Figuur 4.2.3.
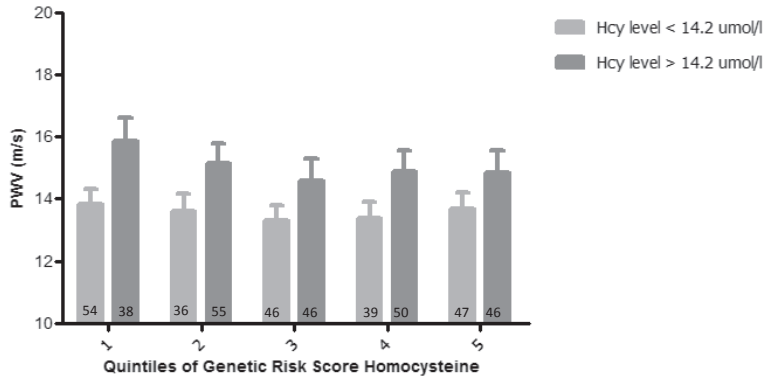
Figuur 4.2.4.

Values are depicted as estimated means ± SE and are adjusted for age, gender, study center, MAP and heart rate. Lowest quintile indicates the lowest chance of having high Hcy levels and the highest quintile indicates the highest chance of having high Hcy levels. Number of subjects are presented in the bars. Abbreviations as in Table 1.

demonstrates the estimated adjusted PWV means per quintile of GRS Hcy, stratified by plasma homocysteine concentration. A trend of higher PWV levels was noticeable within the individuals with low genetic risk of higher homocysteine levels and higher measured plasma homocysteine concentrations. However, the estimated means of PWV per stratum of homocysteine concentrations were not significantly different and the observed trend was

also not significant (p = 0.09 for participants with homocysteine concentrations above the median).

Although the age – GRS Hcy interaction was significant in the association between GRS Hcy and PWV (p < 0.001), age-stratification analysis did not show any associations.

4.2.5   DISCUSSION

Our study demonstrates that in the association between the genetic risk score of hyperhomocysteinemia and PWV, the homocysteine – gene interaction was significant. Therefore, the hypothesis that plasma homocysteine is causally related to arterial stiffness was not confirmed. Furthermore, in multivariable analysis we found no associations between B-vitamins and arterial stiffness and therefore the association between homocysteine and arterial stiffness is unlikely to be driven by B-vitamin levels.

To our knowledge, this is the first study addressing the association between B-vitamin levels and arterial stiffness in elderly. Despite the fact that folate has shown to affect endothelial function in vivo [25], we were not able to demonstrate an association of folate with PWV. Neither were vitamin B12, holoTC or MMA associated with this parameter. All in all, our results suggest that B-vitamins itself do not have a direct effect on the arterial stiffening process, which is supported by the fact that homocysteine is mediating the association between B-vitamin levels and PWV. Although, we should note we investigated these associations cross-sectionally.

Furthermore, we did not find evidence for a causal association between plasma homocysteine and arterial stiffness, as reflected by the lack of an association between the GRS Hcy and arterial stiffness. Although a significant difference between the lowest and the highest quintile of the genetic risk score in PWV level was observed, this trend was opposite to the expected association and the differences in PWV level between the quintiles were small. Thus, higher long-term plasma homocysteine concentrations according to the genetic risk score were associated with lower PWV levels. However, as was shown by stratification, most likely this inverse association was driven by participants with a low GRS Hcy, but with rather high measured plasma homocysteine levels. Since the plasma homocysteine – GRS Hcy interaction

significantly modified the association between GRS Hcy and PWV, this indicates that the association between homocysteine and PWV is not causal. If homocysteine would be a causal factor in the arterial stiffness pathway, one would expect a positive association between GRS Hcy and PWV, instead of the negative association observed. It might be speculated that participants with genetically high homocysteine levels have other, undefined protective mechanisms regarding arterial stiffness.

In our population, the GRS Hcy only explained about 10% of the variance in plasma homocysteine level, which accords with a previous report [22]. This relatively small effect may explain why our hypothesis of a causal effect of plasma homocysteine on arterial stiffness could not be confirmed. Furthermore, as a consequence of the inclusion criteria of the B-PROOF study, our population only consisted of elderly with (mildly) elevated homocysteine levels and therefore is very selected. Because this GRS does not explain all the variance in homocysteine, the lack of an association does not definitely imply there is no causal relation between homocysteine and arterial stiffness. Nevertheless, the fact that the homocysteine – GRS Hcy interaction was clearly present in the association between the GRS and PWV points more towards a non-causal relationship: possibly an unidentified factor may explain the association between homocysteine and arterial stiffness, other than being a direct B-vitamin or homocysteine effect.

Furthermore, the absent association between the GRS Hcy and arterial stiffness may be due to the relatively small sample size in terms of genetic analyses. Previously, we have reported a small effect of plasma homocysteine on the association with PWV [20] and since the GRS only explains 1% of the plasma homocysteine level, a strong association is not likely to be expected. Also, there is still the possibility that existing cardiovascular disease itself would increase plasma homocysteine levels and PWV. This reverse causation has been mentioned previously [2, 18]. Nevertheless, the fact that the homocysteine – GRS Hcy interaction was clearly present in the association between the GRS and PWV points towards a non-causal relationship. Possibly a yet unidentified factor may explain the association between homocysteine and arterial stiffness, other than being a direct B-vitamin or homocysteine effect. For example, via oxidative stress and activation of redox-inflammatory mechanisms, which lead to endothelial dysfunction or via thrombogenecity [8, 12, 15, 26].

Despite the fact that a meta-analysis established an association between MTHFR and risk of cardiovascular disease [24], our study could not find an association with the MTHFR genotype and PWV, as being a preclinical marker for cardiovascular disease. Potentially the found association with clinical outcomes in the meta-analyses was driven by other factors than arterial stiffness. Last, we want to mention that we did not include blood pressure as an outcome variable, but only focused on arterial stiffness.

In conclusion, it is unlikely that homocysteine has a causal effect on arterial stiffness in older individuals. This is based on the lack of an association between genetic determinants of hyperhomocysteinemia and arterial stiffness and in particular on the significant homocysteine – GRS Hcy interaction. Furthermore, B-vitamin levels were also not associated with measures of arterial stiffness. Homocysteine may rather be a risk indicator than a risk factor and potentially, high plasma homocysteine levels reflect an unidentified factor, that causes increased arterial stiffness at older age.

Acknowledgements

4.2.6 REFERENCES

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4.3

Effects of 2-year vitamin B12 and folic acid supplementation in hyperhomocysteinemic elderly on arterial stiffness and cardiovascular outcomes within the B-PROOF trial

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4.3.1 ABSTRACT

Background

Hyperhomocysteinemia is an important cardiovascular risk indicator in the oldest old and is also associated with elevated arterial stiffness in this age group. Since several intervention trials reported a lack of benefit of B-vitamin supplementation on cardiovascular outcomes, we aimed to investigate the effect of B-vitamin supplementation on arterial stiffness and cardiovascular events in hyperhomocysteinemic elderly subjects.

Methods and Results

The B-PROOF study is a double-blind randomized-controlled trial, including 2919 elderly aged ≥ 65, with hyperhomocysteinemia (12-50 µmol/l), who were treated with B-vitamins (500 µg vitamin B12 and 400 µg folic acid) or placebo for 2 years. In a subgroup (n = 569) the effect of vitamin B12 and folic acid supplementation on pulse wave velocity (PWV) was investigated as measurement of arterial stiffness. Applanation tonometry was also used to assess central blood pressure and ultrasonography was used for carotid intima-media thickness measurement. In the total B-PROOF population incidents of cardiovascular and cerebrovascular events were determined via structured questionnaires and blood pressure was measured. Compared to placebo, vitamin B supplementation lowered serum homocysteine by 3.6 µmol/L (p<0.001). Analysis of covariance showed no effect of supplementation on PWV levels, but aortic pulse pressure was higher in the intervention than in the placebo group (49.6 mmHg vs. 47.2 mmHg; p =0.02). Furthermore, a significant reduction of cerebrovascular events in females (OR 0.33 95%CI [0.15 ; 0.71]), but not in males was observed.

Conclusions

Vitamin B12 and folic acid supplementation in hyperhomocysteinemic elderly has no effect on PWV and caused a modest increase in aortic pressure. However, this supplementation also leads to a reduction in cerebrovascular events in females. All in all, arterial stiffness is not likely to be the underlying pathway of the effect of vitamin B12 and folic acid supplementation on the observed treatment effects.

Clinical trial registration: Number: NCT00696514. URL: http://www.clinicaltrials.gov/ct2/show/NCT00696514?term=NCT00696514&rank=1

4.3.2   INTRODUCTION

Hyperhomocysteinemia has been shown to be an independent risk predictor of cardiovascular disease, in particular in the oldest old [1]. However, during the last decade, several trials failed to demonstrate a protective effect of homocysteine-lowering therapy with B-vitamin supplementation on cardio-vascular disease and mortality outcomes, with the exception of stroke [2-4]. Whether this lack of overall benefit has to do with epidemiological and/or methodological issues or with nonexistence of a causal relationship between homocysteine and cardiovascular disease, is still under debate.

A potential link between hyperhomocysteinemia and cardiovascular disease has been suggested to be arterial stiffness. Arterial stiffness is a risk factor for cardiovascular disease [6]. Recently, we have demonstrated that par-ticularly in older individuals with elevated levels of arterial stiffness, that arterial stiffness and homocysteine level are associated [17]. Several trials investigating the effect of B-vitamin supplementation on arterial stiffness showed conflicting results [5-15]. Despite the fact that aortic-femoral pulse wave velocity (PWV) is considered to be the most robust marker of arterial stiffness [16], other measures of arterial stiffness have been used in these trials.

Since in older populations elevated homocysteine levels are associated with both increased cardiovascular risk [1] and arterial stiffness [17], we aimed to investigate the effect of B-vitamin supplementation on arterial stiffness and cardiovascular events. The B-PROOF study, which is primarily designed to investigate the effect of homocysteine-lowering vitamin B12 and folic acid on fracture incidence [18], provided an excellent opportunity to investigate the effect of this intervention on arterial stiffness measures and other car-diovascular outcomes.

4.3.3   METHODS

Study participants
The B-PROOF trial is a multi-center, randomized, placebo-controlled, double-blind, intervention study. B-PROOF is an acronym for 'B-vitamins for the PRe-vention Of Osteoporotic Fractures'. A detailed description of this randomized controlled trial has been reported elsewhere [18]. In short, the B-PROOF trial

included 2919 participants from three areas in the Netherlands from 2008 till 2011 with a follow-up period of 2 years. Inclusion criteria were an age of 65 years or older, and an elevated homocysteine level (12 – 50 µmol/l). Fifty-one percent of the screened population could be included based on their homocysteine level. Main exclusion criteria were renal insufficiency (creatinine level > 150 µmol/l) and presence of a malignancy in the past five years. All participants gave written informed consent before the start of the study. The B-PROOF study has been registered within the Netherlands Trial Register (www.trialregister.nl) under identifier NTR 1333 since June 1, 2008 and with ClinicalTrials.gov under identifier NCT00696514 since June 9, 2008. The Wageningen Medical Ethics Committee approved the study protocol, and the Medical Ethics committees of Rotterdam and Amsterdam gave approval for local feasibility. In all B-PROOF participants, data regarding cardiovascular events and blood pressure were collected and a random subsample of the B-PROOF participants underwent vascular measurements (n = 569) at the Erasmus MC (Rotterdam) or VU University Medical Center (Amsterdam).

Intervention

The intervention period comprised 2 years. Participants were randomly allocated in a 1:1 ratio to the intervention group or to the placebo group. We stratified the randomization for study center, gender, age (65-80 years, ≥ 80 years), and homocysteine concentration (12-18 µmol/L, ≥ 18 µmol/L). The random allocation sequence and randomization were generated and performed using SAS 9.2 by an independent research dietician. The intervention group received a daily tablet with 500 µg vitamin B12 and 400 µg folic acid and the control group received a daily placebo tablet. Both tablets contained 15 µg (600 IU) of vitamin D3 to ensure a normal vitamin D status [19]. The intervention and placebo tablets, were indistinguishable in taste, smell and appearance. Recruitment took place from August 2008 until March 2011.

Baseline and follow-up evaluations

All data and measurements were obtained at baseline and at follow-up. Participants were scheduled for a follow-up visit at 2 years after the intervention period. Adherence was judged by counting tablets, which the participants were asked to send back every 6 months. In case of non-reply, the participants were periodically phoned. Compliance was defined as more than 80% adherence over their two-year period, based on the total amounts of tablets returned during the study.

Clinical characteristics

Height was measured in duplicate to the nearest 0.1 cm, standing erect and wearing no shoes, using a stadiometer. Weight was measured with the participant wearing light garments without shoes and empty pockets to the nearest 0.5 kg using a calibrated weighing device (SECA 761). Body Mass Index (BMI) was calculated as weight divided by squared height and expressed as kg/m^2. All measurements were performed at the baseline visit and at the end of the intervention period after 2 years.

Information regarding alcohol intake and smoking habits were collected using a structured questionnaire, which was administered both at the baseline and follow-up measurements.

Plasma homocysteine and serum vitamin B12, folate, methylmalomic acid (MMA), holo-transcobalamin (HoloTC) and creatinine were measured at baseline. Details regarding all laboratory measurements have been described elsewhere [18, 20]. The estimated glomerular filtration rate (eGFR) was calculated with the Modification of Diet in Renal Disease (MDRD) formula in ml/min/1.73m^2: 186 * (serum creatinine (μmol/l) / 88.4) $^{-1.154}$ * age (years) $^{-0.203}$ * 0.742 (for females) [20].

Vascular measurements

As mentioned in the participant section, a random subsample of participants underwent vascular measurements at baseline (n = 569) at the Erasmus MC (Rotterdam) or VU University Medical Center (Amsterdam). Participants with cardiac arrhythmia were excluded from these additional measurements. During the measurements, participants were situated in supine position on a flat examination couch in a quiet laboratory room for at least 5-10 minutes prior to the measurements, and the participants were not allowed to speak during the measurements. Use of alcohol or coffee during 12 hours before the measurements was prohibited. The vascular measurements were again performed after the intervention period of two years. Also participants who dropped-out were invited to undergo the follow-up measurements. In total, 497 participants had both baseline and follow-up measurements taken.

Applanation tonometry

Arterial tonometry was obtained from the right radial, right carotid and right femoral artery using the Sphygmocor device (Sphygmocor version 7.1, AtCor Medical, Sydney, Australia). An estimate of the corresponding central aortic pulse wave was calculated, using a validated generalized transfer function incorporated in the device. With the integral software, the central augmented

pressure was calculated as the difference between the early and late systolic peaks of the estimated central pressure waveform. Central aortic augmentation index (AIx) was calculated as the augmented pressure expressed as a percentage of the pulse pressure (intra CV = 3%, inter CV = 5%). Aortic pulse wave velocity (PWV) was measured with three channel ECG recording and simultaneously recording of the right carotid artery pulse wave form and subsequently the femoral artery pulse waveform. The PWV was calculated as the delay between the femoral pulse wave and the carotid pulse wave, divided by the transit distance (intra CV = 5 %, inter CV = 8%). Transit distance was assessed with body surface measurement from the carotid artery to the femoral artery [16].

Carotid Intima Media Thickness (Carotid IMT)
For carotid B-mode ultrasonography, the L105 40 mm 7.5 MHz array transducer was used (Picus, Pie Medical Equipment, Maastricht, the Netherlands) on the right carotid artery. IMT is evaluated as the distance luminal - intimal interference and the media - adventitial interface (Art.Lab, Esoate Europe, Maastricht, the Netherlands) at approximately 1 cm from the carotid bifurcation. In order to obtain accurate measurements, the standard deviation of the mean values of 6 independent measurements had to be < 10% of the mean (intra CV = 8%, inter CV = 15%).

Blood pressure measurement
In all participants (n = 2919), blood pressure levels were measured twice with a semi-automatic oscillometer (Omron M1 Plus, OMRON Corporation, Kyoto, Japan) and the levels of the measurement with the lowest diastolic value were used for analysis. The blood pressure measurement was performed at the baseline visit and after the 2-year intervention period. Hypertension was defined as a systolic blood pressure >140 mmHg and/or a diastolic blood pressure >90 mmHg, according to the guidelines of the WHO [21]. Pulse pressure was calculated as the systolic minus the diastolic blood pressure.

At the time of vascular function tests, peripheral blood pressure was measured once with a semi-automatic oscillometric device (Datascope Accurator Plus device, Datascope Corp. New Jersey, USA) after at least five minutes of supine rest. All blood pressure measurements were conducted at the right arm. The mean arterial pressure was measured and the pulse pressure was calculated as the systolic minus the diastolic blood pressure.

Cardiovascular disease incidence

Cardiovascular medical history and potential cardiovascular events of all B-PROOF participants (n = 2919) were determined using a structured questionnaire at both baseline and follow-up measurements by self-report from the participant. Furthermore, data regarding cardiovascular events were obtained prospectively from adverse events reports, which the participants could fill in at their calendars. Any type of cardiovascular disease was defined as myocardial infarction, angina pectoris, heart failure, cardiac valvular disease or arrythmia. Myocardial infarction was also used as a separate entity in our analyses. Cerebrovascular disease was defined as transient ischemic attack (TIA) or stroke.

Statistical analysis

We calculated the vascular subgroup had to include 560 subjects in order to have a more than 90% power for a detection of an improvement of 0.3 standard deviation (~ 2% change in PWV), taking into account an α-level of 0.05.

   With regard to the analyses of the vascular outcomes, participants were included if both baseline and follow-up measurements were available. For cardiovascular disease incidence analysis, all randomized participants were included.

   PWV was defined as the primary outcome. Secondary outcomes included all other arterial stiffness parameters, cardiovascular disease outcomes and blood pressure. Both intention-to-treat (primary) and per protocol analyses (secondary) were performed for all primary and secondary outcome variables. In the intention-to-treat analyses, all participants were included. In the per-protocol analysis, all non-compliant participants were excluded.

   Normal distribution was checked for all outcome variables, and the treatment groups were compared with respect to baseline characteristics. The student's t-test was used for continuous variables and the chi-squared test for categorical variables. Mann-Whitney-U was used for variables, which were not normally distributed.

Differences between treatment groups were analyzed with analyses of covariance (ANCOVA), using the follow-up measurement as the dependent factor, the baseline measurement as covariate and the treatment allocation as the fixed between-subject factor. This analysis was done for all continuous outcome measures. Before performing the ANCOVA, the assumption of linearity and homogeneity of variance was checked. The analysis of covariance was

first done unadjusted (but with adjustment for the baseline measurement). Secondly, covariates were included in the final model as potential confounders when a variable was significantly different between the two treatment groups ($p < 0.2$) and contributed to a more than 10% change in F. Age, gender and study center were all added standard as covariate to the model. To investigate the effect of treatment in specific groups, interaction terms were tested for age, gender, cardiovascular disease history and homocysteine level. If an interaction term was significant ($p < 0.05$), a stratified analysis was done. Strata were created as age <80 vs. ≥ 80; men vs. women; the presence of a cardiovascular disease history vs. no cardiovascular disease history and homocysteine level < 18 μmol/l vs. ≥ 18 μmol/L.

Binary logistic regression analysis was used for dichotomous variables, in which the follow-up measurement was used as the dependent and the treatment group as fixed factor. Also this analysis was first analyzed unadjusted. Secondly, age, gender and study center were added as covariates and additionally, all other covariates that differed between the treatment groups ($p < 0.2$) and led to a more than 10% change of the point estimate. Again, in order to investigate the effect of treatment in specific groups, interaction terms were tested and a stratified analysis was done if an interaction term was significantly associated with the outcome ($p < 0.05$) and strata were created as explained above.

Statistical analyses were done with use of the statistical software package SPSS version 20.0 (SPSS Inc, Chicago, Illinois, USA). Two-sided p-values of < 0.05 were considered statistically significant for both ANCOVA and binary logistic regression analyses.

4.3.4   RESULTS

Characteristics of the study population

Participant disposition is demonstrated in **Figure 4.3.1**. In total, 2919 participants could be included and were randomly allocated to the B-vitamin treatment (n = 1458) or placebo group (n = 1461). The mean age of the study population was 74 ± 6.5 years and 50% were male. Median homocysteine level was 14.4 μmol/l. In **Table 2** the vascular characteristics of the cardiovascular subgroup (n = 569) are depicted per allocation. In total, 497 participants had both baseline and follow-up measurements available.

Mean age of this subpopulation was 73 years and 56% were male. Median homocysteine level was 14.3 µmol/l.

Both treatment groups were well-balanced regarding baseline characteristics and covariates both for the overall population as well as for the vascular subgroup (**Table 1 & 2**). Only holoTC concentrations were slightly different between the two groups in the overall group (p = 0.04) (**Table 1**). There were no differences between the two vascular subgroups with regard to covariates and the arterial stiffness measures (**Table 2**).

Comparing the vascular subgroup with the total B-PROOF population, the participants were somewhat younger (72.5 years vs 74.1 years in the overall population; p < 0.01) and more men were included (48.6% vs. 55.7% in the vascular subgroup; p < 0.01). Also, folic acid and holoTC levels were slightly higher in the vascular subgroup(p < 0.01 for all levels), whereas MMA levels
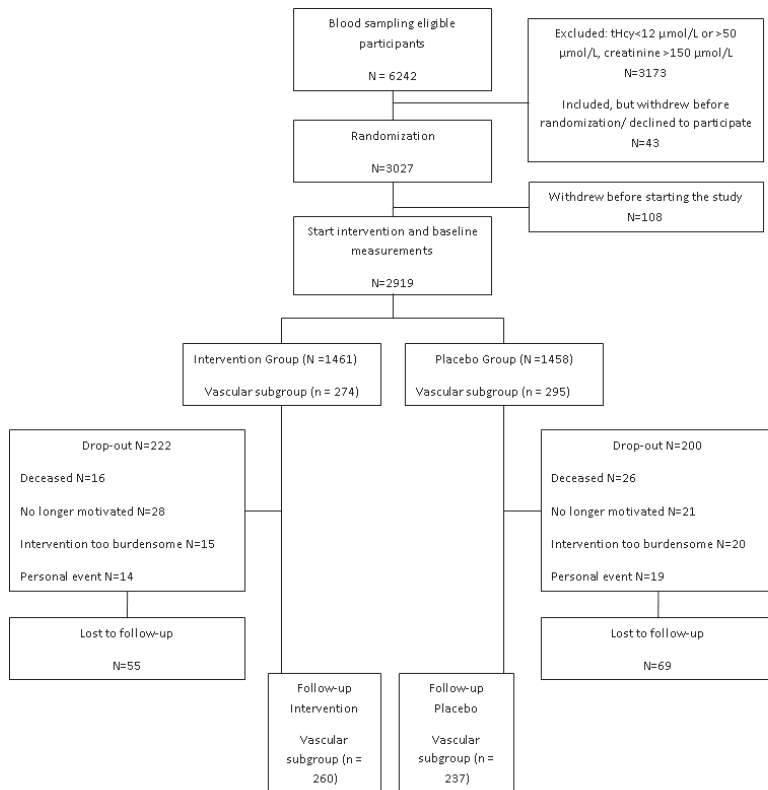
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Table 1. Baseline characteristics of the B-PROOF study population

	Placebo (n = 1458)	Intervention (n = 1461)	p-value
Age (yrs)	74.2 ± 6.4	74.0 ± 6.6	0.60
Male gender	734 (50.3%)	725 (49.6%)	0.70
Homocysteine level (umol/l)	14.5 [13.0 – 16.7]	14.3 [13.0 – 16.5]	0.30
Vitamin B12 level (pmol/l)	265.9 [203.9 – 343.4]	267.3 [212.9 – 341.2]	0.27
Folic acid level (nmol/l)	18.8 [14.7 – 21.2]	18.7 [14.7 – 24.4]	0.50
MMA (µmol/l)	0.23 [0.18 – 0.31]	0.22 [0.18 – 0.30]	0.25
Holo TC (pmol/l)	63.0 [45.0 – 84.0]	65.0 [48.0 – 86.0]	0.03
Creatinine (umol/l)	84.1 ± 18.0	83.9 ± 18.6	0.73
eGFR (ml/min per 1.73m^2)	91.3 ± 37.7	90.9 ± 39.2	0.80
Smoking (n, %)			0.97
Former	821 (56.3%)	828 (56.7%)	
Current	142 (9.7%)	139 (9.5%)	
Alcohol use (n, %)			0.46
Light	972 (66.7%)	994 (68.0%)	
Moderate	422 (28.9%)	417 (28.5%)	
Excessive	57 (3.9%)	43 (2.9%)	
Very excessive	5 (0.3%)	7 (0.5%)	
Supplement use daily (n, %)			
Vitamin B12	216 (14.8%)	207 (14.2%)	0.88
Folic acid	211 (14.5%)	202 (13.8%)	0.87
Self-reported CV medical history (n, %)			
MI	118 (8.1%)	97 (6.6%)	0.46
Any type CVD	279 (19,1%)	272 (18,6%)	0.46
Cerebrovascular event	98 (6.7%)	100 (6.8%)	0.94
Hypercholesterolemia	278 (19.1%)	277 (19.0%)	0.66
Diabetes	105 (7.2%)	128 (8.8%)	0.13
BMI (kg/m^2)	27.2 ± 4.0	27.1 ± 4.0	0.70
Peripheral SBP (mmHg)	147.6 ± 20.1	148.4 ± 19.8	0.31
Peripheral DBP (mmHg)	79.0 ± 10.5	79.2 ± 11.3	0.61
Peripheral PP (mmHg)	68.6 ± 17.5	69.2 ± 16.5	0.39
Hypertension measured (n, %)	703 (48.2%)	756 (51.7%)	0.06

Values are depicted as means ± SD or as n (%), except for homocysteine, vitamin B12, folate, MMA and holoTC level which are provided as median [IQR].

Abbreviations: MMA: methylmalomic acid; holo TC: holotranscobalamin; eGFR: estimated glomerular filtration rate; MI: myocardial infarction; CVD: cardiovascular disease; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; Hypertension measured was defined as SBP > 140 mmHg and/or DBP > 90 mmHg.

Table 2. Population characteristics of the vascular subgroup (n = 569)

	Placebo (n = 274)	Intervention (n = 295)	p-value
Age (yrs)	72.5 ± 5.3	72.5 ± 5.8	0.99
Male gender	152 (55.5%)	165 (55.9%)	0.91
Homocysteine level (umol/l)	14.3 [13.0 – 16.6]	14.2 [13.0 – 16.3]	0.88
Vitamin B12 level (pmol/l)	271.9 [209.7 – 344.0]	272.9 [217.1 – 356.8]	0.67
Folic acid level (nmol/l)	19.2 [15.0 – 24.5]	20.1 [15.5 – 24.4]	0.21
MMA (µmol/l)	0.21 [0.17 – 0.29]	0.21 [0.17 – 0.26]	0.47
Holo TC (pmol/l)	68.0 [50.0 – 89.8]	72.0 [53.8 – 93.0]	0.23
Creatinine (umol/l)	83.7 ± 17.5	83.0 ± 16.8	0.67
eGFR (ml/min per 1.73m^2)	92.1 ± 36.5	90.8 ± 35.1	0.67
Smoking (n, %)	92 (33.6%)	107 (36.3%)	0.80
Former	156 (56.9%)	161 (54.6%)	
Current	26 (9.5%)	27 (9.2%)	
Alcohol use (n, %)			0.70
Light	175 (63.9%)	184 (62.4%)	
Moderate	83 (30.3%)	96 (32.5%)	
Excessive	15 (5.1%)	11 (3.7%)	
Very excessive	2 (0.7%)	4 (1.4%)	
Supplement use daily (n, %)			
Vitamin B12	42 (15.3%)	37 (12.5%)	0.53
Folic acid	37 (13.5%)	36 (12.2%)	0.63
Self-reported CV medical history (n, %)			
MI	28 (10.2%)	18 (6.1%)	0.49
Any type CVD	60 (21.9%)	62 (21.0%)	0.43
Cerebrovascular event	23 (8.4%)	22 (7.5%)	0.69
Hypercholesterolemia	77 (28.1%)	74 (25.1%)	0.44
Diabetes	33 (12.0%)	32 (10.8%)	0.67
BMI (kg/m^2)	27.0 ± 3.8	27.0 ± 3.6	0.93
Peripheral SBP (mmHg)	136.8 ± 18.1	138.8 ± 18.0	0.17
Peripheral DBP (mmHg)	77.2 ± 9.7	77.5 ± 9.7	0.69
Hypertension measured (n, %)	108 (39.4%)	128 (43.4%)	0.34
PWV (m/s)	14.2 ± 4.1	14.5 ± 4.9	0.44
AIx (%)	26.3 ± 9.9	26.1 ± 10.1	0.85
Aortic SBP (mmHg)	127.9 ± 18.5	129.3 ± 18.6	0.38
Aortic PP (mmHg)	49.7 ± 14.9	50.5 ± 15.4	0.52
Carotid IMT (µm)	727.5 ± 172.9	707.3 ± 153.5	0.22

Values are depicted as means ± SD or as n (%), except for homocysteine, vitamin B12, folate, MMA and holoTC level which are provided as median [IQR].

Abbreviations: MMA: methylmalomic acid; holo TC: holotranscobalamin; eGFR: estimated glomerular filtration rate; MI: myocardial infarction; CVD: cardiovascular disease; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; PWV: pulse wave velocity; AIx: augmentation index; IMT: intima media thickness. Hypertension measured was defined as SBP > 140 mmHg and/or DBP > 90 mmHg.

were lower in the vascular subgroup (p < 0.01), but homocysteine levels did not differ. Further, the overall population had less hypertension (47.6% vs. 59.8% in the vascular subgroup; p < 0.01) and DBP levels were lower in the total population (78.5 mmHg vs. 81.0 mmHg; p < 0.01). Other cardiovascular risk factors were similar between the overall population and the vascular subgroup.

Effects on arterial stiffness measurements (vascular subgroup)

Analyzing the effect of the B-vitamin treatment, aortic PP after two years of intervention was significantly higher in the B-vitamin treatment group, compared to placebo (49.6 mmHg vs. 47.2 mmHg; p = 0.02) (**Table 3**). Also the aortic SBP tended to be higher, although not significant. PWV and all other vascular parameters did not differ between the groups.

In per-protocol analysis, 93% of the participants in the intervention group and 90% of the participants in the placebo group remained. The effect on aortic PP pertained in this analysis with 49.9 mmHg in the intervention group versus 47.4 mmHg in the placebo group (p = 0.02). Also, the effects on the other outcomes did not change.

Table 3. Estimated means of blood pressure and arterial stiffness parameters after 2 years of intervention with vitamin B12 and folic acid supplementation investigated with ANCOVA according to the intention-to-treat principle

	Placebo		Intervention		F	p-value
	n	Estimated mean ± SD	n	Estimated mean ± SD		
Peripheral SBP (mmHg)	1284	144.7 ± 0.5	1285	146.0 ± 0.5	3.297	0.07
Peripheral DBP (mmHg)	1284	75.7 ± 0.3	1285	76.1 ± 0.3	0.891	0.35
Peripheral PP (mmHg)	1284	69.0 ± 0.4	1285	69.9 ± 0.4	2.282	0.13
PWV (m/s)	221	14.3 ± 0.3	243	14.2 ± 0.3	0.036	0.85
AIx (%)	233	28.8 ± 0.6	255	27.5 ± 0.6	2.471	0.12
Aortic SBP (mmHg)	234	123.7 ± 0.9	257	126.2 ± 0.9	3.714	0.06
Aortic PP (mmHg)	234	47.2 ± 0.7	257	49.6 ± 0.7	5.446	0.02
Carotid IMT (μm)	177	710.4 ± 11.1	198	705.8 ± 10.5	0.091	0.76

All estimated means are adjusted for the corresponding baseline measurement, age, gender and study center. Peripheral SBP and PP also adjusted for holoTC level, vitamin B12 level and the presence of diabetes. Peripheral DBP is also adjusted for holoTC level and MMA level. PWV, AIx, Aortic SBP and aortic PP are also adjusted for peripheral SBP.

Within the vascular subgroup, the homocysteine level changed from 14.3 µmol/l [IQR 13.0 – 16.6] to 14.9 µmol/l [IQR 12.8 – 17.0] in the placebo group (Δ 0.6 µmol/l) and decreased from 14.2 µmol/l [IQR 13.0 – 16.6] to 10.6 µmol/l [IQR 9.3 – 12.2] in the intervention group (Δ -3.6 µmol/l). Changes in homocyteine levels between female and male participants did not differ.

Blood pressure (overall population)

There were no differences in blood pressure indices between the intervention group and the placebo group (**Table 2**). Despite the fact that kidney function, vitamin B12 level and homocysteine level modified the treatment effect (p < 0.01; p = 0.04 and p = 0.02 respectively), stratified analyses did not show any effect of the intervention on blood pressure. Per protocol analyses did not change these results.

   The incidence of hypertension did not differ between the intervention (287 cases) and placebo (321 cases) group (data not shown). There were no significant interaction-terms with treatment on the outcome of hypertension.

Incidence of cardiovascular events (overall population)

Within the intervention group, the incidence of cerebrovascular events during the intervention period was lower, compared to placebo (46 incidents versus 60 incidents; incidence proportion ratio: 0.75 [95% CI: 0.51 – 1.10]) (**Table 4**). Age was a significant modifier in the treatment effect on cerebrovascular events (p < 0.01). However, stratification for participants under and above the age of 80 showed no differences in the reduction of cerebrovascular events between the age strata. Furthermore, the treatment effect on cerebrovascular events was different between genders (p = 0.048). In women the incidence was lower (OR 0.33 95%CI [0.15 ; 0.71]), with 36 cases in the placebo group, compared to 16 cases in the intervention group (**Figure 4.3.2**).

   There were no differences between the treatment groups in the incidence of myocardial infarction or any other type of cardiac disease (**Table 4**).

Second, a per-protocol analysis was performed with compliant participants only. This comprised 84% of both the placebo and the intervention group. Results were similar to those of the intention-to-treat analyses (data not shown).

Within the total B-PROOF population, the homocysteine level changed from 14.5 µmol/l [IQR 13.0 – 16.7] to 14.3 µmol/l [IQR 12.4 – 17.0] in the placebo group (Δ -0.2 µmol/l) and decreased from 14.3 µmol/l [IQR 13.0 – 16.5] to 10.3 µmol/l [IQR 8.9 – 12.0] in the intervention group (Δ -4.0 µmol/l).

Table 4. The effect of vitamin B12 and folic acid supplementation on self-reported cardiovascular outcomes investigated with logistic regression analysis according to the intention-to-treat principle

	N of cases in placebo group	N of cases in intervention group	Model 1		Model 2	
			OR ± 95% CI	p-value	OR ± 95% CI	p-value
MI	43	45	1.05 [0.68 ; 1.60]	0.84	1.19 [0.66 ; 2.14]	0.56
Any type CVD	170	181	1.07 [0.86 ; 1.35]	0.53	1.08 [0.86 ; 1.36]	0.50
Cerebrovascular event	60	46	0.76 [0.52 ; 1.13]	0.17	0.72 [0.45; 1.15]	0.17

Model 1: unadjusted. Model 2: variables were adjusted for age, gender, study center. MI was also adjusted for holoTC and diabetes. TIA was also adjusted for diabetes.

Abbreviations as in Table 1.OR: odds ratio; CI: confidence interval.
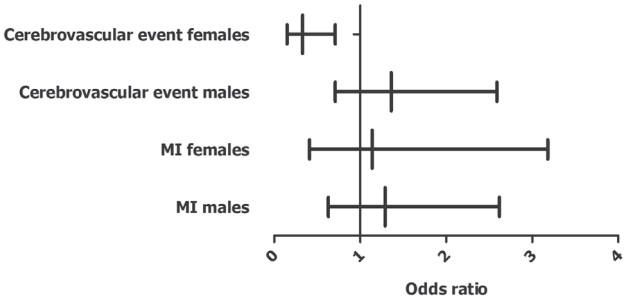
Figuur 4.3.2.

Values are depicted as OR ± 95%CI demonstrating the effect of the B-vitamin intervention. Analyses were performed according to the intention-to-treat principle. Outcomes were only included in the figures when the gender*treatment interaction was significant in the logistic regression analysis.

Abbreviations: as in Table 1.

4.3.5 DISCUSSION

Within this randomized, double-blind, placebo-controlled trial in elderly, hyperhomocysteinemic subjects, we observed that the 2-year intervention with vitamin B12 and folic acid did not affect PWV. Moreover, vitamin B12 and folic acid supplementation modestly raised aortic pulse pressure, whereas, paradoxically, the number of cerebrovascular incidents was reduced, although only in females. Finally, no effect on any type of cardiac event, including myocardial infarction, was observed in females or males after 2-years of B-vitamin supplementation.

Since homocysteine-lowering therapy is thought to have a direct effect on the human vasculature, treatment with B-vitamins [22] is assumed to decrease cardiovascular risk. However, the arterial stiffening process is also known to have a limited reversibility. In concordance with this hypothesis, we did not observe a treatment effect on PWV levels, our main outcome.

Recent meta-analyses have shown contradictory effects of B-vitamin supplementation on cardiovascular disease with a lowering of cerebrovascular events but no effect on coronary heart disease [2-4]. Our finding of a reduction of cerebrovascular events in females after vitamin B12 and folic acid supplementation agrees with these findings. However, in the meta-analyses no difference between males and females with regard to cerebrovascular risk reduction was observed. Differences in age or level of homocysteine could not explain the difference we found for gender, since in the model adjustment for age and homocysteine levels did not modify the treatment effect. However, since this is a sub-group analysis, we need to consider the possibility of this being a chance finding.

Atherosclerosis, thrombo-embolic events and bleedings may all underlie cerebrovascular disease, as a consequence cerebrovascular disease is a much more heterogeneous condition than coronary artery disease, which is due to a local thrombo-embolic process [23]. The difference in etiology between cerebrovascular disease and coronary artery diseases may account for the difference in effect of vitamin B supplementation between the two conditions. Of note, carotid intima-media thickness as a marker of atherosclerosis [24], was not influenced by B-vitamin supplementation.

Regardless of the effect of B-vitamins supplementation on lowering cardiovascular risk, increased homocysteine levels are an important cardiovascular risk predictor in elderly, that can well predict future cardiovascular events in this specific population, in particular compared to the Framingham risk score [1]. Furthermore, homocysteine levels tend to decrease more when hyperhomocysteinemia is present [22]. Therefore, we hypothesized that if homocysteine-lowering therapy indeed lowers cardiovascular risk, this would be most clearly demonstrable in older, hyperhomocysteinemic individuals. However, besides the beneficial effect on cerebrovascular events in females, we did not observe an effect of vitamin B12 and folic acid supplementation on myocardial infarction or other cardiac diseases. However, it needs to be mentioned that our study was not powered to detect differences in cardiovascular events.

Aortic pulse pressure was higher in the intervention than in the placebo group. Remarkably, this rise in aortic pulse pressure occurred despite the fact that PWV levels did not change. An increase in central blood pressure levels without a change in PWV levels can either reflect a cardiac underlying mechanism or a difference in effect between small and large arteries. Central arterial pressure also depends on cardiac performance and peripheral resistance [25, 26]. Possibly, lowering of elevated homocysteine levels is associated with small rise in peripheral resistance resulting in an increase in aortic pressure.

The homocysteine controversy theory needs discussion in light of the paradoxical perspective described above [27]. It has been described that on the one hand there could be a direct harmful effect of B-vitamin supplementation on the vascular wall related to an inflammatory response [27], while on the other hand a beneficial effect is obtained via homocysteine-lowering per se. Although pathophysiological evidence regarding this hypothesis is still lacking, our study adds that arterial stiffness is not involved in this pathophysiological pathway. On the other hand, our finding that central pulse pressure levels were higher in the group treated with B-vitamins despite unchanged PWV may indicate that B-vitamin supplementation has an adverse effect on peripheral resistance. This potential adverse effect on peripheral resistance requires further exploration as it is in line with the paradox hypothesis.

There are several limitations to this study. First, cardiovascular outcomes were not the main outcome of this trial. Therefore the cardiovascular in-

cidents were self-reported with the help of a structured questionnaire and not verified by general practitioner or hospital. This may have lead to an underestimation of the effect, however there is no reason to assume that our structured reporting form of the incidents would differ between the two groups. Second, our population consisted of mildly hyperhomocysteinemic elderly participants because of the anticipated effect of B-vitamin supplementation in this particular group. Results are therefore not generalizable to the general population. However, since almost 50% of the general elderly population in the Netherlands [28] has mildly elevated homocysteine levels, it is a relevant group to address the effect of lowering of homocysteine levels on cardiovascular parameters. Third, we acknowledge that the follow-up period of 2 years is relatively short for cardiovascular outcome parameters. Our study was designed to study the effect of vitamin B12 and folic acid supplementation on PWV and for this parameters a 2 year intervention is considered to be sufficient, because PWV is known to respond quickly as other trials with PWV as outcome variable have shown. Strengths of the study are the multiple cardiovascular outcomes, which makes us able to give a full overview of the effects on arterial stiffness, arteriosclerosis and cardiovascular events. Furthermore, our elderly population is of special interest since it has been shown that with age cardiovascular risk profile changes [1]. Since there are no trials conducted within an elderly population, our study adds knowledge about the homocysteine-lowering effect of this particular population.

Finally, it is important to note that more cancer cases were reported in the participants treated with B-vitamin supplementation compared to participants who received placebo (unpublished data; van Wijngaarden,J.P. *et al*. Effect of daily vitamin B12 and folic acid supplementation on fracture incidence in elderly with an elevated plasma homocysteine level: B-PROOF, a randomized controlled trial. *Submitted)*. Therefore caution is warranted for future research regarding the effects of vitamin B12 and folic acid supplementation.

In conclusion, vitamin B12 and folic acid supplementation did not affect PWV levels after a treatment duration of two years. Furthermore, despite the fact that homocysteine-lowering therapy reduced cerebrovascular incidents in females, it also was associated with an increase aortic pulse pressure. All in all, our findings indicate that arterial stiffness is unlikely the underlying pathway of the effect of vitamin B12 and folic acid supplementation on cerebrovascular or other cardiovascular events. Further studies are still needed

to determine the effects of B-vitamin supplementation on cardiovascular outcomes with more certainty as well as the potential underlying pathways.

Acknowledgements

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4.4

B-vitamins have no effect on biomarkers of endothelial function or inflammation in hyperhomocysteinemic elderly

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4.4.1   ABSTRACT

Introduction

B-vitamin trials failed to demonstrate beneficial effects on cardiovascular outcomes, but hyperhomocysteinemia still stands out as an independent cardiovascular risk indicator, particularly in elderly. B-vitamins may influence early vascular dysfunction, such as endothelial dysfunction, or may have adverse effects, for example on inflammation. We investigated the effect of B-vitamins on endothelial function and inflammation within an intervention study.

Methods

This study was conducted within the framework of the B-PROOF trial, which included 2919 hyperhomocysteinemic elderly, who received daily vitamin B12 (500 µg) and folic acid (400 µg) or placebo for 2 years. Using an electroche-miluminescence platform, we measured Intercellular Adhesion Molecule 1 (ICAM-1), Vascular Adhesion Molecule 1 (VCAM-1), serum amyloid A (SAA), vascular endothelial growth factor (VGEF) and C-reactive protein (CRP) both at baseline and follow-up in a subsample of 522 subjects (271 intervention group; 251 placebo). Treatment effects were analyzed with ANCOVA.

Results

The subjects had a mean age of 72 years and 55% of them was male. With 2-year follow-up, B-vitamins did not change the ICAM-1 (+36% change in the intervention group versus +32% change in the placebo group; $p = 0.72$), VCAM-1 (+27% versus +25%; $p = 0.39$), VEGF (-1% versus +4%; $p = 0.40$), SAA (+34% versus +38%; $p = 0.85$) or CRP levels (+26% versus +36%; $p = 0.70$) as compared to placebo.

Conclusion

In elderly patients with hyperhomocysteinemia, vitamin B12 and folic acid are unlikely to influence either endothelial function or low-grade systemic inflammation.

4.4.2 INTRODUCTION

Hyperhomocysteinemia is an independent risk indicator for cardiovascular disease, in particular among the oldest old [1]. Over the years, several homocysteine-lowering trials with B-vitamins have been performed, however, no beneficial effects on cardiovascular disease or venous thrombosis have been observed [2], with the possible exception of stroke [3, 4]. To date, this overall lack of benefit of lowering homocysteine remains unexplained. One possible explanation is that the association between homocysteine and cardiovascular disease is not causal. Alternatively, it has been proposed that beneficial cardiovascular effects of homocysteine-lowering therapy could be masked by adverse effects of high-dose B-vitamins, such as an increase in proliferation or inflammation [5].

It is possible that B-vitamins only influence vascular dysfunction at an early stage, like endothelial dysfunction, rather than benefit the process of advanced atherosclerosis or arterial stiffness. Earlier, we demonstrated no effects of B-vitamins on arterial stiffness and intima-media thickness, a surrogate measure of atherosclerosis [6]. An increased homocysteine level has previously been related to endothelial dysfunction [7-10]. If homocysteine lowering therapy would have a favorable effect on endothelial function, the time required to see this translated into clinical endpoint may be considerably longer than the 2-5 years follow-up of the current homocysteine lowering intervention trials.

Trials investigating the effect of B-vitamins on endothelial function usually measured flow-mediated dilatation, for which the results are conflicting [11-16]. There were also several small trials investigating the effects of B-vitamins on endothelial biomarkers, again with conflicting results [13, 17-20]. Most of these trials were performed in selected populations, like patients with coronary artery disease or diabetes, and did not select patients with high baseline homocysteine. Also, these trials were of relatively short duration (longest 40 weeks), and included relative few subjects (max 90 subjects). Furthermore, only a small variety of biomarkers were used, and data regarding the more important and relevant markers of endothelial function (ICAM, VCAM, VGEF [21-25]) were often lacking.

As an alternative explanation for the lack of clinical benefit, adverse effects of high doses of B-vitamins on the cardiovascular system have been

postulated. Folate fuels one carbon metabolism, and may stimulate many metabolic active cells, including proliferating or inflammatory cells. Since the atherosclerotic process is partly inflammatory, involving macrophages and T-cells, it is possible that folate enhances inflammation in atherosclerotic lesions, which makes them prone to rupture [5]. Direct assessment of inflammation in atherosclerotic lesions is difficult, but the response of inflammatory biomarkers to B-vitamins may represent a proof-of-concept for pro-inflammatory effects of B-vitamins.

Based on these considerations, we set out to study the effects of B-vitamins on biomarkers of endothelial function and inflammation in a large, population of hyperhomocysteinemic elderly subjects.

4.4.3 METHODS

Study participants

The B-PROOF trial is a multi-center, randomized, placebo-controlled, double-blind, intervention study. B-PROOF is an acronym for 'B-vitamins for the PRevention Of Osteoporotic Fractures'. A detailed description of this randomized controlled trial has been reported elsewhere [26]. In short, the B-PROOF trial included 2919 participants from three areas in the Netherlands. Inclusion criteria were age of 65 years and older, and an elevated homocysteine level (12 – 50 μmol/l). Fifty-one percent of the screened population could be included based on their homocysteine level. The main outcome was osteoporotic fractures. Main exclusion criteria were renal insufficiency (creatinine level > 150 μmol/l) and presence of a malignancy in the past five years. All participants gave written informed consent before the start of the study. The B-PROOF study has been registered with the Netherlands Trial Register http://www.trialregister.nl under identifier NTR 1333 since June 1, 2008 and with ClinicalTrials.gov under identifier NCT00696514 since June 9, 2008. The Wageningen Medical Ethics Committee approved the study protocol. At the baseline measurement, participants were invited for vascular measurements and dual X-ray absorptiometry. In total, 522 participants underwent both additional measurements. Within this group, we measured biomarkers for endothelial function and inflammation.

Intervention

The intervention period comprised 2 years. Participants were randomly allocated in a 1:1 ratio to the intervention group or to the control group. We stratified the randomization for study center, gender, age (65-80 years, ≥ 80 years), and homocysteine concentration (12-18 µmol/L, ≥ 18 µmol/L). The intervention group received a daily tablet with 500 µg vitamin B12 and 400 µg folic acid, and the control group received a daily placebo. Both tablets contained 15 µg (600 IU) vitamin D3 to ensure a normal vitamin D status [27]. The intervention and placebo tablets, produced by MCO Health, Almere, the Netherlands, are indistinguishable in taste, smell and appearance. The random allocation sequence and randomization were generated and performed using SAS 9.2 by an independent research dietician. Recruitment took place from August 2008 until March 2011.

Baseline and follow-up evaluations

All data and measurements were obtained at baseline and at follow-up. Participants were scheduled for a follow-up visit at 2 years after the intervention period. Adherence was judged by counting tablets, which the participants were asked to send back every 6 months. In case of non-reply, the participants were periodically phoned. Compliance was defined as more than 80% adherence over their two-year period, based on the total amounts of tablets provided during the study.

Clinical characteristics

All measurements were performed at the baseline visit and at the end of the intervention period after 2 years. Alcohol intake, smoking habits, cardiovascular history and the presence of diabetes were determined using a structured questionnaire [26], which was requested at both baseline and at the follow-up measurements.

Laboratory measurements

Homocysteine and creatinine

Plasma homocysteine, serum creatinine , vitamin B12, folate, methylmalomic acid (MMA) and holo- transcobalamin (holoTC) were measured. Details regarding laboratory techniques are described elsewhere [26, 28]. The estimated glomerular filtration rate (eGFR) was calculated with the Modification of Diet in Renal Disease (MDRD) formula in ml/min/1.73m^2 [29].

ICAM-1, VCAM-1, SAA, VEGF, CRP

A multi-array electrochemiluminescence platform (MesoScale Discovery, Gaithersburg, MD, USA, www.mesoscale.com) was used to measure Intercellular Adhesion Molecule 1 (ICAM-1), Vascular Adhesion Molecule 1 (VCAM-1), serum amyloid A (SAA), and C-reactive protein (CRP) simultaneously in serum. For vascular endothelial growth factor (VGEF), a single-array platform was used. Each serum sample was frozen after the drawn at baseline or follow-up and both baseline and follow-up samples were measured simultaneously. The samples were measured singular and all measurements were done within a period of 30 days.

Statistical analysis

For comparisons between treatment groups, the student's t-test was used for continuous variables and the chi-square test for categorical variables. Mann-Whitney-U was used for variables which were not normally distributed.

Both intention-to-treat and per-protocol analyses were performed. In the intention-to-treat analyses, all participants for whom both a baseline and follow-up measurement were available were included. In the per-protocol analysis, all non-compliant participants were excluded.

Associations between homocysteine levels and endothelial markers at baseline were tested using multivariable linear regression analysis, with standard adjustment for age, gender, study center and eGFR.

Differences between treatment groups were analyzed with analyses of covariance (ANCOVA), using the follow-up measurement as the dependent factor, the baseline measurement as covariate and treatment allocation as the fixed between-subject factor. This analysis was done for all continuous outcome measures. Before performing the ANCOVA, the assumption of linearity and homogeneity of variance was checked. The ANCOVA was first done unadjusted. Secondly, covariates were included in the final model as potential confounder, if a variable was different between the two treatment groups ($p < 0.2$) and contributed to a more than 10% change in F. Age, gender and study center were all added standard as covariate to the model. To investigate the effect of treatment in specific groups, interaction terms with treatment were tested for age, gender, cardiovascular disease history, homocysteine level, vitamin B12 and folic acid level. If an interaction term

was significant (p < 0.05), stratified ANCOVA was performed. For continuous variables, strata were created based on tertiles.

In order to account for biological variability of the biomarkers of interest, and to increase statistical power, aggregate scores of endothelial function and inflammation were created. First, we calculated the differences between baseline and follow-up of all biomarkers. From this, we calculated overall Z-scores by dividing the difference of the participants individual level minus the mean level, through the standard deviation of the total population. Of these Z-scores, means were calculated. The inflammation score included CRP, SAA and ICAM-1. The endothelial function score included ICAM-1, VCAM-1 and VEGF. ICAM-1 is expressed both in monocytes and the endothelium and was therefore included in both scores [30].

Statistical analyses were done with use of the statistical software package SPSS version 20.0 (SPSS Inc, Chicago, Illinois, USA). Two-sided p-values of < 0.05 were considered statistically significant.

4.4.4   RESULTS

The study population consisted of 522 participants; a subsample of the total B-PROOF study population in whom biomarker data were collected. The characteristics of this subsample are shown in **Table 1**. Of the 522 participants, 271 had been assigned to the intervention group and 251 to the placebo group. Both groups were well-balanced with regard to baseline characteristics and covariates (**Table 1**). Median homocysteine level was 14.2 [interquartile range 12.9-16.4] µmol/l. As compared to the total B-PROOF population, the mean age, homocysteine level and the number of males were similar.

At baseline, there was no association between homocysteine level and CRP, SAA, ICAM-1 or VCAM-1. There was a significant association between homo-cysteine level and VEGF level (beta 132.7 pg/ml [95% CI 55.6 ; 209.8]; p = 0.001) (**Figure 4.4.1**).

B-vitamin treatment had no effect on individual plasma biomarkers of en-dothelial function or on endothelial Z-scores (**Table 2**). With ICAM-1 levels, there was a 36% change in the intervention group and a 32% change in

Table 1. Characteristics of the study population at start of the B-PROOF study

	Placebo group (n = 251)	Intervention group (n = 271)
Age (yrs)	72.3 ± 5.3	72.4 ± 5.7
Male (%)	55.4%	54.6%
Smoking (%)		
Never	35%	38%
Former	56%	54%
Current	10%	7%
Alcohol use (%)		
Light	64%	65%
Moderate	30%	32%
Excessive	6%	3%
Very excessive	1%	1%
Pre-study supplement use, daily (%)		
Vitamin B12	16%	13%
Folic acid	14%	13%
Self-reported CVD history (%)		
Coronary heart disease	13%	9%
TIA / Stroke	8%	6%
Hypertension	40%	35%
Hypercholesterolemia	27%	26%
Diabetes	10%	10%
BMI (kg/m^2)	27.0 ± 3.7	27.1 ± 3.8
SBP (mmHg)	132.0 ± 32.7	135.2 ± 32.8
DBP (mmHg)	96.4 ± 30.8	93.8 ± 29.4
Hypertension (%) *	34.3%	38.7%
Homocysteine (μmol/l)	14.4 [12.9-16.7]	14.1 [13.0-16.2]
Vitamin B12 (pmol/l)	287.9 ± 132.2	293.4 ± 109.7
Folate (nmol/l)	21.1 ± 8.9	22.1 ± 9.8
MMA (μmol/l)	0.2 [0.2 – 0.3]	0.2 [0.2-0.3]
Holo TC (pmol/l)	69.0 [48.8-90.0]	72.0 [54.0-93.0]
Creatinine (μmol/l)	83.8 ± 17.4	82.8 ± 16.3
CRP (ng/ml)	3.1 ·10^4 ± 6.3 ·10^4	3.1 ·10^4 ± 5.6 ·10^4
SAA (ng/ml)	8.8 ·10^4 ± 1.3 ·10^5	9.1 ·10^4 ± 1.3 ·10^5
ICAM-1 (ng/ml)	3.6 ·10^3 ± 3.9 ·10^3	3.4 ·10^3 ± 3.6 ·10^3
VCAM-1 (ng/ml)	6.1 ·10^3 ± 6.6 ·10^3	5.6 ·10^3 ± 6.0 ·10^3
VEGF (pg/ml)	4.1 ·10-3 ±3.1 ·10-3	4.3 ·10-3 ± 3.6 ·10-3

Values are depicted as mean ± SD or as %, with the exception of homocysteine, MMA and ho-loTC which are demonstrated as medians with interquartile ranges.

Abbreviations: MMA: methylmalomic acid; HoloTC: holotranscobalamin; eGFR: estimated glo-merular filtration rate; TIA: transient ischemic attack; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; CRP: c-reactive protein; SAA: serum amyloid A; ICAM-1: intercellular adhesion molecule 1; VCAM-1: vascular adhesion molecule 1; VEGF: vascular endothelial growth factor.

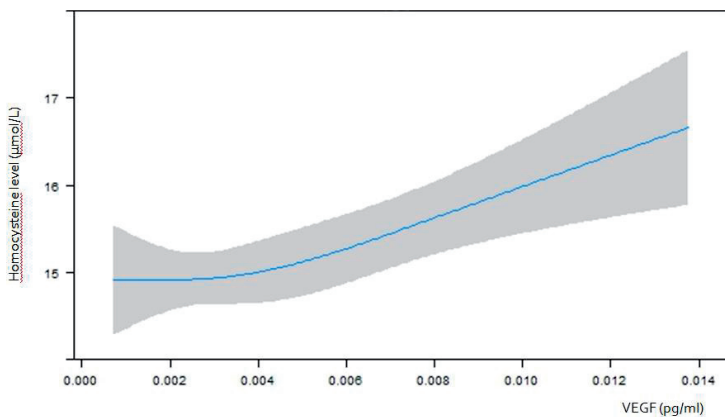*Hypertension was defined as a SBP > 140 mmHg and/or DBP > 90 mmHg [42].

Figuur 4.4.1.

This figure demonstrates the association between homocysteine level and VEGF cross-sectionally at the baseline measurement. Abbreviations as in Table 1.

the placebo group between baseline and follow-up (p = 0.72). For VCAM-1 levels, the intervention group levels changed 27% versus 25% in the placebo group (p = 0.39), and according VEGF levels there was no change between baseline and follow-up in both groups (-1% versus +4%; p = 0.40). In **Table 3** we demonstrate there was no effect of B-vitamin treatment on individual plasma inflammatory markers or on the inflammation Z-score. SAA levels changed with 34% during two year follow-up in the intervention group and with 38% in the placebo group (p = 0.85). CRP levels increased with 26% in the intervention group versus 36% in the placebo group (p = 0.70).

The presence of cardiovascular disease appeared to be a significant effect modifier for the treatment effect on SAA (p = 0.045). Stratified analysis for participants with and without cardiovascular disease did not demonstrate a treatment effect of B-vitamins in one of these strata. A different effect of B-vitamin treatment was seen in the change of SAA levels between follow-up and baseline between the strata. In individuals without cardiovascular disease, B-vitamins caused a 17% increase of SAA levels, whereas in persons with a cardiovascular history, SAA levels increased with 77% (**Figure 4.4.2**).

In per-protocol analysis, 98 % of the participants remained in the intervention group and 95 % of the participants in the placebo group. Also with this analysis we observed no treatment effect of vitamin B12 and folic acid on endothelial function or inflammation.

Table 2. Changes in endothelial markers during two-year follow-up for both placebo and intervention group.

	Placebo group			Intervention group			p-value
	Baseline	Follow-up	change	Baseline	Follow-up	change	
ICAM-1 (ng/ml)	3620.6	4790.6	+32%	3426.3	4646.9	+36%	0.72
VCAM-1 (ng/ml)	6112.6	7668.4	+25%	5610.3	7116.9	+27%	0.39
VEGF (pg/ml)	0.0041	0.0043	+4%	0.0043	0.0043	-1%	0.40
Aggregated endothelial function Z-score			+0.01			-0.02	0.58

Analysis was done with ANCOVA and adjusted for age, gender and study center and baseline value. The aggregated endothelial function score included ICAM-1, VCAM-1 and VEGF and was based on the Z-scores of the change between baseline and follow-up per biomarker. p-values show the outcome for ANCOVA analysis.

Table 3. Changes in inflammation markers during two-year follow-up for both placebo and intervention group.

	Placebo group			Intervention group			p-value
	Baseline	Follow-up	change	Baseline	Follow-up	change	
CRP (ng/ml)	30950.4	42067.9	+36%	31335.2	39611.9	+26%	0.70
SAA (ng/ml)	88301.9	122046.6	+38%	90710.3	121694.4	+34%	0.85
ICAM-1 (ng/ml)	3620.6	4790.6	+32%	3426.3	4646.9	+36%	0.72
Aggregated inflammation Z-score			+0.01			-0.02	0.66

Analysis was done with ANCOVA and adjusted for age, gender and study center and baseline value. The aggregated inflammation score included CRP, SAA and ICAM-1 and was based on the Z-scores of the change between baseline and follow-up per biomarker. p-values show the outcome for ANCOVA analysis.

4.4.5 DISCUSSION

In our study, no effect of vitamin B12 and folic acid on 2-year changes in plasma biomarkers of endothelial function and inflammation was observed in hyperhomocysteinemic elderly subjects.

Other studies on the effect of B-vitamins on endothelial function showed conflicting results. Some suggested a beneficial effect, whereas others showed no effect at all [11-20, 31-33]. Most of these studies had a short follow-up time (maximum of 40 weeks) and were done in specific populations. If a treatment effect of homocysteine-lowering therapy with B-vitamins is present, it would conceivably be observed most clearly in subjects with increased
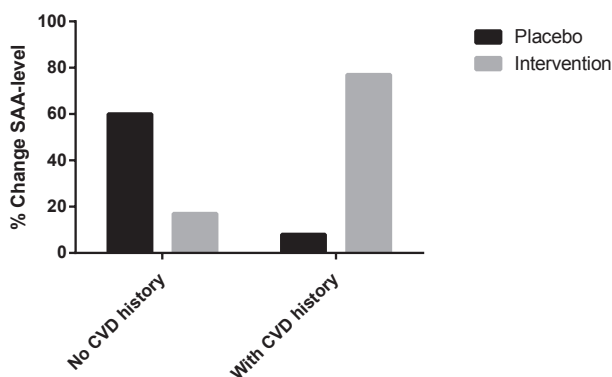
Figure 4.4.2.

Data represent the percentage of change of SAA levels in the placebo and intervention group stratified for participants without cardiovascular history (no CVD history) and with a positive cardiovascular disease history (with CVD history). Differences between placebo and intervention were not statistically significant.

levels of homocysteine. We observed no treatment effect in such subjects after 2 years and also no significant interactions. We believe this provides strong support for a lack of benefit of B-vitamins on endothelial dysfunction.

We selected ICAM-1, VCAM-1 and VEGF as biomarkers for endothelial function. ICAM-1 and VCAM-1 mediate the adhesion of endothelial cells to leukocytes [34]. Because ICAM-1 is also expressed on monocytes, this biomarker is also associated with inflammation [30]. High levels of ICAM-1 and VCAM-1 may be caused by damaged or activated endothelial cells [35]. VEGF induces the migration and proliferation of endothelial cells and is also important for vascular permeability and thrombogeneticy [36]. Low-grade inflammation was assessed with CRP, SAA and ICAM-1 levels. CRP is a widely accepted biomarker for chronic low-grade inflammation and has been consistently associated with cardiovascular disease [37-40]. SAA also predicts cardiovascular disease, but to a lesser extent than CRP [39]. ICAM-1 is expressed both in monocytes and the endothelium and was therefore applied in the inflammatory Z-score as well [30].

Our findings with respect to inflammation argue against the hypothesis that folate could increase systemic inflammation. However, whether a lack of effect on circulating biomarkers excludes an effect on local inflammation in atherosclerotic plaques is unknown. Nevertheless, a small study in stroke patients did not observe a B-vitamin treatment effect on arterial wall inflam-

mation, measured with PET scans [41]. Further research is necessary to determine the intra-atherosclerotic plaque inflammatory process.

The possible difference in treatment effect on SAA levels between individuals with and without history of cardiovascular disease, implicates we cannot completely rule out beneficial effects of B-vitamins in specific subgroups. In general, our study supports previous trials [2] concluding that the cardiovascular system is generally unresponsive to B-vitamin treatment, as well as for cardiovascular disease outcomes, as for arterial stiffness and endothelial function. This does not annihilate the strong association between homocysteine and cardiovascular disease, but further adds to the notion that we need to search for alternative explanations for this association.

This study has its limitations. First, this study was performed in a hyper-homocysteinemic elderly population and our results can therefore not be translated directly to the general population. Nevertheless, when a beneficial treatment effect of homocysteine lowering would be expected, this would be particularly seen in subjects with high homocysteine levels. Also, the bio-markers were only measured once which does not ascertain a perfect quality of the measurements. Third, the use of an aggregated Z-score of endothelial function and inflammation ideally requires that each biomarker has an equal weight. Fourth, the outcome parameters of this report were not assigned as primary outcome of the B-PROOF study at large. Further, circulating biomarkers do not cover the full spectrum of tests for assessing endothelial (dys)function and low-grade inflammation. Hence, we cannot exclude that, for example, tests of endothelial vasomotor function or of other circulating indicators of low-grade inflammation would show different results. However, the tests we used have proven relevance in their respective contexts and are widely used for study purposes similar to ours.

In conclusion, in an elderly population with high-normal homocysteine levels, the use of vitamin B12 and folic acid did not influence endothelial function or inflammation.

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Chapter **5**

General discussion

With advancing age, the predictive value of risk factors for cardiovascular disease change [1]. Classical cardiovascular risk factors included in the Framingham risk score are less predictive at older age. For older persons, an alternative of a risk score is absent. It has been observed that arterial stiffness is an important cardiovascular risk factor in older populations [2-6]. This thesis underlines the importance of arterial stiffness in older individuals, as being an important preclinical marker of cardiovascular disease. Although arterial stiffness has already been associated with many conditions, these associations may well differ in elderly, in particular because of comorbidity and potential modification or mediation.

For this thesis, we studied the possible associations between arterial stiffness and physical function, osteoporosis and homocysteine, using cross-sectional or longitudinal data of the B-PROOF study population. In addition, we researched the effect of 2-year vitamin B12 and folic acid supplementation on arterial stiffness and other cardiovascular-related outcomes within the B-PROOF study. In this chapter, we will give an overview of the observations of this thesis and discuss its implications, and subsequently we will provide suggestions for potential further research.

THE B-PROOF STUDY

The B-PROOF study (B-vitamins for the PRevention Of Osteoporotic Fractures) is a large, multicenter, double-blind, placebo-controlled randomized controlled trial. Individuals with a homocysteine level ≥ 12 – 50 μmol/l could be included. Three centers in the Netherlands conducted this trial: Wageningen University, VU Medical Center at Amsterdam and the Erasmus MC at Rotterdam. We have contacted about 69,000 older individuals aged 65 years and over, to interest them for our trial. Of them, about 10% gave consent and wanted to participate. Blood samples were collected from these participants. About half of them, matched our inclusion criteria and could be included. In total 2919 individuals started with the trial (**Figure 1**, flowdiagram). These participants all underwent the baseline measurements at of close to their home. These baseline measurements included among others: weight and length, blood pressure, physical function tests, cognitive tests and several questionnaires [7].

At the VU Medical Center and Erasmus MC, participants were invited to underwent vascular function tests, all non-invasive. In 567 participants the vascular function tests were performed.

The intervention of the B-PROOF study was vitamin B12 (500 μg) and folic acid (400 μg) once a day. Both the intervention and control group received 15 μg vitamin D daily. After the two-years of follow-up, the homocysteine level decreased with 4.4 μmol/l in the intervention group [8]. Within the vascular subgroup, the homocysteine level decreased with 4.3 μmol/l in the participants included in the intervention arm [8].
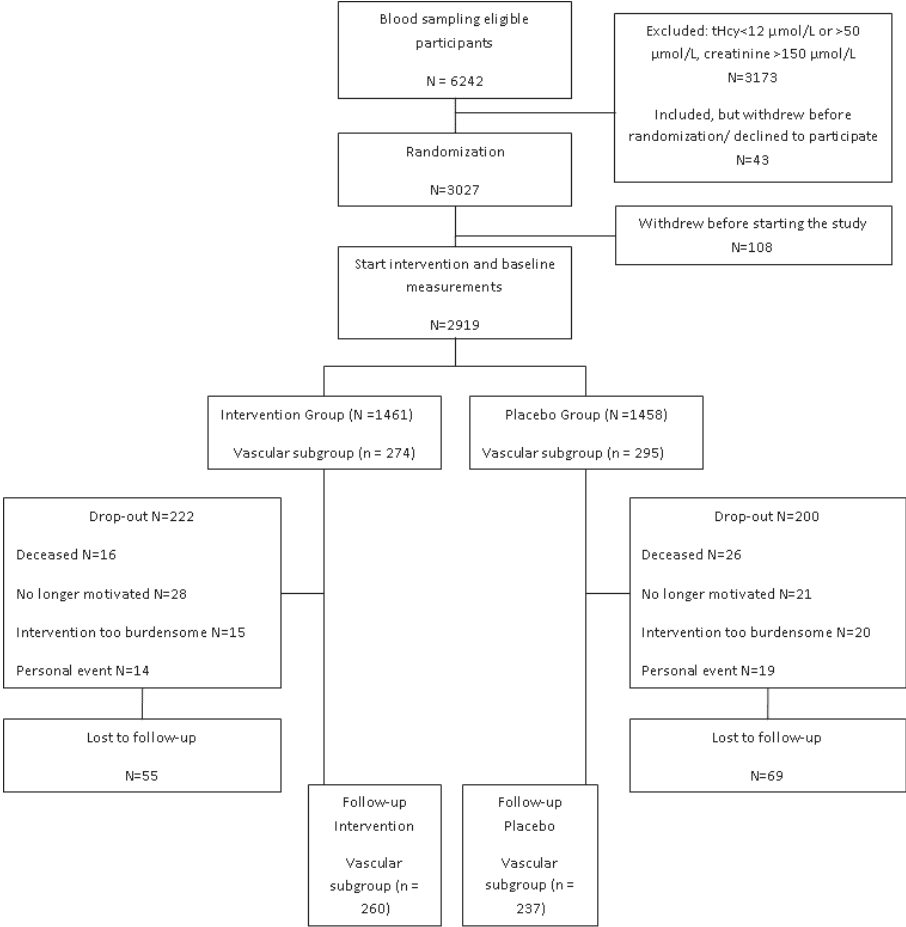
Figuur 5.1.
Flowchart of the inclusion of the B-PROOF study.

MEASURING ARTERIAL STIFFNESS

Because it was not clear whether the applanation tonometry and oscillometry are comparable in terms of arterial stiffness measures, we compared the arterial stiffness measurements between applanation tonometry and the oscillometric method (Chapter 2). As compared to younger individuals, elderly often have increased levels of arterial stiffness being a marker for increased cardiovascular disease risk [2-6]. Therefore this age group was of particular interest, mainly because an accurate measurement of arterial stiffness can be helpful for cardiovascular risk management. We observed that the applanation tonometry and the oscillometric method were not very comparable in PWV levels, in particular not in older individuals with elevated levels of arterial stiffness. Although we did not investigate whether the oscillometric method was capable of predicting cardiovascular risk, we think it might be argued whether the oscillometric method can be used for clinical applications as cardiovascular risk assessment, especially in elderly who are at risk of elevated arterial stiffness levels and therefore higher cardiovascular disease risk. More research will be necessary to ascertain whether the oscillometric method is able to predict cardiovascular disease and mortality. For example, within a longitudinal cohort study as the Rotterdam Study [9], the accuracy of this method for cardiovascular risk prediction could be investigated.

It is also important that researchers are aware that potentially the applanation tonometry and the oscillometric method do not measure similar properties of the vascular tree. It has been reported the oscillometric is sensitive for brachial stiffness [10, 11], because this method measures the PWV in the brachial artery and with use of the central pressure, the central PWV is estimated [12]. This is especially important when measuring arterial stiffness in elderly, since it is thought that the brachial artery is less affected by age-related arterial stiffness [13]. Nevertheless, the applanation tonometry also works with estimations of the central PWV. It therefore remains uncertain whether the applanation tonometry and the oscillometric method measure similar vascular properties. The advice to other researchers is therefore to use the golden standard, applanation tonometry, at least until there are clearer data available with regard to the prediction of cardiovascular risk.

DETERMINANTS OF ARTERIAL STIFFNESS

Arterial stiffness is an abnormality of the vasculature, which co-occurs with advancing age. It has been shown to be an important cardiovascular risk predictor, especially at older age [2-6]. Therefore, arterial stiffness is an interesting phenotype to investigate, mainly to create better insight in prevention of cardiovascular disease. Especially in younger populations, multiple conditions such as diabetes and renal disease have been linked to arterial stiffness [14-20]. Because data are still scarce for older individuals, we have investigated several associations with arterial stiffness in elderly. Elderly have often been excluded in studies, because of among others the presence of co-morbidity, use of medication or cognitive disabilities [21]. Therefore, with older subjects it is more difficult to interpret the results and to evaluate effectiveness. In this perspective, evidence of the beneficial effects of drug treatment or other kind of interventions like physical activity is scarce. However, especially older individuals have a high prevalence of cardiovascular disease, with high health care costs as a consequence. Because with advancing age, the risk determinants of cardiovascular diseases change [1], it is important to get knowledge about other treatable cardiovascular risk factors for this age group in order to create better prevention strategies.

Several associations with arterial stiffness have been described in Chapter 3. All associations have been investigated within the context of the B-PROOF study [8]. Although these participants had mildly elevated homocysteine levels (≥12 µmol/l), this condition has a prevalence of 50%, as in the B-PROOF study half of the elderly could be included. Furthermore, it is worth noticing that in our Dutch population we have no fortification with folate in our foods [7].

Cross-sectional studies cannot prove causality between the association under investigation. Nevertheless, these studies are of importance because they provide information about potential mechanisms, leading to new hypotheses, which in turn can be tested with, for example, a randomized controlled trial, or a genome-wide association study. One of the potential mechanisms that is extensively researched in this thesis is the interaction of the presence of elevated arterial stiffness with the variable of interest. Because arterial stiffness is thought to be an abnormality that is not easily or rapidly reversible, it is plausible there is a difference in associations and effects of treatment between individuals with low levels of arterial stiffness and those with elevated levels.

In order to investigate the underlying mechanisms in which way physical fitness and exercise reduce cardiovascular disease risk, arterial stiffness has already been investigated extensively. Nevertheless, all studies focused on exercise with a short-term follow-up and were mostly assessed within young or middle-aged populations [16, 22-26]. Therefore, we aimed to investigate the associations between physical functioning longitudinally within the B-PROOF study with elderly subjects (Chapter 3). We observed that physical functioning was not related to arterial stiffness in our population and there was no difference between participants with and without elevated arterial stiffness. Our findings indicate that possibly in elderly, physical functioning is not a determinant of arterial stiffness, in contrast to younger individuals [22, 23]. Nevertheless, further research is warranted in the form of an intervention trial to determine whether long-term physical exercise has beneficial effects on arterial stiffness or cardiovascular disease prevention in older persons.

Because both osteoporosis and cardiovascular disease are common among elderly, and an association between both diseases appeared to be present [27-31], we have also investigated the association of bone parameters and fractures with arterial stiffness (Chapter 3). However, we observed no association between these parameters. Recently, other studies became available that question causality between osteoporosis and cardiovascular disease [32, 33], so our study supports the absence of a causal link between bone and vasculature in older individuals.

It is however still possible that there is a common pathway for these diseases that differs between certain populations, explaining a lack of association in certain populations as compared to others. One of the hypothesized overlapping etiologies is homocysteine level [34-37]. That is why we researched the role of homocysteine in this perspective, but we were not able to confirm an overlapping effect of homocysteine. With use of prospective studies in older persons, potential associations between bone disease, fractures and cardiovascular disease may be further determined. Furthermore, genetic risk scores of bone parameters can be used to see whether these genetic markers will be able to predict cardiovascular disease. This would also provide more information regarding causality or overlapping etiology. Potential common pathways between these conditions might be vitamin D levels, calcium levels or maybe even sodium. Sodium is already positively associated with cardiovascular disease, via hypertension, but more recently, studies noticed an association between sodium levels and bone mineral density [38, 39].

Individuals with higher salt intake, have higher blood pressure levels and therefore may have an increased calcium excretion in the urine, which would lower the serum calcium levels and as a consequence this could lead to an increased risk of osteoporosis.

Several studies have demonstrated that individuals with low levels of vitamin D have a higher incidence and prevalence of cardiovascular disease [40-45]. On the other hand, also high vitamin D levels have been suggested to increase cardiovascular risk, potentially due to calcium depositions [46]. Taken these findings together, they point towards a non-linear relation. Our study (Chapter 3) confirmed a non-linear association between vitamin D and arterial stiffness, as well as for arteriosclerosis. In particular normal-high levels of vitamin D were associated with increased arteriosclerosis of the carotid artery. This finding suggests that also normal-high levels of vitamin D may increase cardiovascular risk via increasing arteriosclerosis. This can be of particular interest in the geriatric population, where supplementation with vitamin D is world-wide acknowledged for the prevention of osteoporosis. In the Netherlands, despite the 25-hydroxy vitamin D level, it is recommended for older individuals to use small amounts of vitamin D supplementation additional to daily food consumption. However, if indeed vitamin D levels in the normal-high range already are associated with an increased cardio-vascular risk, one might argue about potential negative consequences of the supplementation recommendations. To get more certainty about these potential disadvantages of vitamin D, and the optimal supplementation dosage, a large prospective study, preferably an intervention trial (regardless of the possible ethics) with different dosing schemes is warranted. With regard to possible ethical problems, it is also possible to do a meta-analysis of all trials that have been using vitamin D supplementation as an intervention and have adverse events reports, including cardiovascular events. It might be more difficult to determine a optimal dosage for supplementation in this way, but it will give us more knowledge about the potential harmful effects of vitamin D supplementation.

HOMOCYSTEINE

Homocysteine forms a key element in this thesis. All variables of interest also have been shown to be associated with homocysteine: physical function [47], measures of osteoporosis [48], and most importantly, its association

with cardiovascular disease. In particular regarding the relation with arterial stiffness, we hypothesized that the association would be predominantly present in a hyperhomocysteinemic population, since this has been shown to form an important cardiovascular risk factor especially in an older age group. As mentioned in the introduction, homocysteine level is an independent risk factor for cardiovascular disease, which is particularly strong in the very old and an even better predictor of cardiovascular disease in comparison to the classical risk factors included in the Framingham score [49]. However, up to now, data were lacking regarding arterial stiffness at older age. Our first cross-sectional analysis, conducted within the framework of the B-PROOF study, was the association between homocysteine level and arterial stiffness (Chapter 4). We observed a positive association between homocysteine level and PWV in elderly, which was strongest in the oldest old. With each point increase of homocysteine level, the PWV increased with 8%. It is plausible that with advancing age, homocysteine becomes a more important cardiovascular risk, as was endorsed by the observed strong association among the oldest old.

However, the question remained whether this observed association between homocysteine and arterial stiffness is truly a causal one. In order to explore potential causality, we investigated the relation between genetic polymorphisms of hyperhomocysteinemia with arterial stiffness and the association between serum vitamin B12 and folate with arterial stiffness (Chapter 4). The negative findings however undermined this hypothesis. In fact, homocysteine levels were significantly mediating the association, indicating that the association with homocysteine and arterial stiffness is not likely to be a causal one.

Moreover, one might even speculate about potential protective mechanisms individuals with a genetically high risk of hyperhomocysteinemia have on the arterial stiffness process. In vitro studies have clearly demonstrated a direct effect of homocysteine on the vasculature. It increases thrombogenecity, oxidative stress and activates redox-sensitive inflammatory pathways [50]. It is possible that individuals with a genetic high risk of having hyperhomocysteinemia do have other genetic factors that compensate the effects of homocysteine or potentially have an activation of processes protecting the vascular wall. Of course this is rather speculative. Because epigenetic research is upcoming, we may expect to get more insights in the processes of genetic risk factors and their true effects in the human body. A more direct way to assess causality are intervention trials, in this case with B-vitamin

supplementation. In this thesis the intervention was also reported and will be discussed in the next subchapter of this discussion.

Next to causality, there was also the possibility that the association between homocysteine and arterial stiffness is driven by B-vitamins itself. Vitamin B12 and folate are co-factors in the re-methylation pathway, converting homo-cysteine into methionine. Methionine is converted to s-adenosylmethionine (SAM), which is a universal methyl-group donor, not only for proteins but also for DNA and RNA. Over the last years, this process has gained interest, including the possible effects on this process with advancing age, since, for several reasons, in older persons B-vitamin deficiencies are common. Within the B-PROOF study, we observed homocysteine mediated the association between B-vitamins and arterial stiffness, making it unlikely that the as-sociation between homocysteine and arterial stiffness is driven by a direct effect of B-vitamin levels. As mentioned before, homocysteine itself has been shown to be an independent risk indicator for many disorders, including cardiovascular disease. High levels of homocysteine could have a direct toxic/negative effect, or negative effects could arise because of less avail-able SAM, leading to hypomethylation. What underlying mechanism explains the association between hyperhomocysteinemia and arterial stiffness and cardiovascular disease is not known. It will be interesting to also measure SAM and S-adenosylhomocysteine (SAH; the break-down product of SAM) within the sera of the individuals of the B-PROOF study, to see whether SAM/SAH ratio drives the association between homocysteine and arterial stiffness in our population.

EFFECT OF B-VITAMINS

Despite the fact that homocysteine has been shown to be an independent risk factor for cardiovascular events [49], several trials with homocysteine-lowering therapy with B-vitamins failed to demonstrate beneficial effects [51]. Obviously, causality of homocysteine on the incidence cardiovascular disease was questioned. However, it has also been brought forward that there are potential epidemiological or methodological issues that may have influenced the outcomes of these trials [52]. Another possible explanation for the lack of benefit of homocysteine-lowering therapy could be arterial stiffness. Because arterial stiffness is an abnormality that is not easily or rapidly reversible, lack of substantial improvement of arterial stiffness might

be the explanation of the non-beneficial effects of B-vitamin supplementation on cardiovascular outcomes. Therefore, we aimed to investigate the effect of vitamin B12 and folic acid supplementation on arterial stiffness (Chapter 4). In concordance with the second hypothesis, we did not observe any treatment effect on arterial stiffness levels; in fact the PWV levels were the same in both the intervention and the placebo group. Furthermore, the results did not differ between participants with and without elevated levels of arterial stiffness at baseline. This last result may imply that homocysteine is not causally associated with arterial stiffness, which was endorsed by the fact that we also did not find an association between genetic polymorphisms of hyperhomocysteinemia and arterial stiffness as we already discussed above.

On the other hand, we did find a beneficial effect on stroke in females. Within the intervention group, we have observed a risk reduction of 67% in the females treated with B-vitamin supplementation. Although we did not power our B-PROOF population size on cardiovascular outcomes, this finding is consistent with literature [53, 54]. Cerebrovascular disease is more heterogeneous as compared to coronary artery disease. It is possible that not only arterial stiffness does not respond to B-vitamin supplementation, but also atherosclerosis does not. We confirmed the latter by showing there was no effect of the intervention with vitamin B12 and folic acid on carotid intima-media thickness. Furthermore, also age did not appear to influence the effects of the intervention. Therefore, we may conclude there is another pathway which should explain the potential beneficial effect of B-vitamins on stroke. Because we only observed this in females, it might be hormonal. However, in literature a gender-specific effect has not yet been described [51, 53, 54]. Another, rather unexpected, finding were the higher blood pressure levels in the individuals treated with vitamin B12 and folic acid, with an approximately 2 mmHg higher level in the intervention group. With the potential beneficial effect of B-vitamins on cerebrovascular disease and the potential harmful effect of B-vitamins on blood pressure, the suggestion arises there are paradoxical effects of B-vitamin supplementation. This has also been described previously [52]. In this perspective our results regarding blood pressure might be a reflection of a cardiac mechanism or of a difference in effect of homocysteine-lowering therapy between small and large arteries. The homocysteine-lowering effect of B-vitamin supplementation would then be partly beneficial, but possibly the treatment as such could lead concurrently lead to adverse effects. Because folate is a fuel source for cells like macrophages and smooth muscle cells, via the one carbon metabolism,

this may lead to increased proliferation of these cells, which are important in the atherosclerotic process [52]. However, only one small study have investigated whether folic acid supplementation does increase the (inflammatory) processes in atherosclerotic plaques [55].

Nevertheless, in the context of the paradoxical hypothesis of B-vitamin effects, we investigated the effect of vitamin B12 and folic acid supplementation on the inflammation status. The paradox hypothesis comprehends the possibility that B-vitamins reduce cardiovascular disease on the one hand, but increase the inflammation status on the other. However, we observed no effect (Chapter 4).

We also investigated the effect on endothelial function, but also B-vitamin supplementation did not improve endothelial function in our population.

We therefore conclude that B-vitamin supplementation may have a beneficial effect on stroke, but no effect on cardiovascular outcomes. Furthermore, regarding potential pathways, B-vitamins did not improve arterial stiffness, endothelial function or the inflammatory status. Why homocysteine is still such a strong indicator for cardiovascular disease in the elderly is therefore remains unknown. Most likely it is not a treatable risk factor, but only a biomarker, able to predict cardiovascular events and mortality. Why stroke and homocysteine appear to have a different etiology has also not yet been elucidated. It is possible that blood pressure may have a role in this perspective, since we observed higher blood pressure levels in individuals treated with B-vitamin supplementation as compared to the individuals with placebo. Because of the heterogeneity of cerebrovascular disease, there is the possibility that effects of B-vitamin supplementation is different between the small and large arteries. In order to elucidate the mechanisms of homocysteine and B-vitamin supplementation further, we may need to focus on the smaller arteries. A potential study with cerebral perfusion scans would be of interest to demonstrate the effects of B-vitamins on the small arteries in the brain.

The lack of beneficial effects of B-vitamin supplementation on cardiovascular disease, also in other trials, also has been explained by the fact that most studies were done in folic-acid fortified populations [52]. The B-PROOF study was conducted in the Netherlands, were we do not have folate-fortification. Another limitation that has been mentioned many times is the relatively short time-period (3-5 years) in which the studies were conducted [52]. It is true that the B-PROOF study had a duration of only 2 years. As cardiovas-

cular disease was a secondary outcome, and the study was designed for the prevention of fractures, it was hypothesized that a period of 2 years would be enough to detect differences in fracture incidence. However, with respect to cardiovascular pathophysiology, it would be expected that differences in the vasculature for the prevention of disease will take longer than 3-5 years. In particular if arterial stiffness is indeed a condition which may be non-rapidly reversible. With other drug treatments, for example statins [56], we were lucky to demonstrate their beneficial effects on a shorter term, but this may not be true for other drug targets as homocysteine-lowering therapy. A larger trial of more than 5 years would therefore be necessary to ascertain effects of B-vitamin supplementation on cardiovascular outcomes. However, more and more discussion arises about the potential harmful effects of folate supplementation; since higher cancer incidences are described. Although a large meta-analyses could not confirm this [51], we may question whether a new trial is ethical.

IMPLICATIONS

This thesis helped us to get more insight into the determinants of arterial stiffness in older individuals. Although arterial stiffness is an important cardiovascular risk predictor, there are some conditions that according to literature seemed to be associated with arterial stiffness, but of which data were scarce, especially in elderly. Therefore we have studied those associations within our elderly, B-PROOF population. We observed that arterial stiffness was not associated with physical functioning and bone parameters in our older population. Furthermore, vitamin D levels appears to associate in a U-shaped curve with carotid intima-media thickness, an important marker for atherosclerosis. Therefore, we must conclude that research is still needed to find other (treatable) cardiovascular risk factors for older individuals in order to decrease mortality and morbidity rates.

Whether our B-PROOF population can be seen as a general elderly population, might be questioned. We have included (based on homocysteine level) approximately 50% of a general elderly population. Therefore, one might say this is a quite well representation of the general population. However, our inclusion is based on mildly elevated homocysteine levels. This can be an indication of certain underlying conditions, as increased cardiovascular risk, but maybe also other, not yet identified conditions which are associated with

hyperhomocysteinemia. Therefore, it is important to interpret the results with caution. We cannot be sure the results are present in the total general population of older individuals.

Furthermore, our studies have improved the understanding in the patho-physiological mechanisms of homocysteine and B-vitamin supplementation regarding cardiovascular outcomes. Although we observed a clear association between homocysteine and arterial stiffness, in particular in the oldest old, we did not find any effects of B-vitamin supplementation on cardiovascular disease, arterial stiffness, endothelial function or inflammation. This lack of benefit in all outcome variables may indicate that homocysteine is indeed not causally associated to cardiovascular outcomes. This hypothesis was also confirmed by a lack of an association with genetic polymorphisms of hyperhomocysteinemia, making this hypothesis even more likely. Why we did see an effect of B-vitamin supplementation on stroke remains unknown. As described before, it is still possible there is a difference in B-vitamin effect between small and large arteries. Nevertheless, considering the sometimes equivocal results of meta-analysis on stroke and the fact that homocysteine-lowering therapy did also not have a large influence on other health outcomes [8], and it might increase the risk of cancer [8], B-vitamin supplementation cannot be seen as a true treatment option for the prevention of stroke. And in the end, as we observed no large treatment effects in any of the outcomes, we can also not suggest to use B-vitamin supplementation for the total elderly population in the Netherlands.

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Chapter **6**

Summary - Samenvatting
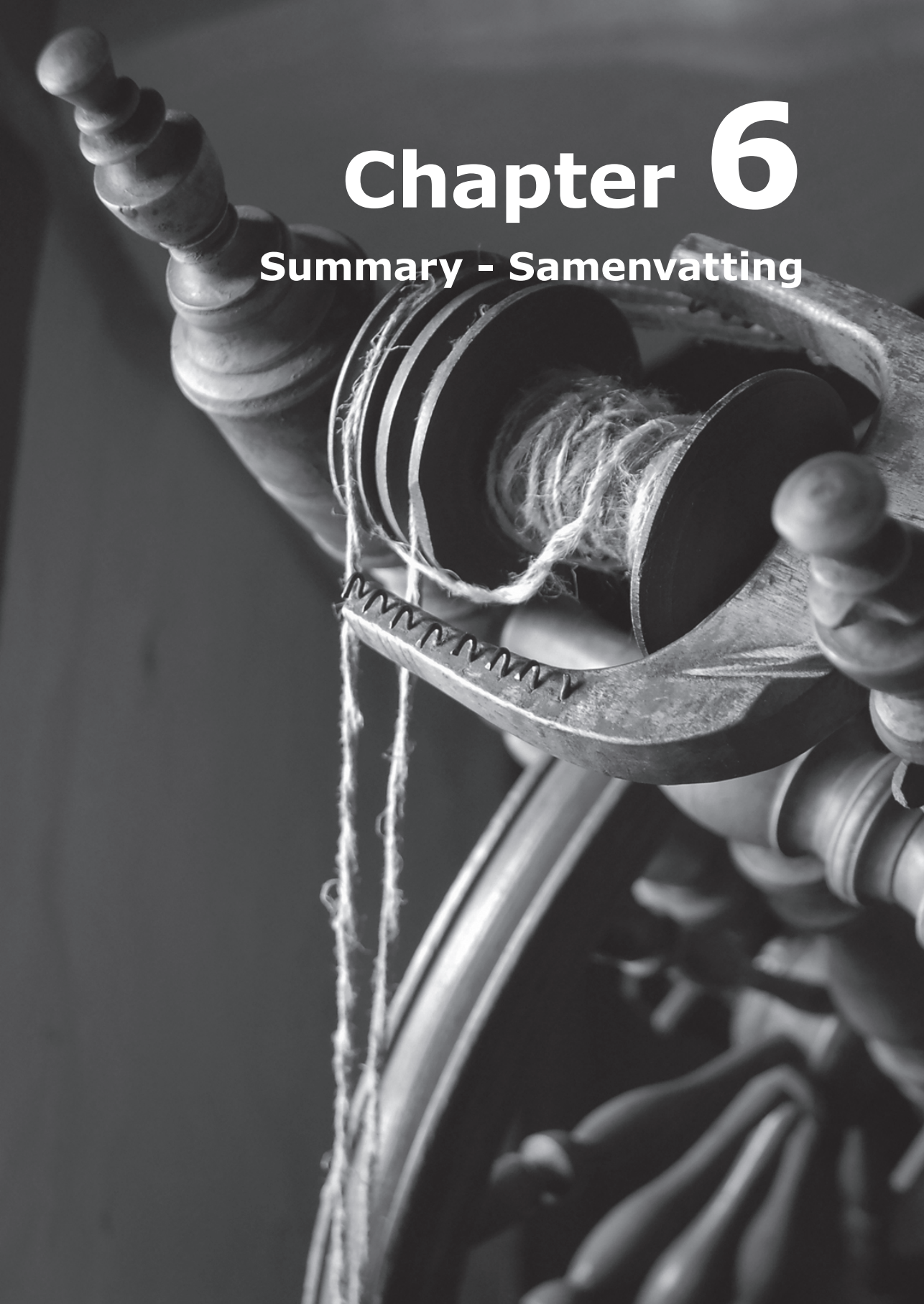
6.1

Summary

In Chapter 1, we describe two cardiovascular risk factors important for an elderly population. Because classical cardiovascular risk factors are less predictive with advancing age, other risk factors become more important. One of the cardiovascular risk factors is pulse wave velocity as measurement of arterial stiffness. Arterial stiffness is a condition, characterized by less elasticity of the arteries and considered a pre-clinical state of cardiovascular disease. In Chapter 1 we explain the etiology of arterial stiffness and its measurements. Also, we introduce another risk factor of cardiovascular disease, homocysteine. However, several trials with homocysteine lowering B-vitamin supplementation as intervention did not observe beneficial effects on the prevention of cardiovascular disease. Numerous hypotheses have been formed to explain the lack of a beneficial effect. One of them is arterial stiffness. Arterial stiffness is a condition that is not easily or rapidly reversible. If an increased homocysteine level would lead to arterial stiffness, this process would be largely irreversible.

In Chapter 1, we also describe the population we have used for this thesis. All subjects are participants of the B-PROOF (B-vitamins for the PRevention Of Osteoporotic Fractures) study, an RCT with 2919 participants, aged 65 years and over, based on their homocysteine level ($\geq$ 12-50 µmol/l).

In Chapter 2 we compared two different methods of assessing PWV within the B-PROOF population. We observed that with oscillometry, the PWV levels in individuals with increased arterial stiffness are not comparable with those of the applanation tonometry. Because applanation tonometry is well validated and has been shown to be associated with cardiovascular risk prediction, we have chosen to use the applanation tonometry based PWV levels to use for the rest of this thesis.

In Chapter 3, we studied potential associations of arterial stiffness with physical function, osteoporosis and vitamin D. Despite the fact that it has been shown that physical function has an influence on arterial stiffness levels in young adults, no effect of physical function or activity was seen in our elderly population (Chapter 3.1). However, many participants in our study population had increased arterial stiffness and it is probable that arterial stiffness is indeed irreversible. However, stratified analysis in individuals without arterial stiffness did also not demonstrate an association between physical function and activity and arterial stiffness.

In Chapter 3.2, we describe the association between arterial stiffness and bone parameters: bone mineral density, bone quality and fractures. Literature demonstrated that osteoporosis and fractures predict cardiovascular events. Homocysteine is a possible underlying common mechanism, since homocysteine is associated with both osteoporosis as well as cardiovascular disease. Within the B-PROOF study however, we did not observe an association between bone parameters and arterial stiffness. Furthermore, homocysteine did not modify or mediate this association and is therefore not plausible to be interacting with this association.

The association between arterial stiffness and vitamin D is described in Chapter 3.3. This relation appears not to be linear but U-shaped. Both a low as well as a high level of 25-OH vitamin D is associated with increased arterial stiffness and in particular with increased intima media thickness, a measure of atherosclerosis. In individuals with a 25-OH vitamin D level ≥ 50 nmol/l, there was a significant increase of the intima-media thickness of 1.24 µm per point of 25-OH vitamin D increase. This finding implies that also normal-high levels of vitamin D may also lead to an increased cardiovascular disease risk.

In Chapter 4 we investigate the relation between homocysteine, arterial stiffness and cardiovascular disease. Firstly in Chapter 4.1, we describe the association between homocysteine and arterial stiffness. This relation is strongest in the oldest old. Nevertheless, such an association study cannot ascertain causality. The relation between homocysteine and arterial stiffness could be driven by B-vitamin levels itself for example. In Chapter 4.2 we therefore tested the association between B-vitamin levels and arterial stiffness, but found no association. With the use of a genetic risk score for hyperhomocysteinemia, we investigated potential causality between homocysteine and arterial stiffness. However, the genetic risk score of hyperhomocysteinemia was not associated with arterial stiffness and was even modified by homocysteine level itself, thus making a causal relationship less likely.

In Chapter 4.3 we assessed effects of B-vitamin supplementation on cardiovascular outcome measures. B-vitamin supplementation did indeed lower levels of homocysteine, but did not decrease arterial stiffness. Furthermore, a stratified analysis of individuals with and without increased arterial stiffness did not show an effect of the B-vitamin supplementation within the individu-

als without arterial stiffness. Therefore we may conclude that homocysteine is not causally associated with arterial stiffness. However, we also observed in Chapter 4.3 that within females receiving the intervention with B-vitamins, less strokes occurred during the two year follow-up. It is not known why we only observed this in females, although, in the entire population there was also a trend towards lower incidence of stroke in the intervention group with B-vitamins. It can be speculated there is a difference between small and large arteries, but further research is warranted.

In order to get more insight in potential underlying pathways in the association between homocysteine and cardiovascular disease, we also investigated the effect of B-vitamin supplementation on endothelial function and inflammation in Chapter 4.4. It has already been described that folic acid is associated with an increased inflammatory status, which might neutralize the potential protective effects of homocysteine-lowering therapy. However, we did not observe any treatment effect on endothelial function or inflammation.

In Chapter 5, we elaborate on the implications of our findings. In conclusion, physical function and osteoporosis were not related to arterial stiffness. Secondly, normal-high levels of vitamin D are potentially associated with increased cardiovascular risk. Thirdly, the association between homocysteine and arterial stiffness is present in elderly, but is most likely not causal. Furthermore, homocysteine can be seen as a biomarker, but not as a treatable risk factor.

6.2

Nederlandse samenvatting

Hart- en vaatziekten zijn veelvoorkomende aandoeningen, met name in ouderen. Dit brengt veel comorbiditeit en gezondheidskosten met zich mee. Omdat traditionele risicofactoren een mindere voorspellende waarde hebben met het toenemen van de leeftijd, is het belangrijk dat er nieuwe risicofactoren worden geïdentificeerd. In hoofdstuk 1 geven we een inleiding over twee factoren die het onderwerp van dit proefschrift zijn.

Een van die factoren is vaatstijfheid. In hoofdstuk 1 vertellen we dat dit een goede voorspeller blijkt te zijn voor hart- en vaatziekten in ouderen. Vaatstijfheid houdt een mindere elasticiteit van de bloedvaten in, wat een voorloper van hart- en vaatziekten blijkt te zijn. Dit wordt vaak gemeten met de zogenaamde: 'pulse-wave-velocity', oftewel de snelheid waarmee een polsgolf van de arteria carotis naar de arteria femoralis beweegt. Aangezien vaatstijfheid een preklinische fase is van hart- en vaatziekten, is het goed om te onderzoeken of dit ook geassocieerd is met diverse aandoeningen of biomarkers, zodat vaatstijfheid mogelijk al vroeg kan worden voorkomen.

De andere risicofactor, die in hoofdstuk 1 wordt besproken, is het homocysteine gehalte. Homocysteine wordt in het lichaam omgezet in methionine en is een aminozuur dat van belang is bij diverse metabole processen in het lichaam. Voor deze omzetting is vitamine B12 en foliumzuur nodig. Een tekort aan deze vitamines kan dus leiden tot een verhoogd homocysteine gehalte. Er zijn vele aandoeningen die geassocieerd lijken te zijn met een verhoogd homocysteine, waaronder dus hart- en vaatziekten.

In hoofdstuk 1 bespreken we ook de reden van dit proefschrift. Het is gebleken dat diverse studies met suppletie van vitamine B12 en foliumzuur, welke het homocysteine dus verlagen, niet konden aantonen dat er hierdoor hart- en vaatziekten voorkomen konden worden. Er zijn diverse hypotheses in omloop over de reden hiervoor. Een ervan is vaatstijfheid. Van vaatstijfheid wordt gedacht dat dit niet snel, of zelfs niet, reversibel is. Wanneer een verhoogd homocysteine zou leiden tot vaatstijfheid, zou dit dus niet meer omkeerbaar kunnen zijn. Aangezien de B-PROOF studie (B-vitamins for the PRevention Of Osteoporotic Fractures) werd opgezet, om te onderzoeken of suppletie van vitamine B12 en foliumzuur osteoporotische fracturen konden voorkomen, was dit een mooie gelegenheid om ook naar de effecten op vaatstijfheid en hart- en vaatziekten te kijken. Deze dubbel-blinde, gerandomiseerde en placebo gecontroleerde trial heeft van 2008 tot 2012, 2919 65+-ers geincludeerd, op basis van hun homocysteine gehalte. Dit moest

≥ 12 - 50 μmol/L zijn. Door personen te selecteren met een iets verhoogd homocysteine gehalte, konden we een verlaging van homocysteine bewerk-stelligen middels suppletie van vitamine B12 (500μg) en foliumzuur (400μg). De proefpersonen werden gerandomiseerd in de interventie groep (suppletie B-vitamines) of in de placebo groep en werden 2 jaar behandeld.

In hoofdstuk 2 onderzochten we twee manieren om de pulse-wave-velocity te meten in ouderen. Dit kan met een tonometer: deze methode maakt gebruik van de drukverschillen van de polsgolf, te voelen bij de arterieen van het lichaam: de carotis- en femoralis arterie. De andere methode maakt gebruik van een oscillometer: een bloeddrukband, welke de druk in de radialis arterie meet en daarmee een inschatting maakt van de carotis-femoralis pulse wave velocity. In hoofdstuk 2 vergelijken we deze metingen in onze B-PROOF onderzoekspopulatie. We vonden dat de oscillometer minder goed hogere PWV-waardes kon bepalen ten opzichte van de tonometer. Mede aangezien de tonometer beter gevalideerd is en tevens is gebleken dat deze waarden hart- en vaatziekten kunnen voorspellen, hebben wij bij de B-PROOF geko-zen om onze PWV metingen uit te voeren met de tonometer.

In hoofdstuk 3 onderzoeken we diverse associaties van vaatstijfheid met: fysiek functioneren, osteoporose en vitamine D. Ondanks dat in jong volwas-senen is beschreven dat fysieke functie van invloed is op de vaatstijfheid, lijkt dit bij ouderen niet het geval (hoofdstuk 3.1). We hebben bekeken of de fysieke functie en de dagelijkse activiteiten van invloed waren op vaatstijfheid en op vaatstijfheid na 2 jaar, echter dit bleek niet het geval. Aangezien veel van onze populatie reeds vaatstijfheid heeft, zou het mogelijk zijn dat vaat-stijfheid inderdaad irreversibel is. Echter, wanneer we onderscheid maakten tussen proefpersonen met en zonder vaatstijfheid, vonden we ook in de proefpersonen die nog geen vaatstijfheid hadden, geen associatie met fysiek functioneren en dagelijkse activiteit. Het is mogelijk dat alleen intensievere activiteit van invloed is op vaatstijfheid bij ouderen, echter dit konden we binnen de B-PROOF niet onderzoeken.

In hoofdstuk 3.2 beschrijven we het onderzoek naar de associatie tussen vaatstijfheid en bot parameters: botdichtheid en fracturen. Er zijn diverse onderzoeken die beschrijven dat osteoporose en fracturen van voorspellende waarde zijn voor het ontstaan van hart- en vaatziekten. Homocysteine is een van de mogelijk onderliggende mechanismen aangezien het zowel geas-socieerd is met hart- en vaatziekten en vaatstijfheid, als met osteoporose en

fracturen. Er bleek binnen de B-PROOF studie echter geen relatie te zijn tussen vaatstijfheid en bot parameters, ook niet met fracturen. Homocysteine bleek ook geen invloed te hebben op deze associatie en is dus waarschijnlijk geen onderdeel van een mogelijk onderliggend mechanisme.

De associatie tussen vaatstijfheid en vitamine D wordt beschreven in hoofdstuk 3.3. Deze relatie blijkt een soort U-shape te hebben en is niet lineair. Zowel een laag als een hoog vitamine D gehalte blijkt dus gerelateerd met een verhoogde vaatstijfheid, maar met name met een verhoogde intima-media dikte van de arteria carotis (IMT), een maat voor atherosclerose. Vanaf een 25-OH vitamine D gehalte van ≥ 50 nmol/l bleek er een significante toename van de IMT te zijn (beta 1.24 μm) per punt stijging van 25-OH vitamine D. Dit zou kunnen impliceren dat ook normaal-hoge waarden van vitamine D een verhoogd risico kunnen geven op hart- en vaatziekten.

Vanaf hoofdstuk 4 onderzoeken we de relatie homocysteine, B-vitamines en vaatstijfheid. Allereerst hebben we in hoofdstuk 4.1 de associatie tussen homocysteine gehalte en vaatstijfheid beschreven. Deze relatie blijkt het sterkst te zijn in de oudste ouderen. Zoals al eerder werd beschreven in de literatuur, dat homocysteine met name in oudste ouderen een goede voorspeller is voor hart- en vaatziekten, ondersteunen we dit met onze studie door de associatie met vaatstijfheid aan te tonen. Echter een associatie studie zegt nog niets over causaliteit. De gevonden relatie homocysteine – vaatstijfheid zou bijvoorbeeld ook gedreven kunnen worden door vitamine B12 en foliumzuur gehaltes. Hierom hebben we in het volgende hoofdstuk 4.2 beschreven of er een relatie is tussen deze vitamines en vaatstijfheid, welke er niet bleek te zijn. Middels een genetische risicoscore voor een verhoogd homocysteine konden we onderzoeken of er aanwijzingen waren voor een causaal verband tussen homocysteine en vaatstijfheid. Echter, er bleek geen verband te zijn tussen de genetische variaties die homocysteine verklaarden en vaatstijfheid. Mogelijk heeft het lichaam een soort protectiemechanisme ontwikkeld, waardoor de schadelijke effecten van een genetisch verhoogd homocysteine teniet worden gedaan. Temeer omdat we vonden dat het homocysteine gehalte zelf de onderzochte associatie modificeerde.

Uiteraard is de uitkomst van het B-PROOF onderzoek een nog betere manier om te zien of homocysteine causaal gerelateerd is aan vaatstijfheid (hoofdstuk 4.3). De suppletie van vitamine B12 en foliumzuur leidde inderdaad tot lagere homocysteine waarden, maar niet tot een vermindering van de vaat-

stijfheid. Dit was in lijn met onze hypothese vooraf: wanneer vaatstijfheid inderdaad een onomkeerbaar proces is, verwacht je geen effect meer van een interventie. Echter, er bleek ook geen effect van de B-vitamine suppletie te zijn in de proefpersonen die vooraf geen vaatstijfheid hadden. Dit maakt dan toch aannemelijk dat het homocysteine gehalte geen causaal verband heeft met vaatstijfheid en hart- en vaatziekten.

Wel hebben we in hoofdstuk 4.3 ook gevonden dat er in vrouwen die B-vitamine suppletie kregen een lagere incidentie was van beroertes. Waarom we dit alleen in vrouwen vonden is niet bekend, al was er gedurende de 2 jaar follow-up in de gehele groep ook een trend naar een lagere incidentie van beroertes in de groep die behandeld werd met B-vitamines. We kunnen speculeren over verschillen in effect van B-vitamine suppletie op kleine en grote arteriën, wat uiteraard nog verder onderzoek nodig heeft.

Om nog meer inzicht te krijgen in het verband tussen homocysteine en hart- en vaatziekten, hebben we in hoofdstuk 4.4 onderzocht wat het effect van de B-vitamine supplementen is op de endotheelfunctie en inflammatie. Er is eerder beschreven dat het zou kunnen dat B-vitamines al werken op het endotheel en dat B-vitamines mogelijk een verhoogde inflammatoire status kunnen induceren, wat de mogelijke positieve effecten van B-vitamines op het voorkomen van hart- en vaatziekten zou kunnen neutraliseren. We hebben echter geen effect van de B-vitamines gevonden op zowel de endotheel-functie als op inflammatie, gemeten met biomarkers.

In hoofdstuk 5 bediscussiëren we onze resultaten. We kunnen uit dit proefschrift concluderen dat vaatstijfheid inderdaad veelvoorkomend is bij ouderen, maar dat de verbanden beschreven in jongere populaties niet altijd overeenkomen met die in ouderen. Zo hebben fysieke functie en osteoporose geen relatie met vaatstijfheid. Van vitamine D echter, lijken normaal-hoge waardes ook een verhoogd risico te geven op hart- en vaatziekten. Wat betreft de relatie homocysteine en vaatstijfheid, hebben we sterke aanwijzingen dat dit verband niet causaal is. Homocysteine kan gezien worden als een goede voorspellende biomarker, maar is geen behandelbare risicofactor.

Chapter **7**

Dankwoord
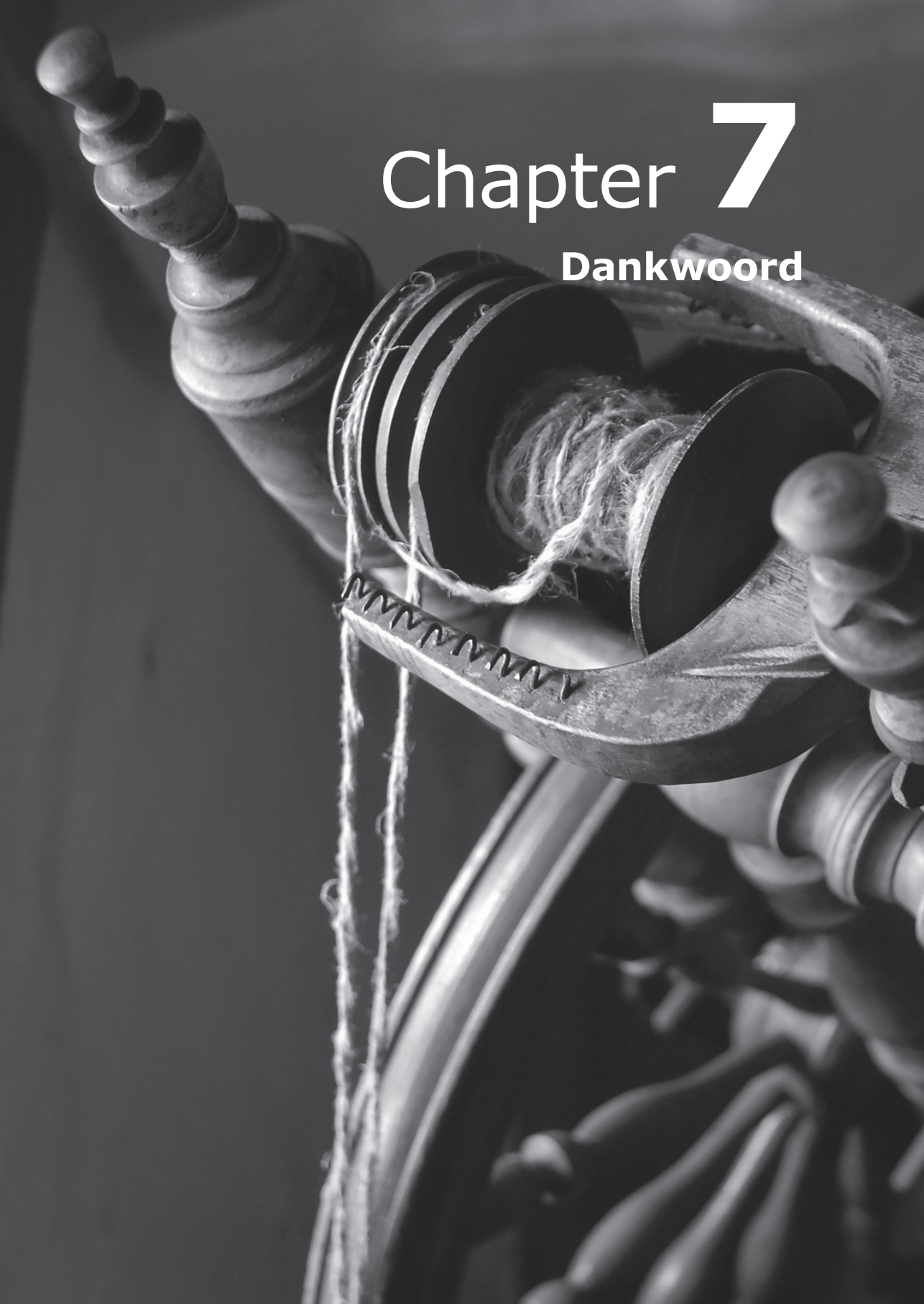
Na al die jaren B-PROOF is het resultaat dan eindelijk daar: mijn proefschrift! En dan te bedenken dat ik nooit wilde promoveren, omdat dat zoveel werk zou zijn.. Nu, veel werk was het zeker, maar ik heb het met veel plezier gedaan en kan terugkijken op een aantal bijzondere jaren. Ik heb ontzettend veel geleerd, op zowel werk als persoonlijk vlak en ben flink wat ervaringen rijker! Uiteraard had ik het niet kunnen doen zonder alle mensen die op een of andere manier betrokken waren met het B-PROOF onderzoek of met mij en zij verdienen dan ook allen mijn hartelijke dank!

Allereerst verdienen de deelnemers aan het B-PROOF onderzoek natuurlijk alle lof, want zonder hen was dit boekje überhaupt niet mogelijk geweest!

Beste proffessor Uitterlinden, beste André, heel erg bedankt voor de wetenschappelijke ruimte en de nodige zetjes voor mijn wetenschappelijke ontwikkeling. Al ligt mijn onderwerp wellicht wat ver af van het dagelijkse werk op jouw afdeling, het lukte je toch altijd om weer te zorgen voor een nieuwe kijk op mijn onderzoek en manuscripten. Dit zorgde dan ook voor de nodige verbeteringen in kwaliteit!

Beste Nathalie, als mijn dagelijkse begeleider en co-promotor hebben we heel wat uurtjes samen doorgebracht. Heel erg bedankt voor de mogelijkheid die jij en Tischa mij in 2009 hebben gegund, om het cardiovasculaire deel van B-PROOF op me te mogen nemen. Ondanks mijn minimale onderzoekservaring heb je me de ruimte gegeven om te groeien in de wereld van onderzoek, waarvoor ik je erg dankbaar ben! Naast het beoordelen van mijn analyses en manuscripten was er ook tijd voor brainstormen, waardoor we op nog meer leuke onderzoeksideeën zijn gekomen en ik hoop dan ook dat we B-PROOF hierna nog kunnen doorzetten!

Francesco, als mijn opleider en tweede co-promotor kan ik je natuurlijk niet vergeten. Grazie mille! Ondanks dat je zelf een zware periode hebt moeten doormaken, stond je toch voor me klaar, waar ik alleen maar bewondering voor kan hebben. Bedankt voor je kritische blik op mijn analyses en manuscripten en de kennismaking met je netwerk, met name in de wereld van arterial stiffness. Gelukkig voor jou ben je nog niet van me af, want nu gaan we door met de opleiding tot klinisch geriater! Ik verheug me nu al op de komende jaren.

Beste Ton, wat gaf jouw bijdrage altijd een enorme verbetering aan mijn onderzoeken! Ik wil je heel erg bedanken voor de kritische noot en je vermogen om alles in een helder licht te zetten en de dingen vooral niet moeilijker te maken dan ze zijn. Waar ik met name wel eens geremd moest worden in het speculeren, kon je me weer met beide benen op de grond zetten waar ik je erg dankbaar voor ben!

Uiteraard had mijn hele onderzoek niet mogelijk geweest zonder het bestaan van het B-PROOF onderzoek en daarvoor wil ik de grondleggers dan ook enorm bedanken. Waar met name Lisette en Rosalie in Wageningen de leidende rol hadden, gaven jullie andere centra toch ook de ruimte voor eigen inbreng en hebben we een prachtig onderzoek neergezet met onze drie centra! Paul en Natasja natuurlijk ook heel erg bedankt voor de fijne samenwerking in de afgelopen jaren! Janneke, als eerste B-PROOF-er had jij de eer je boekje af te hebben. Al duurde het misschien wat langer dan je had gehoopt, het is toch een mooi resultaat van het harde werken aan B-PROOF! Bedankt voor de gezellige vergaderingen en overleggen. Karin, heel erg bedankt voor de leuke jaren, je gezelligheid en altijd nuttige en nuchtere inbreng! Voor jou is ook het einde bijna in zicht! Nikita en Elske, onze samenwerking was altijd erg prettig en fijn te zien dat jullie inmiddels ook jullie verdediging goed hebben afgerond!

Uiteraard heb ik ook veel gehad aan de vasculaire commissie binnen B-PROOF. Yvo, Henk, Edith, Marianne en Renate heel erg bedankt voor jullie tijd en input waarmee we toch een aantal mooie manuscripten hebben kunnen schrijven! Yvo, jij verdient ook een persoonlijke noot aangezien je veel hebt bijgedragen aan de inhoud van mijn stukken en vaak veel tijd nam om te brainstormen over hoe het beter kon. Erg fijn dat je ook zitting hebt willen nemen in mijn promotie commissie. Ik heb veel van je kunnen leren, waarvoor heel erg bedankt!

Ja, en dan zijn daar de vrouwen met wie ik de eer heb gehad dagelijks te mogen werken! Sandra, die ik natuurlijk niet ons B-PROOF moedertje mag noemen, maar dat stiekem toch doe.. Ik heb superfijn met je samen mogen werken de afgelopen jaren en ik denk dat we allemaal, mede door jou, persoonlijk heel erg zijn gegroeid. Je hebt altijd een luisterend oor en als het even minder ging, was jij er om even mee te klagen, om vervolgens weer opbeurend te zijn. Met je praktische kijk en inlevingsvermogen hebben we B-PROOF Rotterdam enorm kunnen laten groeien. Als ik ooit nog een

research nurse nodig heb, dan weet ik je te vinden! We hebben uiteraard ook veel gezelligheid gehad, zoals op de Italiaanse congressen en tijdens onze dinertjes en ik zal me deze tijd nog lang heugen!

Anke, wat heeft het eigenlijk goed uitgepakt we beiden zijn aangenomen op het B-PROOF project Rotterdam! Jouw nuchterheid en doordachtzaam-heid maken het prettig samenwerken en daaraan hebben we waarschijnlijk ook veel deelnemers te danken! Al gingen de afgelopen jaren ook wel eens minder makkelijk, uiteindelijk hebben we het toch maar mooi voor elkaar gekregen. Nu kunnen we het na zoveel jaar bijna tegelijk afsluiten en ik hoop dat je in je volgende baan de uitdaging kunt vinden die je zoekt en natuurlijk heel veel geluk met je gezin!

Annelies, in je eerste jaar zag je mij af en toe in witte jas voorbij vliegen, maar toen ik eenmaal weer terug was konden we het gelijk goed vinden! Al moest je je mindfullness soms even terugvinden, was je open karakter ook erg fijn want iedereen weet meteen wat ze aan je hebben. Bedankt voor de gezelligheid en al onze uurtjes waarin we konden 'sparren'. Ik wens je nog veel succes met de afronding van je eigen onderzoek en weet zeker dat het je gaat lukken!

Sadaf, eigenlijk hebben we maar een relatief korte tijd mogen samenwer-ken. Het was erg leuk om je snel te zien groeien in het onderzoek en hoe meer plezier je eraan beleefde. Ik kan soms met verbazing aanzien hoe jij alles weer voor elkaar krijgt, en bewonder je om je doorzettingsvermogen. Heel veel succes in je verdere carriere, en wie weet kan ik daar ook nog een bijdrage aan leveren!

Een ander die ik wel moet noemen in mijn boekje is natuurlijk Mirjam, want zonder haar stond ik hier nu niet. In de bus naar Zierikzee wees jij mij op een advertentie in het Medisch Contact, wat 'helemaal iets voor mij was'. En zo kwam het dat ik ineens in een AGIKO-constructie zat! Vanaf het eerste jaar geneeskunde mag ik genieten van je gezelligheid, heel erg bedankt daarvoor! Evelyne en Linda, in de huidige drukte zien we elkaar gelukkig nog steeds af en toe. Dus Evelyne, Linda en Mirjam: tot snel!

Ook wil ik mijn andere vrienden en familie bedanken voor hun vriendschap en hun steun. Ik zal mijn best doen genoeg tijd voor jullie te maken nu ik weer in de kliniek zit! Een aantal van hen wil ik speciaal bedanken. Marjolein, zussie, wat fijn eigenlijk dat ik jou gevonden heb en hopelijk volgen er nog vele jaren met uitjes, sporten en feestjes! Ik bewonder je om hoe je in het leven staat en wens je alle geluk en voorspoed! Loek, ik ben er trots

op dat jij mijn paranimf wil zijn en ik hoop dat dit je met name inspireert, want je kunt veel meer dan je denkt! Als het goed is kunnen we samen een belangrijke periode afsluiten en ik ben zeker trots op ons!

Natuurlijk mogen de belangrijkste mensen in mijn leven niet vergeten worden. Pap en mam bedankt wat jullie altijd voor mij hebben gedaan en het mogelijk gemaakt hebben dat ik kon studeren wat ik wilde. Wie had dat toch gedacht dat dat kleinzerige meisje een dokter zou worden?! Ik zal het toch van iemand moeten hebben.. George, bedankt dat je er altijd voor me bent en in me gelooft. Ik verheug me op onze toekomst samen om uiteindelijk te eindigen als grijze geriatrische oudjes! Zoals George Sand ooit al opschreef: "Er is maar één geluk in het leven: iemand liefhebben en die liefde beantwoord zien."

<div align="right">Suzanne</div>

Chapter **8**

About the author
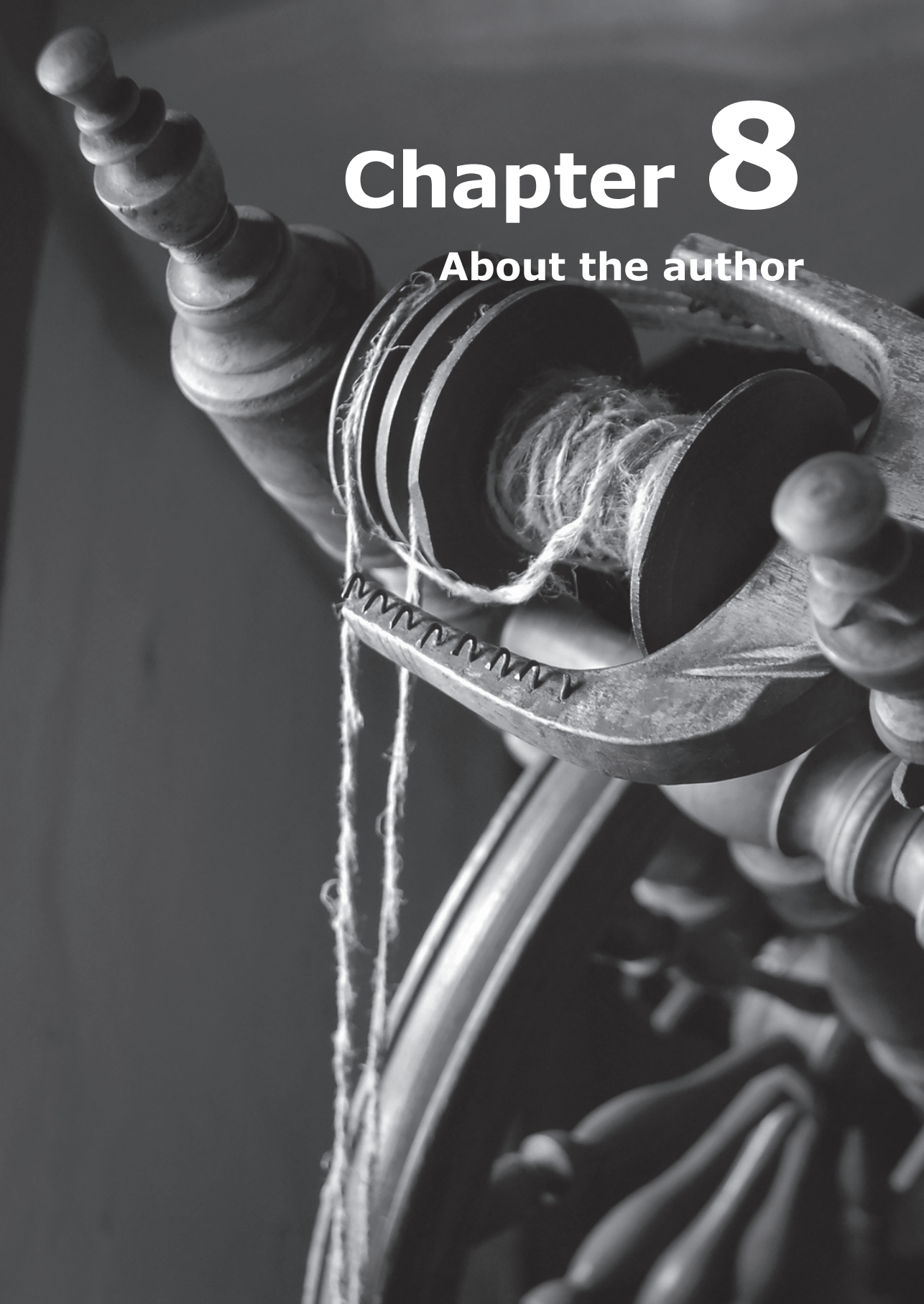
8.1

Curriculum vitae

De auteur van dit proefschrift, Suzanne C. van Dijk, is geboren op 9 februari 1985 in Schiedam. Na het behalen van het VWO diploma in 2003, aan het Maascollege te Maassluis, is zij geneeskunde gaan studeren aan de Erasmus Universiteit in Rotterdam. Gedurende haar studie is zij lang werkzaam geweest in verpleeghuis DrieMaasHave te Maassluis, waar haar voorkeur voor ouderengeneeskunde ontstond. Een keuze-onderzoek in het kader van de opleiding geneeskunde in 2007 aan de afdeling Inwendige Geneeskunde, over de effecten van serotonine op botstofwisseling, zorgde voor een groeiende interesse in wetenschap. Haar arts-examen behaalde zij cum laude in 2009, mede door een oudste co-schap in het Vlietland ziekenhuis op de afdeling Klinische Geriatrie, wat de liefde voor ouderengeneeskunde alleen maar meer aan wakkerde.

Na haar afstuderen in 2009 is zij dan ook gestart met promotieonderzoek bij het B-PROOF onderzoek bij de afdeling geriatrie in het Erasmus MC. Hier heeft zij het cardiovasculaire gedeelte van de studie opgebouwd en geholpen bij de inclusie van zoveel mogelijk deelnemers. In 2011 is zij ook gestart met de opleiding Klinische Geriatrie, waarbij zij als AIOS in dat jaar haar promotieonderzoek heeft gecombineerd. In 2012 is zij fulltime teruggekeerd naar het B-PROOF onderzoek om haar promotie af te ronden.

Per 1 januari 2014 is zij gestart met de vooropleiding interne geneeskunde in het kader van de opleiding Klinische Geriatrie. Zij hoopt in 2018 daadwerkelijk klinisch geriater te zijn en klinische werkzaamheden te combineren met wetenschap.

8.2

Portfolio

Name PhD student: S.C. van Dijk
Erasmus MC Department: Internal Medicine
Research School: MolMed

PhD period: September 2009 – 2014
Promotor(s): A.G. Uitterlinden
Supervisor: N. van der Velde

1. PhD training

	Year	Workload (Hours/ECTS)
General courses		
Biomedical English Writing and Communication	2012	4.0
BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2012	1.0
Specific courses (e.g. Research school, Medical Training)		
NIHES Winter Program (General Statistics and Regression analysis)	2010	3.0
NIHES Summer Program – Genetic Epidemiology	2012	1.2
NIHES Summer Program – Genome Wide Association Analysis	2012	2.0
SNP course 2012	2012	2.0
Seminars and workshops		
Symposium CPO 2009	2009	0.2
PhD Career Day (EPAR)	2012	0.2
Presentations		
Orals		
ARTERY 10, Verona	2010	
Wetenschapsdagen 2011, Antwerpen	2011	
Geriatriedagen 2011, 's Hertogenbosch (2x)	2011	
IAGG 2011, Bologna	2011	
EUGMS 2011 (N. van der Velde)	2011	
Wetenschapsmiddag Opleiding Geriatrie 2011, Breda	2011	
NCHA Outreach meeting 2012, Amersfoort	2012	
IAGG 2013, Seoul	2013	
ARTERY 14, Maastricht	2014	
Poster		
Wetenschapsdagen 2010	2010	
Geriatriedagen 2011, 's Hertogenbosch	2011	
ARTERY 11, Paris	2011	
Wetenschapsdagen 2012 (runner up poster prize)	2012	
MolMed day 2012	2012	
NCHA Outreach meeting 2012, Amersfoort	2012	
EUGMS 2013, Venice	2013	

(Inter)national conferences

CPO Autumn symposium	2009	2.0
Symposium Geriatrie Erasmus	2009	0.2
Wetenschapsdagen, Antwerpen	2010	1.0
Geriatriedagen, Rotterdam	2010	1.0
ARTERY 10, Verona	2010	2.0
Geriatriedagen, 's Hertogenbosch	2011	1.0
Wetenschapsdagen, Antwerpen	2011	1.0
International IAGG 2011, Bologna	2011	3.0
ARTERY 11, Paris	2011	2.0
Wetenschapsdagen, Antwerpen	2012	1.0
Geriatriedagen, 's Hertogenbosch	2012	1.0
MolMed Day 2012, Rotterdam	2012	0.2
NCHA Outreach meeting 2012, Amersfoort	2012	0.5
Osteoporoseles 2012, Baarn	2012	0.2
NCHA Healthy Ageing Congress	2013	0.3
International conference IAGG 2013, Seoul	2013	3.0
EUGMS 2013, Venice	2013	2.0
Valsymposium december 2013	2013	0.2
ARTERY 14, Maastricht	2014	0.2

Other

Organizing committee EUGMS 2014 Rotterdam	2012-14	

2. Teaching

Lecturing

Invited lecture for COEUR 02-03-2012	2012	0.5
Invited lecture for COEUR 20-06-2014	2014	0.5

Supervising practicals and excursions, Tutoring

Master Internal Medicine - Geriatrics	2011	1.0
Master Internal Medicine – Geriatrics	2012	1.0

Supervising Master's theses

Supervising Niels Deenen	2009-10	2.0
Supervising Tjitske van der Vaart	2011	2.0
Supervising Riekske van Zwienen	2012	2.0
Supervising Mehmed Halilovic	2012	2.0
Supervising Colin Bogaard	2012	2.0
Supervising Samantha Jordaans	2012-13	2.0

Other
Awards

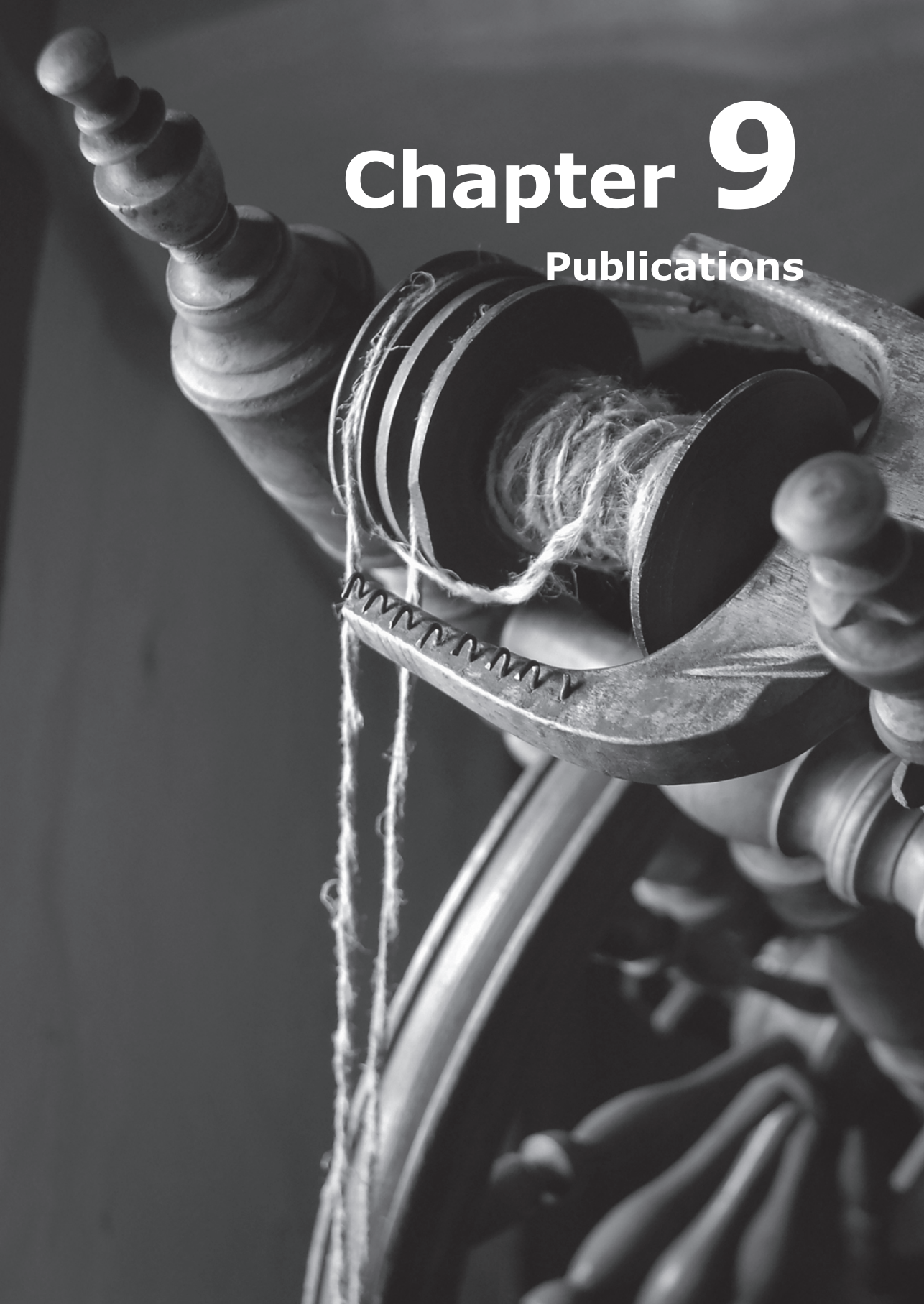|	
Runner up Poster Prize Wetenschapsdagen 2012	2012
Runner up Best Presentation Young Investigator; ARTERY 14	2014

Foundation

Nutricia Research Foundation Cardiovascular biomarkers BPROOF 25k.	2012

Chapter **9**

Publications

Publications based on the studies described in this thesis

van Dijk SC, Smulders YM, Enneman AW, Swart KMA, van Wijngaarden JP, Ham AC, van Schoor NM, Dhonukshe-Rutten RAM, de Groot LCPGM, Lips P, Uitterlinden AG, Blom HJ, Geleijnse JM, Feskens EJ, van den Meiracker AH, Mattace Raso FUS, van der Velde N. Homocysteine level is associated with aortic stiffness in elderly: cross-sectional results from the B-PROOF study. J Hypertens 2013 May;31(5):952-9.

van Dijk SC, Enneman AW, Swart KMA, van Schoor NM, Uitterlinden AG, Smulders YM, van den Meiracker AH, van der Velde N, Mattace Raso FUS. Oscillometry and applanation tonometry measurements in older individuals with elevated levels of arterial stiffness. Blood Press Monit 2013 Dec; 18(6):332-8.

van Dijk SC, Enneman AW, van Meurs J, Swart KMA, Ham AC, van Wijngaarden JP, Brouwer-Brolsma EM, van der Zwaluw NL, van Schoor NM, Dhonukshe-Rutten RAM, de Groot LCPGM, Lips P, Uitterlinden AG, Blom HJ, Geleijnse JM, Feskens EJ, de Jongh RT, Smulders YM, van den Meiracker AH, Mattace Raso FUS, van der Velde N. B-vitamin levels and genetics of hyperhomocysteinemia are not associated with arterial stiffness. Nutr, Metab Cardiovasc Dis 2014; 24: 760-766.

van Dijk SC, Sohl E, Oudshoorn C, Enneman AW, Ham AC, Swart KMA, van Wijngaarden JP, Brouwer-Brolsma EM, van der Zwaluw NL, Uitterlinden AG, de Groot LCPGM, Dhonukshe-Rutten RAM, Lips P, van Schoor NM, Blom HJ, Geleijnse JM, Feskens EJ, Smulders YM, Zillikens MC, de Jongh RT, van den Meiracker AH, Mattace Raso FUS, van der Velde N. Non-linear associations between serum 25-OH vitamin D and indices of arterial stiffness and arteriosclerosis in an older population. Age Ageing 2014, in press

van Dijk SC, Swart KMA, Ham AC, Enneman AW, van Wijngaarden JP, Feskens EJ, Geleijnse JM, de Jongh RT, Blom HJ, Dhonukshe-Rutten RAM, de Groot LCPGM, van Schoor NM, Lips P, Uitterlinden AG, Mattace Raso FUS, Smulders YM, van den Meiracker AH, van der Velde N. Physical fitness, activity and hand-grip strength are not associated wit arterial stiffness in older individuals. J Nutr Health Aging 2014, in press.

Other publications

van Wijngaarden JP, Dhonukshe-Rutten RA, van Schoor NM, van der Velde N, Swart KM, Enneman AW, **van Dijk SC**, Brouwer-Brolsma EM, Zillikens MC, van Meurs JB, Brug J, Uitterlinden AG, Lips P, de Groot LC. Rationale and design of the B-PROOF study, a randomized controlled trial on the effect of supplemental intake of vitamin B12 and folic acid on fracture incidence. BMC Geriatrics 2011, Dec 2; 11-80.

van Dijk SC, de Herder WW, Kwekkeboom DJ, Zillikens MC, Feelders RA, van Schaik RH, van Driel M, van Leeuwen JP. 5-HIAA excretion is not associated with bone metabolism in carcinoid syndrome patients. Bone 2012 Jun;50(6): 1260-5.

van Dijk S, van den Meiracker A, van der Cammen T, Mattace Raso F, van der Velde N. Central but not peripheral fat mass percentage is associated with blood pressure components in the elderly. Age Ageing 2012, Jul 41(4):534-40.

Swart KM, Enneman AW, van Wijngaarden JP, **van Dijk SC**, Brouwer-Brolsma EM, Ham AC, Dhonukshe-Rutten RA, van der Velde N, Brug J, van Meurs JB, de Groot LC, Uitterlinden AG, Lips P, van Schoor NM. Homocysteine and the methylenetetrahydrofolate reductase 677C->T polymorphism in relation to muscle mass and strength, physical performance and postural sway. Eur J Clin Nutr 2013 Jul;67(7):743-8.

A.W. Enneman, K.M. Swart, M.C. Zillikens, **S.C. van Dijk**, J.P. van Wijn-gaarden, E.M. Brouwer-Brolsma, R.A. Dhonukshe-Rutten, A. Hofman, F. Rivadeneira, T.J. van der Cammen, P. Lips, C.P. de Groot, A.G. Uitterlinden, J.B. van Meurs, N.M. van Schoor, N. van der Velde. The association between plasma homocysteine levels and bone quality and bone mineral density parameters in older persons. Bone 2014; Mar; 12(63c): 141-146.

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