The influence of selected biological, lifestyle risk factors and fitness on plasma Hcy and ADMA concentrations and vascular function: The AGAHL-Study

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Innovation through diversity

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

DEDICATION

TO MY PARENTS,

JOHAN & CHARLOTTE HERBST

(MOTHER DECEASED 22/09/'02)

For he will command his angels concerning you to guard you in all your ways (Ps. 91:11)

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AUTHOR'S CONTRIBUTIONS

The study reported in this thesis was planned and executed by a team of researchers. The contribution of each of the researchers is depicted in the table hereafter. Also included in this section is a statement from the co-authors confirming their individual roles in the study and giving their permission that the articles may be part of this thesis.

NAME	ROLE IN THE STUDY
Ms. S.J. Herbst – Spies B.Sc., Hons. (Biokineticist) (South Africa)	Responsible for the execution of the total thesis, some of the data collection, -management and statistical analyses. Main author of the thesis.
Dr. S.J. Moss (Ph.D.) (Biokineticist) (South Africa)	Project co-ordinator and scientist; responsible for all aspects of the study. Significant contribution towards writing of the thesis. Promotor of S.J. Herbst – Spies at the NWU.
Prof. J.W.R. Twisk (Ph.D.) (The Netherlands)	Promotor of S.J. Herbst – Spies in The Netherlands. A significant contribution toward the statistical analyses and writing of the thesis.
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Dr. Y Smulders (Ph.D.) (The Netherlands)	Co-author of Chapter 3. Contribution towards general content of research project, and in writing on article 1.

I declare that I have approved the above mentioned articles and that my role in the study as indicated above is representative of my actual contribution and that I hereby give my consent that it may be published as part of the Ph.D. thesis of S.J. Herbst.

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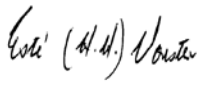

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SUMMARY

The prevalence of cardiovascular disease (CVD) increases with age. It is thus important to study the relationship that might exist between various cardiovascular risk factors within longitudinal studies. Homocysteine (Hcy) and asymmetric dimethylarginine (ADMA) are two of these newly identified risk factors for CVD. They need to be studied in order to understand the underlying mechanisms involved in endothelial function, inflammation and arterial properties.

The objective of this study was to investigate the relationship between (changes in) concentrations of homocysteine (Hcy), asymmetric dimethylarginine (ADMA), fitness, fatness, markers of endothelial function and inflammation, and arterial properties of healthy adults from the Amsterdam Growth and Health Longitudinal Study (AGAHLS).

The AGAHLS is a suitable study to investigate the interactions between cardiovascular risk factors such as Hcy, ADMA, fitness, fatness, markers of endothelial function and inflammation, and arterial properties. The AGAHLS started in 1977 with a group of 13-year-old subjects. They were measured repeatedly over time, of which only the last two measurements (2000 at the age 36 and 2006 at the age of 42) were used in this study.

The following variables were measured:

- Fitness [direct and indirect VO_2max, physical activity (kMETS.min/wk)];
- fatness (trunk fat mass, peripheral fat mass, peripheral lean mass);
- markers of endothelial function [intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), endothelial selectine (E-selectine), plasma selectine (P-selectine), thrombomoduline, Von Willebrand factor (vWf)];
- inflammation markers [C-reactive protein (CRP), serum amyloid A (SAA), tumor necrosis factor α (TNF- α), interleukin-6 (IL-6) and interleukin-8 (IL-8)]; and
- arterial properties [carotid artery intima-media thickness (IMT), carotid artery compliance coefficient (CA CC), carotid artery distensibility coefficient (CA DC),

femoral artery compliance coefficient (FA CC), femoral artery distensibility coefficient (FA DC) and Young's elastic modulus (YEM)].

The relationships between the variables, namely the concentrations of Hcy, ADMA, fitness, fatness, markers of endothelial function and inflammation, and arterial properties were performed with generalized estimating equations (GEE) analyses. Linear regression analyses were applied to analyse the relationships between changes in concentrations of Hcy and ADMA, and changes in fitness, fatness, markers of endothelial function and inflammation, and arterial properties.

Results of the GEE analyses indicated that 1 ml/min/kg higher VO_2max fitness was significantly related to a -0.81 nmol/L lower plasma Hcy concentration (95% confidence interval [-1.53 – -0.09] p=0.03). However, this relationship was attenuated after adjustment for smoking behaviour.

Significant relationships were found between plasma ADMA concentrations and endothelial markers ICAM (B=71.67 [5.01 – 138.33] p=0.04) and thrombomoduline (B=0.89 [0.09 – 1.68] p=0.029). A significant inverse relationship was seen between changes in plasma ADMA concentrations and changes in vWf (B= -42.39 [-82.81 – -1.98] p=0.04).

In conclusion, this study has demonstrated that a significant longitudinal relationship exists between plasma ADMA concentrations and endothelial markers (i.e. ICAM, thrombomoduline). Furthermore, an inverse significant relationship has been found between changes in ADMA concentrations and changes in the vWf.

It is recommended that future investigations include an older population and diverse ethnic groups.

OPSOMMING

Die voorkoms van kardiovaskulêre siektes (KVS) neem toe met toename in ouderdom wêreldwyd. Dus neem die behoefte aan longitudinale studies om die verband te bestudeer wat tussen verskeie kardiovaskulêre risikofaktore bestaan ook toe. Hcy en ADMA is twee van die onlangs geïdentifiseerde merkers wat die risiko om KVS te ontwikkel, beïnvloed. Dit is dus belangrik om die merkers te bestudeer om sodoende die onderliggende meganismes wat betrokke is by endotele funksie, inflammasie en arteriale eienskappe te bepaal.

Die doel van die studie was om die verwantskap tussen Hcy- en ADMA-konsentrasies, fiksheid, vetheid, arteriële eienskappe, asook endotele funksie- en inflammasiemerkers van gesonde volwassenes in die *Amsterdam Growth and Health Longitudinal Study* (AGAHLS) te bepaal.

Die AGAHLS is ’n baie goeie studie waarbinne die verwantskappe tussen kardiovaskulêre risikofaktore ondersoek kon word. Dit sluit die volgende in: Hcy- en ADMA-konsentrasies, fiksheid, vetheid, arteriële eienskappe en endotele disfunksies. Die AGAHLS het in 1977 begin met ’n groep 13-jarige deelnemers. Die deelnemers is herhaaldelik oor ’n tydperk gemeet, en die laaste twee metings word in die studie gebruik (2000 op die ouderdom van 36 en in 2006 op die ouderdom van 42).

In die studie is die volgende veranderlikes gemeet:

- Fiksheid (direkte en indirekte VO_2max);
- fisieke aktiwiteit (kMETS.min/wk);
- vetheid (rompvetmassa, perivere vetmassa, perivere maermassa);
- merkers vir endotele funksie (intersellulêre hegtingsmolekules (ICAM), vaskulêre hegtingsmolekules, (VCAM), endotele selektien (E-selektien), plasmaselektien (P-selektien), trombomodulien, Von Willebrand-faktor (vWf)
- inflammasiemerkers (C-reaktiewe proteïne (CRP), serum-amiloïde A (SAA), tumor-nekrosefaktor (TNF-α), interleukine-6 (IL-6), interleukine-8 (IL-8); en

- arteriële eienskappe (intima-mediadikte (IMD), carotis arterie-konstruksiekoëffisiënt (CA CC), carotis arterie-megewendheidkoëffisiënt (CA DC), femorale arterie-konstruksiekoëffisiënt (FA CC), femorale arterie-megewendheidkoëffisiënt (FA DC) en Young se elastiese modulus (YEM)]

Die verwantskappe tussen Hcy- en ADMA-konsentrasies, fiksheid, vetheid, arteriële eienskappe, asook endotele funksie- en inflammasiemerkers is met behulp van die veralgemeende skattingsvergelykings (VSV) (Engels: *generalized estimating equations*) bepaal. Liniêre regressie-analises is gebruik vir verdere bepaling van verwantskappe tussen die verandering in die Hcy- en ADMA-konsentrasies, fiksheid, vetheid, arteriële eienskappe, asook endotele funksie- en inflammasiemerkers.

Volgens die VSV-analise is ’n 1 ml/min/kg hoër VO_2max-fiksheidsvlak betekenisvol verwant aan -0.81 nmol/l laer plasma Hcy-konsentrasies (95% vertroue-interval [-1.53 – -0.09] p=0.03). Hierdie verwantskap het verlore gegaan nadat aanpassings gemaak is vir rookgewoontes. Volgens die VSV-resultate bestaan daar tog ’n betekenisvolle verwantskap tussen ADMA-konsentrasies en endotele merkers ICAM (β=71.67 [5.01 - 138.33] p=0.04) en trombomodulien (B=0.89 [0.09 - 1.68] p=0.03). ’n Betekenisvolle verband is gevind tussen verandering in ADMA-konsentrasies en verandering in die Von Willebrand-faktor (B= -42.39 [-82.81 - -1.98] p=0.04).

Die gevolgtrekking is dus dat daar ’n betekenisvolle longitudinale verband tussen ADMA-konsentrasies en endotele merkers (ICAM en trombomodulien) bestaan. Daar bestaan ook ’n betekenisvolle omgekeerde verband tussen die verandering in ADMA-konsentrasies en verandering in die Von Willebrand-faktor.

Dit word voorgestel dat toekomstige navorsing aspekte insluit soos ouer populasies en verskillende etniese groepe.

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LIST OF ABBREVIATIONS

A	ADMA	- Asymmetric dimethylarginine
	AGHALS	- Amsterdam Growth and Health Longitudinal Study
	AP	- Arterial Properties
B	BHMT	- Betaine-homocysteine methyltransferase
	BMI	- Body mass index
C	CA	- Carotid artery
	CAD	- Cardio arterial disease
	CBS	- Cystathionine B-synthase
	CC	- Compliance Coefficient
	CHD	- Coronary heart disease
	CI	- Confidence intervals
	CRP	- C-reactive protein
	CVD	- Cardio vascular disease
D	DC	- Distensibility Coefficient
	DD	- Diastolic Diameter
	DDAH	- Dimethylarginine dimethylaminohydrolase
	DXA	- Dual-energy x-ray absorbtiometry
	DM	- Diabetes Mellitus
	DMG	- Dimethylglycine

E	Einc	- Intrinsic-elastic properties
	eNOS	- Endothelial cell nitric oxide synthase
	ER	- Endoplasmic reticulum
F	FA	- Femoral artery
	Fat%	- Percentage body fat
G	GEE	- Generalized estimating equations
H	Hcy	- Homocysteine
	HDL-C	- High density lipoprotein cholesterol
	HHcy	- Hyperhomocysteinemia
I	ICAM	- Intercellular adhesion molecule
	IMT	- Intima-media thickness
	iNOS	- Inducible nitric oxide synthase
	SAA	- Serum amyloid A
	IL-6	- Interleukin-6
	IL-8	- Interleukin-8
K	Kg	- Kilogram
	kPa	- Kilopascal
L	LDL-C	- Low density lipoprotein cholesterol
	L-NMMA	- N-monomethyl-L-arginine
	Lp(a)	- Lipoprotein (a)

M	m	- meter
	MA	- Methylarginine
	Met	- Methionine
	MBP	- Mean blood pressure
	MS	- Methionine synthase
	MTHFR	- 10-methylene tetrahydrofolate reductase
N	n	- Sample size
	NO	- Nitric oxide
	NOS	- Nitric oxide synthase
	NF-$_k$B	- nuclear factor-kappa B
O	OxLDL	- Oxidized low density lipoprotein
P	PA	- Physical activity
	PRMT	- Protein arginine methyltransferase
S	SAA	- Serum amyloid A
	SAH	- S-adenosylhomocysteine
	SAM	- S-adenosylmethionine
	SD	- Standard deviation
	SDMA	- Symmetric dimethylarginine
	SPSS	- Statistical package of social sciences
T	THF	- Tetrahydrofolate
	TNFa	- Tumor recrosis factor a
V	VCAM	- Vascular cell adhesion molecule
	VO_2max	- Fitness (maximal oxygen uptake)
	vWF	- Von Willebrant factor

Y YEM - Young’s elastic modulus

1

INTRODUCTION

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1.1 INTRODUCTION

Cardiovascular disease (CVD) is regarded as one of the leading causes of mortality and morbidity worldwide (Ignarro *et al*., 2007:326). Although plenty of research have been conducted on traditional CVD risk factors (Stampfer *et al*., 1992:878; Welch & Loscalzo, 1998:1042; Ignarro *et al*., 2007:326 & Van Guldener *et al.,* 2007:1684), unfortunately the opposite is true for some recently identified risk factors and/or markers of CVD such as homocysteine (Hcy) and asymmetric dimethylarginine (ADMA).

Hcy is a topic previously more debated than ADMA. Hcy is an amino acid that is metabolised by one of two pathways; the remethylation and transsulfuration pathway (Stampfer *et al*., 1992:877). The suggested mechanism through which Hcy executes its deleterious effects in the body seems to be by means of endothelial dysfunction (Welch & Loscalzo, 1998:1042). The exact role Hcy plays in endothelial dysfunction and the causal pathways of atherosclerosis remains unclear. The link between Hcy and ADMA is based upon interconnections between their respective metabolic pathways (Van Guldener *et al.,* 2007:1683).

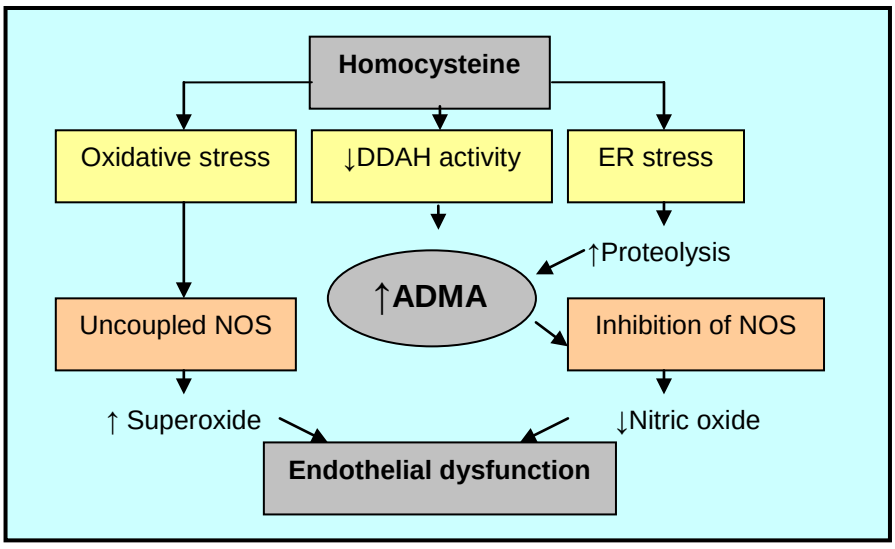

Figure 1: Biosynthesis and metabolism of ADMA (Dayal & Lentz, 2005:531).
(ADMA: asymmetric dimethylarginine; DDAH: Dimethylarginine dimethylamino-hydrolase; ER: Endoplasmic reticulum; NOS: Nitric oxide synthase).

ADMA is an endogenous molecule which is formed from the proteolysis of methylated proteins (Figure 1) (Dayal & Lentz, 2005:531). According to Vallance (2001:160) plasma Hcy concentrations and ADMA concentrations increase the risk of developing atherosclerotic diseases.

According to a number of studies, plasma ADMA concentrations correlate significantly to the severity of atherosclerosis (Song *et al.,* 2007:1530; Lu *et al.,* 2003:463). Attempts to reduce plasma and tissue ADMA concentrations could potentially play an important role in the treatment of endothelial dysfunction and atherosclerosis. The metabolism of ADMA may be affected by Hcy, as Hcy may directly or indirectly inhibit dimethylarginine dimethylaminohydrolase (DDAH) activity.

Hcy induces oxidative stress that leads to elevated ADMA concentrations (MacAllister *et al.,* 1996:1533). Subsequently, elevated ADMA concentrations induce endoplasmic reticulum (ER) stress, in turn leading to increased proteolysis of proteins (Dayal & Lentz, 2005:531). The accumulation of ADMA acts as an endogenous inhibitor of nitric oxide synthase (NOS) that influences relaxation of vascular smooth muscle (vasodilatation) (Vallance *et al.,* 1992:560).

Cardiovascular disease risk factors, Hcy and ADMA have received some attention recently (Vallance *et al.,* 1992:560; Eid *et al.* (2004:1578; Dayal & Lentz, 2005:531). Hcy and ADMA have also been linked to various diseases of CVD risk factors (i.e. lifestyle and biological), for instance renal failure, Type II diabetes mellitus, atherosclerosis and endothelial dysfunction. Keeping in mind that concentrations of Hcy and ADMA contribute to CVD, it is of utmost importance to health care practitioners to find the exact physiological mechanisms through which concentrations of Hcy and ADMA contribute to various diseases.

Existing literature investigating concentrations of Hcy and ADMA is extremely limited, focusing on some critical factors (i.e. biological mechanisms, cardiovascular disease) and yielding rather contradicting results. Two of the factors that need to be addressed in depth are fitness and fatness, and their relationship to concentrations of Hcy and ADMA. According to Eid *et al.* (2004:1578) a strong relationship exists between body mass index (BMI) and ADMA concentrations of elderly high-risk men. To my knowledge no research has illustrated a link between Hcy concentrations and BMI or body fatness. The question can therefore be asked, whether or not there is a relationship between concentrations of Hcy and ADMA, and body fatness.

Physical activity (PA) and the prescription of PA intended for the lowering of coronary heart disease (CHD) risk factors are widely advocated (Blair *et al*., 1995:1097; Ali *et al.,* 1998:1544). Recently the American Collage of Sports and Medicine (ACSM) has launched a campaign known as *Exercise is Medicine* ® (Salis, 2009). Their vision is to make physical activity and exercise a standard part of disease prevention and the treatment medical paradigm in the United States (Salis, 2009).

There is evidence that physical activity may alter Hcy production by either increasing protein and/or methyl group turn over (Joubert & Manore, 2006:344). During exercise, protein turnover could alter Hcy concentrations by increasing methionine catabolism, thus lowering Hcy or by decreasing B-vitamin availability, which would increase Hcy concentrations (Gibala, 2001:87). High-intensity exercise elicits an increase in methyl group turnover, which increases Hcy production (Joubert & Manore, 2006:345). An increase in PA may result in several anti-atherosclerotic effects such as improvement of Nitric Oxide bioavailability, oxidative stress reduction and lipid peroxidation. A limited number of studies investigated the effect of PA on plasma Hcy concentrations and evidence remains controversial (Ali *et al.,* 1998:1544; Wright *et al*., 1998:265; Duncan *et al.*, 2004:900). These inconclusive results indicate that a lack of research on the effect of physical activity on concentrations of both Hcy and ADMA.

No research on the interaction between concentrations of Hcy and ADMA in combination with physical activity could be found in the available literature either. Elevated concentrations of Hcy and ADMA are both identified as biochemical markers that increase the risk of developing CVD (Gomes *et al.,* 2002:575; Matetzky *et al.,* 2003:1933).

Apoptosis of the smooth muscle cells induced by increased Hcy concentrations is related to the stimulation of increased ADMA production, thus affecting arterial properties (i.e. intima-media thickness and stiffness) (Yuan *et al.,* 2007:880). According to Furuki *et al.* (2007:209) ADMA can be regarded as an independent determinant of intima-media thickness (IMT) in subjects without overt cerebro-cardiovascular disease.

A possible mechanism by which ADMA concentration induces its deleterious effects might be through increased methylation of arginine residues within proteins (Furuki *et al.,* 2007:209). Another mechanism might be through the reduction in metabolism of

ADMA by means of the dimethylarginine dimethylaminohydrolase (DDAH) enzymes. These two mechanisms remain mere suggestions, highlighting the importance of more research to be conducted to establish the exact links between circulating Hcy concentrations, ADMA concentrations and arterial properties (i.e. intima-media thickness and stiffness).

Homocysteine and ADMA share numerous presumed patho-physiological mechanisms that link these compounds to vascular disease, as mentioned earlier (Van Guldener *et al.,* 2007:1683). Most of these mechanisms decrease NO production, that leads to endothelial dysfunction. On the other hand, inflammation is an established marker of CVD. Some specific inflammatory markers have been identified to be very useful in the screening and prediction of cardiovascular disorders (Jiang *et al.,* 2007:66; Smith, 2007:1619; Van Guldener *et al.,* 2007:1684).

Evidence exist that both Hcy and ADMA concentrations are linked to inflammation, alluding to the possibility that a relationship exists between concentrations of Hcy and ADMA, and markers of endothelial function. Research investigating the relationship between concentrations of Hcy and ADMA, and markers of endothelial function are limited in the published literature.

As indicated in Figure 1 there is an interrelationship between Hcy and ADMA, as well as Hcy, ADMA and CVD risk factors such as fitness, fatness, arterial properties, endothelial function and inflammation markers. Based on research conducted over the last ten years, no consistent relationship exist between these variables. The objective of this study is, therefore, to determine the relationship between concentrations of Hcy and ADMA, and physical activity/fitness. Both Hcy and ADMA seem to be responsible for endothelial dysfunction that include diminishing of arterial properties but the interrelationship between Hcy and ADMA remains unclear. Lastly, increased concentrations of Hcy and ADMA may also contribute to the development of endothelial dysfunction by means of inflammation markers, although the interrelationship between Hcy and ADMA still needs to be elucidated (Figure 2).

The findings of this study may thus have putative implications for both health sciences and the health of the society, as all of the above-mentioned factors are linked to CVD, and CVD is known as the number one cause of morbidity and mortality worldwide

(Ignarro *et al*., 2007:326). The study may help scientists to have a better insight into the pathological mechanisms of both Hcy and ADMA in relation to cardiovascular health. Health care practitioners may also benefit from these findings, as it may assist in identifying which (and at which critical periods) lifestyle and biological risk factors can be most deleterious in vascular health, thereby informative of which and when primary prevention measures may be more appropriate.

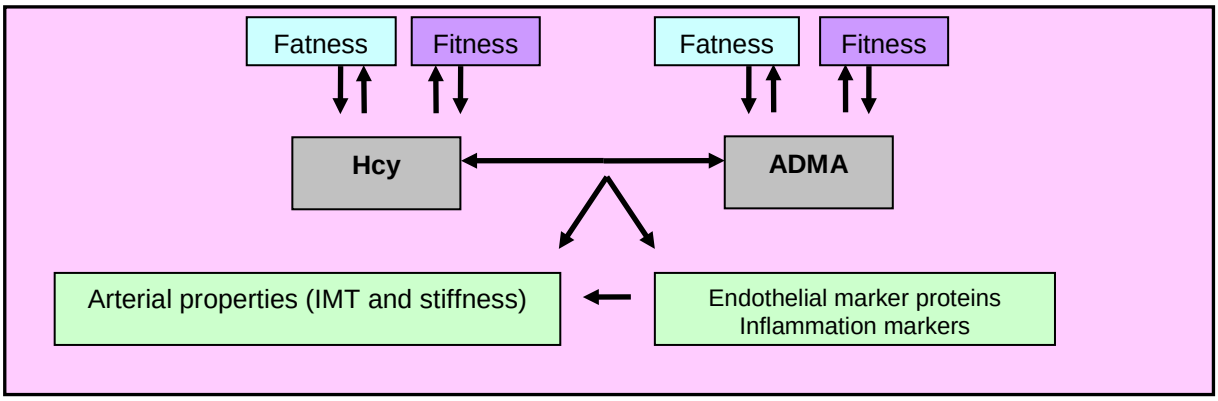

Figure 2: A model to illustrate the proposed causal paths of ADMA, Hcy, fitness, fatness, markers of endothelial function and inflammation, and arterial properties.

Based on the possible relationships mentioned above, the main research question to be answered in this study is whether a relationship exists between (changes in) concentrations of Hcy and ADMA, and fitness, fatness, markers of endothelial function and inflammation, and arterial properties.

This study forms part of the Amsterdam Growth and Health Longitudinal Study (AGAHLS) that has unique characteristics:

1. With the addition of concentrations of homocysteine and ADMA, as well as endothelial function to the already abundant available data from the AGAHLS on fitness, fatness and arterial properties, the causal path to pre-clinical atherosclerosis can be investigated within one study.

2. Because repeated measurements in time are performed, not only the levels but also the changes in Hcy, ADMA, fitness, fatness and endothelial function can be studied in relation to one another, as well as in relation to the levels and changes in arterial properties.

3. The data originate from a relatively young adult population of men and women. Populations like these have scarcely been studied. However, given the prospective dramatic increase in the prevalence of cardiovascular disease (CVD) because of the observed increase in the global prevalence of important risk factors, most benefit can be attained through preventive activities tailored to relatively young, disease free populations.

As the incidence of cardiovascular disease increases with age all around the world, the AGAHLS is a perfect opportunity to investigate the interactions between cardiovascular risk factors such as concentrations of Hcy and ADMA, fitness, fatness, markers of endothelial function and inflammation, and arterial properties. The AGAHLS started in 1977 with a group of 13-year old subjects, initially to investigate the longitudinal relationship between biological and lifestyle variables (Kemper, 2004). The subjects have been measured repeatedly over time and the last measures were done in 2006 at the age of 42.

1.2 AIM AND OBJECTIVES

❖ AIM

The aim of this study is to investigate the relationship between concentrations of Hcy and ADMA, fitness, fatness, markers of endothelial function and inflammation, and arterial properties of healthy adult men and women from the ongoing observational longitudinal study, AGAHLS.

❖ OBJECTIVES

More specifically, this study will investigate**:**

1. The relationship of (changes in) concentrations of Hcy and ADMA with (change in) fitness and fatness.
2. The relationships of (changes in) concentrations of Hcy and ADMA with (changes in) arterial properties.

3. The relationships of (changes in) concentrations of Hcy and ADMA with (changes in) markers of endothelial function and inflammation.

1.3 HYPOTHESIS

This study is based on the following hypotheses:

1. Concentrations of (changes in) Hcy and ADMA are inversely related to (changes in) fitness and fatness.
2. Positive associations are expected between (changes in) concentrations of homocysteine and ADMA, and (changes in) arterial properties.
3. Concentrations (changes in) of homocysteine and ADMA are inversely related to (changes in) endothelial function and inflammation markers.

1.4 STRUCTURE OF THIS THESIS

This thesis is presented in article format. It consists of six chapters, namely an introduction, a literature review (Chapter 2) and three research manuscripts (Chapter 3, 4 and 5). Chapter 6 comprises the summary, conclusions and recommendations for this research. References in Chapter 1, 2 and 6 are done according to the Harvard style of referencing as per the regulations of the North-West University. For the research manuscripts (Chapter 3, 4 and 5), the author's instructions from the respective pre-reviewed journals are followed, as required by the guidelines of the North-West University for a thesis in article format.

Table 1 presents the structure of this thesis in detail, also indicating the journals selected for submission of the manuscripts.

Table 1: The structure of the article format thesis

Chapter 1	Introduction
Chapter 2	Literature review: The putative role of homocysteine and asymmetric dimethylarginine in fitness, fatness, vascular and endothelial function.
Chapter 3	Article 1: Both fitness and fatness are not associated with homocysteine and asymmetric dimethylarginine concentrations: results of the Amsterdam Growth and Health Longitudinal Study (*European Journal of Clinical Nutrition*).
Chapter 4	Article 2: The relationship between concentrations of homocysteine and asymmetric dimethylarginine, and markers of inflammation and endothelial dysfunction (*Atherosclerosis Journal*).
Chapter 5	Article 3: The relationship between concentrations of homocysteine and asymmetric dimethylarginine, and arterial properties (stiffness and thickness) (*Journal of Internal Medicine*).
Chapter 6	Summary, conclusions, limitations and recommendations.

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2

LITERATURE REVIEW: THE PUTATIVE ROLE OF HOMOCYSTEINE AND ASYMMETRIC DIMETHYLARGININE IN FITNESS, FATNESS, VASCULAR AND ENDOTHELIAL FUNCTION

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2.1 Introduction

It is a well-known fact that cardiovascular disease (CVD) is a major cause of mortality worldwide, with traditional cardiovascular risk factors like hypertension, diabetes and smoking contributing significantly to its occurrence (Ignarro *et al.,* 2007:326). While many investigations focused on the traditional risk factors of cardiovascular disease to understand their relationship with and effect on vascular function and arterial properties, the less familiar emerging risk factors such as homocysteine (Hcy) and asymmetric dimethylarginine (ADMA) levels have not received much attention in the available literature.

In this chapter the biological pathways of ADMA and Hcy will be focused on, followed by a discussion of the existing literature with regard to the relationships between concentrations of Hcy and ADMA and their relation to fitness, fatness, markers of endothelial function and inflammation, and arterial properties (stiffness and thickness).

2.2 The biological pathways of ADMA and Hcy

Only more recently researchers agreed that non-traditional risk factors like homocysteine (Hcy) and asymmetric dimethylarginine (ADMA) may be regarded as major role players in the pathogenesis and progression of cardiovascular diseases – particularly in atherosclerosis through endothelial dysfunction (Beltowski & Kedra, 2006:160).

Numerous studies have addressed the possible relationship between concentrations of Hcy and ADMA (Böger *et al.,* 2000:1558; Böger *et al.,* 2001:161; Holven *et al.,* 2003:359; Jonasson *et al.,* 2003:33; Doshi *et al.,* 2005:351 & Ziegler *et al.,* 2005:2125). Hcy and ADMA are biochemically linked in various ways. To begin with, two methyl groups from methionine are used for post-transcriptional methylation of arginine, yielding Hcy and ADMA (Böger *et al.,* 2000:1558).

ADMA inhibits the conversion of arginine to nitric oxide and citrulline. ADMA can be excreted through the urinary tract or it can be degraded to citrulline and methylamine, a process that can be inhibited by Hcy (Van Guldener *et al*., 2007:1684). There are some studies that do not find any significant relationship between concentrations of Hcy and ADMA (Ziegler *et al.,* 2005:2176; Spoelstra-de Man *et al.,* 2006:497 & Schmitt *et al.,* 2007:169). These findings contribute to the controversy and mystery surrounding the

pathological mechanisms and interactions between Hcy and ADMA. More research is eminent.

Based on the possible relationships between Hcy and ADMA, this literature review will examine the relationship that exists between elevated plasma concentrations of Hcy and ADMA and their relationship with some cardiovascular risk factors such as fitness, fatness, arterial properties and inflammation.

ADMA is an endogenous molecule that is synthesised during the methylation of amino acid arginine residues by S-adenosylmethionine protein arginine, after which it is released into the blood plasma (Beltowski *&* Kedra, 2006:176). Normal ADMA concentrations are less than 1 μmol/L but increased concentrations of up to tenfold that of normal concentrations are seen in patients suffering from chronic renal failure (Vallance *et al.,* 1992:61). The excretion of ADMA is primarily through dimethylarginine demethylaminohydrolase (DDAH) metabolism that breaks ADMA down to citrulline and dimethylamine but ADMA can also be excreted through the urinary tract.

ADMA exerts its deleterious biological effects by inhibiting nitric oxide (NO) synthesis (Figure 1) (Beltowski & Kedra, 2006:160). NO plays an important biological role as a mediator and neurotransmitter. NO can be regarded as a key factor in many physiological functions such as regulating vascular tone, neurotransmission in the central and peripheral nervous system, killing invading micro-organisms and regulating mitochondrial respiration (Beltowski & Kedra, 2006:161).

There are numerous speculations regarding treatments for lowering of elevated ADMA concentrations. Research indicated that ADMA concentrations can be reduced by means of angiotensin-converting enzyme inhibitors, angiotensin AT_1 receptor antagonists, and administration of vitamin E and folic acid (Holven *et al*., 2003:1989; Beltowski & Kedra, 2006:160).

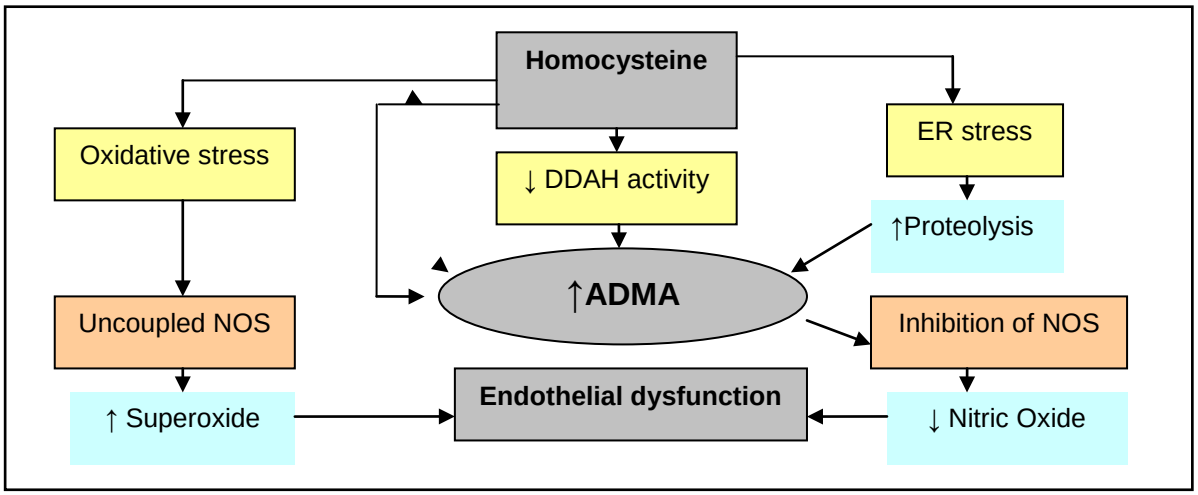

Figure 1: Biosynthesis and metabolism of ADMA (Dayal & Lentz, 2005:531).

(ADMA: Asymmetric dimethylarginine, ER: Endoplasmic reticulum, DDAH: Dimethylarginine dimethylaminohydrolase, NOS: Nitric oxide synthase).

According to Stühlinger *et al.* (2003:936), plasma Hcy stimulates ADMA formation in cultured cells. Plasma Hcy is a non-protein amino acid, an intermediary product of methionine metabolism (Boushey *et al*., 1995:1050). The recommended norm for plasma Hcy concentrations is 5-15 μmol/L (Zamani, 2002:1). According to Zamani (2002:1), plasma Hcy increases the risk of CVD by means of its prothrombotic and atherogenic properties. The metabolism of Hcy in the human body occurs through one of two biological pathways, namely the remethylation and the transsulfuration pathway.

The remethylation of plasma Hcy to methionine is catalysed by the methionine synthase (MS) enzymes. The remethylation requires vitamin B_{12} and 5, 10-methyltetrahydrofolate (5-methyl THF), which is generated by 5, 10-methylene tetrahydrofolate reductase (MTHFR). Some of the Hcy is remethylated to methionine by betaine-Hcy methyltransferase (BHMT) in the liver and kidneys. In this reaction betaine is a methyl donor and generates dimethylglycine (DMG) as a product.

The transsulfuration of Hcy requires vitamin B_6 and the transsulfuration pathway is catalysed by the cystathionine β–synthase (CBS) enzyme to form cystathionine (Figure 2). During the metabolism of Hcy, methionine is activated to S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) as products of the methyl transfer reaction that utilises SAM as a methyl donor (Figure 2).

Hyperhomocysteinemia can be regarded as an independent risk factor for atherosclerosis. The following factors contribute to the increase in plasma Hcy concentrations: enzymatic defects, dietary deficiency of folic acid, vitamins B_{12} and B_6, renal failure, liver disorders, hormonal factors (hypothyroidism), malignancy, drugs, toxins (methotrexate, phenytoin and theophylline) and smoking (Ueland & Refsum, 1989:479; McCully, 1996:389; Welch & Loscalzo, 1998:1050).

The treatment of hyperhomocysteinemia varies with the underlying cause but it generally involves supplementation with folic acid, vitamin B_{12} and pyridoxine (vitamin B_6) (Den Heijer *et al*., 1998:359). A diet rich in fruits, vegetables, low fat dairy products and reduced in saturated and total fat can also lower serum Hcy concentrations (Apple *et al.,* 2000:852). Physical activity (PA) may be regarded as a form of treatment for hyperhomocysteinemia but according to the available literature, opinions on the relationship between PA and Hcy concentrations remain very controversial (Ali *et al.,* 1998:1543; Erikssen *et al*., 1998:353; König *et al*., 2003:115 & Gaume *et al.,* 2005:125).

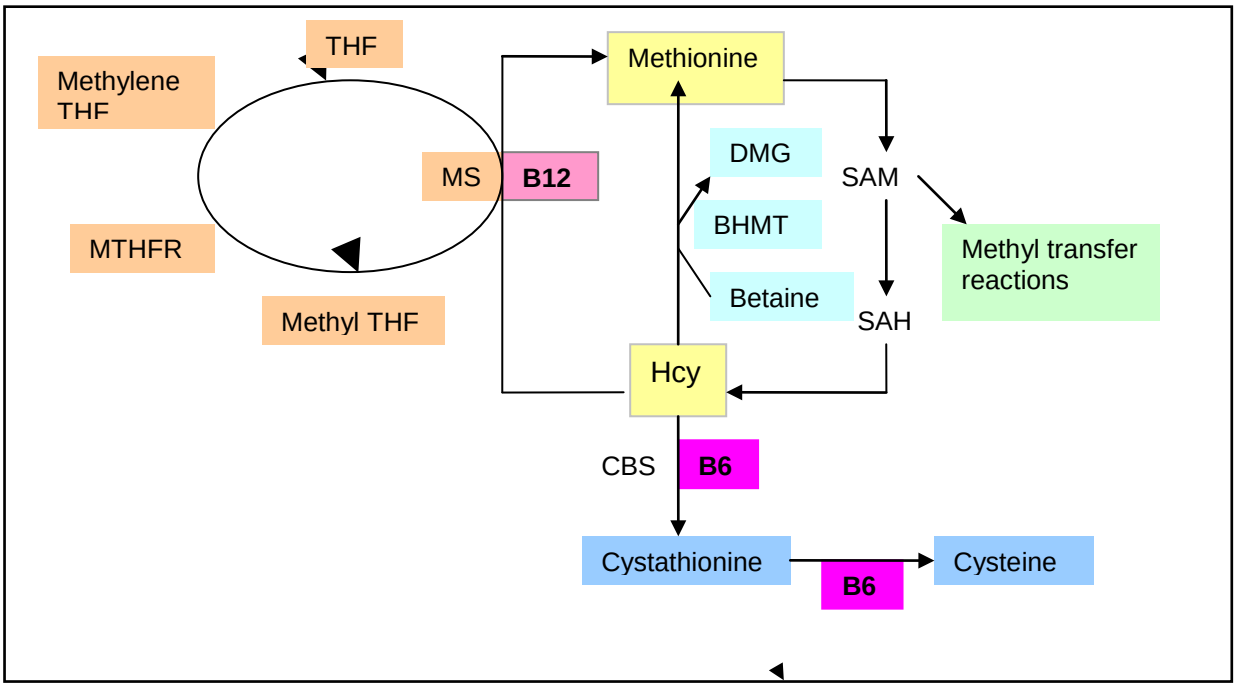

Figure 2: Homocysteine metabolism.

(SAM: S-adenosylmethionine, DMG: Dimethylglycine, SAH: S-adenosylhomocysteine, MS: Methionine synthase, CBS: Cystathionine β-synthase, THF: Tetrahydrofolate, MTHFR: Methylene tetrahydrofolate reductase, BHMT: Betaine homocysteine methyltransferase).

2.3 The relationship between concentrations of Hcy and ADMA, fitness and fatness

Physical fitness is a term that refers to maximal aerobic capacity [maximal oxygen uptake (VO_2max)]. Physical activity and physical fitness are inseparable (Erikssen *et al*., 2001:353). The only way to increase the level of fitness is to increase and structure physical activity. An increase in physical fitness lowers CVD risk factors, such as blood pressure, total cholesterol, obesity and blood lipids (Erikssen *et al*., 2001:354; König *et al*., 2003:116 & Gaume *et al.,* 2005:125).

Research conducted over the past few years found no consistent relationship between physical fitness and plasma Hcy concentrations (Erikssen *et al*., 2001:354; König *et al*., 2003:116; Gaume *et al.,* 2005:125 & Unt *et al*., 2008). Studies that investigated the relationship between physical activity/exercise and Hcy concentrations were either intervention studies (König *et al*., 2003 & Gaume *et al.,* 2005) or epidemiological studies (Gruber *et al.,* 2008; Unt *et al.,* 2008). Although it remains controversial, recent data suggest that physical activity may be associated with decreased Hcy concentrations (König *et al*., 2003; Gaume *et al.,* 2005 & Unt *et al.,* 2008). Some intervention studies have indicated that the relationship between changes in Hcy concentrations and changes in physical fitness are influenced by the duration, intensity, frequency and type of exercise (Erikssen *et al*., 2001; König *et al*., 2003; Gaume *et al.,* 2005 and Unt *et al*., 2008).

On the other hand, studies that investigated the relationship between physical activity/fitness and ADMA concentrations are extremely limited but a positive relationship was found in an intervention study (Richter *et al.,* 2005). Richter investigated the effect of endurance exercise on ADMA as a risk marker. Endurance training reduces circulating ADMA and myloperoxidase levels that may lead to changes in numerous anti-atherosclerotic effects such as improved NO production, a reduction in oxidative stress and lipid peroxidation (Richter *et al.,* 2005:1306). According to the study by Richter *et al.* endurance exercise was significantly related to lower ADMA concentrations. Although some studies found significant differences between concentrations of plasma Hcy and ADMA, these findings remain controversial (Jonasson *et al*., 2003; Wanby *et al*., 2003 and Antoniades *et al*., 2006).

Whether or not exercise improves or modifies recently identified CVD risk factor asymmetric dimethylarginine (ADMA), remains uncertain (Ali *et al.,* 1998:1544; Erikssen *et al*., 2001:353; König *et al*., 2003:115 & Gaume *et al.,* 2005:125).

Although little is known about the modulating effect of physical activity on Hcy and ADMA, in addition to adequate nutrition, there is evidence that physical activity may also alter Hcy production by increasing protein and/or methyl group turnover. During exercise, protein turnover could alter Hcy concentrations by increasing methionine catabolism, consequently lowering Hcy, or decreasing B-vitamin availability, which would increase Hcy concentrations. It is known that prolonged high-intensity exercise increases protein metabolism and alters blood concentrations of certain amino acids (Petrides, 1997:541, Gibala *et al.,* 2001, Fehrenbach & Northoff, 2001:66; McMahon & Jenkins, 2002:761). Reduced methionine availability would promote *de novo* methionine synthesis and thus reduce accumulation of Hcy. In this way, the protein turnover mechanism would lower Hcy concentrations during high intensity prolonged exercise, seeing that folate, vitamins B_6 and B_{12} remain adequate (Joubert & Manore, 2006:341).

Conversely, prolonged exercise, where glycogen reserves are reduced, places an increased demand on vitamin B_6 dependent reaction. In addition, during exercise, glyconeogenesis involves the breakdown of amino acids, with the carbon skeleton used for energy (Joubert & Manore, 2006:341). As exercise intensity increases, the demand for vitamin B_6 increases and less vitamin B_6 would be available for catabolism of Hcy. Subsequently, increased protein turnover during prolonged exercise would lead to an increase in Hcy concentrations (Joubert & Manore, 2006:341).

According to Joubert & Manore (2006:341) high-intensity exercise elicit an increase in methyl group turnover which could increase the production of Hcy. Methionine is converted to s-adenosylmethionine, which is the most important methyl group donor in humans. A sufficient supply of methyl groups is important in several biochemical pathways, of which many are exercise related, such as the synthesis of DNA, RNA carnitine, choline, acetylcholine, phosphatidyl-choline, epinephrine, adrenalin, methylhistadine and creatine (McMahon & Jenkins, 2002:761, Selhub *et al.,* 1999:217). High intensity prolonged physical activity, which increases the demand for creatine, increases Hcy production compared to less intense physical activity of short duration. Thus an increase in methyl group turnover increases Hcy production (Joubert & Manore, 2006).

Very limited research investigating the relationship between fitness and ADMA concentrations were found. As mentioned earlier, endurance training reduces circulating asymmetric dimethylarginine and myloperoxidase levels that may lead to changes in numerous anti-atherosclerotic effects such as improved NO production, a reduction in oxidative stress and lipid peroxidation (Richter *et al.,* 2005:1306). The metabolism of Hcy arginine residues may be another possible pathogenesis. These are residues found in proteins that are methylated by protein arginine methyltransferase (PRMT), which uses S-adenosylmethionine (SAM) as a methyl donor and produces S-adenosylhomocysteine (SAH). Hcy is derived from the hydrolysis of SAH that can be remethylated to methionine (Met); thus, completing the methionine cycle (Figure 2).

ADMA is derived from the proteolysis of proteins that contain methylated arginine residues. It can cause endothelial dysfunction by inhibiting nitric oxide synthase (NOS). The major pathway for the metabolism of ADMA is via the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which produces citrulline and methylamine. A small amount of ADMA is metabolised to alpha keto-acids or excreted by the kidneys.

Alternatively, physical activity can influence concentrations of Hcy and ADMA through an increase in endothelial cell nitric oxide synthase (eNOS). Increasing NOS expression has significant implications on CVD and the anti-atherogenic properties of NO. The impaired NO release that is initiated by elevated concentrations of Hcy and ADMA may be altered by physical activity. Physical activity may to some degree restore the ability of the endothelium to release NO, attenuating endothelial dysfunction (Hayward *et al.,* 2003:209). However, the above-mentioned remains pure speculation and further studies are needed to determine the exact relationship between physical fitness levels and plasma concentrations of Hcy and ADMA. To my knowledge, no studies could be obtained that specifically investigated the relationship between ADMA and physical activity and fitness. A literature search on fitness and fatness in combination to Hcy and ADMA returned no data.

According to McLaughlin *et al.* (2006:1896) plasma ADMA levels were higher in obese insulin resistant women than in the equally obese, insulin sensitive women. Gruber *et al.* (2008:520) concluded that ADMA is slightly increased in obese juveniles without any robust correlations to obesity related disorders.

2.4 The relationship between concentrations of Hcy and ADMA, and arterial properties (stiffness and thickness)

As mentioned previously, hyperhomocysteinemia has been linked to the increased risk of developing atherosclerotic disease. It also appears that impaired endothelial function occurs before the onset of plaque formation in patients suffering from atherosclerosis (Stühlinger & Stanger, 2005:4). Endothelial dysfunction can therefore be regarded as a sensitive indicator of the progression of atherosclerotic lesions and a predictor of vascular events (Stühlinger & Stanger, 2005:4).

Nitric oxide is an endogenous vasodilator and is released by the endothelium. A decrease in NO availability can lead to impaired endothelium-dependent vasorelaxation in patients suffering from hyperhomocysteinemia (Topal *et al.,* 2004:1533; Stühlinger & Stanger, 2005:4).

On the other hand, ADMA is also an NO synthase inhibitor that reduces the production of NO. Subsequently this leads to endothelial dysfunction (Böger, 2003:1468). ADMA is an l-arginine analogue that plays an important role in the endogenous mechanism to regulate NO synthesis and increases adhesion of monocystes to the endothelium (Chan *et al.,* 2000:1040; Böger, 2003:1468). A number of clinical studies suggest that there might be a relationship between hyperhomocysteinemia, ADMA and endothelial function; for instance, elevated ADMA concentrations where related to impaired endothelium-dependent relaxation in patients suffering from hyperhomocysteinemia (Stühlinger & Stanger, 2005:4).

According to Yuan *et al.* (2007:881) apoptosis of vascular smooth muscle cells induced by Hcy is related to the stimulation of ADMA production. A possible mechanism through which ADMA may induce its deleterious effects might be by increasing methylation of arginine residues within proteins, or through a reduction in the metabolism of ADMA by means of the dimethylarginine dimethylaminohydrolase (DDAH) enzymes. However, both of the postulated mechanisms need to be confirmed.

2.5 The relationship between concentrations of Hcy and ADMA, and markers of endothelial function and inflammation

Inflammation markers have received much attention recently. Some of these markers can be used in the screening and prediction of cardiovascular disorders (Smith, 2007:1627; Jiang *et al.,* 2007:67; Van Guldener *et al.,* 2007:1684). Inflammation markers (i.e. C-reactive protein (CRP), serum amyloid A (SAA) and tumor necrosis factor α (TNF-α)) of the vascular system are significant role players in the pathogenesis of atherosclerosis (Huang & Vita, 2005:17; Kaperonis *et al.,* 2006:387).

The process of atherosclerosis is summarised in terms of the "response to injury" and the "lipid infiltration" hypotheses (Thompson & Smith, 1989:90). According to the "response to injury" hypothesis, morphologic changes are observed in endothelial and sub-endothelial layers of arterial walls.

These changes in the endothelial layers are ascribed to an inflammatory response to certain stimuli, i.e. changes in blood flow as observed with turbulence or stagnation and other conditions such as anoxia, hypertension, hypercholesterolemia (Schwartz *et al*., 1991:14), hyperhomocysteinemia (Harker *et al*., 1974:540) and increased ADMA concentrations (Beltowski & Kedra, 2006:159).

Bearing in mind that inflammation is an established marker of cardiovascular disease, and that both plasma Hcy and ADMA are linked to inflammation one would expect to find a relationship between Hcy and ADMA in this regard. There is growing evidence that oxidative stress and vascular inflammation response are both key factors in contributing to the progression of endothelial dysfunction (Blanco *et al.,* 2005:33). ADMA has been associated with oxidative stress and vascular inflammation in general (Böger *et al.,* 2000:2288; Scalera *et al.,* 2004:1817; Goonasekera *et al.,* 2000:18; Holm *et al.,* 2002:1397; Zocalli *et al.,* 2002:494; Nanayakkara *et al.,* 2005:2231).

ADMA and Hcy are biochemically linked in many ways. They share several presumed patho-physiological mechanisms that link them to vascular disease and most of these mechanisms are linked to the decrease in NO production that leads to endothelial dysfunction (Van Guldener *et al.,* 2007:1627). Some studies did not find any significant relationship between concentrations of Hcy and ADMA (Spoelstra-de Man *et al.,* 2006:497; Schmitt *et al.,* 2007:169; Ziegler *et al.,* 2005:2176).

It is evident that these rather controversial findings don't agree on any of the pathological mechanisms and interactions between concentrations of Hcy and ADMA, emphasising the need for more research on this topic.

2.6 Summary

ADMA and Hcy are both recently identified risk factors for endothelial dysfunction that predominantly leads to atherosclerosis. These two cardiovascular risk factors have been studied with reference to their influence on the cardiovascular system, but very little is known about the relationship that exists between them. The same is true for evidence regarding the relationships between concentrations of Hcy and ADMA with other risk factors such as fitness, fatness, arterial properties and markers of endothelial function and inflammation.

From the current literature it is known that a metabolic link exists between Hcy and ADMA. Associations between Hcy and ADMA with fitness and fatness respectively have been identified, although the exact mechanism remains unclear. An increase in physical activity can cause an increase in the consumption of methylated substrates, which may be accompanied by changes in Hcy. The metabolism of Hcy arginine residues may subsequently negatively alter ADMA concentrations. Alternatively, physical activity can influence concentrations of Hcy and ADMA through an increase in endothelial cell nitric oxide synthase (eNOS). Due to the limited availability of studies and the controversy that surrounds the relationship between Hcy, ADMA, fitness and fatness, more studies are required to determine the exact relationship.

The literature also revealed controversial relationships between Hcy, ADMA and arterial properties (stiffness and thickness). As mentioned previously, hyperhomocysteinemia has been linked to the increased risk of developing atherosclerotic disease, as elevated Hcy concentrations decrease the availability of NO, impairing the endothelium-dependent vasorelaxation. On the other hand ADMA is an L-arginine analogue, an eNOS inhibitor that also increases adhesion of monocystes to the endothelium. Both Hcy and ADMA have respectively been linked to atherosclerosis but the underlying mechanism remains to be answered.

According to the literature there are relationships between Hcy, ADMA and inflammation and endothelial markers respectively. Hcy and ADMA are biochemically linked and share several presumed patho-physiological mechanisms that link them to vascular disease. Most of these mechanisms are linked to the decrease in NO production that leads to endothelial dysfunction. Elevated Hcy and ADMA concentrations have been linked to a decrease in eNOS that increases adhesive molecule production (i.e. intracellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM)), stimulating the inflammatory processes. No literature could be obtained that investigated the inter-relationship between Hcy, ADMA, endothelial dysfunction and inflammatory markers.

Studies investigating the relationship between concentrations of Hcy and ADMA, fitness, fatness, markers of endothelial function and inflammation, and arterial properties of healthy adult men and women longitudinally is therefore needed.

The advantage of such studies will assist researchers to establish less controversial conclusions as to whether or not concentrations of Hcy and ADMA can be regarded as primary CVD risk factors. The results may help clarify the exact mechanism through which Hcy and ADMA are linked to cardiovascular disease, assisting in the development of a treatment strategy and ultimately the improvement in CVD management and prevention.

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3

FITNESS AND FATNESS ARE NOT ASSOCIATED WITH CONCENTRATIONS OF HOMOCYSTEINE AND ASYMMETRIC DIMETHYLARGININE: RESULTS OF THE AMSTERDAM GROWTH AND HEALTH LONGITUDINAL STUDY

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Fitness and fatness are not associated with concentrations of homocysteine and asymmetric dimethylarginine: results of the Amsterdam Growth and Health Longitudinal Study

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ABSTRACT

Background: Cardiopulmonary fitness levels, body fatness and body fat distribution are assumed to be associated with concentrations of plasma homocysteine (Hcy) and asymmetric dimethylarginine (ADMA), but little is known of their relationship. The purpose of this study was to investigate the relationship between (changes in) concentrations of plasma Hcy and ADMA, and (changes in) fitness and fatness in adult men and women.

Methods: This study forms part of the Amsterdam Growth and Health Longitudinal Study. The analysis within this ongoing observational longitudinal study included 355 individuals with a complete data set. They were measured in 2000 (at the age of 36) and 293 subjects were measured in 2006 (at age 42). Fitness was assessed by measuring VO_2max and body fatness, and body fat distribution as measured by DXA.

Results: No significant relationships were found between changes in fitness and fatness, and changes in concentrations of plasma Hcy and ADMA from 2000 and 2006. However, a 1 ml/min/kg higher VO_2max was significantly related to a -0.81 nmol/L lower plasma Hcy concentration (95% confidence interval [-1.53 – -0.09] p=0.03). This relationship was attenuated after adjustment for smoking behaviour.

Conclusion: No relationships were found between concentrations of Hcy and ADMA, fitness and fatness. The only significant relationship between Hcy and fitness was partly explained by smoking behaviour.

Key words: Homocysteine, asymmetric dimethylarginine, physical fitness, fatness, aerobic capacity, body mass index.

Introduction

It is a well-known fact that cardiovascular disease (CVD) is the leading cause of mortality and morbidity worldwide (Ignarro *et al.,* 2007). The cardiovascular risk factors that received a lot of interest lately are body fatness and physical fitness. An increase in physical fitness and a decrease in body fatness improve traditional CVD risk factors such as blood pressure, total cholesterol and blood lipids. It remains unclear, however, as to whether or not fitness and fatness modify recently identified CVD risk factors such as circulating levels of homocysteine (Hcy) and asymmetric dimethylarginine (ADMA) (Joubert & Manore, 2006). Research conducted over the last few years found no consistent relationship between fitness, fatness, Hcy and ADMA. Available studies are extremely limited and the available evidence are rather controversial (Erikssen *et al.,* 1998; König *et al.,* 2003; Coombes *et al.,* 2004; Duncan *et al.,* 2004; Eid *et al.,* 2004; Gaume *et al.,* 2005; Richter *et al.,* 2005; Gruber *et al.,* 2008 and Unt *et al.,* 2008).

Studies that investigated the relationship between physical activity/exercise and Hcy concentrations can be divided into two categories, i.e. intervention studies (Ali *et al.,* 1998; Wright *et al.,* 1998; De Crée *et al.,* 2000; De Jong *et al.,* 2001; Herrmann *et al.,* 2003; König *et al.,* 2003; Coombes *et al.,* 2004; Duncan *et al.,* 2004 and Gaume *et al.,* 2005) and epidemiological studies (Nygard *et al.,* 1995; Gruber *et al.,* 2008 and Unt *et al.,* 2008). Although it remains controversial, recent data suggest that physical activity may be associated with decreased Hcy concentrations (Ali *et al.,* 1998; König *et al.,* 2003; Coombes *et al.,* 2004; Gaume *et al.,* 2005 and Unt *et al.,* 2008). On the other hand, studies that investigated the relationship between physical activity/fitness and ADMA concentrations are extremely limited but a positive relationship was found in an intervention study (Richter *et al.,* 2005).

Homocysteine and ADMA are biochemically linked in several ways, and an increase in S-adenosylmethionine (SAM) may alter the protein enzyme methyltransferases (PRMT)-catalyzed methylation of arginine residues in proteins that lead to an increased production of protein-bound ADMA (Teerlink, 2005). Increased SAM levels inhibit the remethylation pathway that leads to an increase in Hcy concentrations. This increase subsequently leads to a shift in the equilibrium of the S-adenosylhomocysteine (SAH) hydrolase and causes an increase in SAH (Teerlink, 2005).

The latter inhibits the transmethylation process which may cause a decrease in the production of protein-bound ADMA. The excretion of ADMA takes place primarily through dimethylarginine dimethylaminohydrolase (DDAH) metabolism that breaks ADMA down to citrulline and dimethylamine but ADMA can also be excreted through the urinary tract (Teerlink, 2005). Finally, Hcy stimulates proteolysis and inhibits DDAH activity that can lead to an increase in ADMA concentrations (Teerlink, 2005).

A number of studies have demonstrated that elevated Hcy concentrations stimulate the formation of ADMA (Böger *et al.,* 2000; Böger *et al.,* 2001; Holven *et al.,* 2003; Jonasson *et al.,* 2003; Doshi *et al.,* 2005 and Ziegler *et al.,* 2005). Plasma ADMA concentrations are elevated in patients suffering from various diseases like chronic renal failure, pulmonary hypertension and chronic heart failure (Dückelmann *et al.,* 2008; Kato *et al.,* 2009 and Young *et al.,* 2009). Normal Hcy and ADMA concentrations are 5 µmol/L and 1 µmol/L respectively. Information about the relationship between physical fitness and fatness, and concentrations of Hcy and ADMA may give indications of how elevated concentrations of Hcy and ADMA can be prevented. Within the Amsterdam Growth and Health Longitudinal Study (AGAHLS) it is possible to investigate these relationships longitudinally.

In the AGAHL-Study, measurements of cardiopulmonary fitness, total fatness and central fatness have been gathered longitudinally from adolescence into adulthood. Assessment of concentrations of plasma Hcy and ADMA was performed for this study at the ages of 36 and 42.

The purpose of this study was firstly to investigate the (longitudinal) relationship between concentrations of Hcy and ADMA, fitness and fatness, and secondly, to investigate the relationship between changes in these variables from 2000 until 2006.

Subjects and Methods

Subjects

This study is part of the AGAHL-Study (approved by the medical ethics committee of the Vrije University Medical Center, Amsterdam, The Netherlands), an observational longitudinal study that started in 1977 with n = 698 adolescents with a mean age of 13. Since 1977 measurements have been obtained from participants two to eight times during a 29-year follow-up period.

At each measurement, anthropometrical variables (stature, body mass and skin folds), biological (serum lipoprotein levels, blood pressure and physical fitness) and lifestyle (smoking behaviour, alcohol consumption, daily physical activity) were assessed. A more detailed description of the study is provided elsewhere (Kemper, 2004).

Because concentrations of Hcy and ADMA were only assessed in 2000 and 2006 (i.e. at the age of 36 and 42 respectively), only those measurements were used in this study. All participants (47.1% men) completed an informed consent before any assessments were performed. All the initial subjects of the AGAHL-study that could be found were included in this study (Kemper, 1985).

Methods

Fitness and Fatness

Body fatness and body fat distribution of each individual were measured with dual-energy X-ray absorbtiometry (DXA) (Ferreira *et al.,* 2004). Total and regional (arms, legs, trunk and head) body fat and body lean mass were measured with a whole body DXA scanner (Hologic QDR-2000, software version V5.67A; Hologic Inc, Walthan, Massachussetts, USA). Peripheral fat was calculated by adding the fat mass of the legs to that of the arms, and peripheral lean mass was calculated by adding the lean mass of the legs to that of the arms. Whole body fat mass was used as an estimate of total body fatness, and trunk fat mass was used as an estimate of a central pattern of fat (visceral and subcutaneous) distribution. Anthropometric measurements of body height and body mass were performed according to standard procedures.

Body mass index (BMI), being the ratio between body mass (kg) and body height squared (m^2), was calculated as an indicator of obesity. The cardiopulmonary fitness of the participants was assessed with a maximal running test (VO_2max) on the treadmill (Kemper *et al.,* 2001) in 2000 and with the sub-maximal Chester Step Test in 2006 (Sykes & Roberts, 1998). The Chester Step Test data were converted to VO_2max values. Because of the different equipment used, gender specific z-scores were determined and used for statistical analysis.

Biochemical Analyses of Homocysteine and ADMA

Concentrations of total plasma Hcy and ADMA were determined from venous blood that was taken between 0800 and 0900 from the antecubital vein. Plasma was separated within 60 minutes after sampling. Total plasma Hcy was determined with an automated fluorescence polarization immunoassay on an Abbott IMx analyser (Shipchandler & Moore, 1995) with an inter-assay coefficient of variation of 4%. Plasma concentrations of ADMA were measured by high-performance liquid chromatography with fluorescence detection as described earlier (Teerlink *et al.,* 2002), using modified chromatographic separation conditions (De Jong and Teerlink, 2006). The intra-assay and inter-assay coefficients of variation were 1.5% and 3% respectively. Samples from individual patients were analyzed in the same analytical series.

Covariates

The level of habitual physical activity was assessed in a structured interview that covered the activities of the previous three months (Kemper *et al.,* 2001). In this interview the intensity, frequency and duration of all daily physical activities (at school, at work, in organized and unorganized sports, during leisure time, climbing stairs and transportation) were assessed. Only those activities with duration of at least five minutes and more, and an intensity of more than four times the basal metabolic rate (METs) were considered. The physical activities were classified as light (4–7 METs), medium-heavy (7–10 METs) and heavy (10 METs), based on values found in the literature (Ainsworth *et al.,* 1993). The average weekly time spent on the different activities were then multiplied by their intensity to calculate a total weighted activity metabolic score (expressed in metabolic equivalents - $MET \cdot wk^{-1}$).

Alcohol consumption data were obtained with a quantity-frequency questionnaire and expressed as standard units consumed per day (1 unit equals 10 g of pure alcohol) (Koppes *et al.,* 2002). The smoking habit was determined by means of a questionnaire. In the analysis smoking behaviour was dichotomised into smoking and non-smoking, and into how much tobacco was smoked.

Statistical Analyses

To investigate the longitudinal relationship between Hcy and ADMA on the one hand, and fatness and fitness on the other, generalized estimating equations (GEE) were used. This

method adjusts to the correlation between repeated observations taken in the same subject, and it has the advantage of handling longitudinal data on participants with a varying number of observations.

All the relationships were first analysed in a crude analysis (Model 1) with adjustment for gender. Model 2 was adjusted for either fitness or fatness, and in Model 3 an additional adjustment was made for smoking, alcohol consumption and physical activity. In all GEE analyses an exchangeable correlation structure was assumed.

The relationships between changes in concentrations of Hcy and ADMA, and changes in fitness and fatness were determined by means of linear regression analyses. The same three models were used as those used for the GEE analyses. All analyses were carried out with the statistical package of Social Sciences, 16 for Windows (SPSS. Inc. Chicago, Illinois, USA).

Results

Table 1 shows descriptive information of the AGAHLS population.

Table 1 Characteristics of the study population

Variable	n	Year 2000	N	Year 2006
Physical activity (METs.min/wk)[†]	377	4135.0 [2567.7 – 6326.7]	293	2727.6 [2222.2 – 3229.3]
Alcohol (glasses/wk) [†]	373	3.4 [0.8 – 6.3]	340	5.5 [0.8 – 11.3]
Smoking (%)[o]	408	23.0[a]	343	15.5[a]
Homocysteine (nmol/L)	378	11.5 ± 6.6	335	12.1 ± 5.5
ADMA (nmol/L)	378	0.41 ± 0.06	336	0.42 ± 0.06
VO_2max (mL/min.kg$^{-2/3}$)	364	34.6 ± 9.1[b]	342	38.9 ± 7.3[b]
Height (cm)	378	176.7 ± 9.3	343	177.2 ± 8.9
Trunk fat mass (kg)	356	8.2 ± 4.1[c]	294	8.9 ± 3.9[c]
Peripheral fat mass (kg)	355	11.0 ± 4.0[d]	294	10.1 ± 4.3[d]
Peripheral lean mass (kg)	355	21.5 ± 5.9[e]	294	25.4 ± 5.8[e]

[†] Data are shown as median and [25 – 75] percentiles; [o] data shown as percentage, other data shown as means ± SD; a – e: similar letters indicate significant difference between 2000 and 2006 values. (VO_2max; physical fitness)

According to Table 1 significant differences where found in baseline characteristics between 2000 and 2006. These variables include smoking, VO_2max, trunk fatness, peripheral fat mass and peripheral lean mass.

Table 2 Relationship between concentrations of Hcy and ADMA, and fitness and fatness adjusted for lifestyle and biological risk factors.

Variable	Hcy			ADMA		
Model 1	B	95% CI	P	B	95% CI	p
Fitness (mL/min.kg$^{-2/3}$)	-0.07	-0.50 to 0.37	0.77	0.000	-0.005 to 0.005	0.98
Trunk fat mass (kg)	-0.04	-0.48 to 0.42	0.87	0.002	-0.003 to 0.007	0.42
Peripheral fat mass (kg)	-0.12	-0.49 to 0.25	0.53	0.003	0.000 to 0.008	0.12
Peripheral lean mass (kg)	0.07	-0.29 to 0.45	0.69	-0.003	-0.008 to 0.002	0.25
Model 2						
Fitness (mL/min.kg$^{-2/3}$)						
Trunk fat mass (kg)	-0.04	-0.59 to 0.51	0.89	0.000	-0.007 to 0.006	0.88
Peripheral fat mass (kg)	-0.14	-0.68 to 0.41	0.62	0.005	0.000 to 0.011	0.06
Peripheral lean mass (kg)	0.13	-0.29 to 0.56	0.53	-0.005	-0.010 to 0.001	0.09
Model 3						
Fitness (mL/min.kg$^{-2/3}$)	-0.12	-0.56 to 0.33	0.61	0.000	-0.005 to 0.005	0.93
Trunk fat mass (kg)	0.01	-0.55 to 0.58	0.96	0.000	-0.008 to 0.006	0.85
Peripheral fat mass (kg)	-0.18	-0.78 to 0.43	0.57	0.005	0.00 to 0.011	0.07
Peripheral lean mass (kg)	0.12	-0.30 to 0.55	0.57	-0.005	-0.010 to 0.001	0.09

B states differences in concentrations of Hcy and ADMA by one unit difference in the particular fitness or fatness parameters as estimated by GEE analysis.

Model 1, adjusted for gender.

Model 2, model 1 + additional adjustment for fitness (VO_2max) and fat mass.

Model 3, model 2 + physical activity + smoking + alcohol.

Table 2 shows the results of the GEE analyses that revealed no significant relationships between concentrations of Hcy and ADMA, on the one hand, and fitness and fatness on the other. In models 1 and 3 there seems to be a borderline significant relation between peripheral mass (both fat and lean) and ADMA concentrations.

The results of the linear regression analysis (Table 3) indicate that a crude significant association (Model 1) was found between changes in plasma Hcy concentrations and changes in fitness (VO_2max). However, adjustment for physical activity, smoking and

alcohol consumption (Model 3) attenuated this association. In additional analyses it was found that all attenuation was caused by smoking.

Table 3 Results of the linear regression analysis regarding the relationship between changes in concentrations of Hcy and ADMA, and changes in fitness and fatness

Variable	Hcy			ADMA		
Model 1	B	95% CI	P	B	95% CI	p
Fitness (mL/min.kg$^{-2/3}$)	-0.81	-1.53 to -0.09	0.03*	-0.00	-0.01 to 0.01	0.75
Trunk fat mass (kg)	-0.54	-2.45 to 1.38	0.58	-0.01	-0.03 to 0.01	0.54
Peripheral fat mass (kg)	0.58	-0.93 to 2.09	0.45	-0.01	-0.02 to 0.01	0.43
Peripheral lean mass (kg)	-0.82	-2.61 to 0.97	0.37	0.00	-0.02 to 0.02	0.93
Model 2						
Fitness, (mL/min.kg$^{-2/3}$)						
Trunk fat mass (kg)	-0.99	-3.14 to 1.16	0.37	-0.00	-0.03 to 0.02	0.77
Peripheral fat mass (kg)	0.94	-0.75 to 2.63	0.27	-0.01	-0.02 to 0.01	0.57
Peripheral lean mass (kg)	0.75	-2.55 to 1.05	0.41	0.00	-0.02 to 0.02	0.97
Model 3						
Fitness, (mL/min.kg$^{-2/3}$)	-0.59	-1.41 to 0.22	0.15	-0.007	-0.003 to 0.017	0.67
Trunk fat mass (kg)	-0.07	-2.86 to 2.72	0.96	-0.002	-0.032 to 0.027	0.87
Peripheral fat mass (kg)	0.89	-2.07 to 3.85	0.55	-0.006	-0.037 to 0.025	0.69
Peripheral lean mass (kg)	-0.81	-2.73 to 1.12	0.41	0.000	-0.020 to 0.020	1.00

B states differences in changes in concentrations of Hcy and ADMA by one unit difference in changes in the particular fitness or fatness indicator, as estimated by linear regression analyses.

Model 1, adjusted for gender.

Model 2, model1 + fitness (VO_2max) and fat mass.

Model 3, model 2 + physical activity + smoking + alcohol, * = significance ($p<0.05$).

Discussion

In this study the relationships between (changes in) concentrations of Hcy and ADMA, and (changes in) fitness and fatness were investigated. This study forms part of the AGAHL-study, focusing on the two consecutive data sets of 2000 and 2006 respectively. Some significant differences were found between the baseline characteristics in 2000 compared to 2006 (smoking, VO_2max, trunk fatness, peripheral fat mass and peripheral lean mass). According to the GEE analysis, used to determine the relationship between the concentrations of Hcy and ADMA, and fitness and fatness, no significant relationship was

found. The results of the linear regression analysis revealed a crude significant association between changes in plasma Hcy concentrations and changes in fitness (VO_2max). However, this significant association was attenuated when an adjustment was made for smoking behaviour.

In order to draw a better conclusion, a comparison was made between the results found by our study and other studies that investigated the same variables. To our knowledge no longitudinal observational studies have investigated the relationship between fitness, fatness, Hcy and ADMA concentrations. Some intervention studies have indicated that the relationship between changes in Hcy concentrations and changes in physical fitness are influenced by the duration, intensity, frequency and type of exercise (Erikssen *et al.,* 1998; König *et al.,* 2003; Coombes *et al.,* 2004; Gaume *et al.,* 2005 and Unt *et al.,* 2008).

According to Coombes *et al.* (2004), there has been conflicting reports of the relationship between physical activity and Hcy concentrations. Cross-sectional associations between cardio-respiratory fitness (VO_2max) and plasma Hcy concentrations indicated that elevated cardio-respiratory fitness was associated with decreased Hcy concentrations in women.
Gaume *et al. (*2005) found that incremental exercises induced a decrease in Hcy and cysteine concentrations*.* According to Unt *et al.* (2008) both current physical activity and cardio-respiratory fitness are significantly inversely associated with elevated Hcy concentrations in middle-aged former athletes. De Luis *et al.* (2005) analyzed the relationship between Hcy concentrations and body composition and found that elevated Hcy concentrations were not associated with fat mass but rather with high levels of fibrinogen, lipoprotein (a), microalbuminuria, and blood pressure levels.

The availability of research on ADMA concentrations in relation to physical fitness is very limited (Richter *et al.,* 2005). According to Richter *et al.* (2005) endurance exercise was significantly related to lower ADMA concentrations. Although some studies found significant differences between concentrations of plasma Hcy and ADMA, these findings remain controversial (Jonasson *et al.,* 2003; Wanby *et al.,* 2003 and Antoniades *et al.,* 2006).

It has to be kept in mind that the majority of the available studies included participants who suffered from ailments such as renal failure, diabetes mellitus, hypertension, chronic heart

failure and lipid disorders, to name a few, whereas the AGAHL-Study participants were healthy free-living participants. This difference in participant health makes it virtually impossible to compare the studies with one another. This in particular might explain the difference in the findings between this study and some previous studies (Asagami *et al.,* 2002; Achan *et al.,* 2003 and Böger *et al.,* 2003).

According to our knowledge this study is the first to investigate the longitudinal relationship between the following variables: concentrations of Hcy and ADMA, and fatness. A limited number of studies investigated the relationship between Hcy and body fatness. Most of the available research has been done on diabetic patients or patients suffering form other diseases (De Luis *et al.,* 2005; McLaughlin *et al.,* 2006) and most of these studies investigated the relationship between ADMA and Hcy, and body composition (i.e. body weight, body fat mass and BMI). Gruber *et al.,* (2008) investigated ADMA in obese juveniles and found that ADMA is slightly increased in obese juveniles without any robust correlations to obesity related disorders.

Most of the studies yielded greatly contradicting results regarding the relationship between concentrations of Hcy and ADMA, and body composition (Eid *et al.,* 2004; Russo *et al.,* 2004; De Luis *et al.,* 2005; Soedamah-Muthu *et al.,* 2005; McLaughlin *et al.,* 2006; Gruber *et al.,* 2008). According to Szuba and Podgorski (2006) plasma concentrations of ADMA were 20% higher in patients with CVD and increased with the number of traditional risk factors, i.e. body mass index. A possible mechanism that might link body fatness to elevated ADMA concentrations can be through the increase in obesity related disorders (i.e. diabetes mellitus) and other diseases (De Luis *et al.,* 2005; McLaughlin *et al.,* 2006) or secondly, through the biochemical link between ADMA and Hcy (Russo *et al.,* 2004; Soedamah-Muthu *et al.,* 2005).

As the Hcy concentrations increase with the increase in cardiovascular risk factors, so will the ADMA concentrations increase through the biochemical link that has been established (Böger *et al.,* 2000; Böger *et al.,* 2001; Holven *et al.,* 2003; Jonasson *et al.,* 2003; Doshi *et al.,* 2005 and Ziegler *et al.,* 2005). Homocysteinemia is a metabolic abnormality that is associated with endothelial dysfunction and increased cardiovascular disease risk, but the underlying mechanisms of these effects, remain obscure (Antoniades *et al.,* 2006).

The increase in traditional risk factors leads to an increase in Hcy concentrations, and ultimately an increase in ADMA concentrations. In the present study, however, no association was found between any of the variables and concentrations of plasma Hcy and ADMA. A possible reason might be that the participants in this study were young healthy adult men and women.

A small number of other studies indicated a significant relationship between Hcy concentrations and smoking behaviour (Folsum *et al.,* 1998; Ganje & Kafai*,* 2003; Chrysohoou *et al.,* 2004; Qiao *et al.,* 2008 and Khedr *et al.,* 2009). It has been suggested that exposure to tobacco smoke may negatively impact on vitamin B_6 and folic acid levels in the body, both of which are involved in the metabolism of Hcy (Folsum *et al.,* 1998; Ganje & Kafai, 2003; Chrysohoou *et al.,* 2004; Qiao *et al.,* 2008 and Khedr *et al.,* 2009).

Additional research, especially more large longitudinal and epidemiological studies, may lead to a more in-depth understanding of the relationships between concentrations of plasma Hcy and ADMA, and their relationship with lifestyle and biological risk factors. In general, no relationships were found between (changes in) concentrations of Hcy and ADMA, and (changes in) fitness and fatness. The only significant relationship between changes in plasma Hcy and changes in fitness was partly explained by smoking behaviour.

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4

THE RELATIONSHIP BETWEEN CONCENTRATIONS OF HOMOCYSTEINE AND ASYMMETRIC DIMETHYLARGININE, AND MARKERS OF ENDOTHELIAL FUNCTION AND INFLAMMATION

ABSTRACT..... 55

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The relationship between concentrations of homocysteine and asymmetric dimethylarginine, and markers of endothelial function and inflammation

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ABSTRACT

Objective: Inflammation has received much attention as a cardiovascular risk factor. Concentrations of homocysteine (Hcy) and asymmetric dimethylarginine (ADMA) have recently been linked to inflammation. The purpose of this study was therefore to investigate the relationship between concentrations of Hcy and ADMA with markers of endothelial function and inflammation.

Methods: This study is part of the Amsterdam Growth and Health Longitudinal Study. The analysis within this ongoing observational longitudinal study consisted of 378 subjects who were measured in 2000 (at the age of 36 with n = 178 men) and 343 subjects measured in 2006 (at the age of 42 with n = 158 men). Markers of endothelial function and inflammation include E- and P-selectine, thrombomoduline, C-reactive protein (CRP), serum amyloid A (SAA), interleukin-6 (IL-6), interleukin-8 (IL-8), Von Willebrand factor (vWF), intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), and tumor necrosis factor alpha (TNF-α).

Results: Generalised estimating equations (GEE) analyses showed significant relationships between ADMA concentrations and inflammation factors ICAM (regression coefficient (b) = 71.7 [5.0 - 138.3] p=0.03) and thrombomoduline (b=0.89 [0.09 - 1.68] p=0.03). A significant relationship was also found between changes in ADMA concentrations and changes in the Von Willebrand factor (b = -42.4 [-82.8 – -2.0] p=0.04).

Conclusions: ADMA concentrations were related to inflammation cytokines ICAM and thrombomoduline, and the change in ADMA is related to the change in the vWF. Hcy was not related to markers or changes in markers of endothelial function or inflammation in apparently healthy adult men and women.

Key words: Homocysteine, asymmetric dimethylarginine, inflammation markers, endothelial function markers.

1. Introduction

Recently markers of endothelial function and inflammation have received much attention as cardiovascular risk factors [1, 2, 3]. Some of the endothelial function and inflammation markers have been recommended to be very useful in the screening and prediction of cardiovascular disorders [1, 2, 3]. Endothelial dysfunction and low grade inflammation play an important role in the onset of atherothrombosis [4, 5]. Most of the existing research focuses on either markers of endothelial function or markers of inflammation [1, 2, 3, 4, 5]. A vascular phenotype known as endothelial dysfunction is prone to atherogenesis, and might therefore be regarded as a marker of a person's atherosclerotic risk.

There are indications that ADMA is a forceful endogenous inhibitor of nitric oxide synthase (NOS) that may lead to endothelial dysfunction [6, 7]. There is emerging evidence that oxidative stress and vascular inflammation response are key factors contributing to the progression of endothelial dysfunction [8]. ADMA has also been associated with oxidative stress and vascular inflammation [9, 10, 11 12, 13, 14]. The inflammation-atherosclerosis process may play out as follows: firstly the endothelium is damaged by unhealthy conditions such as hyperhomocysteinemia, high plasma levels of ADMA, or smoking. These variables injure the artery and lead to the formation of fatty deposits (plaque). This fatty deposits activate the inflammatory response through the activation of platelets and other blood cells to the site of the plaque deposit in the intima [6].

As the blood cells and other substances continue to accumulate, the plaque grows and digs deeper into the layers of the vessel wall. More immune cells are drawn to the injured vessel, fuelling the inflammation process. Increasing inflammation can cause the plaque to break apart or rupture. Blood clots from the ruptured plaque can block blood flow to or within the heart causing a myocardial infarction or stroke.

Homocysteine (Hcy) is a sulfhydryl containing amino acid, which is formed by demethylation of methionine. Hcy is metabolised by one of two pathways, namely the remethylation or the transsulferation pathway. Hcy and ADMA share numerous presumed patho-physiological mechanisms that link both of these compounds to

vascular disease [3]. Most of these mechanisms are linked to the decrease in NO production that lead to endothelial dysfunction [3]. A number of studies did not find any significant relationship between concentrations of Hcy and ADMA with endothelial dysfunction [10, 15, 16, 17]. According to Goonasekera *et al.* [10], raised levels of ADMA concentrations and VCAM were found in the plasma of hypertensive subjects compared to those of normotensives.

It is speculated that endothelial dysfunction occurs due to an increase in concentrations of Hcy that also increase ADMA concentrations. This increase in ADMA concentrations is linked to a decrease in NOS, resulting in an increase in adhesive molecules (e.g. ICAM and VCAM). Hcy is linked to increased pro-inflammatory cytokines that also result in an increase in adhesion molecules. The endothelial activation is involved in the permeability of the vascular system that controls the homeostasis of coagulation and fibrinolysis. Increased concentrations of ADMA were observed in hypertensive children [10]. In order to investigate the relationship between concentrations of Hcy and ADMA in endothelial function, plasma levels of the recognised markers of endothelial activation such as P-selectin, E-selectin, ICAM, VCAM, thrombomoduline and vWF should be studied.

In the Amsterdam Growth and Health Longitudinal Study (AGAHLS) it is possible to investigate these relationships longitudinally. In the AGAHLS, longitudinal measurements of concentrations of Hcy and ADMA, and markers of endothelial function and inflammation were measured. Therefore, the purpose of this study is to investigate the longitudinal relationship between concentrations of Hcy and ADMA, and markers of endothelial function and inflammation. The results of this study will contribute to the possible interaction and understanding of the mechanisms involved in the link between concentrations of Hcy and ADMA, and changes in concentrations of Hcy and ADMA in healthy persons and endothelial activation and inflammation.

2. Materials and methods

2.1 Study design

This study is part of the AGAHLS (approved by the medical ethics committee of the Vrije University Medical Center, Amsterdam, The Netherlands), a longitudinal study that started in 1977 with n = 698 adolescents with a mean age of 13 [18]. Since 1977

measurements have been obtained two to eight times during a 29-year follow-up period. At each measurement, anthropometrical (stature, body mass, skin folds), biological (serum lipoprotein levels, blood pressure and physical fitness), and lifestyle (smoking behaviour, alcohol consumption, daily physical activity) variables were assessed. A more detailed description of the study is provided elsewhere [18].

Because concentrations of Hcy and ADMA, and markers of endothelial function were only assessed at the 2000 and 2006 measurements (i.e. at the ages of 36 and 42 respectively), only those measurements were used in this study. All the subjects (47.1% men) completed an informed consent before any analyses were performed.

2.2 Endothelial function and inflammation markers

Several plasma biomarkers of endothelial function [intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), endothelial selectine (E-selectine), plasma selectine (P-selectine), thrombomoduline, Von Willebrand factor (vWf)] and the inflammation markers C-reactive protein (CRP), serum amyloid A (SAA), tumor necrosis factor α (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) were assessed.

Venous blood was taken between 0800 and 0930 AM from the antecubital vein, in which plasma was separated within 60 minutes of sampling. Measurements were performed with an electro-chemiluminescence detection system using multi-array technology (SECTOR Imager 2400, Meso Scale Discovery, USA). In brief, this system uses multi-array plates fitted with four electrodes per cell, each electrode coated with a different catching antibody.

The assay procedure then follows that of a classic sandwich ELISA with any of the analytes of interest captured on the relevant electrode. These captured analytes are then detected by a secondary analyte-specific ruthenium conjugated antibody, which is capable of emitting light after electrochemical stimulation. The latter allows for the determination of the biomarker concentration of interest [19]. A particular advantage of this system is the ability to measure different biomarkers of endothelial function and/or inflammation simultaneously in relatively small serum samples (25-50 µl).

2.3 Homocysteine and asymmetric dimethylarginine

Concentrations of total plasma Hcy and ADMA were determined from venous blood that was taken between 0800 and 0900 AM from the antecubital vein. Plasma was separated within 60 minutes after sampling. Total plasma homocysteine was determined with an automated fluorescence polarization immuno-assay on an Abbott IMx analyser [20] with an inter-assay coefficient of variation of 4%. Plasma concentrations of ADMA were measured by high-performance liquid chromatography with fluorescence detection as earlier described [21], using modified chromatographic separation conditions [22]. The intra-assay and inter-assay coefficients of variation were 1.5% and 3% respectively. Samples from individual patients were analyzed in the same analytical series.

2.4 Covariates

Body fatness and body fat distribution of each individual were measured with the dual-energy X-ray absorbtiometry (DXA) [23]. Total and regional (arms, legs, trunk and head) body fat and body lean mass were measured with a whole body DXA scanner (Hologic QDR-2000, software version V5.67A; Hologic Inc., Walthan, Massachussetts, USA). Trunk fat mass was used as an estimate of a central pattern of visceral and subcutaneous fat distribution. Triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) concentrations were determined in serum drawn between 0800 and 0930 AM from the antecubital vein.

The TC, HDL-C and LDL-C concentrations were measured using standard methods with a precision of 0.01 mmol/L (0.39 mg/dl) for HDL-C/LDL-C and 0.1 mmol/L (8.86 mg/dl) for TG. External quality control were applied with target samples. Cardiopulmonary fitness was assessed with a maximal running test (VO_2max) on the treadmill in 2000 [24] and with the sub-maximal Chester step test in 2006 [25]. The Chester step test data were converted to VO_2max values, and the z-scores were statistically determined and used for analysis.

Alcohol consumption data were obtained with a quantity frequency questionnaire and are expressed as standard units consumed per day (1 unit equals 10 g of pure alcohol) [26]. A questionnaire was used to ask whether and how much tobacco was smoked by the participants. The level of habitual physical activity was assessed in a structured

interview that covered the activities of the previous three months. In this interview the intensity, frequency and duration of all daily physical activities were assessed. The average weekly time spent on the different activities were multiplied by their intensity to calculate a total weighted activity metabolic score (expressed in metabolic equivalents — MET/wk).

2.5 Statistical analysis

To investigate the longitudinal relationship between concentrations of Hcy and ADMA on the one hand, and markers of endothelial function and inflammation on the other, generalised estimating equations (GEE) were used. This method adjusts for the correlation between repeated observations taken in the same subject, and has the advantage of handling longitudinal data on subjects with varying numbers and unequally spaced observations. Interleukin-6, SAA and CRP were log transformed due to the fact that they were not normally distributed. All the relationships were first analysed in a crude analysis with adjustment for gender (model 1).

Model 2 was additionally adjusted for biological risk factors HDL-C, LDL-C, triglyceride, fitness and trunk fatness. In Model 3 additional adjustments were performed for lifestyle factors of smoking, alcohol consumption and physical activity. In all GEE analyses an exchangeable correlation structure was assumed. Linear regression analyses were used to investigate the relationships between changes in concentrations of Hcy and ADMA, and changes in markers of endothelial function and inflammation. The same three models were analysed than those for the GEE analyses. All analyses were carried out with the statistical package of Social Sciences, 16 for Windows (SPSS. Inc. Chicago, Illinois, USA).

3. Results

Table 1 shows descriptive information of the study population. According to the results of the GEE analysis (Table 2) significant positive relationships were found between plasma ADMA concentrations and markers of endothelial function, ICAM and thrombomoduline. These relationships were not influenced by other biological risk factors or lifestyle factors. When changes were analysed, the only significant relationship observed was an inverse relationship between changes in ADMA

concentrations and changes in vWF (Table 3). This relationship was also not influenced by any of the covariates.

TABLE 1 Characteristics of the participants (n=293)

Variable	36 years	42 years
Homocysteine (nmol/L)	11.5 ± 6.6	12.1 ± 5.5
Asymmetric dimethylarginine (nmol/L)	0.4 ± 0.1	0.4 ± 0.1
C-reactive protein (mg/L)*	782.5 [323.7 – 2239.3]	748.63 [340.1 – 1780.3]
Serum amyloid A (ng/ml)*	1293.9 [734.7 – 2316.6]	1344.3 [738.8 – 2340.8]
Interleukin-6 (pg/ml)*	2.4 [1.7 – 3.8]	2.4 [1.8 – 3.8]
Interleukin-8 (pg/ml)	9.3 ± 3.5	9.6 ± 3.9
Von Willebrand factor (%)	85.4 ± 33.6[a]	92.2 ± 34.3[a]
Intercellular adhesion molecule (ng/ml)	204.3 ± 51.2	199.5 ± 40.2
Vascular cell adhesion molecule (ng/ml)	333.2 ± 82.6	331.9 ± 67.7
E-selectine (ng/ml)	10.5 ± 4.8	10.1 ± 4.5
P-selectine (ng/ml)	60.2 ± 23.1[b]	71.2 ± 25.3[b]
Thrombomoduline (ng/ml)	2.5 ± 0.6	2.5 ± 0.6
Tumor necrosis factor α (pg/ml)	9.4 ± 3.4[c]	8.9 ± 3.4[c]
HDL-C (mmol/L)	1.4 ± 0.4[d]	1.7 ± 0.4[d]
LDL-C (mmol/L)	3.0 ± 0.9[e]	2.8 ± 0.8[e]
Triglyceride (mmol/L)	1.3 ± 0.9[f]	1.2 ± 0.8[f]
Physical activity (METs.min.wk^{-1}) *	4135.0 [2567.7 - 6326.7]	2727.6 [2222.2 - 3229.3]
Alcohol (glasses.wk^{-1})*	34 [8 - 63]	55 [8 – 113]
Smoking (%)	23.0[g]	15.5[g]
VO_2max (mL.min^{-1}.kg$^{-2/3}$)	34.6 ± 9.1[h]	38.9 ± 7.3[h]
Height (cm)	176.7 ± 9.3	177.2 ± 8.9
Trunk fat mass (kg)	8.2 ± 4.1[i]	8.9 ± 3.9[i]
Peripheral fat mass (kg)	11.0 ± 4.0[j]	10.1 ± 4.3[j]
Peripheral lean mass (kg)	21.5 ± 5.9[k]	25.4 ± 5.8[k]

* Data are shown as median and [25 - 75] percentiles; other data shown as means ± SD

a-k: similar letter indicate significant differences, p<0.05

TABLE 2 The relationship between concentrations of Hcy and ADMA, and markers of endothelial function and inflammation

Variable	Hcy			ADMA		
Model 1	**B**	**95% CI**	**p**	**β**	**95% CI**	**P**
SAA (ng/ml)	0.004	-0.01 to 0.02	0.54	1.70	-0.03 to 3.44	0.06
CRP (mg/l)	0.006	-0.01 to 0.02	0.49	0.28	-1.54 to 2.09	0.77
TNF-α (ng/ml)	0.001	-0.03 to 0.03	0.95	1.35	-3.06 to 5.75	0.55
IL-6 (pg/ml)	0.001	-0.01 to 0.01	0.89	-0.29	-1.12 to 0.53	0.48
IL-8 (pg/ml)	-0.024	-0.06 to 0.01	0.18	1.99	-2.75 to 6.73	0.41
Thrombomoduline (ng/ml)	0.002	-0.01 to 0.01	0.51	0.89	0.09 to 1.68	0.03*
P-selectine (ng/ml)	0.211	-0.02 to 0.44	0.07	2.09	-28.18 to 32.35	0.89
E-selectine (ng/ml)	-0.008	-0.05 to 0.03	0.65	4.24	-1.55 to 10.03	0.15
VCAM (ng/ml)	-0.568	-1.39 to 0.25	0.18	60.66	-50.90 to 172.22	0.29
ICAM (ng/ml)	-0.344	-0.84 to 0.15	-0.18	71.67	5.01 to 138.33	0.04*
vWF (%)	-0.004	-0.46 to 0.45	0.99	-6.33	-53.56 to 40.89	0.79
Model 2						
SAA	0.005	-0.01 to 0.02	0.54	1.57	-0.05 to 3.19	0.06
CRP	0.009	-0.01 to 0.03	0.35	0.21	-1.34 to 1.77	0.79
TNF-α (ng/ml)	0.005	-0.02 to 0.03	0.74	1.46	-2.88 to 5.79	0.51
IL-6 (pg/ml)	0.001	-0.01 to 0.01	0.85	-0.31	-1.13 to 0.52	0.47
IL-l8 (pg/ml)	-0.021	-0.05 to 0.01	0.20	2.09	-2.74 to 6.92	0.39
Thrombomoduline (ng/ml)	0.003	-0.00 to 0.01	0.44	0.84	0.07 to 1.61	0.03*
P-selectine (ng/ml)	0.182	-0.08 to 0.44	0.17	-1.38	-31.43 to 28.68	0.93
E-selectine (ng/ml)	-0.004	-0.05 to 0.04	0.84	4.09	-1.67 to 9.85	0.16
VCAM (ng/ml)	-0.461	-1.32 to 0.39	0.29	60.80	-47.15 to 168.76	0.27
ICAM (ng/ml)	-0.334	-0.81 to 0.14	0.17	75.49	10.33 to 140.66	0.02*
vWF (%)	0.005	-0.44 to 0.45	0.98	-9.14	-56.74 to 38.46	0.71
Model 3						
SAA (ng/ml)	0.005	-0.01 to 0.02	0.49	1.33	-0.25 to 2.90	0.09
CRP (mg/l)	0.009	-0.001 to 0.03	0.29	-0.05	-1.60 to 1.51	0.95
TNF-α (ng/ml)	0.005	-0.02 to 0.03	0.74	1.61	-2.61 to 5.84	0.46
IL-6 (pg/ml)	0.002	-0.01 to 0.01	0.14	-0.46	-1.27 to 0.36	0.27
IL-8 (pg/ml)	-0.022	-0.06 to 0.01	0.18	2.52	-2.31 to 7.34	0.31
Thrombomoduline (ng/ml)	0.003	-0.00 to 0.01	0.41	0.89	0.12 to 1.67	0.02*
P-selectine (ng/ml)	0.196	-0.06 to 0.45	0.13	0.00	-29.14 to 29.14	1.00
E-selectine (ng/ml)	-0.002	-0.04 to 0.04	0.92	4.33	-1.512 to 10.18	0.15
VCAM (ng/ml)	-0.488	-1.31 to 0.33	0.24	50.88	-56.15 to 157.89	0.35
ICAM (ng/ml)	-0.278	-0.73 to 0.18	0.23	64.89	5.70 to 124.07	0.03*
vWF (%)	0.038	-0.41 to 0.48	0.87	-15.62	-61.28 to 30.04	0.50

B is differences in concentrations of Hcy and ADMA by one unit in the particular endothelial marker, as estimated by GEE analysis.
Model 1, adjusted for gender.
Model 2, model 1 + fitness (VO_2max), HDL-C, LDL-C, triglycerides, trunk fat mass.
Model 3, model 2 + physical activity + smoking+ alcohol.
ADMA - Asymmetric dimethylarginine; CRP - C-reactive protein; Hcy - Homocysteine; ICAM - Intercellular adhesion molecule; IL-6 - Interleukin-6; IL-8 - Interleukin-8; SAA - Serum amyloid A; TNF-α - Tumor necrosis factor A; VCAM - Vascular cell adhesion molecule; vWF - Von Willebrand factor; HDL-C - High density lipoprotein cholesterol; LDL-C - Low density lipoprotein cholesterol;
* = significance [p<0.05]

TABLE 3 Relationship between changes in concentrations of Hcy and ADMA, and changes in markers of endothelial function and inflammation

Variable	Hcy			ADMA		
Model 1	**B**	**95% CI**	**p**	**β**	**95% CI**	**P**
SAA (ng/ml)	49.805	-147.81 to 247.42	0.62	-9626.27	-29980.09 to 10727.55	0.35
CRP (ng/l)	42.989	-72.69 to 158.67	0.47	-3561.98	-15494.08 to 8370.12	0.56
TNF-α (ng/ml)	0.012	-0.02 to 0.04	0.59	0.72	-2.58 to 4.02	0.67
IL-6 (pg/ml)	-0.006	-0.04 to 0.03	0.73	1.39	-1.79 to 4.59	0.39
IL-8 (pg/ml)	-0.000	-0.06 to 0.06	0.99	2.17	-3.86 to 8.19	0.48
Thrombomoduline (ng/ml)	0.001	-0.00 to 0.01	0.62	0.01	-0.58 to 0.59	0.98
P-selectine (ng/ml)	-0.043	-0.30 to 0.22	0.75	-4.67	-31.18 to 21.85	0.73
E-selectine (ng/ml)	-0.007	-0.05 to 0.03	0.72	1.03	-2.96 to 5.02	0.61
VCAM (ng/ml)	-0.545	-1.48 to 0.39	0.25	-42.49	-139.04 to 54.05	0.39
ICAM (ng/ml)	-0.032	-0.45 to 0.52	0.89	-2.18	-52.17 to 47.82	0.93
vWF (%)	-0.251	-0.65 to 0.14	0.21	-42.39	-82.81 to -1.98	0.04*
Model 2						
SAA (ng/ml)	57.065	-170.32 to 284.45	0.62	-11692.36	-34255.12 to 10870.40	0.31
CRP (mg/l)	45.073	-87.74 to 177.88	0.50	-4458.96	-17659.54 to 8741.62	0.51
TNF-α (ng/ml)	0.014	-0.02 to 0.05	0.41	0.89	-2.51 to 4.29	0.61
IL-6 (pg/ml)	-0.007	-0.04 to 0.03	0.70	1.88	-1.58 to 5.34	0.29
IL-8 (pg/ml)	-0.021	-0.09 to 0.04	0.52	0.89	-5.59 to 7.38	0.79
Thrombomoduline (ng/ml)	0.002	-0.01 to 0.01	0.58	0.09	-0.54 to 0.71	0.79
P-selectine (ng/ml)	-0.004	-0.29 to 0.28	0.98	-5.83	-34.19 to 22.53	0.69
E-selectine (ng/ml)	0.000	-0.04 to 0.04	0.96	0.44	-3.66 to 4.55	0.83
VCAM (ng/ml)	-0.616	-1.66 to 0.43	0.25	-37.32	-141.42 to 66.78	0.48
ICAM (ng/ml)	0.021	-0.50 to 0.55	0.94	-2.44	-54.69 to 49.82	0.93
vWF (%)	-0.105	-0.50 to 0.29	0.60	-41.76	-80.85 to -2.66	0.04*
Model 3						
SAA (ng/ml)	49.885	-227.17 to 326.94	0.72	-21308.29	-38709.19 to 14092.62	0.36
CRP (mg/l)	43.443	-112.71 to 199.59	0.58	-6468.15	-21359.08 to 8422.79	0.39
TNF-α (ng/ml)	0.010	-0.03 to 0.05	0.59	0.88	-2.68 to 4.44	0.63
IL-6 (pg/ml)	-0.005	-0.05 to 0.04	0.82	1.81	-2.04 to 5.65	0.36
IL-8 (pg/ml)	-0.029	-0.10 to 0.04	0.43	0.79	-6.23 to 7.82	0.82
Thrombomoduline (ng/ml)	0.003	-0.00 to 0.01	0.35	0.19	-0.43 to 0.81	0.54
P-selectine (ng/ml)	-0.086	-0.41 to 0.23	0.59	2.06	-28.43 to 32.55	0.89
E-selectine (ng/ml)	-0.005	-0.05 to 0.04	0.82	1.23	-2.82 to 5.29	0.55
VCAM (ng/ml)	-0.660	-1.76 to 0.44	0.24	-54.83	-159.89 to 50.23	0.31
ICAM (ng/ml)	0.05	-0.55 to 0.56	0.99	-10.16	-63.16 to 42.85	0.71
vWF (%)	-0.257	-0.66 to 0.15	0.21	-53.98	-92.09 to -15.88	0.01*

B is differences in concentrations of Hcy and ADMA by one unit in the particular endothelial marker, as estimated by linear regression analysis.
Model 1, adjusted for gender.
Model 2, model 1 + fitness (VO_2max), HDL-C, LDL-C, triglycerides, trunk fat mass.
Model 3, model 2 + physical activity + smoking+ alcohol.
ADMA - Asymmetric dimethylarginine; CRP - C-reactive protein; Hcy - Homocysteine; ICAM - Intercellular adhesion molecule; IL-6 - Interleukin-6; IL-8 - Interleukin-8; SAA - Serum amyloid A; TNF-α - Tumor necrosis factor A; VCAM - Vascular cell adhesion molecule; vWF - Von Willebrand factor, HDL-C - High density lipoprotein cholesterol; LDL-C - Low density lipoprotein cholesterol;
* = significance [p<0.05]

4. Discussion

The objective of this study was to investigate the relationships between (changes in) concentrations of Hcy and ADMA, and changes in markers of endothelial function and inflammation. This study forms part of the overarching AGAHL-study, and focuses only on the two consecutive datasets (2000 and 2006 respectively). The results of this study indicate a significant relationship between ADMA concentrations, and thrombomoduline and ICAM. This relationship is constant when adjusted for gender, fitness, fatness and lifestyle factors (smoking, alcohol and physical activity).

The relationship indicates that an increase in ADMA results in an increase in thrombomoduline and ICAM. Thrombomoduline is a luminal surface molecule that inhibits coagulation and stimulated fibrinolysis in the circulatory system [27]. ICAM on the other hand is a cell adhesion molecule that indicates an increase in concentration with an increase in ADMA concentrations. Nanayakkara *et al.* [14] found an inverse positive association between ADMA and plasma VCAM concentrations, in patients with mild to moderate renal failure. According to Goonasekera et al. [10] higher VCAM and vWF concentrations were significantly associated with higher plasma ADMA concentrations in the hypertensive subjects.

The increase in ADMA concentration inhibits NOS, which is indicated to inhibit endothelial activation, favouring coagulation and increasing permeability of the vascular system [28]. The increased permeability of the endothelium allows for the inflow of leukocytes in order to initiate the inflammatory response. The increase in thrombomoduline complements the increase in permeability with an increase in the binding sites for thrombin as part of the haemostatic cascade [27].

The hypothesised pathway through which Hcy is linked to endothelial function is via an increase in pro-inflammatory cytokines (i.e. TNF-α) or an increase in oxidative stress [29]. Oxidative stress is accompanied by a decrease in DDAH enzyme activity that inhibits NOS, that once again results in endothelial activation [30].

The relationship between changes in concentrations of Hcy and ADMA with endothelial and inflammatory markers revealed a significant inverse relationship between changes in ADMA concentrations and changes in vWf.

Von Willebrand Factor is a marker of endothelial function and is involved in coagulation and platelet adhesiveness [31]. There are only a small number of studies that investigated the relationship between concentrations of Hcy and ADMA, and markers of endothelial function and inflammation [3, 4, 5]. The findings of this study suggest that ADMA concentrations may be involved in the inflammation processes linked to endothelial dysfunction. According to Zocalli [12] accumulation of ADMA is an important risk factor for cardiovascular disease in chronic renal failure patients, and suggests a possible link between ADMA and inflammation.

In comparison to the above-mentioned study, our study was conducted in a young healthy population and therefore it might be the reason for the limited significant relationships found, since our subjects were healthy and free living young adults in comparison with the unhealthy subjects included in most of the other studies.

From the results of the AGAHLS, concentrations of Hcy and changes in concentrations of Hcy are not related to endothelial function. However, ADMA concentrations and changes in ADMA concentrations indicate significant relationships with markers of endothelial dysfunction, namely ICAM and vWF. These results allude to the fact that an increase in ADMA concentrations activates fibrinolysis. This may be a compensatory response in anticipation of the procoagulatory state induced by increased ADMA concentrations.

In conclusion, the present study showed a significant relationship between ADMA concentrations and endothelial markers ICAM and thrombomoduline, as well as a significant relationship between changes in ADMA and changes in vWF between healthy persons 36 and 42 years of age. No relationship was found between (changes in) concentrations of Hcy, and (changes in) endothelial function and inflammation markers.

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5

THE RELATIONSHIP BETWEEN CONCENTRATIONS OF HOMOCYSTEINE AND ASYMMETRIC DIMETHYLARGININE AND ARTERIAL PROPERTIES (STIFFNESS AND THICKNESS)

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The relationship between concentrations of homocysteine and asymmetric dimethylarginine, and arterial properties (stiffness and thickness)

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ABSTRACT

Objective: Changes in concentrations of homocysteine (Hcy) and asymmetric dimethylarginine (ADMA) are assumed to be related to atherosclerosis, but little is known about the relationship between Hcy and ADMA and arterial properties in healthy adults. The purpose of this study was to investigate the relationships between (changes in) concentrations of Hcy and ADMA and (changes in) arterial properties in adulthood.

Design: This study is part of the Amsterdam Growth and Health Longitudinal Study.

Participants and methods: The analyses were performed on 293 participants who were measured in both 2000 and 2006 (n=138 men and n=155 women). GEE analysis was used to analyse the relationships of Hcy and ADMA concentrations with arterial properties (carotid intima-media thickness, carotid and femoral artery distensibility and compliance, and carotid Young's elastic modulus). Linear regression analyses were used to analyse the relationships between changes in concentrations of Hcy and ADMA and changes in arterial properties. Analyses were performed without and with adjustment for traditional biochemical cardiovascular disease risk factors and lifestyle factors.

Results: No significant relationships were found between Hcy and ADMA absolute values or changes in concentrations of Hcy and ADMA, and arterial properties.

Conclusion: Concentrations of Hcy and ADMA and changes in concentrations of Hcy and ADMA are not related to arterial properties (stiffness and thickness) in healthy men and women.

Key words

Homocysteine, asymmetric dimethylarginine, mean blood pressure, intima-media thickness, carotid artery, femoral artery.

Introduction

Elevated concentrations of plasma homocysteine (Hcy) and asymmetric dimethylarginine (ADMA) are identified as biochemical markers of increased risk for development of cardiovascular disease (CVD) [1, 2]. Hcy is an amino acid that is formed during the metabolism of methionine and it is metabolised by one of two pathways, the remethylation or transsulfuration pathway [3]. Elevated concentrations of Hcy, i.e. hyperhomocysteinemia (HHcy), have been linked to increased risk of developing atherosclerotic diseases [1, 2]. The impact of elevated Hcy concentrations might be clinically relevant in view of the fact that the total cardiovascular risk that is associated with hyperhomocysteinemia may be comparable to the risk associated with for instance smoking or hyperlipidemia [4].

Homocysteine is a nitric oxide synthase (NOS) inhibitor that reduces the production of nitric oxide (NO), subsequently leading to endothelial dysfunction [5]. A reduction in the availability of NO may cause impaired endothelium-dependent vaso-relaxation in HHcy patients [4, 5]. Impaired endothelial function occurs before the onset of overt plaques in humans who suffer from atherosclerosis [4].

Asymmetric dimethylarginine is an L-arginine analogue that plays an important role in the endogenous mechanism that regulates NO synthase and increases adhesion of monocytes to the endothelium [6, 7]. An interrelationship between elevated concentrations of Hcy and ADMA, and endothelial dysfunction has been suggested [8, 9, 10, 11, 12].

Similar to elevated concentrations of Hcy, elevated ADMA concentrations were also related to impaired endothelium-dependent relaxation in HHcy patients [4]. Elevated concentrations of Hcy and ADMA have thus been acknowledged to deteriorate endothelial function, eventually leading to atherosclerosis [9, 10, 13, 14, 15, 16]. Hardly any studies have investigated arterial properties in relation to concentrations of Hcy and ADMA. The available studies focused on mostly investigated intima media thickness (IMT), whereas distensibility, compliance and Young’s elastic modulus rarely shed additional light on the relationship of Hcy and DAMA with arterial properties, nor any of the other arterial properties measured in this study [17, 18, 19, 20].

In the Amsterdam Growth and Health Longitudinal Study (AGAHLS) it is possible to investigate these relationships longitudinally. In the AGAHLS, arterial properties as well as concentrations of Hcy and ADMA were measured in the same men and women at the age of 36 and 42 years respectively. The purpose of this study was therefore to investigate the (longitudinal) relationship between concentrations of Hcy and ADMA with regard to the arterial properties of the common carotid and femoral arteries.

Participants and methods

Study Design

This study forms part of the overarching AGAHLS (approved by the medical ethics committee of the Vrije University Medical Center, Amsterdam, The Netherlands), an observational longitudinal study that started in 1977 with n=698 adolescents with a mean age of 13 years. Since 1977, nine measurement waves have been performed during a 29-year follow-up period. At each measurement, anthropometrical (stature, body mass, skin folds), biological (serum lipoprotein levels, blood pressure and physical fitness), and lifestyle (smoking behaviour, alcohol consumption and daily physical activity) variables were assessed. A more detailed description of the study is provided elsewhere [21].

Hcy, ADMA and arterial properties were only assessed at the 2000 and 2006 measurement waves (i.e. at the age of 36 and 42 years respectively). Only those measurements were used in the present study. All the participants (n = 293; 47.1% men) completed an informed consent before any assessment was performed.

Arterial Properties

Arterial properties of the right common carotid and femoral arteries were measured by means of ultrasound scanner equipment with a 7.5-MHz linear array probe (Pie Medical, Maastricht, The Netherlands). The scanner was connected to a computer equipped with an acquisition system and vessel wall movement detector software system (Wall Track System 2, Pie Medical, Maastricht, The Netherlands) (WTS_2). This device enables measurements of arterial diameter, distention and intima-media

thickness [22]. Temporarily, after fifteen minutes of rest, a visual image was taken of the specific artery in B-mode after which an M-line perpendicular was placed at the measured site. Whilst in M-mode, data acquisition was enabled after the lumen of the specific artery was identified (with the mouse) in real time A-mode presentation on the computer.

Ultrasound data were taken for a period of four seconds (including 3 - 7 heartbeats) that was stimulated by the R-top of the concurrently recorded electrocardiograph. The radio frequency signal on the screen made it possible to see if the markers that were automatically placed by the WTS_2 matched the anterior (adventitia-media) and the posterior (media-adventitia) vessel wall reflections in the diastolic phase of the cardiac cycle. The cumulative radio frequency signals were then digitised and stored on the computer.

Changes in diameter as a function of time (distension [ΔD]) were estimated and presented on the computer (distension wave form). The diastolic diameter (D) was calculated as the difference in position between anterior and posterior vessel wall markers. Based on the radio frequency signals of the carotid posterior wall, the distance from the leading edge interface between the lumen and the intima to the leading edge interface between the media and the adventitia was automatically calculated as the intima-media complex. The mean diameter, distension and intima-media thickness (IMT) of the three consecutive measurements were used in the analysis.

Measurement of the carotid artery was taken 10 mm proximal to the beginning of the bulb. The femoral artery measurement was taken 20 mm proximal to the flow divider. Systolic and diastolic blood pressure was assessed in the left arm at 5-minute intervals with an oscillometric device during the entire period of the ultrasound imaging (Colin Press-Mate, Komaki City, Japan; model BP-8800). Pulse pressure of the carotid and femoral arteries were calculated by calibration on the distension wave forms.

Diameter, distension and mean pulse pressure of the three measurements, as described above, were used to estimate the distensibility (DC) and compliance coefficients (CC) as follows:

$$DC = (2\Delta D.D+\Delta D^2)/(PP \cdot D^2) \text{ in } 10^{-3}kPa^{-1}$$

$$CC = \pi\ (2D \cdot \Delta D+\Delta D^2)/4PP \text{ in } mm^2kPa^{-1}$$

Distensibility, as defined above, reflects the elastic properties of the artery, whereas the compliance reflects the buffering capacity of the artery. From IMT, diameter and distensibility coefficient of the carotid artery, the Young elastic modulus (YEM) was calculated. YEM is indicative of the intrinsic elastic properties of the vessel wall.

$$YEM = D/(IMT \cdot DC) \text{ in kPa}$$

To obtain an overall measure for arterial properties, the sum of the z-scores for all six indicators ((IMT); carotid artery (CC) (DC); femoral artery (CC) (DC); YEM - Young's elastic modulus) were calculated. Z-score of peripheral lean mass, the peripheral fat mass, and trunk fat mass were inversed because high values indicate bad-good arterial properties.

Biochemical Analysis of Hcy and ADMA

Concentrations of total plasma Hcy and ADMA were determined from venous blood that was taken between 0800 and 0900 from the antecubital vein. Plasma was separated within 60 minutes after sampling. Total plasma Hcy was determined with an automated fluorescence polarization immunoassay on an Abbott IMx analyser [23] with an inter-assay coefficient of variation of 4%. Plasma concentrations of ADMA were measured with high-performance liquid chromatography with fluorescence detection as earlier described [24], using modified chromatographic separation conditions [25]. The intra-assay and inter-assay coefficients of variation were 1.5% and 3%, respectively. Samples from individual patients were analyzed in the same analytical series.

Covariates

Body fatness and body fat distribution of each individual were measured with dual-energy X-ray absorbtiometry (DXA) [26]. Total and regional (arms, legs, trunk and head) body fat and body lean mass were measured with a whole body DXA scanner (Hologic QDR-2000, software version V5.67A; Hologic Inc, Walthan,

Massachussetts, USA). Peripheral fat was calculated by adding the fat mass of the legs to the fat mass of the arms, and peripheral lean mass was calculated by adding the lean mass of the legs to the lean mass of the arms. Whole body fat mass was used as an estimate of total body fatness and trunk fat mass was used as an estimate of a central pattern of fat (visceral and subcutaneous) distribution. Body height was measured according to standard procedures.

The cardiopulmonary fitness of the participants was assessed with a maximal running test (VO_2max) on a treadmill [27] in 2000 and with the sub-maximal Chester step test in 2006 [28]. The Chester step test data were converted to VO_2max values, and the z-scores were statistically determined and used in the analysis. The level of habitual physical activity was assessed in a structured interview that covered the activities of the previous three months. In this interview the intensity, frequency and duration of all daily physical activities (at school, at work, in organised and unorganised sports, during leisure time, climbing stairs and transportation) were assessed. Only those activities with duration of at least five minutes and intensity of more than four times the basal metabolic rate (METs) were considered. The physical activities were classified as light (4–7 METs), medium-heavy (7–10 METs) and heavy (>10 METs), based on values found in the literature [29]. The average weekly time spent on the different activities was multiplied by their intensity to calculate a total weighted activity metabolic score (expressed in metabolic equivalents — MET·wk^{-1}).

Alcohol consumption data were obtained with a quantity frequency questionnaire and are expressed as standard units consumed per day (1 unit equals 10 g of pure alcohol) [30]. Non-fasting triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) cholesterol concentrations were determined in serum drawn between 0800 and 0930 am from the antecubital vein. The TC, HDL-C and LDL-C levels were measured using standard methods with a precision of 0.01 mmol/L (0.39 mg/dL) for HDL and LDL cholesterol and 0.1 mmol/L (8.86 mg/dL) for TG [22]. External quality control was applied with target samples.

Statistical Analysis

To investigate the longitudinal relationship of concentrations of plasma Hcy and ADMA on the one hand and arterial properties (IMT and arterial stiffness) on the other, generalised estimating equations (GEE) were used. GEE adjust for the correlation between repeated observations taken in the same participants and have the advantage of handling longitudinal data on participants with varying numbers and unequally spaced observations.

All the relationships were first analysed with adjustment for gender, height and mean blood pressure (Model 1). Model 2 was additionally adjusted for the biological CVD risk factors HDL-C, LDL-C, triglyceride, fitness and trunk fatness. In Model 3 additional adjustments were performed for the lifestyle factors such as smoking, alcohol consumption and physical activity. In all GEE analyses an exchangeable correlation structure was assumed. Linear regression analyses were used to investigate the relationships between changes in concentrations of Hcy and ADMA, and changes in arterial properties. The same three models were analysed as those for the GEE analyses. All analyses were carried out with the statistical package of Social Sciences, 16 for Windows (SPSS. Inc. Chicago, Illinois, USA).

Results

Table 1 shows the descriptive information of the participants at age 36 and 42 years respectively. Significant differences at baseline were observed for the measurements that were taken in 2000 and 2006. Significant increases were observed in HDL-C, trunk fat mass and peripheral lean mass. Significant decreases were observed in; LDL-C, triglyceride concentrations, number of cigarettes smoked, VO_2max and peripheral fat mass.

The results of the GEE analysis (Table 2) showed no significant relationships of concentrations of plasma Hcy and ADMA with arterial properties. The relationship found is, however, inverse for concentrations of ADMA with the arterial properties. Higher beta scores were also observed for concentrations of ADMA with arterial properties than with concentrations of Hcy with arterial properties.

Table 3 shows the results of the linear regression analysis regarding changes in concentrations of plasma Hcy and ADMA, and changes in arterial properties. No significant associations were found.

Table 1 Characteristics of the participants mean ± SD (n = 293)

Variable	36 years	42 years
Hcy (nmol/L)	11.5 ± 6.6	12.1 ± 5.5
ADMA (nmol/L)	0.4 ± 0.1	0.4 ± 0.1
HDL-C (mmol/L)	1.4 ± 0.4[a]	1.7 ± 0.4[a]
LDL-C (mmol/L)	3.0 ± 0.9[b]	2.8 ± 0.8[b]
Triglyceride (mmol/L)	1.3 ± 0.9[c]	1.2 ± 0.8[c]
MBP (mmHg)	82.0 ± 8.6	85.0 ± 9.9
Carotid IMT (mm)	0.6 ± 0.1	0.7 ± 0.1
CA DC (10^{-3} kPa^{-1})	26.6 ± 6.1	25.6 ± 7.7
CA CC ($mm^{2.}$ kPa^{-1})	0.9 ± 0.3	1.0 ± 0.4
FA DC (10^{-3} kPa^{-1})	7.2 ± 3.8	8.4 ± 4.5
FA CC ($mm^{2.}$ kPa^{-1})	0.5 ± 0.2	0.6 ± 0.3
YEM (kPa)	0.4 ± 0.1	0.5 ± 0.2
PA (kMETs.min/wk)*	4.1 [2.6 – 6.3]	2.7 [2.2 – 3.2]
Alcohol (units/wk)*	3.4 [0.8 – 6.3]	5.5 [0.8 – 11.3]
Smoking (%)	23.0[d]	15.5[d]
VO_2max ($mL/min.kg^{-2/3}$)	34.6 ± 9.1[e]	38.9 ± 7.3[e]
Height (cm)	176.7 ± 9.3	177.2 ± 8.9
Trunk fat mass (kg)	8.2 ± 4.1[f]	8.9 ± 3.9[f]
Peripheral fat mass (kg)	11.0 ± 4.0[g]	10.1 ± 4.3[g]
Peripheral lean mass (kg)	21.5 ± 5.9[h]	25.4 ± 5.8[h]

* Data are shown as median and [25 – 75] percentiles;
a-h: similar letters indicate significant differences, $p<0.05$.
ADMA - Asymmetric dimethylarginine; HDL-C - High density lipoprotein cholesterol; LDL-C – low density lipoprotein cholesterol; MBP - mean blood pressure; IMT - intima-media thickness; CA - carotid artery; FA - femoral artery; CC - compliance coefficient; DC - distensibility coefficient; YEM - Young's elastic modulus; PA – Physical activity.

Table 2 The relationship between concentrations of Hcy and ADMA, and arterial properties (n = 293)

Variable	Hcy			ADMA		
Model 1	**B**	**95% CI**	**P**	**B**	**95% CI**	**P**
CA DC (10^{-3} kPa^{-1})	-0.003	-0.01 to 0.01	0.54	0.85	-0.28 to 1.98	0.14
CA CC (mm^2. kPa^{-1})	-0.006	-0.01 to 0.00	0.14	0.58	-0.45 to 1.61	0.27
FA DC (10^{-3} kPa^{-1})	0.004	-0.01 to 0.02	0.54	-0.50	-1.78 to 0.77	0.44
FA CC (mm^2.kPa^{-1})	0.000	-0.01 to 0.01	0.98	-0.75	-1.93 to 0.44	0.22
YEM (kPa)	0.000	-0.01 to 0.01	0.89	-1.09	-2.39 to 0.19	0.09
Σ AP	-0.002	-0.01 to 0.01	0.59	0.19	-0.61 to 0.99	0.64
IMT (mm)	-0.005	-0.01 to 0.00	0.23	0.82	-0.31 to 1.95	0.16
Model 2						
CA DC (10^{-3} kPa^{-1})	-0.002	-0.01 to 0.01	0.60	0.73	-0.34 to 1.86	0.21
CA CC (mm^2. kPa^{-1})	-0.006	-0.01 to 0.00	0.19	0.53	-0.50 to 1.57	0.31
FA DC (10^{-3} kPa^{-1})	0.003	-0.01 to 0.02	0.63	-0.58	-1.83 to 0.67	0.37
FA CC (mm^2. kPa^{-1})	0.000	-0.01 to 0.01	0.96	-0.81	-1.93 to 0.32	0.16
YEM (kPa)	0.000	-0.01 to 0.01	0.91	-1.04	-2.31 to 0.23	0.11
Σ AP	-0.002	-0.01 to 0.01	0.58	0.13	-0.65 to 0.91	0.74
IMT (mm)	-0.006	-0.02 to 0.00	0.22	0.86	-0.26 to 1.98	0.13
Model 3						
CA DC (10^{-3} kPa^{-1})	-0.002	-0.01 to 0.01	0.69	0.75	-0.37 to 1.88	0.19
CA CC (mm^2. kPa^{-1})	-0.005	-0.01 to 0.00	0.21	0.48	-0.54 to 1.52	0.37
FA DC (10^{-3} kPa^{-1})	0.003	-0.01 to 0.02	0.64	-0.54	-1.82 to 0.73	0.40
FA CC (mm^2. kPa^{-1})	0.000	-0.01 to 0.01	0.93	-0.71	-1.85 to 0.42	0.22
YEM (kPa)	-0.001	-0.01 to 0.01	0.79	-1.07	-2.31 to 0.18	0.09
Σ AP	-0.002	-0.01 to 0.01	0.61	0.15	-0.62 to 0.91	0.71
IMT (mm)	-0.005	-0.01 to 0.00	0.25	0.79	-0.33 to 1.94	0.17

B are differences in arterial properties by one unit difference in concentrations of Hcy and ADMA, as estimated by GEE analysis.
Model 1, adjusted for gender, mean-blood pressure, height.
Model 2, Model 1 + fitness (VO_2max), HDL-C, LDL-C, triglycerides, sum of trunk fat mass.
Model 3, Model 2 + physical activity + smoking + alcohol.
(CI – Confidence Interval; ADMA - Asymmetric dimethylarginine; IMT - Intima-media thickness; CA - Carotid artery; FA - Femoral artery; CC - Compliance coefficient; DC - Distensibility coefficient; YEM - Young's elastic modulus; PA – Physical activity, Σ AP - Sum of arterial properties; HDL-C - high density lipoprotein cholesterol; LDL-C - Low density lipoprotein cholesterol).

Table 3 Relationship between changes in concentrations of Hcy and ADMA, and changes in arterial properties (n = 293)

Variable	Hcy			ADMA		
Model 1	**B**	**95% CI**	**P**	**B**	**95% CI**	**P**
CA DC (10^{-3} kPa^{-1})	-0.003	-0.02 to 0.01	0.66	0.85	-0.28 to 1.98	0.14
CA CC (mm^2. kPa^{-1})	-0.004	-0.02 to 0.01	0.53	0.53	-0.85 to 1.91	0.45
FA DC (10^{-3} kPa^{-1})	0.003	-0.01 to 0.02	0.73	-0.82	-2.32 to 0.68	0.28
FA CC (mm^2. kPa^{-1})	0.002	-0.01 to 0.02	0.73	-0.71	-2.09 to 0.66	0.31
YEM (kPa)	0.004	-0.01 to 0.02	0.57	-0.55	-2.07 to 0.97	0.48
Σ AP	-0.002	-0.01 to 0.01	0.73	0.04	-0.97 to 1.04	0.95
IMT (mm)	-0.005	-0.02 to 0.01	0.48	0.12	-1.33 to 1.58	0.87
Model 2						
CA DC (10^{-3} kPa^{-1})	-0.003	-0.02 to 0.01	0.73	0.83	-0.81 to 2.47	0.32
CA CC (mm^2. kPa^{-1})	-0.004	-0.02 to 0.01	0.65	0.50	-0.99 to 1.99	0.51
FA DC (10^{-3} kPa^{-1})	-0.002	-0.02 to 0.02	0.86	-0.84	-2.46 to 0.78	0.31
FA CC (mm^2. kPa^{-1})	-0.002	-0.02 to 0.01	0.84	-0.76	-2.24 to 0.72	0.31
YEM (kPa)	0.007	-0.01 to 0.02	0.43	-0.79	-2.45 to 0.85	0.34
Σ AP	-0.004	-0.02 to 0.01	0.53	0.05	-1.04 to 1.14	0.93
IMT (mm)	-0.009	-0.03 to 0.01	0.23	0.26	-1.26 to 1.78	0.74
Model 3						
CA DC (10^{-3} kPa^{-1})	-0.004	-0.02 to 0.02	0.69	0.91	-0.88 to 2.69	-0.87
CA CC (mm^2. kPa^{-1})	-0.004	-0.02 to 0.01	0.67	0.59	-0.99 to 2.17	0.47
FA DC (10^{-3} kPa^{-1})	0.002	-0.02 to 0.02	0.87	-1.09	-2.81 to 0.64	0.22
FA CC (mm^2. kPa^{-1})	-0.003	-0.02 to 0.02	0.85	-0.93	-2.48 to 0.63	0.24
YEM (kPa)	0.002	-0.02 to 0.02	0.85	-1.01	-2.79 to 0.77	0.27
Σ AP	-0.001	-0.01 to 0.01	0.82	0.05	-1.08 to 1.19	0.93
IMT (mm)	-0.006	-0.02 to 0.01	0.47	0.88	-0.71 to 2.46	0.28

B are differences in changes in arterial properties by one unit difference in changes in concentrations of Hcy and ADMA, as estimated by linear regression analysis.
Model 1, adjusted for gender, mean blood pressure, height.
Model 2, Model1 + fitness (VO_2max); HDL-C, LDL-C, triglycerides, sum of trunk fat mass.
Model 3, Model 2 + physical activity + smoking + alcohol.
(CI – Confidence Interval, ADMA - Asymmetric dimethylarginine; IMT - Intima-media thickness; CA - Carotid artery; FA - Femoral artery; CC - Compliance coefficient; DC - Distensibility coefficient; YEM - Young's elastic modulus; PA – Physical activity; Σ AP - Sum of arterial properties; HDL-C - high density lipoprotein cholesterol; LDL-C - Low density lipoprotein cholesterol, * = significance [$p<0.05$]).

Discussion

The objective of this study was to investigate the relationships between concentrations of Hcy and ADMA, and arterial properties of the common carotid and femoral arteries. Significant differences between the baseline measurements at age 36 and 42 years were found for HDL-C, LDL-C, triglycerides, smoking, VO_2max, trunk fat mass, peripheral fat mass and peripheral lean mass. No significant relationships were found between concentrations of Hcy and ADMA with arterial properties or between the changes in these variables. Although very limited, there are studies that found a relationship between the Hcy, ADMA and arterial properties (especially IMT) [31, 32, 33].

Some evidence indicated that concentrations of Hcy and ADMA might be closely related to endothelial function [8, 9, 10, 11, 12, 31, 32, 33]. ADMA acts as a NOS inhibitor that promotes atherogenesis by impairing vasodilator function and opposing the vasoprotective effects of NO [9]. Interestingly Juonala *et al.* [34] found that elevated plasma ADMA concentrations are associated with decreased brachial flow-mediated vasodilatation responses in healthy adults, and also found no significant association to the carotid or femoral arteries. These data can be considered to provide evidence at the population level that ADMA levels are associated with endothelial function [34]. As the same results were found by Juonala *et al*. [34], ADMA concentrations are therefore concluded to be associated with endothelial function at the population level.

According to Furuki *et al.* [17] plasma levels of ADMA can be regarded as a strong and independent determinant of IMT of the carotid artery in the large number of subjects without overt cerebro-cardiovascular diseases. Cable *et al.* [33] investigated the direct vaso-active properties of ADMA in different arterial beds and found that endothelium-dependent relaxation mediated by ADMA is more marked in femoral and renal arteries than in coronary arteries. These responses in coronary arteries may be overall protective, considering the different effects in various artery types. Therefore the role of ADMA as a confinable and specific cardiovascular risk factor is thus questionable.

According to available literature, endothelial dysfunction is the major mechanism through which Hcy induces its deleterious effect that ultimately leads to atherosclerosis [1, 2, 4, 5]. Atherosclerosis, on the other hand, can be explained in terms of the "response to injury" and the "lipid infiltration" hypotheses [37]. According to the "response to injury"' hypothesis, morphological changes occur in endothelial and sub-endothelial layers of arterial walls. These changes are ascribed to an inflammatory reaction to certain stimuli (i.e. changes in blood flow, hypercholesterolemia [14], hyperhomocysteinemia [15] and increased ADMA concentrations [38]. The "lipid infiltration" hypothesis [39] postulates that increased circulating levels of LDL-cholesterol lead to an increase in transcytosis of LDL-cholesterol to the intima, and the endothelial injury is secondary to sub-endothelial events [16].

It is difficult to compare our study with existing studies. Most of the other studies are intervention studies [8, 11, 12, 13, 17, 31, 32, 33, 34]. Also, relationships in the literature search between arterial properties, and concentrations of Hcy and ADMA were consistently reported in participants at an older age than the age of the participants of the AGAHLS [17, 18, 34]. Another reason for the lack of association might be that all of the AGAHLS participants (n=293) were healthy and free living in comparison to other studies that only included diseased participants [13, 19, 20].

The fact that the vaso-active properties of ADMA is dissimilar in different arterial beds and that the endothelium-dependent relaxation mediated by ADMA is more marked in femoral and renal arteries than in coronary arteries, has to be kept in mind [33]. The responses in the coronary arteries may be protective and different effects are found in different types of arteries. The fact that only the carotid and femoral arteries where measured within the AGAHLS may in part also explain our results, and future research might include additional types of arteries (femoral, carotid, brachial and renal arteries etc.). As established through the literature, the apoptosis of the smooth muscle cells induced by the increase in Hcy concentrations is related to the stimulation of increased ADMA production, thus affecting arterial properties.

Hcy and ADMA concentrations in relation to the diminishment of arterial properties might therefore only be detected at an older age and/or in combination with the onset

of other CVD diseases. Therefore it remains essential to re-evaluate the subjects of the AGAHLS over time, in an effort to determine the exact age at which arterial properties are diminished and whether it can be related to the deleterious effects of changes in Hcy and ADMA concentrations. A study by Cable *et al.* [33] found a relationship between concentrations of ADMA and arterial properties of the coronary arteries, but no relationship with femoral and renal arteries in canines. The AGAHLS included human participants, while most other studies reported findings in animals such as rats [11], mice [32] and canines [33].

Regardless of all the proposed mechanisms and the controversial results of the limited studies, the exact mechanism through which elevated concentrations of Hcy and ADMA influence arterial properties remains unanswered. It is therefore important that more longitudinal epidemiological studies are undertaken, in order to determine the exact relationships between the arterial properties and these recently identified cardiovascular disease risk factors (i.e. Hcy and ADMA).

Conclusion

The conclusion that can be drawn from the results of this study, is that no significant (longitudinal) relationships between concentrations of Hcy and ADMA, and arterial properties in apparently healthy, young adult human participants were observed. There was also no significant relationship observed in the changes in concentrations of Hcy and ADMA with changes in arterial properties.

Acknowledgements

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Acknowledgement is given for the financial support towards this study by both the National Research Foundation of South Africa and the Vrije University of Amsterdam, The Netherlands.

Abbreviations

Hcy: homocysteine; ADMA: asymmetric dimethylarginine; HHcy: hyperhomocysteinemia; NO: nitric oxide; DDAH: dimethylarginine dimethylaminohydrolase; AGAHLS: Amsterdam Growth and Health Longitudinal Study; D: diastolic diameter; IMT: intima-media thickness; MBP: mean blood pressure; DC: distensibility coefficient; CC: compliance coefficient; YEM: Young's elastic modulus; GEE: generalised estimating equations; CA: carotid artery; FA: femoral artery, HDL: high density lipoprotein; LDL: low density lipoprotein; PA: physical activity; VO_2max: physical fitness; AP: arterial properties.

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6

CONCLUSIONS

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6.1 INTRODUCTION

The general purpose of this study was to investigate the relationship between concentrations of homocysteine (Hcy) and asymmetric dimethylarginine (ADMA), fitness, fatness, endothelial function, arterial properties and inflammation markers in healthy adults from the ongoing observational longitudinal study, AGAHLS (Amsterdam Growth and Health Longitudinal Study). As the incidence of cardiovascular disease is increasing with age all around the world, the AGAHLS was deemed a perfect study to investigate the interactions between cardiovascular risk factors such as concentrations of Hcy and ADMA, fitness, fatness, markers of endothelial function and inflammation, and arterial properties.

The AGAHLS started in 1977 with a group of 13-year old subjects, initially to investigate the longitudinal relationship between biological and lifestyle variables. The subjects have been measured repeatedly over time and the last measurements were done in 2006 when the study population was 42 years old. For purposes of this thesis, the longitudinal analyses of the variables that were measured in 2000 (aged 36) and 2006 (aged 42) respectively were used. This chapter summarised the findings of the relationship between Hcy and ADMA concentrations and fitness, fatness, markers of endothelial function, inflammation and arterial properties. The conclusions of this study are presented as derived from the stated hypotheses. The limitations of this investigation are also presented, together with recommendations for future investigations.

6.2 SUMMARY

Chapter 1 introduces the problem statement, aim and hypothesis of the study, and explained the structure of the study. Chapter 1 also aids as an introduction to the study, by giving a short description of the various variables and the objectives of the various studies to be undertaken. Although plenty of research were conducted on traditional CVD risk factors, the opposite is true for some recently identified risk factors and/or markers of CVD such as Hcy and ADMA. Hcy and ADMA increase the risk of developing atherosclerotic diseases. According to the available literature there is a metabolic link between Hcy and ADMA, but interestingly enough the inter-relationship between Hcy and ADMA in relation to lifestyle and biological risk factors has not yet been studied.

The investigation of the above-mentioned relationship are very important if one is to understand the underlying mechanisms involved in endothelial function, inflammation and arterial properties, as it might assist in the prevention of CVD and therefore improve the health care of patients that suffer from CVD. Various studies have suggested that elevated concentrations of Hcy are related to increased concentrations of ADMA. ADMA on the other hand, had been related to nitric oxide (NO) concentrations that regulate the dilatation of the vascular system. Therefore it has been suggested that Hcy concentrations might be related to ADMA concentrations and changes in Hcy concentrations might be related to changes in ADMA concentrations. Hcy and ADMA might be influenced by fatness and fitness, and might also be related to endothelial function and inflammation as well as arterial properties.

The objective of this study was thus to investigate the relationship between (changes in) concentrations of Hcy, ADMA, fitness, fatness, markers of endothelial function and inflammation, and arterial properties of healthy adults from the AGAHLS. The study of the currently available literature (Chapter 2) gave an in-depth explanation of the biological pathways of the two recently identified CVD risk factors Hcy and ADMA and their respective relationships with some lifestyle (fitness and fatness) and biological risk factors (endothelial function, arterial properties and inflammation markers), separately and in relationship with each other.

Hcy is an amino acid that is metabolised by one of two pathways; the remethylation and transsulfuration pathway. The suggested mechanism through which Hcy executes its deleterious effects in the body seems to be that of endothelial dysfunction, although the exact role of Hcy in endothelial dysfunction and atherosclerosis remains unclear. As mentioned earlier, the link between Hcy and ADMA is based upon interconnections between their respective metabolic pathways.

ADMA is an endogenous molecule which is formed from the proteolysis of methylated proteins, and metabolism of ADMA may be affected by Hcy. Hcy may directly or indirectly inhibit dimethylarginine dimethylaminohydrolase (DDAH) activity, and Hcy may also induce oxidative stress that can lead to elevated ADMA concentrations. ADMA acts as an endogenous inhibitor of nitric oxide synthase (NOS) that influences vasodilatation etc. Although Hcy has been researched more than ADMA, the existing literature on Hcy and ADMA is extremely limited as far as some critical factors (i.e. biological

mechanisms, cardiovascular disease) are concerned, and has yielded rather contradicting results. General tendencies that can be derived from the literature review, are that concentrations of Hcy and ADMA are related to CVD risk factors in persons with an older age and in persons with chronic diseases.

The lifestyle factors influencing Hcy and ADMA include measurements of fitness (i.e. VO_2max and physical activity) and fatness [i.e. fat percentage, fat distribution and body mass index (BMI)]. The biological factors include endothelial function [such as intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), Von Willebrand factor (vWf), inflammation markers such as C-reactive protein (CRP) and arterial properties, intima-media thickness (IMT) and Young's elastic modulus (YEM)]. The literature study has indicated that a very limited number of studies investigated the relationship of Hcy with regard to fatness, endothelial and inflammation markers as well as arterial properties. It became clear that ADMA has not been studied in-depth with regard to all the above-mentioned variables, including fitness. The influences of ADMA on cardiovascular health are less debated than its relationship with Hcy.

The age at which these relationships become apparent is not known. Therefore, it is necessary to investigate the relationship in concentrations of Hcy and ADMA, and the changes over age in these risk factors with relation to lifestyle risk factors, inflammation and arterial properties longitudinally in healthy subjects. Thus the research question, to investigate the relationships that might exist between (changes in) concentrations of Hcy, ADMA, fitness, fatness, markers of endothelial function and inflammation, and arterial properties of healthy adults from the AGAHLS. The answer to this question was addressed within the three manuscripts presented in Chapters 3-5.

The AGAHLS was an ideal study to investigate the above-mentioned research problem because of the fact that repeated measurements in time are performed, and not only the levels but also the changes in Hcy, ADMA, fitness, fatness and endothelial function can be studied in relation to one another, as well as in relation to the levels and changes in arterial properties. The subjects have been measured repeatedly over time and the last measurements were done in 2006 at the age of 42.

With the addition of concentrations of Hcy and ADMA, as well as endothelial function to the already abundant available data from the AGAHLS on fitness, fatness and arterial

properties, the causal path to pre-clinical atherosclerosis was investigated within one study. The data originated from a relatively healthy adult population of men and women and populations like these have scarcely been studied.

The investigation into the relationship between Hcy, ADMA, fitness and fatness has been documented in Chapter 3. Physical activity (PA) is widely advocated for the lowering of coronary heart disease (CHD) risk factors due to the fact that an increase in PA may result in several anti-atherosclerotic effects, for instance the improvement of NO bioavailability, reduction in oxidative stress and lipid peroxidation. Although the effect of PA on Hcy concentrations yields rather controversial results, the relationship between (changes in) concentrations of plasma Hcy and ADMA, and their association with (changes in) fitness and fatness determinants in adult men and women were investigated.

The GEE analysis indicated no significant positive relationships between changes in concentrations of plasma Hcy and ADMA, and fitness and fatness from the 2000 and 2006 data sets. The only significant relationship between Hcy concentrations and fitness was partly explained by the increase in the smoking behaviour which increased between 2000 and 2006.

As mentioned before, Hcy and ADMA share numerous presumed patho-physiological mechanisms, linking them to vascular disease. Most of these patho-physiological mechanisms decrease NO production, leading to endothelial dysfunction on the one hand and inflammation, an established marker of CVD, on the other. Both Hcy and ADMA have been linked to inflammation, eluding to the possibility that an inter-relationship might exist between Hcy, ADMA and markers of endothelial function. In Chapter 4 the interrelationship between concentrations of Hcy and ADMA, and their association with markers of endothelial dysfunction and inflammation were investigated.

The markers of endothelial function were measured by means of several variables [intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), endothelial selectine (E-selectine), plasma selectine (P-selectine), thrombomoduline, Von Willebrand factor (vWf)], and inflammation markers were supported by means of various variables [C-reactive protein (CRP), serum amyloid A (SAA), tumor necrosis factor α (TNF- α), interleukin-6 (IL-6) and interleukin-8 (IL-8)]. According to the GEE analysis, a significant positive longitudinal relationship was found between plasma

ADMA concentrations and inflammation cytokines (i.e. CRP, ICAM and thrombo-moduline). Furthermore, an inverse significant relationship was found between changes in ADMA concentrations and changes in the Von Willebrand factor.

The apoptosis of the smooth muscle cells induced by increased Hcy concentrations is related to the stimulation of increased ADMA production, thus affecting arterial properties (i.e. intima-media thickness and stiffness). There are two suggested mechanisms through which ADMA induces its deleterious effects. One mechanism might be through increased methylation of arginine residues within proteins and the other through the reduction in metabolism of ADMA by means of the dimethylarginine dimethylaminohydrolase (DDAH) enzymes.

The investigation with regard to the relationships between (changes in) concentrations of Hcy and ADMA, and (changes in) arterial properties in Chapter 5, included the biochemical variables of concentrations of Hcy and ADMA, high density lipoprotein cholesterol, low density lipoprotein cholesterol and triglycerides. Arterial properties were determined by means of a range of variables [carotid artery intima-media thickness (IMT), carotid artery compliance coefficient (CA CC), carotid artery distensibility coefficient (CA DC), femoral artery compliance coefficient (FA CC), femoral artery distensibility coefficient (FA DC) and Young's elastic modulus (YEM)]. The arterial properties measured were carotid intima-media thickness, carotid and femoral artery distensibility and compliance, and carotid Young's elastic modulus. No significant relationships were found between concentrations of Hcy and ADMA on the one hand, and arterial properties on the other. No significant relationships were found in the changes in concentrations of Hcy and ADMA and arterial properties either.

6.3 CONCLUSION

The conclusion of this study is derived from the stated hypotheses.

6.3.1 Hypothesis 1

Concentrations of (changes in) Hcy and ADMA are inversely related to (changes in) fitness and fatness.

The results indicate no significant relationship between (changes in) fitness and fatness, and (changes in) concentrations of Hcy and ADMA. The only significant relationship between plasma Hcy concentrations and fitness was partly explained by the smoking behaviour of the study population that increased between 2000 and 2006 (β = -0.81; p = 0.03). Hypothesis 1 is therefore rejected.

6.3.2 Hypothesis 2

Positive associations are expected between (changes in) concentrations of homocysteine and ADMA, and (changes in) arterial properties.

The results indicate a significant relationship between plasma ADMA concentrations and endothelial markers ICAM (β = 71.7; p = 0.03) and thrombomoduline (β = 0.89; p = 0.03) as well as a significant relationship between the changes in ADMA concentrations and the Von Willebrand factor (β = -42.4; p = 0.04). Hypothesis 2 is therefore accepted.

6.3.3 Hypothesis 3

Concentrations (changes in) of homocysteine and ADMA are inversely related to (changes in) endothelial function and inflammation markers.

According to the results, no significant relationships exist between concentrations of Hcy and ADMA on the one hand, and arterial properties on the other. Hypothesis 3 is therefore rejected. No significant relationship was found between changes in concentrations of Hcy and ADMA, and changes in arterial properties.

In light of the above-mentioned, the overall conclusion that can be drawn is that lifestyle risk factors (especially smoking) have been shown to influence Hcy concentrations, and

although the same reaction was predicted for ADMA and lifestyle factors, no significant relationships were established in this regard. ADMA concentrations, however, seemed to be positively related to endothelial markers (ICAM and thrombomoduline) and inversely related to inflammation that leads to endothelial dysfunction. To the contrary, ADMA did not lead to the diminishment of arterial properties. These findings might be explained by the fact that the subjects were relatively healthy, free living, young adults and that the onset of the diminishment of arterial properties might only be detected at an older age or in combination with diseases. Therefore it remains vital to follow these subjects within the AGAHLS over time, to determine whether an increase in either/both Hcy and AMDA concentrations leads to endothelial dysfunction at a later age than was investigated with this study.

6.4 LIMITATIONS AND RECOMMENDATIONS

The study has demonstrated that relationships indeed exist between concentrations of Hcy and ADMA, and lifestyle risk factors. The study also illustrated a relationship between ADMA concentrations and markers of endothelial function and inflammation. It may therefore be accepted that ADMA induces its deleterious effects on cardiovascular health and consequently endothelial function. The limitations that were experienced in the study together with the recommendations that can improve future outcomes of longitudinal studies on concentrations of Hcy and ADMA are:

- Although the AGAHLS is a longitudinal study, only two years of follow-up data (2000 and 2006 respectively) were available. It is recommended that the relationships over a longer period should be investigated, since the risk of developing cardiovascular disease increases with an increase in age.
- The adaptations made to methods influenced by an increase in age, for example, changing direct VO_2max measurements in the AGAHLS 2000 to the indirect VO_2max Chester step test in 2006 can be indicated as a limitation as it may have influenced the outcome of the study. Future fitness testing should be standardised to a single measurement protocol.
- In the AGAHLS the measurements were not taken by the same investigators over the consecutive years (2000 - 2006) and this might also be regarded as a limitation. The recommendation, if possible, is to have at least the same person taking two consecutive measurements.

- All the subjects in the study are Caucasian. It can be recommended that future investigations should include an older population and other ethnic groups to determine whether similar results will be obtained.
- A final limitation of this study was that NO was not measured. It is recommended that future investigation include the measurement of NO in order to obtain clarity on the pathways linking relationships between Hcy, ADMA and endothelial dysfunction.

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A

APPENDIX A: GUIDELINE TO AUTHORS EUROPEAN JOURNAL OF CLINICAL NUTRITION

Guidelines for Authors

European Journal of Clinical Nutrition

Welcome to the electronic manuscript submission website for European Journal of Clinical Nutrition. The instructions below are structured so you can quickly and easily answer the following questions:

1. Is my manuscript suitable for *European Journal of Clinical Nutrition*? (Scope + Editorial Note)
2. How do I format my manuscript for *European Journal of Clinical Nutrition*? (Format of Papers)
3. How do I submit my manuscript to *European Journal of Clinical Nutrition*? (Submission of Papers)

1.1.1 OTHER LINKS

- About the journal
- Format of papers
  - Preparation of Original Articles
  - House style
- Submission of papers
- Editorial policy
  - Ethics and bioethics
  - Peer review policy
  - Corrections
  - About npg online services

1.1.2 ABOUT THE JOURNAL

1.1.3 Scope

The *European Journal of Clinical Nutrition* is an international, peer-reviewed journal publishing articles related to human and clinical nutrition. We aim to cover all aspects of human and clinical nutrition:

- Clinical and whole body metabolic investigations
- Epidemiological, social and behavioural studies in nutrition
- Nutritional determinants of growth, development and health
- Assessment and measurement of nutritional status and indicators, and their relation to function and health
- Nutritional causes and effects of disease
- Community nutrition and education
- Nutrition, population health and health promotion

Topics Covered

The scope of the journal includes publishing original articles, short communications and case reports based on clinical, metabolic and epidemiological studies that describe methodologies, mechanisms, relationships and benefits of nutritional interventions for disease and health promotion. Editorials, commentaries, reviews, book reviews and letters to the editor will also feature in EJCN

Editor	Professor Prakash S Shetty, Institute of Human Nutrition, School of Medicine, University of Southampton, Southampton, UK
Frequency	12 issues a year
Abstracted in	Current Contents Current Contents Clinical Medicine Current Contents Life Sciences EMBASE/Excerpta Medica Elsevier BIOBASE/Current Awareness in Biological Sciences Index medicus Science Citation Index BIOSIS CAB Abstracts CAB Health and Nutrition Newsletter

1.1.4 Editorial Note

Manuscripts based on animal nutrition and in vitro studies will not be considered. Papers reporting validation of generally accepted methodologies in specific population groups and prevalence or incidence data on nutritional problems from countries have very low priority. When validation studies and prevalence or incidence data specific to countries are submitted for publication to EJCN, they will be processed only if they are submitted as a short communication with the clear understanding that supplementary data will be made available by the authors to anyone interested in compiling regional or global comparisons. EJCN also does not encourage submissions based on testing clinical or commercial food, and nutritional or clinical products. If a manuscript, previously considered for publication in another journal, is submitted to EJCN, we would appreciate receiving copies of the comments from reviewers and Editors. This will enable an early decision by the Editorial team and the possibility to fast track the same with fewer peer reviews. Finally, please make sure covering letters highlight the unique features of the submitted manuscript and make the case why this manuscript deserves publication in EJCN.

1.1.5 FORMAT OF PAPERS

Article Types Table

Article Type	Description	Approximate Word Count
Original Article		
These are reports of current basic or clinical research and should follow the structure outlined below this table.Reports of Randomised controlled trials (RCTs) submitted to EJCN must adhere to the CONSORT statement, (CONsolidated Standards Of Reporting Trials). This guideline provides an evidence-based, minimum set of recommendations for reporting RCTs, it comprises a list of items to report and a patient flow diagram. For other study designs, EJCN strongly encourages authors to consult reporting guidelines relevant to their specific research design: studies of diagnostic accuracy (STARD); observational studies in epidemiology (STROBE); systematic reviews and meta-analyses (QUOROM); and meta-analyses of		Word limit: 3,000 words excluding abstract, references, figures and tables. The abstract should be no longer than 250 words and structures, as outlined in the Abstract and Keywords section below. References: 50 references maximum (as far as possible only recent references). Display items: No more than six display items (e.g. figures and/or tables) should accompany the manuscript. Multiple figures (1a, 1b, 1c etc.) will count as individual figures.

observational studies in epidemiology (MOOSE). All these reporting guidelines can be found at http://www.equator-network.org	
Short Communications	
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- In the Methods section, specify the type of equipment (microscopes/objective lenses, cameras, detectors, filter model and batch number) and acquisition software used. Although we appreciate that there is some variation between instruments, equipment settings for critical measurements should also be listed.

- The display lookup table (LUT) and the quantitative map between the LUT and the bitmap should be provided, especially when rainbow pseudocolour is used. It should be stated if the LUT is linear and covers the full range of the data.
- Processing software should be named and manipulations indicated (such as type of deconvolution, three-dimensional reconstructions, surface and volume rendering, 'gamma changes', filtering, thresholding and projection).
- Authors should state the measured resolution at which an image was acquired and any downstream processing or averaging that enhances the resolution of the image.

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For primary research manuscripts reporting experiments on live vertebrates and/or higher invertebrates, the corresponding author must confirm that all experiments were performed in accordance with relevant guidelines and regulations. The manuscript must include in the Supplementary Information (methods) section (or, if brief, within of the print/online article at an appropriate place), a statement identifying the institutional and/or licensing committee approving the experiments, including any relevant details regarding animal welfare, patient anonymity, drug side effects and informed consent.

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Accept, with or without editorial revisions. Invite the authors to revise their manuscript to address specific concerns before a final decision is reached.
Reject, but indicate to the authors that further work might justify a resubmission.
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B

APPENDIX B: GUIDELINES FOR AUTHORS ATHEROSCLEROSIS JOURNAL

Guidelines for Authors

Atherosclerosis Journal

International Journal for Research and Investigation on Atherosclerosis and Related Diseases. Official Journal of the European Atherosclerosis Society. Affiliated with the International Atherosclerosis Society and the Society of Atherosclerosis Imaging and Prevention

Atherosclerosis is a fully electronic journal; all manuscripts are to be submitted via the internet. To submit your paper **online**, click on the link http://ees.elsevier.com/ath/. This will take you to the *Atherosclerosis* Editorial Manager home page. The **Author Information** box to the right of the page provides relevant information, including a tutorial on how to submit your manuscript. Authors must select an appropriate Associate Editor from the list shown on the website, the expertise terms for each Editor are shown to assist with this choice. Authors must suggest four potential reviewers for their paper and to avoid delay in processing your submission please ensure that email addresses given for reviewers are correct. The Editorial Board reserves the right to decide whether or not the suggested reviewers are used. Please note the Associate Editors will not act as reviewers. Authors may also indicate if a particular reviewer should not be approached.

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b) *Clinical Research Papers* reporting results of original clinical research or investigation in human subjects. Clinical Research Papers should not exceed **4000** words (including tables and legends to figures) and no more than **30** references. Basic and Clinical Research papers must have no more than **5** figures and tables in total (e.g., 1 figure consisting of panels A and B, and 4 tables). Authors are encouraged to include additional figures and tables as supplementary appendixes, and these will be considered for Web-only publication.

c) *Fast-track submission*, for new findings of sufficient importance to justify accelerated review and publication, a fast-track submission process for original articles is available. In the submission letter, authors should explicitly request this option and provide credit card information (number, expiration date, name as it appears on the card, related invoice address and email address). If the editors agree that the manuscript is worthy of fast-track publication, the fee of 600 Euros will be automatically charged to the credit card. If accepted for fast-track submission, an article will be reviewed within 72 hours (otherwise, authors will be informed that the paper will be handled within the normal peer-review process). If accepted, a fast-track submission will appear in the first available issue of the journal. PLEASE SELECT THE "FAST TRACK" OPTION FROM THE DROP DOWN MENU OF PAPER TYPES WHEN YOU SUBMIT YOUR MANUSCRIPT.

d) *Rapid Communications*. These papers should provide a brief but complete account of important new observations which merit urgent publication. The papers should be less than **5** printed pages (8-10 double-spaced typed pages) including figures and tables and should be concisely but adequately referenced. Authors should state in the comments section during the submission process why the paper merits urgent publication. Papers requiring revision will not be considered as Rapid Communications. The Editor-in-Chief will normally reach a decision on these papers within one month.

e) *Short Communications*. These papers should include original data of basic or clinical

research. The following word limits apply: abstract **150** words, main text **1500** words, up to 2 figures and or tables and a maximum of **15** references. Authors maybe invited to submit a short communication by the editorial team.

f) *Review Articles and Mini-Reviews*, usually by invitation. Mini-Reviews should normally consist of current short reviews of topical information. Word limit: **3500**, **25** references and up to **3** tables and or figures. Full reviews may contain up to 6 tables and or figures, authors are encouraged to include a "mechanism/overview" figure. Word limit **5000** and **60** references. Exceptions to these limits should be discussed with the Reviews Editor before submission.

All Reviews should be submitted for handling by the Reviews Editor, Arnold von Eckardstein.
g) *Hypotheses and Viewpoints* of up to **1500** words are published occasionally. These contributions are subject to the normal editorial procedure. These should be submitted to the Editor-in-Chief.

h) *Commentary*. If you wish to comment on work published in Atherosclerosis, please submit your opinions as a Commentary. The original Author(s) will have the opportunity to respond to your comments in the same issue of the journal.

i) *Letters to the Editor* are welcomed. Letters to the Editor reporting research findings that do not include novel data are unlikely to be published. Letters should not exceed **1200** words and should be submitted to the Editor-in-Chief.

j) *Announcements* of meetings, workshops, courses etc. are welcomed subject to available space.

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Abstracts

A structured abstract (objective, methods, results and conclusion) of 50-250 words must be included.

Keywords
A keyword summary must be provided; normally 3-7 items should be included. Authors are encouraged to choose their own keywords but, if in grave doubt which items to select, *Medical Subject Headings* (issued with the January *Index Medicus*, 1969) may be used as a guideline.

Illustrations
Figures should ideally be submitted in high-resolution TIF format, or alternatively in GIF, JPEG/JPG, or EPS format. The figures should be placed in separate files, named purely with the figure numbers (e.g. "Figure1.tif"). Legends for figures should be on separate pages within the main manuscript. The cost of colour figures will be paid by the Author.

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C

APPEDIX C: GUIDELINES TO AUTHORS JOURNAL OF INTERNAL MEDICINE

Guidelines for Authors

Journal of Internal Medicine

The *Journal of Internal Medicine* (JIM) publishes Original Articles on clinical and experimental research related to the broad field of medicine. JIM also welcomes Review Articles at the forefront of science and research in medicine, and also supports and organizes Workshops and Symposia on topics within the scope of the journal. Case reports on unique clinical observations may also be considered but only exceptionally.

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All manuscripts should be written in British English. Use only few abbreviations. An abbreviation list should be included, if needed.

The SI system must be used; for guidance see Units, Symbols, and Abbreviations, 4th edn (London: Royal Society of Medicine Services, 1988).

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The manuscript should include the following: (i) title page, (ii) abstract, (iii) main text (introduction, materials and methods, results, discussion), (iv) references, (v) figure legends, (vi) tables, (vii) figures.

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The first page should contain: title of paper, suggested running headline of not more than 30 characters (including both letters and spaces) and author's name, department, institution, city and country.

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For all 'Original Articles' but not for 'Case Reports' and 'Reviews' (which should have a short, conventional abstract of approximately 100 words) a structured abstract of approximately 250 words should be given. The subtitles shown below should be used, whenever applicable:

Objectives

Give a clear statement of the main aim of the study and the main hypothesis tested, if any.

Design

Describe the design of the study and mention, as appropriate, randomization, blinding, placebo-control, case-control, cross-over, criterion standards for diagnostic tests, and so on.

Setting

Describe the setting of the study, including the level of clinical care (primary care, etc.) and the number of participating centres.

Subjects

State the entry requirements, selection procedures and the number of subjects approached, entering and completing the study.

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Describe the main features of any interventions, including the method and duration of their use.

Main outcome measures. State the primary outcome measures as planned before the data were collected. If the paper does not emphasize the planned main outcome measures, this should be stated and explained.

Results

Give the main results of the study.

Conclusions

State the primary conclusions of the study and their clinical implications. Suggest areas for further research, if appropriate.

Keywords

We encourage the authors to list at least two, but no more than six keywords below the abstract. Preferably use the keywords listed at http://jim.manuscriptcentral.com/, or use terms from the 'Medical Subject Headings' list from *Index Medicus*.

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Acknowledgements

Financial support, technical and other assistance of importance for the study may be acknowledged.

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List all authors when seven or less; when more, give three followed by *et al*.

Examples of correct forms of references are given below.

1 Standard journal article

Carlson LA, Hamsten A, Asplund A. Pronounced lowering of serum levels of lipoprotein Lp(a) in hyperlipidaemic subjects treated with nicotinic acid. *J Intern Med* 1989; **226:** 271-6.

2 Chapter in book

Weinstein L, Swartz MN. Pathogenic properties of invading microorganisms. In: Sodeman WA Jr, Sodeman WA, eds, *Pathologic Physiology: Mechanisms of Disease*. Philadelphia: W. B. Saunders. 1974; 457-72.

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specified when ordered. Note that it is not uncommon for printed offprints to take up to eight weeks to arrive after publication of the journal.

REVIEW AND ACTION

Manuscripts are examined by the editorial group and are usually sent to outside reviewers. *To be considered for publication the author should suggest at least four reviewers.*

RAPID COMMUNICATION

Concise papers (not exceeding 1500 words) representing new but completed work which is of unusually high priority and significance, as clearly indicated by the authors in the covering letter, will be considered as Rapid communications. Papers will be reviewed immediately and publication should occur within 8 weeks of acceptance of the manuscript, with a few exceptions.

FURTHER INFORMATION

For more information please contact the Editorial Office in Stockholm (e-mail: edit.off@jim.se)

D

APPENDIX D: INFORMED CONSENT FORM

Informed consent form

Uitleg en toestemmingsverklaring meting

Met deze brief willen we je informeren over de meting van het Amsterdams Groei- en GezondheidsOnderzoek. Na aankomst tussen 8.00 en 8.30u. op het medisch centrum van de Vrije Universiteit nemen we eerst de ingevulde vragenlijsten in ontvangst, alsmede het meegebrachte activiteitenmetertje en voeding- en activiteitendagboekje. Vervolgens wordt van ieder wat veneus bloed afgenomen voor de bepaling van glucose, cholesterol, en andere indicatoren van hart- en vaatziekten, waarna kan je aanschuiven aan de ontbijttafel die door ons verzorgd wordt.

Gedurende de dag (tussen 9.00 en 17.00 u.) ga je de volgende meetstations langs:

1. Lichaamsbouw en –samenstelling (lengte, gewicht, omvang, huidplooien).
2. Wiebelstoel, meting van de houdingscontrole van je romp.
3. Meting van je hersenen en hersenfuncties
4. Cognitietests
5. Uithoudingsvermogen (geen lopende band, maar een minder inspannende test).
6. Spierkracht, lenigheid en ademhalingssnelheid.
7. Vragenlijsten over persoonlijkheid en alcoholgebruik voeding en activiteit.
8. Sterkte van je botten (hiel, lendenwervels, heup en hele lichaam).
9. Kwaliteit van je aderen.

- Tussen 12.00 en 14.00 u. verzorgen wij de lunch voor je.

  De meeste metingen hebben jullie de laatste keer (in 2000) al meegemaakt. Dit geldt niet voor:
- Meting van houdingscontrole van de romp. Dit is een veelbelovend nieuw aspect in het onderzoek naar rugklachten. Bij deze nieuwe meting ga je zitten op een stoel die slechts op één punt onder het zitvlak ondersteund wordt (vallen is echter niet mogelijk). De manier waarop je balans houdt wordt aan de stoel geregistreerd.
- De kwaliteit van kleine aderen. Evenals bij de meting van de grote aderen die in 2000 wel heeft plaatsgevonden wordt in deze meting met geluidsgolven de elasticiteit en de stevigheid bepaald. Dit vindt plaats in de haarvaten van een vinger. De kwaliteit van je aderen is een indicator van het risico voor hart- en vaatziekten en diabetes.
- Meting van je hersenen en hersenfuncties middels magneto-encefalografie

(MEG). Dit is een techniek die veel overeenkomsten vertoont met het “klassieke” hersenfilmpje (EEG). In plaats van de elektrische signalen worden bij MEG de magnetische signalen gemeten die je hersenen produceren. Bij dit onderzoek worden alleen signalen opgevangen die je lichaam zelf produceert. Het MEG apparaat zelf ziet er uit als een soort haardroogkap, waar je in zittende houding onder plaatsneemt. Er worden drie kleine spoeltjes met tape op je hoofd geplakt. De MEG registratie duurt ongeveer een half uur. Tijdens de registratie hoef je weinig anders te doen dan rustig stilzitten en knopje indrukken wanneer je een bepaalde toon hoort.

- Cognitietests (deels uitgevoerd op een computer). Het gaat om één test die verschillende aspecten van je geheugen meet en één test die je vermogen tot plannen en vooruitkijken bepaalt. Verder krijg je nog een test op het gebied van taalvaardigheid en tests over logisch redeneren en visueel inzicht. Het uitvoeren van deze testen neemt in totaal zo’n drie kwartier in beslag.

Meting van je botmassa wordt wederom met behulp van een zeer geringe dosis\ röntgenstraling gedaan (10,8 µSv). Dit is te vergelijken met de hoeveelheid straling die je in het dagelijks leven in twee dagen tot je krijgt door de straling die altijd in de atmosfeer ‘hangt’ (de zogenaamde achtergrondstraling). Als je in verwachting bent doen we deze meting niet. Dit kun je op de dag zelf kenbaar maken. Met alle resultaten zal, zoals alle jaren hiervoor, vertrouwelijk worden omgegaan. De gegevens zullen anoniem (d.w.z. onder deelnemernummer) worden opgeslagen en geanalyseerd. Publicatie van de resultaten wordt alleen op groepsniveau uitgevoerd.

Alleen voor de persoonlijke resultaten van de metingen die je ongeveer zes maanden na de meting ontvangt, worden de gegevens aan je naam gekoppeld. Indien wij bijzondere bevindingen doen die een indicatie kunnen zijn dat je een verhoogd gezondheidsrisico hebt, dan zullen wij je daar zo spoedig mogelijk over benaderen. We hopen je voldoende te hebben geïnformeerd over de meting in 2006. Mocht je nog vragen hebben, dan kun je contact opnemen met Lando Koppes, tel: 020 - 444
8196 of 06 – 5187 1371. Hiermee gaat ondergetekende akkoord dat bovengenoemde metingen bij hem/haar worden uitgevoerd.

N.B. Het ondertekenen van deze brief betekent niet dat je verplicht bent aan alle metingen mee te doen. Het is op elk moment mogelijk om van een bepaalde meting af te zien.

Getekend,

…….	……	…….
Handtekening	Naam	Datum

E

APPENDIX E: ACTIVITY QUESTIONNAIRE

Deelnemernummer: …….. Datum: ……..

Lichamelijke activiteit

Neem in uw gedachten een normale week in de afgelopen maanden. Wilt u aangeven **hoeveel dagen per week** u de onderstaande activiteiten verrichtte, hoeveel minuten u daar dan **gemiddeld** op zo'n dag mee bezig was en hoe inspannend deze activiteit was.

1. WOON-WERK VERKEER (heen en terug)	**Aantal dagen per *week***	**Gemiddelde tijd per *dag***	**Inspanning**		
			langzaam	gemiddeld	snel
Lopen van/naar werk	☐☐ dagen	☐☐ uur ☐☐ minuten	☐☐	☐☐	☐☐
Fietsen van/naar werk	☐☐ dagen	☐☐ uur ☐☐ minuten			

2. LICHAMELIJKE ACTIVITEIT OP WERK	**Gemiddelde tijd per week**
Licht inspannende activiteit (Bijvoorbeeld; zittend/staand werk, met af en toe lopen, zoals bureauwerk etc.)	☐☐ uur ☐☐ minuten
Zwaar inspannend activiteit (Bijvoorbeeld: lopend werk, waarbij regelmatig zware dingen moeten worden opgetild).	☐☐ uur ☐☐ minuten

3. HUISHOUDELIJKE ACTIVITEITEN	**Aantal dagen Per *week***	**Gemiddelde tijd per *dag***
Licht inspannend huishoudelijke activiteit (Bijvoorbeeld: staand werk, zoals koken, afwassen, strijken,kind eten geven/in bad doen, stofzuigen, boodschappen doen)	☐ dagen	☐ uur ☐☐ minuten
Zwaar inspannend huishoudelijke activiteit (Bijvoorbeeld: vloer schrobben, tapijt uitkloppen, met zware boodschappen lopen)	☐ dagen	☐ uur ☐☐ minuten

4. VRIJE TIJD	**Aantal dagen per *week***	**Gemiddelde tijd per *dag***	**Inspanning**		
			Langzaam	Gemiddeld	Snel
Wandelen	☐☐ dagen	☐☐ uur ☐☐ minuten	☐☐	☐☐	☐☐
Fietsen	☐☐ dagen	☐☐ uur ☐☐ minuten			
			Licht	Gemiddeld	Zwaar
Tuinieren	☐☐ dagen	☐☐ uur ☐☐ minuten	☐☐	☐☐	☐☐
Klussen/Doe-het-zelven	☐☐ dagen	☐☐ uur ☐☐ minuten			

5. ACTIEVE SPORTEN (Hier maximaal 4 opschrijven) *Bijv.: tennis, handbal, gymnastiek, fitness, schaatsen, zwemmen*			**Inspanning**		
			Licht	Gemiddeld	Zwaar
1.	☐☐ dagen	☐☐ uur ☐☐ minuten	☐☐	☐☐	☐☐
2.	☐☐ dagen	☐☐ uur ☐☐ minuten			
3.	☐☐ dagen	☐☐ uur ☐☐ minuten			
4.	☐☐ dagen	☐☐ uur ☐☐ minuten			
TOTAAL					

Op gemiddeld hoeveel dagen per week bent u, alles bij elkaar opgeteld, Tenminste een half uur bezig met fietsen, klussen, tuinieren of sporten?	☐☐ dagen per week

F

APPENDIX F: ALCOHOL QUESTIONNAIRE

Alcoholconsumptie Vragenlijst

Onderstaande vragen hebben betrekking op je patroon van alcoholconsumptie en mogelijke problemen a.g.v. alcoholgebruik.

1. Hoe vaak neem je één of meer alcoholische drankjes?

[ ] Nooit ➔ einde vragenlijst.
[ ] Maandelijks of minder vaak
[ ] 2 tot 4 maal per maand
[ ] 2 tot 3 maal per week
[ ] 4 maal per week of vaker.

2. Waar dronk je het laatste jaar meestal?

[ ] Thuis
[ ] In restaurant
[ ] In café
[ ] Elders, namelijk: .....

3. Hoeveel glazen alcoholische drank drink je op een doorsnee drinkdag?

[ ] 1
[ ] 2 of 3
[ ] 4 of 5
[ ] 6 – 8
[ ] 9 of meer.

4. Hoe vaak drink je 6 of meer glazen alcoholische drank op één gelegenheid (binnen 8 uur)?

[ ] Nooit
[ ] Minder dan 1 maal per maand
[ ] Maandelijks
[ ] Wekelijks
[ ] Meerdere keren per week.

5. Het grootste aantal glazen alcoholische drank dat je de afgelopen maand op één gelegenheid hebt gedronken is:

[ ] 0 glazen
[ ] 1 glas
[ ] 2 of 3 glazen
[ ] 4 of 5 glazen
[ ] 6 – 9 glazen
[ ] 10 – 15 glazen

[ ] 16 – 24 glazen
[ ] 25 of meer glazen.

6. Hoe dronk je het laatste jaar meestal?
[ ] Altijd alleen
[ ] Meestal alleen
[ ] Even vaak alleen als in gezelschap
[ ] Meestal in gezelschap
[ ] Altijd in gezelschap.

6. Heb je ooit het gevoel gehad dat je niet meer kon stoppen met drinken toen je begonnen was?
[ ] Nee, nooit
[ ] Ja, maar niet in het afgelopen jaar
[ ] Ja, in het afgelopen jaar.

7. Heb jij jezelf of iemand anders ooit schade berokkend of bezeerd als gevolg van jouw alcoholgebruik?
[ ] Nee, nooit
[ ] Ja, maar niet in het afgelopen jaar
[ ] Ja, in het afgelopen jaar.

8. Heb je ooit problemen in een relatie gehad als gevolg van je alcoholgebruik?
[ ] Nee, nooit
[ ] Ja, maar niet in het afgelopen jaar
[ ] Ja, in het afgelopen jaar.

9. Heb je ooit problemen in je opleiding of op je werk gehad als gevolg van je alcoholgebruik?
[ ] Nee, nooit
[ ] Ja, maar niet in het afgelopen jaar
[ ] Ja, in het afgelopen jaar.

10. Hebben je partner, naaste familieleden en/of vrienden zich ooit zorgen gemaakt over jouw drankgebruik?
[ ] Nee, nooit
[ ] Ja, maar niet in het afgelopen jaar
[ ] Ja, in het afgelopen jaar.

11. Heb je ooit onder (lichte) invloed van alcohol een auto of motor bestuurd?

[ ] Nee, nooit

[ ] Ja, maar niet in het afgelopen jaar

[ ] Ja, in het afgelopen jaar.

12. Heb je de dag nadat je gedronken had wel eens gemerkt dat je niet meer wist wat er tijdens of vlak na het drinken gebeurd was?

[ ] Nee, nooit

[ ] Ja, maar niet in het afgelopen jaar

[ ] Ja, in het afgelopen jaar.

13. Heb je wel eens het gevoel gehad dat je minder zou moeten gaan drinken?

[ ] Ja

[ ] Nee.

14. Ben je wel eens geërgerd/geïrriteerd geraakt door aanmerkingen van anderen over je drankgebruik?

[ ] Ja

[ ] Nee.

15. Heb je je wel eens vervelend of schuldig gevoeld over je drinken?

[ ] Ja

[ ] Nee.

16. Heb je ooit direct na het opstaan gedronken om de zenuwen de baas te worden of om van een kater af te komen?

[ ] Ja

[ ] Nee.

G

APPENDIX G: SMOKING QUESTIONNAIRE

ROOKVRAGENLIJST

1. Rook je? Ja θ

Nee θ

1 ***Indien nee, ga naar vraag 18***

2. Rook je sigaretten? Ja θ

Nee θ

2 Indien nee, ga naar vraag 3

- Hoeveel sigaretten rook je gemiddeld per dag? . . . . . sigaretten

(N.B. Als je minder dan 1 sigaret per dag rookt, vul dan een getal kleiner dan 1 in)

- Welk(e) merk(en) sigaretten rook je? . . . . . .

. . . . . .

- Inhaleer je de rook? Nee θ

Ja, een klein beetje θ

Ja, normaal θ

Ja, heel diep θ

- Soort tabak? Extra licht θ

Licht θ

Halfzwaar θ

Zwaar θ

Weet niet θ

- Filter? Met θ

Zonder θ

- Gedurende hoeveel jaren rook je dit aantal sigaretten per dag? gedurende . . . . . jaar

- Voor die tijd rookte ik Niet θ

Wel θ

n.l. . . . . sigaretten per dag

3. Rook je shag? Ja θ

Nee θ

3 ***Indien nee, ga naar vraag 4***

- Hoeveel pakjes shag rook je gemiddeld per week? . . . . . pakjes shag

(N.B. Als je minder dan 1 pakje shag per week rookt, vul dan een getal in kleiner dan 1 in)

- Welk(e) merk(en) shag rook je? . . . . . .
. . . . . .

- Inhaleer je de rook? Nee θ
Ja, een klein beetje θ
Ja, normaal θ
Ja, heel diep θ

- Soort tabak? Extra licht θ
Licht θ
Halfzwaar θ
Zwaar θ
weet niet θ

- Gedurende hoeveel jaren rook je dit aantal pakjes shag per week? gedurende . . . . . jaar

- Voor die tijd rookte ik Niet θ
Wel θ
n.l. . . . . pakjes shag per week

4. Rook je sigaren en/of cigarillo's? Ja θ
Nee θ

4 ***Indien nee, ga naar vraag 5***

- Hoeveel sigaren/cigarillo's rook je gemiddeld per week? . . . . . per week
(N.B. Als je minder dan 1 sigaar/cigarillo per week rookt, vul dan een getal kleiner dan 1 in)

- Welk(e) merk(en) sigaren/cigarillo's rook je? . . . . . .
. . . . . .

- Inhaleer je de rook? Nee θ
Ja, een klein beetje θ
Ja, normaal θ
Ja, heel diep θ

- Dikte sigaar? Dun θ
Normaal θ
Dik θ

- Soort tabak? Extra licht θ

Licht θ

Halfzwaar θ

Zwaar θ

Weet niet θ

- Gedurende hoeveel jaren rook je dit aantal sigaren/ cigarillo's per week? gedurende . . . . . jaar

- Voor die tijd rookte ik Niet θ

Wel θ

n.l. . . . . sigaren/cigarillo's per week

5. Rook je pijptabak? Ja θ

Nee θ

5 ***Indien nee, ga naar vraag 6***

- Hoeveel pakjes pijptabak (à 50 gram) rook je gemiddeld per week? . . . . . pakjes

(N.B. Als je minder dan 1 pakje pijptabak per week rookt, vul dan een getal kleiner dan 1 in)

- Welk(e) merk(en) pijptabak rook je? . . . . . .

. . . . . .

- Inhaleer je de rook? Nee θ

Ja, een klein beetje θ

Ja, normaal θ

Ja, heel diep θ

- Soort tabak? Extra licht θ

Licht θ

Halfzwaar θ

Zwaar θ

Weet niet θ

- Gedurende hoeveel jaren rook je dit aantal pakjes pijptabak per week? gedurende . . . . . jaar

- Voor die tijd rookte ik Niet θ

Wel θ

n.l. . . . . pakjes pijptabak per week

6. Indien ik belemmerd word in mijn rookgedrag, Ja θ

ga ik op andere momenten gewoon meer roken. Nee θ

7. Hoe lang na het ontwaken steek je je eerste sigaret of shagje op?
   - binnen 5 minuten θ
   - 6-30 minuten θ
   - 31-60 minuten θ
   - na 60 minuten θ
   - n.v.t. θ

8. Welke sigaret of welk shagje zou je het moeilijkst kunnen opgeven?
   - de eerste ‘s morgens θ
   - een andere θ
   - n.v.t. θ

9. Vind je het moeilijk om niet te roken op plaatsen waar het verboden is (bijv. bioscoop, bibliotheek, kerk, school, ziekenhuis)?
   - Ja θ
   - Nee θ

10. Rook je in de eerste uren na het opstaan meer per uur dan gedurende de rest van de dag?
   - Ja θ
   - Nee θ

11. Rook je als je ziek bent en het grootste deel van de dag in bed ligt?
   - Ja θ
   - Nee θ

12. Hoe oud was je toen je begon met roken? . . . . . jaar

13. Heb je wel eens geprobeerd te stoppen met roken vanaf het moment dat je regelmatig rookte?
   - Ja θ
   - Nee θ

INDIEN NEE, EINDE VRAGENLIJST

14. Hoe vaak ben je gestopt met roken? . . . . . keer

15. Hoe lang hield je het toen gemiddeld vol? . . . . . .

16. Hoe oud was je toen je voor de eerste keer probeerde te stoppen met roken? . . . . . jaar

17. Hoe oud was je toen je voor de laatste keer probeerde te stoppen met roken? . . . . . jaar

6 EINDE VRAGENLIJST VOOR DE ROKERS

7 HET VOLGENDE DEEL IS ALLEEN VOOR NIET-ROKERS

18. Heb je ooit gerookt?
   - Ja θ
   - Nee θ

8 ***Indien nee, ga naar vraag 29***

19. Hoeveel **sigaretten** rookte je gemiddeld per dag? . . . . . per dag
(N.B. Als je minder dan 1 sigaret per dag rookte, vul dan een getal kleiner dan 1 in)

20. Hoeveel **pakjes shag** rookte je gemiddeld per week? . . . . . per week
(N.B. Als je minder dan 1 pakje shag per week rookte, vul dan een getal kleiner dan 1 in)

21. Hoeveel **sigaren/cigarillo's** rookte je gemiddeld per week? . . . . . per week
(N.B. Als je minder dan 1 sigaar/cigarillo per week rookte, vul dan een getal kleiner dan 1 in)

22. Hoeveel **pakjes pijptabak** rookte je gemiddeld per week? . . . . . per week
(N.B. Als je minder dan 1 pakje pijptabak per week rookte, vul dan een getal kleiner dan 1 in)

23. Hoeveel jaren rookte je dit aantal - sigaretten . . . . . jaar
- pakjes shag . . . . . jaar
- sigaren/cigarillo's . . . . . jaar
- pakjes pijptabak? . . . . . jaar

24. Voor die tijd rookte ik Niet θ
Wel θ
n.l. sigaretten . . . . . dag
pakjes shag . . . . . week
sigaren/cigarillo's . . . . . week
pakjes pijptabak . . . . . week

25. Hoe oud was je toen je begon met roken? . . . . . jaar

26. Hoe oud was je toen je voor de laatste keer stopte met roken? . . . . . jaar

27. Hoe vaak ben je eerder gestopt? . . . . . keer

28. Hoe lang hield je het toen gemiddeld vol? . . . . . .

29. Woon je samen met een roker? Ja θ
Nee θ
Indien ja,
- deze persoon rookt Heel weinig θ
Af en toe θ
Veel θ
Heel veel θ

30. Zit je veel in rokerige ruimtes (bijv. café, Ja θ
trein, kantine) ? Nee θ

H

APPENDIX H: PROOF OF SUBMISSION