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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12093106

Thesis submitted for the degree Doctor of Philosophy in Human Movement Studies at the Potchefstroom Campus of the North-West University

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Assistant promotor: Dr. L.L.J. Koppes, VU

May 2010

Innovation through diversity
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)
ACKNOWLEDGEMENTS

I want to thank the North-West University (South Africa) and Vrije University of Amsterdam (The Netherlands) for providing the infrastructure in which I could complete my PhD study. I would also like to convey my gratitude to the following people who supported and assisted me in the completion of this study:

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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.

<table>
<thead>
<tr>
<th>NAME</th>
<th>ROLE IN THE STUDY</th>
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<tr>
<td>Ms. S.J. Herbst – Spies B.Sc., Hons. (Biokineticist) (South Africa)</td>
<td>Responsible for the execution of the total thesis, some of the data collection, management and statistical analyses. Main author of the thesis.</td>
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<td>Promotor of S.J. Herbst – Spies in The Netherlands. A significant contribution toward the statistical analyses and writing of the thesis.</td>
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</tr>
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<td>Co-author of Chapter 3. Contribution towards general content of research project, and in writing on article 1.</td>
</tr>
</tbody>
</table>
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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___________________    __________________
Prof. H.H. Voster (Ph.D.)    Dr. L.L.J. Koppes (Ph.D.)

___________________
Dr. Y. Smulders (Ph.D.)
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$_2$max, 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),

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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$_2$max 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.
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 geïdentifieerde merkers wat die risiko om KVS te ontwikkel, beïnvloed. Dit is dus belangrik om die merkers te bestudeer om sodoende die onderliggende mekanismes 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, vettheid, arteriële eienskappe, asook endotele funksie- en inflammasie -merkers 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, vettheid, arteriële eienskappe en endotele dysfunksies. Die AGAHLS het in 1977 begin met 'n groep 13-jarige deelnemers. Die deelnemers is herhaaldelik oor 'n tydperk gemete, 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$_2$max);
- fisieke aktiwiteit (kMETS.min/wk);
- vettheid (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
OPSOMMING

- 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, vettheid, 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, vettheid, arteriële eienskappe, asook endotele funksie- en inflammasiemerkers.

Volgens die VSV-analise is 'n 1 ml/min/kg hoër VO$_2$max-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 verlo of gegaan nadat aanpassings gemaak is vir rookgewoontes. Volgens die VSV-resultate bestaan daar tog 'n betekenisvolle verwantskap tussen ADMA-konsentrasies en endotele merkers ICAM ($\beta=71.67$ [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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<td>ADMA</td>
<td>Asymmetric dimethylarginine</td>
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<td>A</td>
<td>AGHALS</td>
<td>Amsterdam Growth and Health Longitudinal Study</td>
</tr>
<tr>
<td>A</td>
<td>AP</td>
<td>Arterial Properties</td>
</tr>
<tr>
<td>B</td>
<td>BHMT</td>
<td>Betaine-homocysteine methyltransferase</td>
</tr>
<tr>
<td>B</td>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>C</td>
<td>CA</td>
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<td>Dimethylarginine dimethylaminohydrolase</td>
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<td>Dual-energy x-ray absorbiometry</td>
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<td>Dimethylglycine</td>
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<td>M</td>
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<td>MA</td>
<td>MA - Methylarginine</td>
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<td>Met - Methionine</td>
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<td>MBP</td>
<td>MBP - Mean blood pressure</td>
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<td>MS</td>
<td>MS - Methionine synthase</td>
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<td>MTHFR</td>
<td>MTHFR - 10-methylene tetrahydrofolate reductase</td>
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<td>n - Sample size</td>
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<tr>
<td>NO</td>
<td>NO - Nitric oxide</td>
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<td>NOS</td>
<td>NOS - Nitric oxide synthase</td>
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<td>NF-κB</td>
<td>NF-κB - nuclear factor-kappa B</td>
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<td>O</td>
<td>OxLDL - Oxidized low density lipoprotein</td>
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<tr>
<td>P</td>
<td>PA - Physical activity</td>
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<tr>
<td>PRMT</td>
<td>PRMT - Protein arginine methyltransferase</td>
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<tr>
<td>S</td>
<td>SAA - Serum amyloid A</td>
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<tr>
<td>SAH</td>
<td>SAH - S-adenosylhomocysteine</td>
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<td>SAM - S-adenosylmethionine</td>
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<td>SD - Standard deviation</td>
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<td>SDMA - Symmetric dimethylarginine</td>
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<tr>
<td>SPSS</td>
<td>SPSS - Statistical package of social sciences</td>
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<tr>
<td>T</td>
<td>THF - Tetrahydrofolate</td>
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</tr>
<tr>
<td>TNFa</td>
<td>TNFa - Tumor recrosis factor a</td>
<td></td>
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<tr>
<td>V</td>
<td>VCAM - Vascular cell adhesion molecule</td>
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<tr>
<td></td>
<td>VO\textsubscript{2} max - Fitness (maximal oxygen uptake)</td>
<td></td>
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<tr>
<td></td>
<td>vWF - Von Willebrant factor</td>
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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).](image)

(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 turnover (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**

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# 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 AT₁ 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).


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 B₁₂ 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 B₈ 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 B₁₂ and B₆, 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 B₁₂ and pyridoxine (vitamin B₆) (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.

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 (VO₂max)]. 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 B₆ and B₁₂ remain adequate (Joubert & Manore, 2006:341).

Conversely, prolonged exercise, where glycogen reserves are reduced, places an increased demand on vitamin B₆ 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 B₆ increases and less vitamin B₆ 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 myeloperoxidase 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 monocytes 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 monocytes 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.
REFERENCES


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$_2$max 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$_2$max 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 (AGAHL-S) 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 absorptiometry (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, Massachusetts, 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$_2$max) 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$_2$max 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·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>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of the study population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>n</td>
</tr>
<tr>
<td>Physical activity (METs.min/wk)†</td>
<td>377</td>
</tr>
<tr>
<td>Alcohol (glasses/wk) †</td>
<td>373</td>
</tr>
<tr>
<td>Smoking (%) o</td>
<td>408</td>
</tr>
<tr>
<td>Homocysteine (nmol/L)</td>
<td>378</td>
</tr>
<tr>
<td>ADMA (nmol/L)</td>
<td>378</td>
</tr>
<tr>
<td>VO₂max (mL/min.kg²/³)</td>
<td>364</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>378</td>
</tr>
<tr>
<td>Trunk fat mass (kg)</td>
<td>356</td>
</tr>
<tr>
<td>Peripheral fat mass (kg)</td>
<td>355</td>
</tr>
<tr>
<td>Peripheral lean mass (kg)</td>
<td>355</td>
</tr>
</tbody>
</table>

† 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.

(VO₂max; physical fitness)
According to Table 1 significant differences where found in baseline characteristics between 2000 and 2006. These variables include smoking, VO$_2$max, 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.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hcy</th>
<th>ADMA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fitness (mL/min.kg$^{2/3}$)</td>
<td>B</td>
<td>95% CI</td>
</tr>
<tr>
<td></td>
<td>-0.07</td>
<td>-0.50 to 0.37</td>
</tr>
<tr>
<td>Trunk fat mass (kg)</td>
<td>-0.04</td>
<td>-0.48 to 0.42</td>
</tr>
<tr>
<td>Peripheral fat mass (kg)</td>
<td>-0.12</td>
<td>-0.49 to 0.25</td>
</tr>
<tr>
<td>Peripheral lean mass (kg)</td>
<td>0.07</td>
<td>-0.29 to 0.45</td>
</tr>
<tr>
<td><strong>Model 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fitness (mL/min.kg$^{2/3}$)</td>
<td>B</td>
<td>95% CI</td>
</tr>
<tr>
<td></td>
<td>-0.04</td>
<td>-0.59 to 0.51</td>
</tr>
<tr>
<td>Trunk fat mass (kg)</td>
<td>-0.14</td>
<td>-0.68 to 0.41</td>
</tr>
<tr>
<td>Peripheral fat mass (kg)</td>
<td>0.13</td>
<td>-0.29 to 0.56</td>
</tr>
<tr>
<td><strong>Model 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fitness (mL/min.kg$^{2/3}$)</td>
<td>B</td>
<td>95% CI</td>
</tr>
<tr>
<td></td>
<td>-0.12</td>
<td>-0.56 to 0.33</td>
</tr>
<tr>
<td>Trunk fat mass (kg)</td>
<td>0.01</td>
<td>-0.55 to 0.58</td>
</tr>
<tr>
<td>Peripheral fat mass (kg)</td>
<td>-0.18</td>
<td>-0.78 to 0.43</td>
</tr>
<tr>
<td>Peripheral lean mass (kg)</td>
<td>0.12</td>
<td>-0.30 to 0.55</td>
</tr>
</tbody>
</table>

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$_2$max) 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$_2$max). 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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hcy</th>
<th>ADMA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fitness (mL/min.kg(^{2/3}))</td>
<td>B</td>
<td>95% CI</td>
</tr>
<tr>
<td></td>
<td>-0.81</td>
<td>-1.53 to -0.09</td>
</tr>
<tr>
<td>Trunk fat mass (kg)</td>
<td>-0.54</td>
<td>-2.45 to 1.38</td>
</tr>
<tr>
<td>Peripheral fat mass (kg)</td>
<td>0.58</td>
<td>-0.93 to 2.09</td>
</tr>
<tr>
<td>Peripheral lean mass (kg)</td>
<td>-0.82</td>
<td>-2.61 to 0.97</td>
</tr>
<tr>
<td><strong>Model 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fitness, (mL/min.kg(^{2/3}))</td>
<td>B</td>
<td>95% CI</td>
</tr>
<tr>
<td>Trunk fat mass (kg)</td>
<td>-0.99</td>
<td>-3.14 to 1.16</td>
</tr>
<tr>
<td>Peripheral fat mass (kg)</td>
<td>0.94</td>
<td>-0.75 to 2.63</td>
</tr>
<tr>
<td>Peripheral lean mass (kg)</td>
<td>0.75</td>
<td>-2.55 to 1.05</td>
</tr>
<tr>
<td><strong>Model 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fitness, (mL/min.kg(^{2/3}))</td>
<td>B</td>
<td>95% CI</td>
</tr>
<tr>
<td>Trunk fat mass (kg)</td>
<td>-0.07</td>
<td>-2.86 to 2.72</td>
</tr>
<tr>
<td>Peripheral fat mass (kg)</td>
<td>0.89</td>
<td>-2.07 to 3.85</td>
</tr>
<tr>
<td>Peripheral lean mass (kg)</td>
<td>-0.81</td>
<td>-2.73 to 1.12</td>
</tr>
</tbody>
</table>

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, model 1 + fitness (VO\(_2\)max) 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\(_2\)max, 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 (VO₂max). 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 (VO₂max) 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 from 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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REFERENCES


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

ABSTRACT

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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 absorptiometry (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, Massachusetts, 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 (VO₂max) 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 VO₂max 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 thrombomodulin. 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)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>36 years</th>
<th>42 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine (nmol/L)</td>
<td>11.5 ± 6.6</td>
<td>12.1 ± 5.5</td>
</tr>
<tr>
<td>Asymmetric dimethylarginine (nmol/L)</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)*</td>
<td>782.5 [323.7 – 2239.3]</td>
<td>748.63 [340.1 – 1780.3]</td>
</tr>
<tr>
<td>Serum amyloid A (ng/ml)*</td>
<td>1293.9 [734.7 – 2316.6]</td>
<td>1344.3 [738.8 – 2340.8]</td>
</tr>
<tr>
<td>Interleukin-6 (pg/ml)*</td>
<td>2.4 [1.7 – 3.8]</td>
<td>2.4 [1.8 – 3.8]</td>
</tr>
<tr>
<td>Interleukin-8 (pg/ml)</td>
<td>9.3 ± 3.5</td>
<td>9.6 ± 3.9</td>
</tr>
<tr>
<td>Von Willebrand factor (%)</td>
<td>85.4 ± 33.6ª</td>
<td>92.2 ± 34.3ª</td>
</tr>
<tr>
<td>Intercellular adhesion molecule (ng/ml)</td>
<td>204.3 ± 51.2</td>
<td>199.5 ± 40.2</td>
</tr>
<tr>
<td>Vascular cell adhesion molecule (ng/ml)</td>
<td>333.2 ± 82.6</td>
<td>331.9 ± 67.7</td>
</tr>
<tr>
<td>E-selectine (ng/ml)</td>
<td>10.5 ± 4.8</td>
<td>10.1 ± 4.5</td>
</tr>
<tr>
<td>P-selectine (ng/ml)</td>
<td>60.2 ± 23.1ªb</td>
<td>71.2 ± 25.3ªb</td>
</tr>
<tr>
<td>Thrombomodulin (ng/ml)</td>
<td>2.5 ± 0.6</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>Tumor necrosis factor α (pg/ml)</td>
<td>9.4 ± 3.4ªc</td>
<td>8.9 ± 3.4ªc</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.4 ± 0.4ªd</td>
<td>1.7 ± 0.4ªd</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>3.0 ± 0.9ªe</td>
<td>2.8 ± 0.8ªe</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>1.3 ± 0.9ªf</td>
<td>1.2 ± 0.8ªf</td>
</tr>
<tr>
<td>Physical activity (METs.min.wk⁻¹) *</td>
<td>4135.0 [2567.7 – 6326.7]</td>
<td>2727.6 [2222.2 – 3229.3]</td>
</tr>
<tr>
<td>Alcohol (glasses.wk⁻¹)*</td>
<td>34 [8 – 63]</td>
<td>55 [8 – 113]</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>23.0ª</td>
<td>15.5ª</td>
</tr>
<tr>
<td>VO₂max (mL.min⁻¹.kg⁻²/³)</td>
<td>34.6 ± 9.1ªh</td>
<td>38.9 ± 7.3ªh</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>176.7 ± 9.3</td>
<td>177.2 ± 8.9</td>
</tr>
<tr>
<td>Trunk fat mass (kg)</td>
<td>8.2 ± 4.1ªi</td>
<td>8.9 ± 3.9ªi</td>
</tr>
<tr>
<td>Peripheral fat mass (kg)</td>
<td>11.0 ± 4.0ªj</td>
<td>10.1 ± 4.3ªj</td>
</tr>
<tr>
<td>Peripheral lean mass (kg)</td>
<td>21.5 ± 5.9ªk</td>
<td>25.4 ± 5.8ªk</td>
</tr>
</tbody>
</table>

* 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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model 1 B</th>
<th>95% CI</th>
<th>p</th>
<th>β</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA (ng/ml)</td>
<td>0.004</td>
<td>-0.01 to 0.02</td>
<td>0.54</td>
<td>1.70</td>
<td>-0.03 to 3.44</td>
<td>0.06</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.006</td>
<td>-0.01 to 0.02</td>
<td>0.49</td>
<td>0.28</td>
<td>-1.54 to 2.09</td>
<td>0.77</td>
</tr>
<tr>
<td>TNF-α (ng/ml)</td>
<td>0.001</td>
<td>-0.03 to 0.03</td>
<td>0.95</td>
<td>1.35</td>
<td>-3.06 to 5.75</td>
<td>0.55</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>0.001</td>
<td>-0.01 to 0.01</td>
<td>0.89</td>
<td>-0.29</td>
<td>-1.12 to 0.53</td>
<td>0.48</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>-0.024</td>
<td>-0.06 to 0.01</td>
<td>0.18</td>
<td>1.99</td>
<td>-2.75 to 6.73</td>
<td>0.41</td>
</tr>
<tr>
<td>Thrombomodulin (ng/ml)</td>
<td>0.002</td>
<td>-0.01 to 0.01</td>
<td>0.51</td>
<td>0.89</td>
<td>0.09 to 1.68</td>
<td>0.03*</td>
</tr>
<tr>
<td>P-selectine (ng/ml)</td>
<td>0.211</td>
<td>-0.02 to 0.44</td>
<td>0.07</td>
<td>2.09</td>
<td>-28.18 to 32.35</td>
<td>0.89</td>
</tr>
<tr>
<td>E-selectine (ng/ml)</td>
<td>-0.008</td>
<td>-0.05 to 0.03</td>
<td>0.65</td>
<td>4.24</td>
<td>-1.55 to 10.03</td>
<td>0.15</td>
</tr>
<tr>
<td>VCAM (ng/ml)</td>
<td>-0.568</td>
<td>-1.39 to 0.25</td>
<td>0.18</td>
<td>60.66</td>
<td>-50.90 to 172.22</td>
<td>0.29</td>
</tr>
<tr>
<td>ICAM (ng/ml)</td>
<td>-0.344</td>
<td>-0.84 to 0.15</td>
<td>-0.18</td>
<td>71.67</td>
<td>5.01 to 138.33</td>
<td>0.04*</td>
</tr>
<tr>
<td>vWF (%)</td>
<td>-0.004</td>
<td>-0.46 to 0.45</td>
<td>0.99</td>
<td>-6.33</td>
<td>-53.56 to 40.89</td>
<td>0.79</td>
</tr>
</tbody>
</table>

**Model 2**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model 2 B</th>
<th>95% CI</th>
<th>p</th>
<th>β</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA (ng/ml)</td>
<td>0.005</td>
<td>-0.01 to 0.02</td>
<td>0.54</td>
<td>1.57</td>
<td>-0.05 to 3.19</td>
<td>0.06</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.009</td>
<td>-0.01 to 0.03</td>
<td>0.35</td>
<td>0.21</td>
<td>-1.34 to 1.77</td>
<td>0.79</td>
</tr>
<tr>
<td>TNF-α (ng/ml)</td>
<td>0.005</td>
<td>-0.02 to 0.03</td>
<td>0.74</td>
<td>1.46</td>
<td>-2.88 to 5.79</td>
<td>0.51</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>0.001</td>
<td>-0.01 to 0.01</td>
<td>0.85</td>
<td>-0.31</td>
<td>-1.13 to 0.52</td>
<td>0.47</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>-0.021</td>
<td>-0.05 to 0.01</td>
<td>0.20</td>
<td>2.09</td>
<td>-2.74 to 6.92</td>
<td>0.39</td>
</tr>
<tr>
<td>Thrombomodulin (ng/ml)</td>
<td>0.003</td>
<td>-0.00 to 0.01</td>
<td>0.44</td>
<td>0.84</td>
<td>0.07 to 1.61</td>
<td>0.03*</td>
</tr>
<tr>
<td>P-selectine (ng/ml)</td>
<td>0.182</td>
<td>-0.08 to 0.44</td>
<td>0.17</td>
<td>-1.38</td>
<td>-31.43 to 28.68</td>
<td>0.93</td>
</tr>
<tr>
<td>E-selectine (ng/ml)</td>
<td>-0.004</td>
<td>-0.05 to 0.04</td>
<td>0.84</td>
<td>4.09</td>
<td>-1.67 to 9.85</td>
<td>0.16</td>
</tr>
<tr>
<td>VCAM (ng/ml)</td>
<td>-0.461</td>
<td>-1.32 to 0.39</td>
<td>0.29</td>
<td>60.80</td>
<td>-47.15 to 168.76</td>
<td>0.27</td>
</tr>
<tr>
<td>ICAM (ng/ml)</td>
<td>-0.334</td>
<td>-0.81 to 0.14</td>
<td>0.17</td>
<td>75.49</td>
<td>10.33 to 140.66</td>
<td>0.02*</td>
</tr>
<tr>
<td>vWF (%)</td>
<td>0.005</td>
<td>-0.44 to 0.45</td>
<td>0.98</td>
<td>-9.14</td>
<td>-56.74 to 38.46</td>
<td>0.71</td>
</tr>
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</table>

**Model 3**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model 3 B</th>
<th>95% CI</th>
<th>p</th>
<th>β</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA (ng/ml)</td>
<td>0.005</td>
<td>-0.01 to 0.02</td>
<td>0.49</td>
<td>1.33</td>
<td>-0.25 to 2.90</td>
<td>0.09</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.009</td>
<td>-0.001 to 0.03</td>
<td>0.29</td>
<td>-0.05</td>
<td>-1.60 to 1.51</td>
<td>0.95</td>
</tr>
<tr>
<td>TNF-α (ng/ml)</td>
<td>0.005</td>
<td>-0.02 to 0.03</td>
<td>0.74</td>
<td>1.61</td>
<td>-2.61 to 5.84</td>
<td>0.46</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>0.002</td>
<td>-0.01 to 0.01</td>
<td>0.14</td>
<td>-0.46</td>
<td>-1.27 to 0.36</td>
<td>0.27</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>-0.022</td>
<td>-0.06 to 0.01</td>
<td>0.18</td>
<td>2.52</td>
<td>-2.31 to 7.34</td>
<td>0.31</td>
</tr>
<tr>
<td>Thrombomodulin (ng/ml)</td>
<td>0.003</td>
<td>-0.00 to 0.01</td>
<td>0.41</td>
<td>0.89</td>
<td>0.12 to 1.67</td>
<td>0.02*</td>
</tr>
<tr>
<td>P-selectine (ng/ml)</td>
<td>0.196</td>
<td>-0.06 to 0.45</td>
<td>0.13</td>
<td>0.00</td>
<td>-29.14 to 29.14</td>
<td>1.00</td>
</tr>
<tr>
<td>E-selectine (ng/ml)</td>
<td>-0.002</td>
<td>-0.04 to 0.04</td>
<td>0.92</td>
<td>4.33</td>
<td>-1.512 to 10.18</td>
<td>0.15</td>
</tr>
<tr>
<td>VCAM (ng/ml)</td>
<td>-0.488</td>
<td>-1.31 to 0.33</td>
<td>0.24</td>
<td>50.88</td>
<td>-56.15 to 157.89</td>
<td>0.35</td>
</tr>
<tr>
<td>ICAM (ng/ml)</td>
<td>-0.278</td>
<td>-0.73 to 0.18</td>
<td>0.23</td>
<td>64.89</td>
<td>5.70 to 124.07</td>
<td>0.03*</td>
</tr>
<tr>
<td>vWF (%)</td>
<td>0.038</td>
<td>-0.41 to 0.48</td>
<td>0.87</td>
<td>-15.62</td>
<td>-61.28 to 30.04</td>
<td>0.50</td>
</tr>
</tbody>
</table>

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 (VO₂ max); 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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model 1 B (95% CI)</th>
<th>p</th>
<th>β (95% CI)</th>
<th>P</th>
<th>Model 2 B (95% CI)</th>
<th>p</th>
<th>β (95% CI)</th>
<th>P</th>
<th>Model 3 B (95% CI)</th>
<th>p</th>
<th>β (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA (ng/ml)</td>
<td>49.805 (-147.81 to 247.42)</td>
<td>0.62</td>
<td>-9626.27</td>
<td>0.35</td>
<td>57.065 (-170.32 to 284.45)</td>
<td>0.62</td>
<td>-11692.36</td>
<td>0.31</td>
<td>49.885 (-227.17 to 326.94)</td>
<td>0.72</td>
<td>-21308.29</td>
<td>0.36</td>
</tr>
<tr>
<td>CRP (ng/l)</td>
<td>42.989 (-72.69 to 158.67)</td>
<td>0.47</td>
<td>-3561.98</td>
<td>0.56</td>
<td>45.073 (-87.74 to 177.88)</td>
<td>0.50</td>
<td>-4458.96</td>
<td>0.51</td>
<td>43.443 (-112.71 to 199.59)</td>
<td>0.58</td>
<td>-6468.15</td>
<td>0.39</td>
</tr>
<tr>
<td>TNF-α (ng/ml)</td>
<td>0.012 (-0.02 to 0.04)</td>
<td>0.59</td>
<td>0.72</td>
<td>0.67</td>
<td>0.014 (-0.02 to 0.05)</td>
<td>0.41</td>
<td>0.89</td>
<td>0.61</td>
<td>0.010 (-0.03 to 0.05)</td>
<td>0.59</td>
<td>0.88</td>
<td>0.63</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>-0.006 (-0.04 to 0.03)</td>
<td>0.73</td>
<td>1.39</td>
<td>0.39</td>
<td>-0.007 (-0.05 to 0.03)</td>
<td>0.72</td>
<td>1.03</td>
<td>0.61</td>
<td>-0.005 (-0.05 to 0.04)</td>
<td>0.82</td>
<td>1.81</td>
<td>0.66</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>-0.000 (-0.06 to 0.06)</td>
<td>0.99</td>
<td>2.17</td>
<td>0.48</td>
<td>-0.021 (-0.09 to 0.04)</td>
<td>0.52</td>
<td>0.89</td>
<td>0.79</td>
<td>-0.029 (-0.10 to 0.04)</td>
<td>0.43</td>
<td>0.79</td>
<td>0.82</td>
</tr>
<tr>
<td>Thrombomodulin (ng/ml)</td>
<td>0.001 (-0.00 to 0.01)</td>
<td>0.62</td>
<td>0.01</td>
<td>0.98</td>
<td>0.002 (-0.01 to 0.01)</td>
<td>0.58</td>
<td>0.09</td>
<td>0.79</td>
<td>0.003 (-0.00 to 0.01)</td>
<td>0.35</td>
<td>0.19</td>
<td>0.54</td>
</tr>
<tr>
<td>P-selectine (ng/ml)</td>
<td>-0.043 (-0.30 to 0.22)</td>
<td>0.75</td>
<td>-4.67</td>
<td>0.73</td>
<td>-0.004 (-0.29 to 0.28)</td>
<td>0.98</td>
<td>-5.83</td>
<td>0.69</td>
<td>-0.006 (-0.04 to 0.04)</td>
<td>0.96</td>
<td>0.44</td>
<td>0.83</td>
</tr>
<tr>
<td>E-selectine (ng/ml)</td>
<td>-0.007 (-0.05 to 0.03)</td>
<td>0.72</td>
<td>1.03</td>
<td>0.61</td>
<td>-0.016 (-1.66 to 0.43)</td>
<td>0.25</td>
<td>-37.32</td>
<td>0.48</td>
<td>-0.000 (-0.04 to 0.04)</td>
<td>0.46</td>
<td>0.44</td>
<td>0.63</td>
</tr>
<tr>
<td>VCAM (ng/ml)</td>
<td>-0.545 (-1.48 to 0.39)</td>
<td>0.25</td>
<td>-42.49</td>
<td>0.39</td>
<td>-0.411 (-1.34 to 0.52)</td>
<td>0.25</td>
<td>-42.14</td>
<td>0.29</td>
<td>-0.021 (-0.50 to 0.55)</td>
<td>0.94</td>
<td>-2.44</td>
<td>0.93</td>
</tr>
<tr>
<td>ICAM (ng/ml)</td>
<td>-0.032 (-0.45 to 0.52)</td>
<td>0.89</td>
<td>-2.18</td>
<td>0.93</td>
<td>0.021 (-0.50 to 0.55)</td>
<td>0.94</td>
<td>-2.44</td>
<td>0.93</td>
<td>0.015 (-0.05 to 0.04)</td>
<td>0.59</td>
<td>-1.25</td>
<td>0.82</td>
</tr>
<tr>
<td>vWF (%)</td>
<td>-0.251 (-0.65 to 0.14)</td>
<td>0.21</td>
<td>-42.39</td>
<td>0.04*</td>
<td>-0.105 (-0.50 to 0.29)</td>
<td>0.60</td>
<td>-41.76</td>
<td>0.04*</td>
<td>-0.086 (-0.41 to 0.23)</td>
<td>0.59</td>
<td>2.06</td>
<td>0.89</td>
</tr>
<tr>
<td>selectine (ng/ml)</td>
<td>-0.004 (-0.29 to 0.28)</td>
<td>0.98</td>
<td>-5.83</td>
<td>0.69</td>
<td>-0.006 (-0.05 to 0.04)</td>
<td>0.82</td>
<td>1.81</td>
<td>0.66</td>
<td>-0.005 (-0.05 to 0.04)</td>
<td>0.82</td>
<td>1.23</td>
<td>0.55</td>
</tr>
</tbody>
</table>

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 (VO₂ max), 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 thrombomodulin 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 thrombomodulin and ICAM. Thrombomodulin 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 thrombomodulin 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.
Acknowledgements

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

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</tbody>
</table>
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) (WTS₂). 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 [\(\Delta D\)]) 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:
\[ DC = \frac{(2\Delta D \cdot D + \Delta D^2)}{(PP \cdot D^2)} \text{ in } 10^{-3}\text{kPa}^{-1} \]

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

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.

\[ YEM = \frac{D}{(IMT \cdot DC)} \text{ in kPa} \]

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 absorptiometry (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,
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$_2$max) 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$_2$max 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, VO₂max 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)

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

* 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)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hcy</th>
<th>ADMA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>95% CI</td>
</tr>
<tr>
<td><strong>Model 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA DC (10⁻³ kPa⁻¹)</td>
<td>-0.003</td>
<td>-0.01 to 0.01</td>
</tr>
<tr>
<td>CA CC (mm². kPa⁻¹)</td>
<td>-0.006</td>
<td>-0.01 to 0.00</td>
</tr>
<tr>
<td>FA DC (10⁻³ kPa⁻¹)</td>
<td>0.004</td>
<td>-0.01 to 0.02</td>
</tr>
<tr>
<td>FA CC (mm². kPa⁻¹)</td>
<td>0.000</td>
<td>-0.01 to 0.01</td>
</tr>
<tr>
<td>YEM (kPa)</td>
<td>0.000</td>
<td>-0.01 to 0.01</td>
</tr>
<tr>
<td>Σ AP</td>
<td>-0.002</td>
<td>-0.01 to 0.01</td>
</tr>
<tr>
<td>IMT (mm)</td>
<td>-0.005</td>
<td>-0.01 to 0.00</td>
</tr>
<tr>
<td><strong>Model 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA DC (10⁻³ kPa⁻¹)</td>
<td>-0.002</td>
<td>-0.01 to 0.01</td>
</tr>
<tr>
<td>CA CC (mm². kPa⁻¹)</td>
<td>-0.006</td>
<td>-0.01 to 0.00</td>
</tr>
<tr>
<td>FA DC (10⁻³ kPa⁻¹)</td>
<td>0.003</td>
<td>-0.01 to 0.02</td>
</tr>
<tr>
<td>FA CC (mm². kPa⁻¹)</td>
<td>0.000</td>
<td>-0.01 to 0.01</td>
</tr>
<tr>
<td>YEM (kPa)</td>
<td>0.000</td>
<td>-0.01 to 0.01</td>
</tr>
<tr>
<td>Σ AP</td>
<td>-0.002</td>
<td>-0.01 to 0.01</td>
</tr>
<tr>
<td>IMT (mm)</td>
<td>-0.006</td>
<td>-0.02 to 0.00</td>
</tr>
<tr>
<td><strong>Model 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA DC (10⁻² kPa⁻¹)</td>
<td>-0.002</td>
<td>-0.01 to 0.01</td>
</tr>
<tr>
<td>CA CC (mm². kPa⁻¹)</td>
<td>-0.005</td>
<td>-0.01 to 0.00</td>
</tr>
<tr>
<td>FA DC (10⁻³ kPa⁻¹)</td>
<td>0.003</td>
<td>-0.01 to 0.02</td>
</tr>
<tr>
<td>FA CC (mm². kPa⁻¹)</td>
<td>0.000</td>
<td>-0.01 to 0.01</td>
</tr>
<tr>
<td>YEM (kPa)</td>
<td>-0.001</td>
<td>-0.01 to 0.01</td>
</tr>
<tr>
<td>Σ AP</td>
<td>-0.002</td>
<td>-0.01 to 0.01</td>
</tr>
<tr>
<td>IMT (mm)</td>
<td>-0.005</td>
<td>-0.01 to 0.00</td>
</tr>
</tbody>
</table>

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 (VO₂max), 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)

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

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 (VO2max); 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, VO2max, 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

The AGAHLS was financially supported by Dutch Heart Foundation grants, Dutch Prevention Fund grants, a Dutch Ministry of Well-being and Public Health grant, the Dairy Foundation on Nutrition and Health, the Dutch Olympic Committee/Netherlands Sports Federation, Heineken, Inc., and the Scientific Board of Smoking and Health. This study forms part of the Amsterdam Growth and Health Longitudinal Study (AGAHLS). Therefore, the authors want to thank the participants from the AGAHLS for all their sacrifices to take part in the measurements.

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; VO₂max: physical fitness; AP: arterial properties.
References


38 Beltowski J, Kedra A. Asymmetric dimethylarginine (ADMA) as a target for pharmacotherapy. Pharmacological Reports 2006; 58: 159-178.

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_{2}\text{max} 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 thrombomoduline). 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 ($\beta = -0.81; p = 0.03$). 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 ($\beta = 71.7; p = 0.03$) and thrombomoduline ($\beta = 0.89; p = 0.03$) as well as a significant relationship between the changes in ADMA concentrations and the Von Willebrand factor ($\beta = -42.4; p = 0.04$). 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 thrombomodulin) 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_{2}\text{max} measurements in the AGAHLS 2000 to the indirect VO_{2}\text{max} 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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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

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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

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<td>Original Article</td>
<td>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</td>
<td>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.</td>
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observational studies in epidemiology (MOOSE). All these reporting guidelines can be found at [http://www.equator-network.org](http://www.equator-network.org).

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invitation of the Editors)

Commentaries discuss a paper published in a specific issue and should set the problems addressed by the paper in the wider context of the field. Invited commentaries may also provide a 'point-of-view' related to a controversy or debate in nutrition. Debates will address an area of research that is of major present interest and for which there are substantially different views. The subject to be debated and the authors producing the opposing views will be selected by the Editors.

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Continuing Educational/Guidelines/Nutrition in Clinical Practice (only by invitation of the Editors)

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| Word limit: 3,000 words maximum excluding the abstract, references, figures and tables. Abstract: Unstructured abstract of 250 words maximum. References: 50 references maximum (as far as possible only recent references). Display items: No more than 8 display items (e.g. figures and/or tables) should accompany the manuscript. |

**Preparation of Original Articles**

1. Cover letter (must include a Conflict of Interest statement)
2. Title page (excluding acknowledgements)
3. Abstract and keywords
4. Introduction
5. Materials (or patients) and methods
6. Results
7. Discussion
8. Acknowledgements
9. Conflict of Interest
10. References
11. Tables
12. Figures

**Cover letter**

The uploaded covering letter must state the material is original research, has not been previously published and has not been submitted for publication elsewhere while under consideration. The covering letter must also contain a Conflict of Interest statement (see Editorial Policy section).

**Title page**
The title page should bear the title of the paper, the full names of all the authors, highest academic degree obtained, and their affiliations, together with the name, full postal address, telephone and fax numbers and e-mail address of the author to whom correspondence and offprint requests are to be sent (This information is also asked for on the electronic submission form). The title should be brief, informative, of 150 characters or less and should not make a statement or conclusion. The running title should consist of not more than 50 letters and spaces. It should be as brief as possible, convey the essential message of the paper and contain no abbreviations. Authors should disclose the sources of any support for the work, received in the form of grants and/or equipment and drugs.

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Articles must be prepared with a structured abstract designed to summarize the essential features of the paper in a logical and concise sequence under the following headings, which are mandatory.

- **Background/Objectives**: What was the main question or hypothesis tested?
- **Subjects/Methods**: How many subjects were recruited, how many dropped out? Was the study randomized, case-controlled etc? Interventions/methods used and duration of administration.
- **Results**: Indicate 95% confidence intervals and exact $P$ value for effects.
- **Conclusions**: Answer (significant or not) to main question.
- **Keywords**: Preferably use up to 6 words chosen from MEDLINE MeSH, which best describe your paper. These will be used for indexing your paper in EJCN and also help retrieval from computer databases.

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The Introduction should assume that the reader is knowledgeable in the field and should therefore be as brief as possible but can include a short historical review where desirable.

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This section should contain sufficient detail, so that all experimental procedures can be reproduced, and include references. Methods, however, that have been published in detail elsewhere should not be described in detail. Authors should provide the name of the manufacturer and their location for any specifically named medical equipment and instruments, and all drugs should be identified by their pharmaceutical names, and by their trade name if relevant.

Results and Discussion

The Results section should briefly present the experimental data in text, tables or figures. Tables and figures should not be described extensively in the text, either. The discussion should focus on the interpretation and the significance of the findings with concise objective comments that describe their relation to other work in the area. It should not repeat information in the results. The final paragraph should highlight the main conclusion(s), and provide some indication of the direction future research should take.

Acknowledgements

These should be brief, and should include sources of support including sponsorship (e.g. university, charity, commercial organization) and sources of material (e.g. novel drugs) not available commercially.

Conflict of interest
Authors must declare whether or not there is any competing financial interests in relation to the work described. This information must be included at this stage and will be published as part of the paper. Conflict of interest should also be noted on the cover letter and as part of the submission process. See the Conflict of Interest documentation in the Editorial Policy section for detailed information.

References

References in text are indicated in the text by name and date e.g. (Pampiglione & Ricciardi, 1986) and (Kusin et al., 1994), and listed at the end of the paper in alphabetical order of first author. References should be listed and journal titles abbreviated according to the style used by Index Medicus, examples are given below. All authors should be quoted for papers with up to six authors; for papers with more than six authors, the first six only should be quoted, followed by et al. Please use recent references wherever possible.

Examples of article references:


Articles in books:


Personal communications must be allocated a number and included in the list of references in the usual way or simply referred to in the text; the authors may choose which method to use. In either case authors must obtain permission from the individual concerned to quote his/her unpublished work.

Tables

These should be labelled sequentially and cited within the text. Each table should be presented on its own page, numbered and titled. Reference to table footnotes should be made by means of Arabic numerals. Tables should not duplicate the content of the text. They should consist of at least two columns; columns should always have headings. Authors should ensure that the data in the tables are consistent with those cited in the relevant places in the text, totals add up correctly, and percentages have been calculated correctly. Unlike figures or images, tables may be embedded into the word processing software if necessary, or supplied as separate electronic files.

Figures

Figures and images should be labelled sequentially, numbered and cited in the text. Figure legends should be brief, specific and appear on a separate manuscript page after the References section. Refer to (and cite) figures specifically in the text of the paper. Figures should not be embedded within the text. If a table or figure has been published before, the authors must obtain written permission to reproduce the material in both print and electronic formats from the copyright owner and submit it with the manuscript. This follows for quotes, illustrations and other materials taken from previously published works not in the public domain. The original source should be cited in the figure caption or table footnote. The use of three-dimensional histograms is strongly discouraged when the addition of the third dimension gives no extra information. Scale markers should be used in the image for electron micrographs, and
indicate the type of stain used. Detailed guidelines for submitting artwork can be found by downloading the Artwork Guidelines PDF.

Supplementary information

Supplementary information (SI) is peer-reviewed material directly relevant to the conclusion of an article that cannot be included in the printed version owing to space or format constraints. The article must be complete and self-explanatory without the SI, which is posted on the journal's website and linked to the article. SI may consist of data files, graphics, movies or extensive tables, view the Artwork Guidelines PDF for more information on accepted file types. Authors should submit documents in their FINAL format as they are not edited, typeset or changed, and will appear online exactly as submitted. When submitting SI authors are required to:

- Include a text summary (no more than 50 words) to describe the contents of each file.
- Identify the types of files (file formats) submitted.
- Include the text "Supplementary information is available at [the journal's name]'s website" at the end of the article and before the references.

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General

- Do not make rules thinner than 1pt (0.36mm)
- Use a coarse hatching pattern rather than shading for tints in graphs
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- Use SI units throughout
- Text should be double spaced with a wide margin
- At first mention of a manufacturer, the town (and state if USA) and country should be provided

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An inherent principle of publication is that others should be able to replicate and build upon the authors' published claims. Therefore, a condition of publication is that authors are required to make materials, data and associated protocols available in a publicly accessible database (as detailed in the sections below on this page). Where one does not exist, the information must be made available to referees at submission and to readers promptly on request. Any restrictions on materials availability or other relevant information must be disclosed in the manuscript's methods section and should include details of how materials and information may be obtained.

Sequences, structures and 'omics': Papers reporting protein or DNA sequences and molecular structures will not be accepted without an accession number to Genbank/EMBL/DDBJ, Protein DataBank, SWISS-PROT or other appropriate, identified, publicly available database in general use in the field that gives free access to researchers from the date of publication.

Authors of papers describing structures of biological macromolecules must provide experimental data upon the request of editors if they are not already freely accessible in a publicly available database such as Protein DataBank, Nucleic Acids Database or Biological Magnetic Resonance Databank. Five separate copies of these data should be provided to the editors in an appropriate format (for example, CD or DVD) for the purposes of peer-review.

Statistical methods: For normally distributed data, mean (SD) is the preferred summary statistic. Relative risks should be expressed as odds ratios with 95% confidence interval. To
APPENDIX A

compare two methods for measuring a variable the method of Bland & Altman (1986, Lancet 1, 307–310) should be used; for this, calculation of P only is not appropriate.

Units and Abbreviations: Use metric units (SI units) as fully as possible. Preferably give measurements of energy in kiloJoules or MegaJoules with kilocalories in parentheses (1 kcal = 4.186kJ). Use % throughout. Very common abbreviations such as FFA, RNA, need not be defined; on first using an abbreviation place it in parentheses after the full item. Note these abbreviations: gram g; litre l; milligram mg; kilogram kg; kilojoule kJ; megajoule MJ; weight wt; seconds s; minutes min; hours h. Do not add s for plural units.

1.1.6 SUBMISSION OF PAPERS
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For optimum performance, your browser should be either Netscape 4.7 or above, or Internet Explorer 5.0 and above. Make sure that your browser is set to accept cookies. Our tracking system requires cookies for proper operation. (If you have Windows XP the defaults will need changing. For more details on this, please refer to the 'Tips' function on this site.)

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When you first access our tracking system, you will be taken to your Home page, where different categories of tasks are listed. If you are required to perform a pending action item or task, there will be a red arrow ➤ next to a 'Manuscript' link. Throughout the system, red arrows ➤ reflect pending action items which you should address. If there are no red arrows visible on your Home page, then you are finished and have no outstanding tasks to complete. At any time please press HOME to go to the submission home page.

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- Fax Numbers

In addition you will need:

- Covering letter (including Conflict of Interest statement)
- Title and Running Title (you can copy and paste this from your manuscript)
- Abstract (you can copy and paste this from your manuscript)
- Manuscript files in Word, WordPerfect, text or any RTF format
- Figures/Images in external files in TIFF or JPEG, in either grayscale or CMYK colour, not in RGB
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The manuscript submission process is broken down into a series of 4 primary tasks that gather detailed information about your manuscript and allow you to upload the pertinent text and figure/image files. The sequence of screens is as follows:
1. The 'Files' primary task allows you to select the actual file locations (via an open file dialogue). You will be able to 'Browse' for the relevant files on your computer. **Please include the figure number in the title line for each figure.** On the completion screen, you will be asked to specify the order in which you want the individual files to appear in the merged document. Editors and/or reviewers will also be able to look at the individual PDF files if necessary. For more information on accepted file types, view the [Artwork Guidelines PDF](#).

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**Setting**

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**Subjects**

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**Interventions**

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

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

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**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 activiteitenmeter 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:

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).
7. Vragenlijsten over persoonlijkheid en alcoholgebruik voeding en activiteit.
8. Sterkte van je botten (hiel, lendenwervels, heup en hele lichaam).

- 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

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(MEG). Dit is een techniek die veel overeenkomsten vertoont met het "klassieke" hersenfilmje (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 redden 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
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.
### APPENDIX E

#### 1. WOON-WERK VERKEER (heen en terug)

<table>
<thead>
<tr>
<th>Activity</th>
<th>Aantal dagen per week</th>
<th>Gemiddelde tijd per dag</th>
<th>Inspanning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lopen van/naar werk</td>
<td>dagen</td>
<td>uur</td>
<td>minuten</td>
</tr>
<tr>
<td>Fietsen van/naar werk</td>
<td>dagen</td>
<td>uur</td>
<td>minuten</td>
</tr>
</tbody>
</table>

#### 2. LICHAMELIJKE ACTIVITEIT OP WERK

- **Licht inspannende activiteit** (Bijvoorbeeld: zittend/staand werk, met af en toe lopen, zoals bureauwerk etc.)
- **Zwaar inspannend activiteit** (Bijvoorbeeld: lopend werk, waarbij regelmatig zware dingen moeten worden opgetild).

#### 3. HUISHOUDELIJKE ACTIVITEITEN

- **Licht inspannende huishoudelijke activiteit** (Bijvoorbeeld: staand werk, zoals koken, afwassen, strijken, kind eten geven/in bad doen, stofzuigen, boodschappen doen)
- **Zwaar inspannend huishoudelijke activiteit** (Bijvoorbeeld: vloer schrobben, tapijt uitkloppen, met zware boodschappen lopen)

#### 4. VRIJE TIJD

<table>
<thead>
<tr>
<th>Activity</th>
<th>Aantal dagen per week</th>
<th>Gemiddelde tijd per dag</th>
<th>Inspanning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wandelen</td>
<td>dagen</td>
<td>uur</td>
<td>minuten</td>
</tr>
<tr>
<td>Fietsen</td>
<td>dagen</td>
<td>uur</td>
<td>minuten</td>
</tr>
<tr>
<td>Tuinieren</td>
<td>dagen</td>
<td>uur</td>
<td>minuten</td>
</tr>
<tr>
<td>Klussen/Doe-het-zelven</td>
<td>dagen</td>
<td>uur</td>
<td>minuten</td>
</tr>
</tbody>
</table>

#### 5. ACTIEVE SPORTEN (Hier maximaal 4 opschrijven)

<table>
<thead>
<tr>
<th>Activity</th>
<th>Aantal dagen</th>
<th>Gemiddelde tijd</th>
<th>Inspanning</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>dagen</td>
<td>uur</td>
<td>minuten</td>
</tr>
<tr>
<td>2.</td>
<td>dagen</td>
<td>uur</td>
<td>minuten</td>
</tr>
<tr>
<td>3.</td>
<td>dagen</td>
<td>uur</td>
<td>minuten</td>
</tr>
<tr>
<td>4.</td>
<td>dagen</td>
<td>uur</td>
<td>minuten</td>
</tr>
</tbody>
</table>

**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
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
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.
APPENDIX G: SMOKING QUESTIONNAIRE
**ROOKVRAGENLIJST**

1. Rook je?  
   - Ja  
   - Nee  

2. Rook je sigaretten?  
   - Ja  
   - Nee  

3. Rook je shag?  
   - Ja  
   - Nee  

---

1. *Indien nee, ga naar vraag 18*

2. *Indien nee, ga naar vraag 3*

- Hoeveel sigaretten rook je gemiddeld per dag?  
  - Nee  
  - Ja

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

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

- Soort tabak?  
  - Extra licht
  - Licht
  - Halfzwaar
  - Zwaar
  - Weet niet

- Filter?  
  - Met
  - Zonder

- Gedurende hoeveel jaren rook je dit aantal sigaretten per dag?  
  - Nee

- Voor die tijd rookte ik  
  - Niet
  - Wel

3. *Indien nee, ga naar vraag 4*

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

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
APPENDIX G

Licht
Halfzwaar
Zwaar
Weet niet

- Gedurende hoeveel jaren rook je dit aantal sigaren/cigarillo’s per week?

gedurende . . . . . . jaar

- Voor die tijd rookte ik

Niet
Wel
n.l. . . . . . . sigaren/cigarillo’s per week

5. Rook je pijptabak?

Ja
Nee

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
Ja, een klein beetje
Ja, normaal
Ja, heel diep

- Soort tabak?

Extra licht
Licht
Halfzwaar
Zwaar
Weet niet

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

gedurende . . . . . . jaar

- Voor die tijd rookte ik

Niet
Wel
n.l. . . . . . . pakjes pijptabak per week

6. Indien ik belemmerd word in mijn rookgedrag,

Ja
ga ik op andere momenten gewoon meer roken. Nee

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

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

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

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

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

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
    Nee

    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
    Nee

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
   Wel
   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
   Nee

   Indien ja,
   - deze persoon rookt
     Heel weinig
     Af en toe
     Veel
     Heel veel

30. Zit je veel in rokerige ruimtes (bijv. café, trein, kantine) ?
   Ja
   Nee
APPENDIX H: PROOF OF SUBMISSION