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Plasma Asymmetric Dimethylarginine (ADMA) in Relation to Cardiovascular Physiology and Risk Factors of Atherosclerosis

■

to my family

ABSTRACT

Background Atherosclerosis may be manifested as an endothelial dysfunction with a concurrent reduced bioavailability of nitric oxide (NO). Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of NO synthase that can modulate NO production and thereby affect endothelial function. ADMA has been observed to associate with several cardiovascular risk factors and it may be a circulating marker of endothelial dysfunction and thus subclinical atherosclerosis. However, the role of ADMA in the development of vascular disease is still largely unclear.

Objectives To study how plasma ADMA is related to early atherosclerotic changes like carotid intima-media thickness (IMT), indices of vascular function, and atherosclerosis risk factors. Furthermore, the association of dietary factors with ADMA and the effect of statin therapy on plasma concentration of ADMA were studied.

Subjects and Methods The study was based on five separate study populations (studies **I-V**), consisting of 373 patients or controls. In study **I**, there were forty-seven healthy controls, 16 men with borderline hypertension and 14 men with heterozygous familial hypercholesterolemia. This study evaluated the association between plasma ADMA and myocardial blood flow measured by positron emission tomography (PET) as well as carotid IMT measured by ultrasound. Study **II** included eighty-six type 2 diabetic patients and 65 controls with a comparable distribution of age and gender. In this study, plasma levels of ADMA were compared between subjects with type 2 diabetes and non-diabetic controls and plasma ADMA concentrations were related to glycemic control and glomerular filtration rate (GFR). In study **III**, forty-four mild to moderately hypercholesterolemic subjects (29 men and 15 women), participated in a randomised, double-blind, placebo-controlled trial to determine the effect of high dose atorva- or simvastatin therapy on plasma arginine derivatives. Thirty-four subjects (14 men and 20 women) participated in study **IV** to determine the impact of dietary factors and alcohol consumption on the plasma concentrations of arginine derivatives. In that study, seven-day food records were used to analyze diet and alcohol intake. Study **V** evaluated, in sixty-seven men, how plasma ADMA relates to blood pressure and other non-invasively studied indices of hemodynamics.

Results There was a significant direct association between plasma ADMA and carotid IMT as well as an inverse association to the reduced dipyridamole induced vasodilatory function in young men as measured by PET (study I). In this study, borderline hypertensive subjects not on antihypertensive treatment had higher plasma ADMA levels than controls. In study II, type 2 diabetic subjects had significantly lower plasma ADMA levels compared to controls, possibly due to increased kidney GFR. There was no significant correlation between plasma ADMA and blood pressure in studies IV and V or other measured indices of hemodynamics (study V). In study V, subjects on antihypertensive treatment had lower plasma ADMA levels than non-treated subjects. There were no significant association between plasma ADMA and risk factors of atherosclerosis such as hypercholesterolemia or smoking in any of the studies. In study III, high dose simvastatin and atorvastatin treatment effectively decreased plasma total and LDL cholesterol but had no influence on plasma ADMA concentration. In dietary study IV, high amounts of energy derived from carbohydrates were significantly associated with low plasma ADMA levels and ADMA concentration was significantly higher in alcohol drinkers than in abstainers.

Conclusions Plasma ADMA was associated with early atherosclerotic changes like carotid IMT and hyperemic myocardial blood flow measured by PET but not with risk factors of atherosclerosis. High amounts of energy received from carbohydrates were strongly associated with low plasma ADMA concentrations but statin treatment had no influence on plasma ADMA.

TIIVISTELMÄ

Tausta Valtimokovettumatauti voi tulla esiin endoteelin toimintahäiriönä, johon liittyy alentunut typpioksidin (NO) saatavuus. Asymmetrinen dimetyyliarginiini (ADMA) on typpioksidia syntetisoivan entsyymin toimintaa estävä aine, joka voi vaikuttaa typpioksidin muodostumiseen ja siten endoteelin toimintaan. ADMA:n on todettu liittyvän useisiin sydänverisuonisairauksien riskitekijöihin ja sen on ajateltu olevan endoteelin toimintahäiriön ja piileväoireisen valtimokovettumataudin markkeri. ADMA:n rooli verisuonisairauden kehittymisessä on kuitenkin pitkälti epäselvä.

Tavoitteet Tutkittiin ADMA:n suhdetta alkuvaiheen valtimokovettumataudin muutoksiin, kuten karotisvaltimon intima-media paksuuntumaan (IMT), verisuonitoimintaa kuvaaviin muuttujiin ja valtimokovettumataudin riskitekijöihin. Lisäksi tutkittiin ravintotekijöiden ja ADMA:n suhdetta sekä korkea-annoksisen statiinilääkityksen vaikutusta ADMA:an.

Tutkittavat ja menetelmät Tutkimuksessa oli viisi tutkimusryhmää (tutkimukset **I-V**)**,** käsittäen yhteensä 373 potilasta tai kontrollihenkilöä. Tutkimuksessa **I** oli neljäkymmentäseitsemän tervettä kontrollihenkilöä, 16 miestä, joilla oli raja-arvoinen verenpainetauti ja 14 miestä, joilla oli heterotsykoottinen familiaalinen hyperkolesterolemia. Tutkimus selvitti ADMA:n suhdetta positroni emissio tomografialla (PET) mitattuun sydänverisuonten verenvirtaukseen ja ultraäänellä mitattuun IMT:hen. Tutkimuksessa **II** oli kahdeksankymmentäkuusi tyypin 2 diabetestä sairastavaa henkilöä ja 65 saman ikä- ja sukupuolijakauman täyttävää kontrollihenkilöä. Tutkimuksessa vertailtiin diabetestä sairastavien ja kontrollihenkilöiden ADMA tasoja sekä ADMA tasojen suhdetta sokeritasapainoon ja glomerulussuodoksen määrään (GFR). Tutkimukseen **III** otettiin neljäkymmentäneljä lievästi hyperkolesterolemista henkilöä (29 miestä ja 15 naista). Tutkimus oli randomisoitu, kaksoissokkoutettu ja plasebokontrolloitu tutkimus, jossa selvitettiin suuriannoksisen atorva- ja simvastatiinihoidon vaikutusta plasman arginiinijohdannaisiin. Kolmekymmentäneljä henkilöä (14 miestä ja 20 naista) osallistui tutkimukseen **IV**, jossa selvitettiin ravintotekijöiden ja alkoholin käytön vaikutusta plasman arginiinijohdannaisiin. Ruokavalio ja alkoholin käyttö arvioitiin seitsemän päivän ruokapäiväkirjan perusteella. Tutkimuksessa **V** oli mukana kuusikymmentäseitsemän miestä ja siinä selvitettiin ADMA:n suhdetta verenpaineeseen ja muihin ei-kajoavasti mitattuihin hemodynaamisiin muuttujiin.

Tulokset Nuorilla miehillä ADMA oli merkitsevässä suhteessa karotiksen IMT:hen sekä käänteisessä suhteessa alentuneeseen dipyridamolilla aikaansaatuun ja PET:llä mitattuun verisuonen laajenemiseen (tutkimus I). Tässä tutkimuksessa ei-lääkityillä raja-arvoisesti verenpainetautisilla henkilöillä oli korkeammat plasman ADMA tasot kuin kontrollihenkilöillä. Tutkimuksessa II tyypin 2 diabetestä sairastavilla henkilöillä oli merkitsevästi matalammat plasman ADMA tasot kuin kontrolleilla, mahdollisesti lisääntyneestä munuaisten GFR:stä johtuen. Plasman ADMA ei korreloinut merkitsevästi verenpaineeseen tutkimuksissa IV ja V tai muihin hemodynaamisiin muuttujiin (tutkimus V). Tutkimuksessa V verenpainelääkitystä käyttävillä henkilöillä oli matalammat plasman ADMA tasot kuin ei-hoidetuilla henkilöillä. Yhdessäkään tutkimuksessa ADMA ei assosioitunut valtimokovettumataudin riskitekijöihin, kuten hyperkolesterolemiaan tai tupakointiin. Tutkimuksessa III korkea-annoksinen simvastatiini- ja atorvastatiinihoito laskivat tehokkaasti plasman kokonais- ja LDL kolesterolia, mutta plasman ADMA pitoisuuteen hoidoilla ei ollut vaikutusta. Ruokavalioon liittyvässä tutkimuksessa IV runsas hiilihydraattien käyttö oli merkitsevässä suhteessa mataliin plasman ADMA tasoihin ja ADMA pitoisuus oli merkitsevästi korkeampi alkoholin käyttäjillä kuin alkoholista pidättäytyvillä.

Yhteenveto Plasman ADMA assosioitui aikaisiin valtimokovettumatautiin liittyviin muutoksiin, kuten karotiksen IMT:hen ja PET:llä mitattuun sydänlihaksen verenvirtaukseen, mutta ei valtimokovettumataudin riskitekijöihin. Runsas hiilihydraattien käyttö oli vahvassa suhteessa mataliin plasman ADMA pitoisuuksiin, mutta statiinihoidolla ei ollut vaikutusta plasman ADMA:an.

CONTENTS

ABBREVIATIONS

ACE — angiotensin converting enzyme
ADMA — asymmetric dimethylarginine
BHT — borderline hypertension
BMI — body mass index
BP — blood pressure
CFR — coronary flow reserve
CO — cardiac output
DBP — diastolic blood pressure
DM — diabetes mellitus
FH — familial hypercholesterolemia
GFR — glomerular filtration rate
$GHbA_1c$ — glycosylated hemoglobin
HDL — high density lipoprotein
HPLC — high-performance liquid chromatography
IMT — intima-media thickness
LCW — left cardiac work
LDL — low density lipoprotein
LMMA — monomethylarginine
MAP — mean arterial pressure
NO — nitric oxide
NOS — nitric oxide synthase
OPA — o-pthalaldehyde
PET — positron emission tomography
PRMT — protein arginine methyltransferase
PWV — pulse wave velocity
RANOVA — variance for repeated measurements
ROI — region of interest
SBP — systolic blood pressure
SDMA — symmetric dimethylarginine
SV — stroke volume
SVR — systemic vascular resistance
SVRI — systemic vascular resistance index
TC — total cholesterol
TG — triglycerides
TNF — tumor necrosis factor
UV — ultraviolet
WHO — World Health Organization

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, which are referred to in the text by their Roman numerals I-V. In addition, some unpublished data are presented.

I   Päivä H, Laakso J, Laine H, Laaksonen R, Knuuti J, Raitakari OT (2002): Plasma asymmetric dimethylarginine (ADMA) and hyperemic myocardial blood flow in young subjects with borderline hypertension or familial hypercholesterolemia. J Am Coll Cardiol 40:1241-1247.

II   Päivä H, Lehtimäki T, Laakso J, Ruokonen I, Rantalaiho V, Wirta O, Pasternack A, Laaksonen R (2003): Plasma concentrations of asymmetric-dimethyl-arginine in type 2 diabetes associate with glycemic control and glomerular filtration rate but not with risk factors of vasculopathy. Metabolism 52:303-307.

III   Päivä H, Laakso J, Lehtimäki T, Isomustajärvi M, Ruokonen I, Laaksonen R (2003). Effect of high-dose statin treatment on plasma concentrations of endogenous nitric oxide synthase inhibitors. J Cardiovasc Pharmacol 41:219-222.

IV   Päivä H, Lehtimäki T, Laakso J, Ruokonen I, Tervonen R, Lehtinen S, Nikkilä M, Wuolijoki E, Laaksonen R (2004). Dietary composition as a determinant of plasma asymmetric dimethylarginine (ADMA) in subjects with mild hypercholesterolemia. Metabolism 53:1072-1075.

V   Päivä H, Laakso J, Kähönen M, Turjanmaa V, Kööbi T, Majahalme S, Lehtimäki T, Ruokonen I, Laaksonen R (2004). Asymmetric dimethylarginine (ADMA) and hemodynamic regulation in middle-aged men. (submitted for publication).

INTRODUCTION

Atherosclerosis related vascular disease is the main cause of morbidity and mortality in Finland (Pyörälä et al. 1985) and in other Western countries (Tunstall-Pedoe et al. 1994). Traditional risk factors for the development of atherosclerosis, such as hypertension, hypercholesterolemia, smoking and diabetes mellitus have been shown to cause endothelial vasodilator dysfunction and, together have an additive effect in relation to cardiovascular disease risk. Treatment of these factors restores endothelial function and decreases cardiovascular morbidity and mortality (Böger et al. 1998b, Miyazaki et al. 1999, Chan et al. 2000, De Vriese et al. 2000).

There is abundant evidence that the endothelium plays a crucial role in the maintenance of vascular tone and structure. Early functional changes, eventually leading to atherosclerotic plaque formation, happen in the endothelial cells (Vane et al. 1990). One of the major endothelium-derived vasoactive mediators is nitric oxide (NO). NO is formed in the endothelium by the endothelial isoform of nitric oxide synthase (NOS). NO is the most potent vasodilatator and plays a significant role in regulating cardiovascular functions (Furchgott and Zawadzki 1980, Hattenbach et al. 2000) and inhibiting key processes in atherogenesis (Böger et al. 1998b, Ito et al. 1999, Ding et al. 2000, Fujiwara et al. 2000). Thus, endothelial dysfunction is not only a marker of atherosclerosis but may also be an important regulator of atherogenic processes (Laufs et al. 1998). Generally, atherosclerosis has been associated with a reduced bioavailability of NO. Experimental studies have indicated that both enhanced NO degradation by reactive oxygen species as well as decreased NO production are possible causes for reduced NO bioavailability (Wever et al. 1999).

Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of NOS that can modulate NO production and thereby endothelial function (Vallance et al. 1992). A number of cells

elaborate ADMA, and it is thought to be derived from proteins that have been posttranslationally methylated and subsequently hydrolyzed (Leiper and Vallance 1999).

In earlier studies, elevated plasma ADMA had been linked to risk factors of atherosclerosis as well as to endothelial dysfunction. Plasma ADMA levels were found to correlate with hypercholesterolemia (Böger et al. 1998b), ageing (Miyazaki et al. 1999), hypertension (Goonasekera et al. 1997, Miyazaki et al. 1999, Surdacki et al. 1999) and glucose balance (Goonasekera et al. 1997, Miyazaki et al. 1999, Surdacki et al. 1999, Abbasi et al. 2001). Elevated plasma levels of ADMA were also reported to associate with impaired endothelium-dependent brachial artery vasodilatation in young hypercholesterolemic individuals (Böger et al. 1998b) and carotid intima-media thickness (IMT) in healthy subjects (Miyazaki et al. 1999).

Specifically, we observed that high ADMA was a potent predictor of acute coronary events in non-smoking middle-aged men (Valkonen et al. 2001). In that study, high plasma ADMA seemed to independently raise the risk of coronary events in men with a family history of coronary heart disease after adjusting for other atherosclerosis risk factors such as elevated low density lipoprotein (LDL) cholesterol, diabetes, and hypertension. Based on these previous findings, it has been suggested that ADMA may represent a circulating marker of endothelial dysfunction and subclinical atherosclerosis.

Despite these earlier studies, the causes of variation in plasma ADMA levels and its clinical significance are largely unknown. Thus, the present study was designed to further evaluate the role of plasma ADMA in the development of early atherosclerotic changes (i.e., carotid IMT), regulation of vascular functions and the relationship between plasma ADMA concentrations and risk factors of clinical and subclinical atherosclerosis.

In theory, ADMA lowering treatment could have some clinical significance. Thus, the effect of lipid-lowering therapy with high dose statins on plasma ADMA was studied as well as the association

of dietary factors on plasma ADMA. The final goal of the study is to gain a better knowledge of the

pathophysiological role of ADMA in endothelial function and development of atherosclerosis.

REVIEW OF THE LITERATURE

1. Atherosclerosis and vascular function in relation to nitric oxide pathway and ADMA

1.1. Atherosclerosis, vascular function and L-arginine-nitric oxide pathway

Atherosclerosis related vascular disease has been associated with a reduced bioavailability of NO (Cosentino and Lüscher 1998, Wever et al. 1999, De Vriese et al. 2000). Several factors with a large overlap may affect the risk of atherosclerosis (Figure 1). Endothelium-derived NO, an important signal-transduction molecule, plays a significant role in regulating cardiovascular functions (Furchgott and Zawadzki 1980, Hattenbach et al. 2000). Besides being a potent vasodilatator (Furchgott and Zawadzki 1980, Hattenbach et al. 2000), NO also inhibits key processes in atherogenesis, such as monocyte adhesion, platelet adhesion and aggregation, vascular smooth muscle proliferation as well as interference with leukocyte-endothelial cell interaction (Böger et al. 1998b, Ito et al. 1999). NO also reduces vascular production of superoxide radicals and thus acts as an inhibitor of LDL oxidation (Hogg et al. 1993).
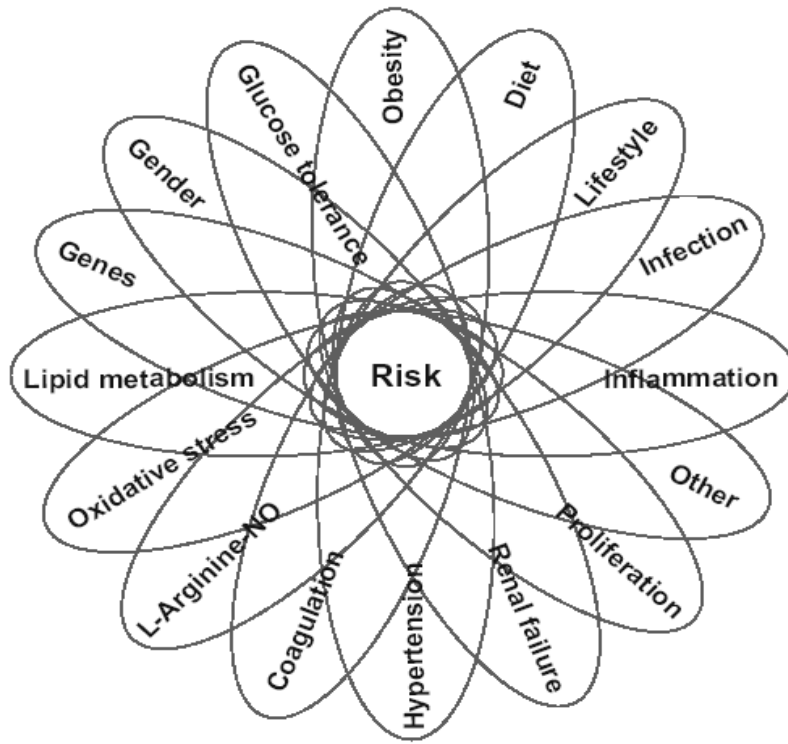
Figure 1. Several cardiovascular risk factors show large overlap, making it difficult to assess their singular contribution to overall cardiovascular risk (Maas and Böger 2003).

An amino acid, L-arginine, is a precursor for NO synthesis. In the endothelium, L-arginine is converted to NO and L-citrulline by the endothelial isoform of NOS (Figure 2), one of the three NO synthases (Moncada and Higgs 1993, Böger et al. 1998b, Hattenbach et al. 2000). It has been shown that dietary supplementation of L-arginine enhances endothelial function and reduces the progression of atherosclerosis in cholesterol-fed rabbits (Bode-Böger et al. 1996, Böger et al. 1998a). This therapeutic benefit of supplemental L-arginine has also been observed in humans with endothelial dysfunction. Lerman et al. (1998) demonstrated that long-term L-arginine supplementation improves the endothelial function of small vessel coronaries in humans. Another study demonstrated that intravenous L-arginine can normalize defective insulin-mediated vasodilatation in obese patients and patients with type 2 diabetes. Also, L-arginine can improve insulin sensitivity in obese and diabetic patients (Wascher et al. 1997). In addition, L-arginine supplementation normalises coronary

vasomotion in long term smokers (Campisi et al. 1999). However, in healthy young men, oral L-arginine did not enhance endothelium dependent vasodilation (Adams et al. 1995). One possible mechanism for the beneficial effect of L-arginine to endothelial function could be an enhanced production of NO. ADMA is an endogenous NOS inhibitor that competes with arginine for the active site of NO synthase leading to reduced NO generation (Vallance et al. 1992, Leiper and Vallance 1999) (see Figure 2). An other hypothetical mechanism whereby L-arginine administration could indirectly affect the NO production is that large doses of L-arginine might overcome the effect of ADMA (Lerman et al. 1998).
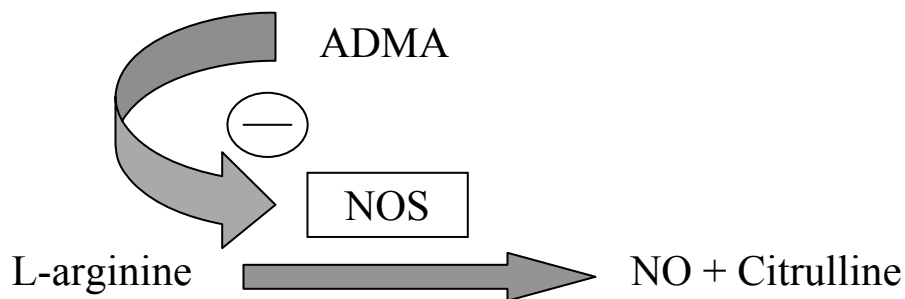
Figure 2. L-arginine is converted to nitric oxide (NO) and citrulline by the enzyme nitric oxide synthase (NOS). Asymmetric dimethylarginine (ADMA) competes with arginine for the active site of NOS leading to reduced NO formation.

1.2. Atherosclerosis and vascular function in relation to ADMA

Elevated concentrations of plasma ADMA have been linked to endothelial dysfunction and atherosclerosis. In 1992, Calver et al. (1992) demonstrated in healthy volunteers that infusion of ADMA into the brachial artery reduced forearm blood flow and caused endothelial dysfunction.

Elevated plasma levels of ADMA are also associated with impaired endothelium-dependent brachial artery vasodilation in young hypercholesterolemic individuals (Böger et al. 1998b), as well as in hypertensive patients (Takiuchi et al. 2004). Coronary flow reserve (CFR) in hypertensive patients (Takiuchi et al. 2004) and carotid IMT in healthy subjects (Miyazaki et al. 1999) have also been associated with plasma ADMA. Zoccali et al. (2002) reported that in subjects with end-stage renal disease, concentrations of plasma ADMA were directly associated with carotid IMT in baseline and also with the carotid IMT change after 15 months follow-up. They also reported that plasma ADMA was an independent predictor of the progression of intimal lesions in patients with initially normal carotid IMT. Another study demonstrated (Kielstein et al. 1999) that the mean concentration of ADMA in plasma was higher in patients with end-stage renal disease and atherosclerotic vascular disease than in subjects without vascular complications. Furthermore, patients with peripheral arterial occlusive disease (Böger et al. 1997b, Böger et al. 1998c) or patients with ischemic stroke (Yoo and Lee 2001), were reported to have significantly higher plasma ADMA concentrations than control subjects.

Piatti et al. (2003) observed that patients with cardiac syndrome X have increased concentrations of plasma ADMA. They suggested that increased ADMA levels play a role in the abnormal vascular reactivity that is observed in these patients. Along with this, patients with syndrome X have significantly reduced CFR, reduced L-arginine to ADMA ratio, reduced levels of plasma nitrate and nitrite and increased levels of plasma ADMA, suggesting that high ADMA and diminished NO bioavailability may be related to reduced vasodilator response in these patients (Chen et al. 2002).

Cardiovascular disease is the major cause of death in patients with end-stage renal disease (Anderstam et al. 1997). The role of ADMA in this was clarified by Zoccali et al. (2001) who demonstrated in a prospective study that ADMA is a stronger independent predictor of all-cause mortality and cardiovascular outcome in patients with chronic renal failure than some traditional risk factors. In a nested case-control study (Valkonen et al. 2001), we demonstrated that middle-aged non-

smoking men who had plasma ADMA concentrations in the highest quartile had a 3.9-fold increased risk for acute coronary events compared to others. As well, Nijveldt et al. (2003) recently reported that ADMA is the strongest predictor of death of patients in an intensive care unit, with a 17-fold excess in mortality for patients in the highest ADMA quartile compared to those in the lowest quartile. Results of the prospective studies suggest that plasma ADMA may be a significant predictor of vascular diseases.

2. Metabolism of ADMA and other methylarginines

2.1. Synthesis, metabolism and excretion of methylarginines

L-arginine analogues, ADMA and the guanidino-methylated arginine analogue NG-monomethyl-L-arginine (LMMA), are endogenous competitive inhibitors of all three isoforms of NOS while symmetric dimethylarginine (SDMA) is biologically inactive (Miyazaki et al. 1999, Surdacki et al. 1999). Chemical structures of L-arginine and its methylated derivatives are presented in Figure 3. Free biologically active methylarginines compete with arginine for the active site of NOS leading reduced NO generation. LMMA has been a standard NOS inhibitor used to evaluate the role of the L-arginine-NO pathway in cardiovascular, nervous and immune systems in experimental studies. However, recent clinical interest has focused on ADMA because it is found in plasma in far higher concentrations than LMMA and may therefore be a major endogenous NOS inhibitor (Vallance 2001).
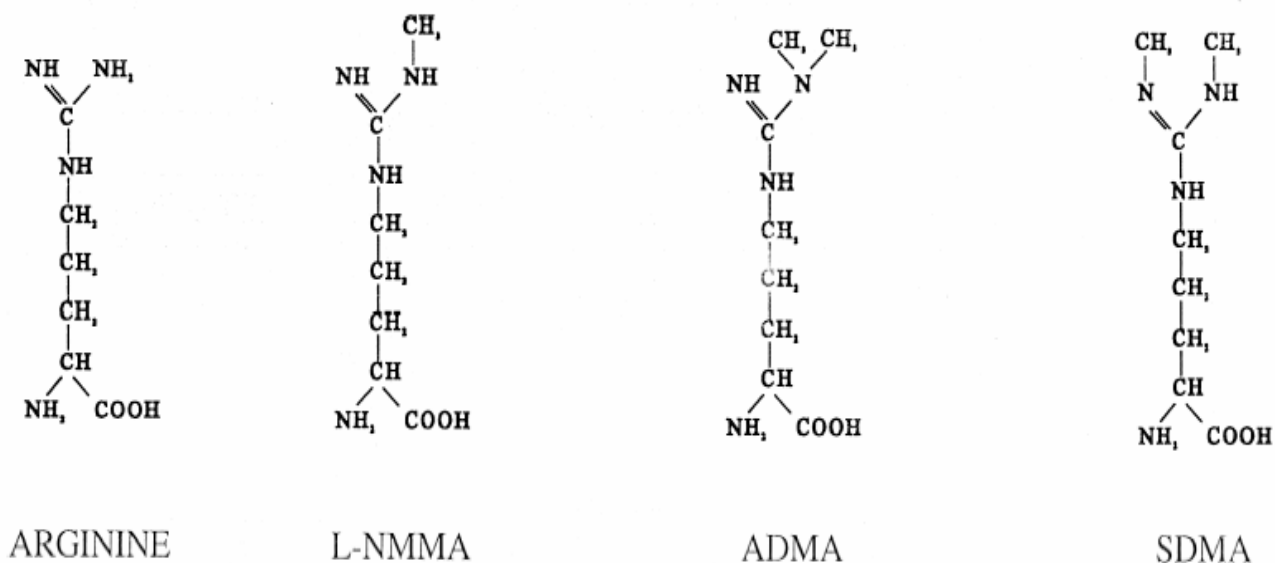
Figure 3. Chemical structures of L-arginine, asymmetric dimethylarginine (ADMA), monomethylarginine (LMMA), and symmetric dimethylarginine (SDMA). ADMA and LMMA are competitive inhibitors of NO synthase, where as SDMA is biologically inactive (Leiper and Vallance 1999).

Synthesis, metabolism and excretion of methylarginines are presented in Figure 4. Endogenous methylarginines (ADMA, LMMA and SDMA) are synthetized in vivo by the action of enzymes known as protein arginine methyltransferases (PRMTs) (Cooke 2000). PRMT activity was initially identified by Paik and Kim (Paik and Kim 1968) and denoted protein methylase I activity. Although the existence of protein-arginine methylation has been known for over 30 years, the genes encoding PRMTs have only been identified in the last decade, and their cellular functions are only beginning to be understood (Tran et al. 2003). There are two types of enzymes that methylate arginine residues (Ghosh et al. 1988, Tang et al. 2000). PRMT type I forms ADMA and LMMA, whereas PRMT type II forms SDMA and LMMA (Cooke 2000). There are a number of type I PRMTs, with a specificity for different proteins whereas the only known substrate for type II PRMT is the myelin basic protein (Najbauer et al. 1993).

21

Proteolysis of proteins containing methylated arginine residues releases free methylarginine residues into the cytosol, plasma and tissues. Thus, free ADMA, LMMA and SDMA are released during proteolytic breakdown (Cooke 2000, Böger 2003).

ADMA, LMMA and SDMA are eliminated from the body by renal excretion. Several studies have demonstrated elevated plasma concentrations of ADMA and SDMA in patients with renal failure (Vallance et al. 1992, MacAllister et al. 1996b, Kielstein et al. 1999, Kielstein et al. 2001). Reduced NO elaboratorion secondary to the accumulation of ADMA may be an important pathogenic factor for atherosclerosis in patients with chronic renal failure (Kielstein et al. 1999). Recently, this was supported by Cross et al. (2001a) who demonstrated that hemodialysis-treatment significantly reduced plasma ADMA levels and this occurred concomitant with improved endothelium-dependent vasodilation. However, it has been shown that dialytic clearance of ADMA in patients with chronic renal failure undergoing hemodialysis is lower than predicted. This has been partly explained by protein binding of plasma ADMA (MacAllister et al. 1996b, Kielstein et al. 1999). Interestingly, the dialysis treatment method of end-stage renal disease also has an influence on plasma ADMA and SDMA levels. Hemodialysis-treated patients had a plasma predialysis ADMA concentrations approximately sixfold higher and significantly lower plasma nitrate concentrations than control subjects, suggesting that ADMA may inhibit NO synthase in hemodialysis-treated subjects (Kielstein et al. 1999). In contrast, plasma ADMA levels and nitrate concentrations in peritoneal dialysis-treated patients were similar to those in control subjects. That study demonstrated that high plasma SDMA levels were accompanied by low ADMA levels in patients undergoing peritoneal dialysis-treatment, suggesting that renal excretion is the only elimination pathway for SDMA whereas ADMA may be eliminated also by other metabolic pathways (Kielstein et al. 1999).

ADMA and LMMA, but not SDMA, are also metabolized to L-citrulline and either di- or monomethylamine by the enzyme dimethylarginine dimethyl-aminohydrolase (DDAH), which is

widely distributed in vascular and extravascular tissues (Ogawa et al. 1989, Kimoto et al. 1993, Kimoto et al. 1995, MacAllister et al. 1996a). Over 90 % of ADMA may be metabolized by DDAH (Tran et al. 2003) of which two different isoforms have been characterised (Leiper et al. 1999). DDAH I is predominate in tissues that express neuronal NOS and DDAH II in tissues expressing endothelial NOS (Leiper and Vallance 1999, Leiper et al. 1999). In isolated blood vessels, inhibition of DDAH leads to an increase in ADMA levels and inhibition of NO synthase causing vasoconstriction that can be reversed by L-arginine (MacAllister et al. 1996a). The activity of this enzyme seems to be regulated by complex mechanisms, which are only partly understood. Ito et al. (1999) demonstrated that oxidative stress induced by oxidized LDL or TNF-α may increase endothelial elaboration of ADMA by reducing DDAH activity, but not its protein expression, in cultured endothelial cells. Moreover, Stühlinger et al. (2001) demonstrated that homocysteine increased ADMA levels by reducing DDAH activity via redox-mediated mechanism, and directly interfered with isolated DDAH in a cell-free system. Recently, was shown *in vitro* that NO donors reversibly inhibit DDAH activity by s-nitrosylation of DDAH providing a potential feedback mechanism to regulate NO production (Leiper et al. 2002). Moreover, it has been observed that impaired liver function may lead to elevated plasma ADMA levels by reduced DDAH activity (Nijveldt et al. 2003, Tsikas et al. 2003). In summary, DDAH enzyme may be of critical importance in affecting NO pathway, but further clinical studies are needed to clarify the regulation of this enzyme and its role in health and disease.
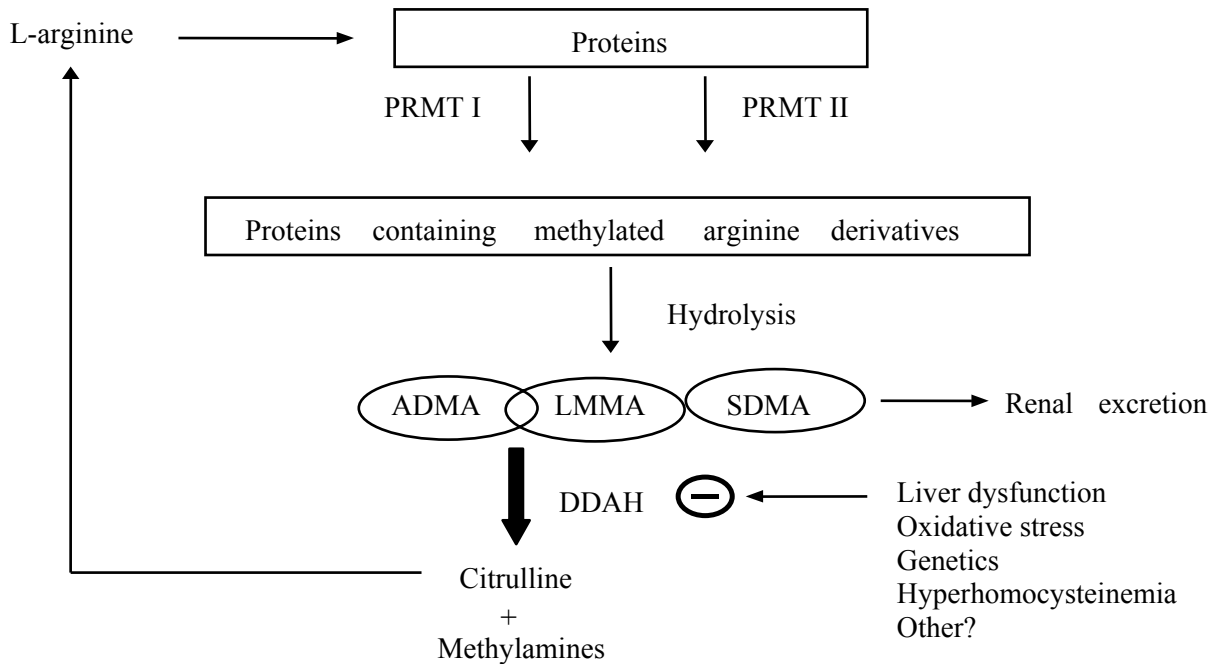
Figure 4. Metabolism of asymmetric dimethylarginine (ADMA), monomethylarginine (LMMA), and symmetric dimethylarginine (SDMA). Methylated arginines are derived from the breakdown of proteins that have been acted on by enzymes known as protein arginine methyltransferases (PRMT I and II). PRMT I methylates proteins that, when hydrolyzed, release SDMA and LMMA whereas PRMT II methylates protein that release LMMA and SDMA. All methylated arginines are excreted to urine but the major methabolic pathway for ADMA and LMMA is enzyme dimethylarginine dimethylaminohydrolase (DDAH), which metabolizes them to citrulline and monomethyamines. Impaired liver function, oxidative stress, genetics, hyperhomocysteinemia and possibly other unknown factors may reduce DDAH activity.

2.2. Plasma concentrations and analysis of ADMA and other methylarginines

Table 1 summarises reported concentrations and analysis methods of plasma ADMA in healthy human beings as well as in subjects with various diseases. Several studies have measured concentrations of plasma arginine and its derivatives. There is a wide observed range for plasma arginine derivatives which, in part, may be explained by differences in determination methodologies. Plasma ADMA levels have been determined by employing a variety of methods including high-performance liquid chromatography (HPLC) with ultraviolet (UV) or with fluorescence detection after a derivatization step (Böger et al. 1998b, Böger et al. 2000) or without derivatization (Vallance et al. 1992, Fard et al. 2000), capillary electrophoresis with laser-induced fluorescence detection (Causse et al. 2000), electrochemical detection (MacAllister et al. 1996b) and HPLC tandem mass spectrometry (Meyer et al. 1997).

Table 1. Summary of reported mean plasma ADMA concentrations and analysis methods in various groups of subjects.

Population	Arginine and its derivatives (µmol/l)				Method	Reference
N/group	ADMA	SDMA	LMMA	Arginine		
31/H	1.03	0.60		49.8	A	(Böger et al. 1998b)
118/H	0.51				A	(Miyazaki et al. 1999)
29/H	0.43		0.10	96.1	A	(Meyer et al. 1997)
7/H	0.30	0.34	0.31	60.7	A	(Pi et al. 2000)
20H	0.12	0.16	0.01	52.1	B	(Vishwanathan et al. 2000)
27/H	1.1	0.8		75.5	A	(Kielstein et al. 1999)
47/YH	1.25	0.71		83.2	A	(Böger et al. 1997b)
37/EH	1.01	0.83		75.5	A	(Böger et al. 1997b)
24/EH	2.77	0.56		56.2	A	(Kielstein et al. 2003)
9/HCH	0.10	1.18	0.18		B	(Goonasekera et al. 1997)
49/HC	2.17	0.73		52.4	A	(Böger et al. 1998b)
15/HTG	1.13				A	(Lundman et al. 2001)
77/PAOD	2.80	2.30		80.1	A	(Böger et al. 1997b)
24/EHT	3.53	0.57		60.0	A	(Kielstein et al. 2003)
24/EHTA	1.86				C	(Fujiwara et al. 2000)
19/EHTAM	2.4	1.4			A	(Surdacki et al. 1999)
38/HACH	0.23	1.37	0.21		B	(Goonasekera et al. 1997)
50/DM2	1.04	0.73		49.8	D	(Fard et al. 2000)
16/DM2	1.59				A	(Abbasi et al. 2001)
43/HD	7.3	5.2		75.9	A	(Kielstein et al. 1999)
37/PD	3.7	5.1		84.0	A	(Kielstein et al. 1999)
19/HD	0.59				A	(Anderstam et al. 1997)
11/PD	0.70				A	(Anderstam et al. 1997)

ABBREVIATIONS: H, healthy; YH, young healthy; EH, elderly healthy; HCH, healthy children; HC, hypercholesterolemic; HTG, hypertriglyceridemic; PAOD, peripheral arterial occlusive disease; EHT, elderly hypertensive; EHTA, essential hypertonia arterialis; EHTAM, essential hypertonia arterialis men; HACH, hypertensive children; DM2, diabetes mellitus typus 2; HD, hemodialysis; PD, peritoneal dialysis; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; LMMA, monomethylarginine. A=HPLC-FL, high performance liquid chromatography with fluorescence detector; B=HPLC-MS/MS, high performance liquid chromatography with tandem mass spectrometry; C=HPLC-not given, high performance liquid chromatography, detector not given; D=HPLC-UV, high performance liquid chromatography with ultraviolet detection.

3. ADMA in relation to atherosclerosis risk factors

3.1. ADMA and hypertension

Elevated ADMA levels may play a role in the development of essential hypertension. Miyazaki et al. (1999) observed in subjects (n = 114, age range 26 - 77 years) without any known vascular disease that plasma ADMA levels were associated with mean arterial pressure (MAP) (r = 0.38, p < 0.0001). Furthermore, Surdacki et al. (1999) demonstrated that men with newly diagnosed and untreated hypertension had increased plasma ADMA levels and depressed systemic NO formation tested by urinary excretion rate of nitrite plus nitrate (Unox), an index of endogenous NO production. As well, Kielstein et al. (2003) demonstrated that elderly hypertensive subjects had significantly higher plasma ADMA levels than normotensive elderly controls whereas levels of SDMA, a biologically inactive stereoisomere of ADMA, were comparable in both groups. In contrast, Delles et al. (2002) did not find any correlation between plasma ADMA concentration and blood pressure (BP) in young men with a mild essential hypertension (n = 20). Increased plasma ADMA concentrations have been reported in women with pregnancy induced hypertension (Holden et al. 1998, Pettersson et al. 1998), where as a decreased BP seen in normal pregnancy is accompanied by a significant fall in plasma ADMA concentration (Holden et al. 1998). An observation showing increased plasma levels of ADMA in young hypertensive children (Goonasekera et al. 1997) also indicate that elevations in ADMA levels may play a role in the early stages of hypertension. Since methylarginines are, in part, eliminated by renal excretion, they accumulate in the plasma of patients with end-stage renal failure (MacAllister et al. 1996b, Anderstam et al. 1997, Schmidt et al. 1999). Accumulation of ADMA may contribute to the development of hypertension in these patients (Vallance et al. 1992). Taken together, it seems that plasma ADMA is, in some part, associated with BP, but the relationship between ADMA and

cardiovascular physiology is not fully established. Therefore, it was important to further study the role of plasma ADMA in the regulation of BP and vascular functions.

3.2. ADMA and diabetes mellitus

Vascular diseases are a major cause of morbidity and mortality in type 2 diabetes (Pyörälä et al. 1987, Haffner et al. 1998), which is an increasingly common disease with a large impact on society in Western countries. Considering plasma ADMA as a marker of artherosclerosis, it is not surprising that previous studies have found an association between diabetes mellitus and plasma ADMA. A significant association has been observed between glucose balance and ADMA both in animal models (Xiong et al. 1997, Masuda et al. 1999) and in healthy humans (Miyazaki et al. 1999). As well, a recent study found that type 2 diabetic subjects have elevated plasma ADMA levels (Abbasi et al. 2001). Moreover, normoglycaemic women with previous gestational diabetes have been observed to have increased serum ADMA levels (Mittermayer et al. 2002). This increase was independent from other risk factors or surrogate markers for diabetes or cardiovascular events. Also, it has been reported that in healthy subjects without diabetes, a significant direct relationship between insulin resistance and plasma ADMA concentration exists (Stühlinger et al. 2002). Lin et al. (2002) demonstrated in an animal model that hyperglycemia elevates ADMA by impairing DDAH activity in vascular smooth muscle and endothelium. Elsewhere, it was hypothesised that dysregulation of the enzyme DDAH could be responsible for an increase in plasma ADMA concentrations in diabetic subjects (Abbasi et al. 2001).

Taken together, diabetic vascular disease seems to be related to a reduced NO bioavailability and elevated plasma ADMA concentrations. However, NO may also be a harmful agent in diabetic patients by inducing renal vasodilatation and hyperfiltration, phenomena seen in early diabetic nephropathy (Wirta et al. 1996, Chaiken et al. 1998, Chiarelli et al. 2000). This hyperfiltration could

affect ADMA concentrations due to increased renal excretion. Previous studies have not clarified how plasma ADMA relates to glycemic control and renal function, especially in early diabetic hyperfiltration. Therefore, this issue was addressed in study II.

3.3. ADMA and dyslipidemia

Plasma levels of ADMA and its biologically inactive structural isomer SDMA have been shown to be elevated in hypercholesterolemic rabbits (Bode-Böger et al. 1996, Böger et al. 1998a) and monkeys (Boger et al. 2000). In dietary intervention models, a diet enriched with cholesterol was shown to increase plasma and serum ADMA levels in rabbits (Yu et al. 1994, Böger et al. 1997a). However, in humans the relationship between hypercholesterolemia and ADMA is not established. Some clinical studies have found an association (Böger et al. 1998b, Eid et al. 2003), while others have not (Miyazaki et al. 1999, Cardinale 2001, Stühlinger et al. 2002). Statin therapy incontrovertibly decreases plasma cholesterol levels (Scandinavian Simvastatin Survival Study Group 1994, Shepherd et al. 1995, 2002, Heart Protection Study Collaborative Group 2002, Sever et al. 2003). Therefore, statins could lower plasma ADMA if there is a true association between LDL cholesterol and ADMA.

In young men, hypertriglyceridemia was associated with increased concentrations of plasma ADMA (Lundman et al. 2001) along with fasting triglyceride levels associated with an increase of plasma ADMA concentrations in healthy subjects (Stühlinger et al. 2002). In addition, a recent study demonstrated elevated plasma ADMA levels in type 2 diabetic patients after eating a high-fat meal (Fard et al. 2000). An elevation of plasma ADMA levels occurred in association with increased plasma levels of triglycerides. These findings raised the idea to study whether or not dietary composition has an influence on plasma ADMA levels in the circulation (study IV).

3.4. ADMA and smoking

Cigarette smoking is a well-recognized risk factor of atherosclerosis. Some studies (Miyazaki et al. 1999, Eid et al. 2003) have not found an association between tobacco use and plasma ADMA levels, but in those studies amount and duration of smoking were not evaluated. Nicotine may contribute to endothelium dysfunction in smokers and acute local exposure to nicotine is associated with an impaired response to endothelium-derived NO in human veins (Chalon et al. 2000). In rabbits, long-term oral nicotine impaired NO production, which may be due to increases in LMMA, ADMA and endothelin-1 concentrations (Hamasaki et al. 1997).

3.5. ADMA and ageing

In an animal model, a significant association has been recorded between plasma ADMA concentrations and ageing (Xiong et al. 2001). As well, Miyazaki et al. (1999) observed that age was a significant correlate of plasma ADMA (r = 0.54, p < 0.0001) in healthy subjects with a mean age of 52 ± 1 years (range 26 to 77 years). Similarly, this was demonstrated in a recent study in which normotensive, nonsmoking elderly (69 ± 1 years) subjects had significantly higher plasma ADMA levels than young (25 ± 1 years) control subjects (Kielstein et al. 2003). Contrary to that, one study found no association between plasma ADMA concentration and age (Eid et al. 2003). Furthermore, it was found that elderly subjects have even lower plasma ADMA levels than other control subjects (Böger et al. 1997b). Thus, association between plasma ADMA and ageing is not clear.

3.6. ADMA and homocysteine

Hyperhomocysteinemia impairs vascular function and is a putative risk factor for cardiovascular diseases (Welch and Loscalzo 1998). A significant positive association has been detected between plasma homocysteine and ADMA concentrations both in animal models (Böger et al. 2000) and in humans (Yoo and Lee 2001, Holven et al. 2003). In an experimental study, Böger et al. (2001) demonstrated in young healthy subjects that elevation of plasma homocysteine levels was associated with increased plasma concentrations of ADMA. Stühlinger et al. (2001) showed that homocysteine post-translationally inhibits DDAH enzyme activity, causing ADMA to accumulate and inhibit NO synthesis. Thus, currently available data suggest an association between plasma ADMA and homocysteine.

4. Plasma ADMA concentration lowering drugs and dietary supplements

Several studies attempting to lower plasma ADMA levels have been published lately. L-arginine is a precursor for NO synthesis and it has been demonstrated that L-arginine enhances endothelial function and improves the clinical status of patients with a cardiovascular disease in many studies (Bode-Böger et al. 1996, Wascher et al. 1997, Böger et al. 1998a, Böger et al. 1998b, Böger et al. 1998c, Campisi et al. 1999, Bode-Böger et al. 2003), but not in all (Cross et al. 2001b, Walker et al. 2001).

L-arginine supplementation. L-arginine supplementation trials have shown to improve endothelium-dependent, NO mediated vascular symptoms and increase arginine to ADMA ratio. However, plasma ADMA levels were not lowered (Böger et al. 1998b, Böger et al. 1998c, Bode-Böger et al. 2003). These findings suggest that L-arginine administration can indirectly affect the NO production by overcoming the effect of ADMA (Lerman et al. 1998).

Folic acid, vitamins B$_6$ and B$_{12}$. Folic acid treatment has been observed to reduce plasma levels of ADMA in hyperhomocysteinemic subjects (Holven et al. 2003). In contrast, another study observed that combined treatment with folic acid, vitamin B$_6$ and B$_{12}$ did not change plasma ADMA concentrations in elderly subjects with hyperhomocysteinemia and vascular disease (Sydow et al. 2003).

Estrogen replacement therapy. Estrogen replacement therapy has been reported to lower plasma levels of ADMA in healthy postmenopausal women while arginine and SDMA levels remain unchanged (Teerlink et al. 2003).

Metformin and rosiglitazone. Metformin treatment lowered ADMA concentrations in patients with type 2 diabetes (Asagami et al. 2002). In that study, metformin treatment led to both an improvement in glycemic control and a decrease in plasma ADMA concentrations. Thus, it is not clear how much of a change in plasma ADMA concentration was secondary to the decrease in plasma glucose concentration, as compared to the direct effect of metformin. A cross sectional study in healthy subjects without diabetes demonstrated a significant relationship between insulin resistance and plasma ADMA levels and that intervention with rosiglitazone enhanced insulin resistance and reduced plasma ADMA levels (Stühlinger et al. 2002).

Renin-angiotensinogen system inhibitors. A blockade of angiotensin II effects has been found to improve endothelial function (Mancini 2000, Prasad et al. 2000). In a randomized, double-blind, fourfold cross-over study, Delles et al. (2002) found that levels of plasma ADMA were reduced with an angiotensin converting enzyme (ACE) inhibitor, enalapril, and angiotensin II AT 1 receptor blockade eprosartan therapy. Changes in ADMA levels were independent of the drug action on BP. Ito et al. (2002) observed in patients with non-complicated type 2 diabetes that 4 weeks of therapy with the ACE inhibitor perindropril significantly decreased serum ADMA concentrations but did not affect BP or glucose metabolism. Furthermore, Chen et al. (2002) reported in a randomised placebo control study

that an eight week long ACE inhibitor therapy with enalapril significantly reduced plasma ADMA concentrations in patients with syndrome X.

Statin therapy. A previous finding, which found that elevated plasma ADMA is associated with hypercholesterolemia (Bode-Böger et al. 1996, Böger et al. 1998a, Böger et al. 1998b, Böger et al. 2000, Eid et al. 2003) raises an interesting question of whether or not statin therapy could lower not only plasma cholesterol but also plasma ADMA levels and thereby contribute to the improvement of endothelial function that can be seen during statin treatment (Leung et al. 1993, Egashira et al. 1994, Anderson et al. 1995, Treasure et al. 1995). Thus, one of the aims of this study was to evaluate the effect of statin treatment on plasma ADMA levels (study III).

AIMS OF THE STUDY

Based on our earlier findings and a critical review of the literature, the aim of this study was to evaluate the following questions:

1. What is the role of ADMA in cardiovascular physiology? The relationship between ADMA and carotid IMT, myocardial blood flow and coronary reactivity as well as BP regulation and other vascular functions were studied.

2. How does type 2 diabetes, kidney glomerular filtration rate (GFR), glycemic control, and other atherosclerosis risk factors relate to plasma ADMA?

3. Can lipid lowering treatment with high-dose statins decrease ADMA concentrations and is dietary composition associated with plasma ADMA concentrations?

SUBJECTS AND METHODS

1. Subjects, study design and ethics

The study was based on five separated study populations (studies I-V). The characteristics of these subjects are summarised in Table 2.

Table 2. Characteristics of separate study populations (studies I-V). Values are shown as mean±SD.

Study	Subgroups	N	Age (years)	Sex (M/F)	BMI (kg/m^2)	TC (mmol/l)	HDL-C (mmol/l)	TG (mmol/l)	SBP (mmHg)	DBP (mmHg)
I	Controls	47	35±4	47/-	24.6±2.2	4.90±1.17	1.24±0.28	1.06±0.53	114±12	63±8
	BHT-group	16	37±4	16/-	26.1±2.5	5.55±1.03	1.11±0.17	1.95±0.98	137±22	82±10
	FH-group	14	31±8	14/-	26.2±2.9	7.58±1.88	0.96±0.17	1.32±0.39	122±8	63±10
II	ND patients	65	65±7	36/29	27.8±3.9	5.61±1.11	1.20±0.46	1.54±0.80	152±19	87±9
	Diabetic patients	86	64±7	52/34	29.7±4.7	5.27±1.00	1.09±0.40	2.06±1.39	161±21	88±9
III	Placebo-group	14	56±9	9/5	25.4±3.7	5.85±0.91	1.41±0.43	1.79±0.89	_	_
	Simvastatin-group	15	57±9	9/6	28.2±3.5	5.90±1.00	1.24±0.36	1.86±0.87	_	_
	Atorvastatin-group	15	56±8	11/4	26.7±4.9	5.88±0.87	1.26±0.39	1.84±0.94	_	_
IV	Healthy HC	34	46±7	14/20	25.7±3.9	6.26±0.89	1.72±0.44	1.36±0.82	132±20	83±12
V	PREHT-group	23	50±4	23/-	28.4±3.2	5.63±1.10	1.30±0.37	1.72±0.99	130±7	82±6
	ST1HT-group	29	52±4	29/-	26.7±3.6	5.44±1.14	1.41±0.34	1.67±0.93	147±6	91±5
	ST2HT-group	15	51±4	15/-	28.1±4.1	5.39±0.94	1.41±0.30	1.35±0.57	160±12	100±9

ABBREVIATIONS: BHT, borderline hypertension; FH, familial hypercholesterolemia; ND, non-diabetic; HC, hypercholesterolemic; PREHT, prehypertension; ST1HT, stage 1 hypertension; ST2HT, stage 2 hypertension; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides; SBP, systolic blood pressure; DBP, diastolic blood pressure; -, not measured

1.1. Study I

Forty-seven healthy controls (control-group), 16 men with borderline hypertension (BHT)-group and 14 men with heterozygous familial hypercholesterolemia (FH)-group participated in the study focused on investigating the association of plasma ADMA with myocardial blood flow and BP (study I).

The inclusion criteria for healthy controls were: age < 45 years, body mass index (BMI) < 27 kg/m^2, BP < 150/90 mmHg, non-smoking, no history of diabetes and no history of atherosclerotic disease.

The BHT-group included healthy men with several BP values during the previous 2 - 3 years as defined according to the Joint National Committee VI classification (The sixth report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure 1997) as high normal (systolic blood pressure (SBP) 130 - 140 mmHg or diastolic blood pressure (DBP) 85 - 89 mmHg in all previous measurements). Otherwise, the inclusion criteria for BHT-group were the same as for the controls.

The diagnosis of FH was confirmed by through DNA tests in five men, seven men had either clinical signs of tendon xanthomas or a positive finding in ultrasound scanning of the Achilles tendon, and three men had at least one first degree relative with hypercholesterolemia and tendon xanthomata. Subjects with a clinical history or evidence of coronary artery disease or other cardiac diseases, diabetes, systemic hypertension or current smoking were excluded. Twelve FH patients had been on cholesterol-lowering medication for several years. Stress echocardiographic examinations were performed on all subjects with relatively low coronary perfusion reserve values (< 3.0) to rule out significant coronary heart disease. All these men had normal exercise capacity, were asymptomatic during the test, had no diagnostic ST-segment changes in electrocardiography and no wall motion disturbances in echocardiography either at rest or after the exercise test.

The positron emission tomography (PET) study was done for every study subject and myocardial perfusion was measured twice, once at rest and once after administration of dipyridamole using PET and [^{15}O] labelled tracers. Heart rate and electrocardiogram were monitored continuously and BP was monitored with an automatic oscillometric BP monitor during the PET study.

Carotid artery IMT measurements were performed by ultrasonography. Venous blood samples for plasma lipid, lipoprotein, arginine, ADMA and SDMA assays were taken after a 12-hour overnight fast during the same week as the PET study was performed.

1.2. Study II

Eighty-six type 2 diabetic patients and sixty-five non-diabetic controls with a comparable distribution of age and gender were studied to compare the plasma ADMA levels in type 2 diabetic and controls, as well as investigating the determinants of plasma ADMA (study II).

The patients fulfilled the World Health Organization (WHO) diagnostic criteria for type 2 diabetes (WHO Expert Committee on Diabetes Mellitus: second report 1980) and had a known disease duration of no more than one year. The study participants were evaluated after a mean period of a 9.0 year (range, 7.4 to 10.7 years) follow-up. At the evaluation time, 45 diabetic subjects had diet and oral drug therapy, 17 had insulin and 10 subjects had combined oral drug and insulin therapy as a treatment for diabetes.

BMI was calculated (kg/m^2) and blood pressure was measured with a sphygmomanometer. Hypertension was defined as SBP > 160 mmHg or DBP > 95 mmHg (Arterial hypertension. Report of a WHO expert committee 1978). Also, the patients on antihypertensive treatment were classified as hypertensive. Plasma lipids, lipoproteins, glucose, glycosylated hemoglobin and endogenous

dimethylated arginine derivatives, ADMA and SDMA were assayed. Renal function was tested by measuring GFR.

1.3. Study III

Forty-four mildly or moderately hypercholesterolemic subjects (29 men and 15 women), aged 31 to 69 years were recruited to a randomised, double-blind, placebo-controlled trial to clarify the effect of high dose statin therapy on plasma arginine derivatives (study III).

The study consisted of three treatment groups: placebo (n = 14), simvastatin 80 mg/day (n = 15) and atorvastatin 40 mg/day (n = 15). Duration of the follow-up was eight weeks. Inclusion criteria were: age 20 - 70 years and LDL cholesterol 3.2 - 5.2 mmol/l. Patients on antihypertensive treatment were classified as hypertensive and patients on oral antiglycemic treatment were classified as patients with type 2 diabetes. Subjects with previous myocardial infarction or by clinical ergometric test or angiographically documented disease were classified as patients with coronary artery disease. None of the participants received any concurrent lipid-altering medications. They had no significant renal or hepatic dysfunction or medications known to inhibit metabolism of atorvastatin or simvastatin. Lipids, lipoproteins and arginine derivatives, ADMA and LMMA were collected before treatment and at 1 and 8 weeks during follow-up.

1.4. Study IV

Thirty-four mildly or moderately hypercholesterolemic participants without any severe disease were recruited in the study to assess the association of diet on plasma arginine derivatives (study IV).

Study subjects included 14 men and 20 women aged between 35 and 65 years. The inclusion criteria were: aged 30 - 70 years, clinically normal liver, kidney and thyroid function, plasma total cholesterol (TC) > 5.0 mmol/l and BMI 18 - 30. None of subjects received any lipid altering medication, systemic cortisone medication or antihypertensive treatment. They had no renal or hepatic dysfunction, diabetes, cancer or other metabolic diseases and they were not vegetarians.

In this study (IV), seven-day food records were used to analyze diet and alcohol intake. Dietary analysis was done by a professional nutritionist as described in detail in study IV. The study subject's BP was determined with an automatic BP gauge. Height and weight were recorded and BMI was calculated as weight/height square (kg/m^2). Blood samples were drawn for plasma lipid, lipoprotein and arginine derivatives, ADMA and SDMA assays.

1.5. Study V

Sixty-seven men with or without known hypertension who were otherwise healthy were studied. The aim of this study was to define the role of ADMA in the regulation of vascular function in men (study V).

Using Joint National Committee VII criteria (Chobanian et al. 2003), the participants were classified on the basis of repeated casual BP measurements. Twenty-three of the participants were pre-hypertensive (PREHT) (SBP 120 - 139 or DBP 80 - 89 mmHg, or both), 29 belonged to the stage 1

hypertensive group (ST1HT) (SBP 140 - 159 or DBP 90 - 99 mmHg, or both) and 15 belonged to the stage 2 hypertensive group (ST2HT) (SBP $\geq$ 160 or DBP $\geq$ 100 mmHg, or both).

In addition to casual BP measurements, BP was also measured by 24-hour ambulatory monitoring. All subjects participated in a dynamic exercise test that was performed in an upright position using a bicycle ergometer and also in non-invasive haemodynamic study. The NO production was estimated based on plasma nitrate (NO_3) determination.

1.6. Ethical considerations

Study I was approved by the Joint Commission on Ethics of Turku University and the Turku University Central Hospital. Study II was approved by the ethics committees of the University of Tampere and the Health Care Center of Tampere. The protocol of studies III, IV and V was approved by the ethics committee of the University Hospital of Tampere. All participants in all studies gave written informed consent.

2. Methods

2.1. Analysis of arginine and its endogenous methylated derivatives

Plasma arginine and its dimethylated endogenous derivatives, ADMA and SDMA were determined using a HPLC method in studies I and II. Briefly, 200 µl serum was diluted with 160 µl of distilled water and a solution was mixed with 40 µl of internal standard (homo-arginine, 400 µmol/l). Arginine, ADMA and SDMA were then absorbed on an 100 mg Bond Elut silica solid phase extraction column (Varian, Harbor City, CA, USA), pretreated with 800 µl of methanol and with 800 µl of distilled water.

After washing, arginine, ADMA and SDMA were eluted from the SPE column with o-pthalaldehyde (OPA) derivatizing reagent. The OPA reagent was modified by adding acetonitrile and tetrahydrofuran (2:1:1). The stable OPA derivatives of arginine, ADMA and SDMA were then separated with HPLC on a Waters Bondapak phenyl column (5 μm, 150 x 4.6 mm) with a 10 mmol/l phosphate buffer containing 870 mg/l of hexanesulphonic acid as a mobile phase. Fluorescence was monitored at 328 nm (exitation) and 445 nm (emission). The HPLC equipment consisted of a LKB Pharmacia 2248 pump, a Hewlett-Packard 1050 autosampler, a Shimadzu RF-551 fluorescence detector. Total imprecision (CV %) for ADMA, SDMA and arginine was no more than 12 % as studied by repeated analysis of a pooled sample during the analysis of the actual samples.

In the other studies (III, IV, V), ADMA and SDMA determination was carried out using HPLC tandem mass spectrometry. A 200 μl aliquot of plasma was diluted and applied on a solid phase silica column. After washing with methanol arginine and its methylated derivatives were eluted into 4 mol/l NH_4OH in 50 % acetonitrile and eluent was evaporated with N_2. Prior analysis dry residue was dissolved in HPLC mobile phase. Transitions used were *m/z* 203 → 70 for ADMA and SDMA, m/z 189 → 70 for (monomethylarginine) LMMA and m/z 175 → 60 for arginine. Analysis of every sample was carried out in parallel with and without added SDMA. Area normalized difference in SDMA peaks of samples run with and without added SDMA was used as an internal standard. The level of SDMA in each sample was, in turn, determined by the method of standard additions, using the normalized SDMA values. For ADMA total CV % was 10.3 % and intra- and inter-day component CVs were 7.0 and 7.6 %, respectively (Vishwanathan et al. 2000).

2.2. Analysis of lipids and other laboratory methods

In study I, plasma TC, high density lipoprotein (HDL) cholesterol and triglyceride (TG) concentrations were measured using standard enzymatic methods (Boehringer Mannheim GmbH) with a fully automated analyzer (Hitachi 704; Hitachi Ltd, Tokyo, Japan). HDL cholesterol was measured after polyethyleneglycol precipitation. Apolipoprotein B concentrations were measured using an immunonephelometric method (Behring BNA).

In study IV, the concentrations of plasma TG, TC, and HDL cholesterol were determined using Cobas Integra automatic analyser and reagents and calibrators as recommended by the manufacturer (Roche Diagnostics, Basel, Switzerland).

In the other studies, plasma cholesterol and triglycerides were determined using the dry slide technique (Ektachem 700 analyzer, Johnson & Johnson Clinical Diagnostics, Rochester, NY, USA). HDL cholesterol was measured with the same technique after precipitation of LDL.

In all studies, LDL cholesterol was calculated using Friedewald`s formula (Friedewald et al. 1972), since plasma triglyceride levels did not exceed 4.0 mmol/l.

In study II, blood glucose concentrations were measured with a Hitachi 717 analyser by the enzymatic method of Merck and glycosylated hemoglobin (GHbA$_1$c) measurements were made using a Mono S HR 5/5 column and lithium malonate buffers, pH 5.7, according to the manufacturer's instructions (Jeppsson et al. 1986).

GFR was determined by the plasma clearance of 51 Cr-EDTA assessed by the single-injection method.

In study V, NO production in plasma was monitored by The Nitric Oxide Quantitation Kit (Active Motif, California, USA) according to manufacturer's instructions. The kit is based on nitrate

and nitrite determination. The absorbances were detected with Multiskan Ascent spectrophotometer (Thermo Labsystems, Vantaa, Finland). The detection limit of the assay is < 1 µmol of nitrite/nitrate.

2.3. Ultrasound imaging of carotid artery intima-media thickness

The common carotid artery far-wall IMT were measured from both sides in three different cardiac cycles approximately 10 mm distally from the carotid bulb and the values of twelve measurements were averaged (Toikka et al. 2000). All IMT measurements were performed using Acuson 128XP/10 (Acuson Inc., California) ultrasonography, using a 7 MHz scanning frequency linear array transducer.

Interobserver and intraobserver coefficients of variation of carotid IMT measurements were 5.2 ± 4.1 % and 4.0 ± 3.2 %, respectively (Toikka et al. 2000). IMT measurements were available from the majority of trial I subjects: control subjects (43/47), BHT-group (16/16) and FH-group (7/14).

2.4. Myocardial blood flow measurements by using positron emission tomography

After 12 hours of fasting, myocardial perfusion was measured twice, once at rest and once after the administration of dipyridamole using PET and [^{15}O] labelled tracers. To calculate myocardial perfusion non-invasively without arterial blood sampling, a mathematical model was used that corrects for the spillover of radioactivity both from the myocardium into the left ventricle region of interest (ROI) and the blood into the myocardial ROI. The method requires the measurement of a time-activity curve in the left ventricle chamber during the dynamic [^{15}O]H$_2$O study and the measurement of the recovery coefficient of the left ventricle ROI using a [^{15}O]-carbon monoxide scan and venous blood sampling (Iida et al. 1992)**.**

In brief, the subjects were positioned supine in a 15-slice ECAT 931/08-12 tomograph (Siemens/CTI, Knoxville, TN) with a measured axial resolution of 6.7 mm and in a plane resolution of 6.5 mm. The subjects inhaled [^{15}O]-carbon monoxide for two minutes (mean dose 3400 ± 410 Mbq). After inhalation, two minutes were allowed for the carbon monoxide to combine with hemoglobin in red blood cells before a static scan was started for four minutes. During the scan period, three blood samples were drawn at two-minute intervals for blood radioactivity. A 10-minute period was given for the radioactive decay of [^{15}O]-carbon monoxide flow measurements. Flow was measured at the baseline and two minutes after the end of intravenous administration of dipyridamole (0.56 mg per kilogram of body weight over a period of 4 minutes). 1650 ± 110 MBq of [^{15}O]H$_2$O was injected intravenously over two minutes and a dynamic scan was started for six minutes. Previous data have shown that resting and hyperemic myocardial blood flow can be measured reproducibly with PET (Nagamachi et al. 1996).

Calculation of regional blood flow. Regions of interest were placed on representative transaxial ventricular slices in each study covering the anterior, septal and lateral free wall of the left ventricle. The regions of interest were drawn on the images obtained at rest and copied to the images obtained after dipyridamole administration. Values of regional myocardial blood flow (expressed in milliliters per gram of tissue per minute) were calculated according to the previously published method employing the single compartment model (Iida et al. 1992, Iida et al. 1995).

Qualitative analysis of the PET data did not reveal any regional differences in the distribution of blood flow. Therefore, to enhance the accuracy and statistics of flow measurements, the average flow of the global left ventricular myocardium was calculated, and no detailed regional analysis was carried out.

2.5. Blood pressure measurements

In study I, BP was monitored with an automatic oscillometric BP monitor (OMRON HEM-705C, Omron corporation, Japan). In study II, BP was measured from both the subjects` arms by a sphygmomanometer and the mean of the two recordings was calculated to the nearest 2 mmHg after 10 minutes` rest in the supine position. The mean arterial blood pressure was calculated as twice the DBP plus the SBP divided by three. In study IV, BP was determined with an automatic BP gauge and the mean of the two recordings was calculated after 10 minutes` rest in the supine position. In study V, BP was measured using both casual and ambulatory BP measurements. Casual BP measurements were assessed with the participants in the sitting position after 10 minutes of rest, using a calibrated aneroid barometer (Speidel and Keller). Systolic BP was read at the first Korotkoff sound and diastolic BP at the disappearance of the Korotkoff sounds (phase V). The deflation rate was 2 mmHg/s. BP was recorded on two consecutive days, before the ambulatory recording (three measurements at least 1 minute apart) and after (two measurements at least 1 minute apart). The average of the five readings was used for analysis.

Ambulatory BP monitoring was performed with the previously validated (O'Brien et al. 1991a, O'Brien et al. 1991b) DIASYS 200 device (Novacor SA). BP was measured at 15-minute intervals between 6:00 AM and 10:00 PM and at 30-minute intervals between 10:00 PM and 6:00 AM. 24-hour BP was calculated using hourly means. Only recordings with less than 10 % missing or inappropriate values were accepted. The raw data were controlled manually and inappropriate readings removed (Devereux et al. 1993).

2.6. Other hemodynamic measurements

In study V, a hemodynamic profile was monitored using non-invasive methods. BP was monitored using the finger BP measurement method (Finapres^{TM} 2300, series FAX, Ohmeda, Louisville, CO, USA), a device that provides continuous non-invasive monitoring of beat-to-beat BP, and is therefore a useful non-invasive alternative to intra-arterial BP measurements (Imholz et al. 1988, Imholz et al. 1990).

Cardiac output (CO) is heart rate x stroke volume (SV) and was measured using a whole-body impedance cardiography (CircMon^{TM}, Model B202, JR Medical, Tallinn, Estonia), which is based on the Tishchenko (Tishchenko 1973) SV equation with a correction factor for tetrapolar registration, and also includes a correction of SV by haematocrit and BMI. Disposable electrocardiography electrodes (Blue sensor type R-00-S, Medicotest A/S, Denmark) were used. A pair of electrically connected current electrodes were placed on extremities, just proximal to wrists and ankles. Voltage electrodes were placed proximal to the current electrodes, with a 5 cm distance between the centres of the electrodes. The whole-body impedance cardiography reliably measures CO, and has an excellent agreement with invasive thermodilution and direct oxygen Fick methods for measuring CO in subjects without cardiac shunts and valvular lesions (Kööbi et al. 1997a, Kööbi et al. 1997b). Thus, this method is a feasible and practical technique for a non-invasive and continuous analysis of CO. Systemic vascular resistance (SVR) was calculated from CO and MAP as SVR = MAP / CO x 80. Left cardiac work (LCW) was calculated from the equation LCW = 0.0143 x MAP x CO. In addition, total arterial compliance was estimated by SV-to-pulse pressure ratio, which has been proposed as a rough measure for total arterial compliance (de Simone et al. 1997, Chemla et al. 1998). Arterial pulse wave velocity (PWV) was obtained from the time delay between simultaneously recorded flow pulses and the distance between recording sites, for example between the root of aorta and popliteal artery, a method

that we have found to show good agreement with measurements of pulse transition in the arterial tree by the ultrasound-determined doppler-flow method (Kööbi et al. 2003). Arterial pulse waves were recorded with voltage sensing channels of the CircMon^{TM} B202 and analysed automatically with the same device. Pulse transition in aortic root was estimated from the whole-body impedance cardiogram at the point where a sharp systolic upstroke commenced. The pulse wave arrival to the popliteal artery was similarly estimated from the sharp systolic upstroke of the second channel with the active electrode placed at the knee joint level. The distance between the aortic root and the knee joint was estimated from the subject's height by using a ratio H/1.61.

SVR was related to the surface area of the subjects, and it was transformed to the respective index - systemic vascular resistance index (SVRI = SVR x m^2)

Dynamic exercise testing was performed in an upright position using a bicycle ergometer (Siemens Elema, Germany®). The starting work load was 50 W, and the work load was increased in a stepwise manner with increments of 50 W/4 minutes until 85 % of the age-specific maximum heart rate was reached. The pedalling frequency was 60 r/min. The mean values of BP during the final minute of the pre-exercise period (the subject sitting on the bicycle ergometer before test initiation), the second workload, the final workload, and 10 minutes after the exercise testing were used for comparisons.

2.7. Statistical analyses

The data were analysed with the Statistica for Windows (studies II, III, IV, V) statistics program (StatSoft Inc, Tulsa, Oklahoma, USA) or SAS software (study I). Data comparison among the study subgroups was based on analysis of variance (studies II, III, IV, V) with respective all pairwise multiple comparison post-hoc analysis using the Bonferroni method (study I). Categorical variables (e.g., smoking and gender) were compared using χ^2–test.

Univariate correlation analysis was carried out with Pearson`s correlation test for normally distributed variables and Spearman`s correlation test for non-normally distributed variables (e.g. GHbA$_1$c). The normality of each variable distribution was studied using the Kolmogorov-Smirnow test. Multivariate-regression analysis was used to find out the determinants of plasma ADMA levels. In the dynamic exercise test, overall differences in responses of BP over time between ADMA subgroups were compared with analysis of variance for repeated measurements (RANOVA). RANOVA was also used to compare overall differences in responses of lipids, ADMA, LMMA, and SDMA over time between the statin and placebo treatment groups. The carry-over effect in trial IV was tested using RANOVA that used a crossover design in which in the independent factor was the treatment group, the dependent factors were changes in studied dietary factors and BP, and the repeated measure factors were the first and second periods of the crossover intervention. Data are presented as mean ± SD unless otherwise stated. A p-value of less than 0.05 was considered statistically significant.

RESULTS

1. Plasma arginine derivative levels in different studies (I-V) and subgroups

Plasma arginine and arginine derivative concentrations in different studies (I-V) are presented together in Table 3. In addition, plasma ADMA results were pooled together to evaluate the effect of DM, age, BMI, hypertension, hypercholesterolemia and smoking on plasma ADMA concentrations in the whole study material (Table 4). Hyperfiltration in diabetic subjects (study II, see 1.3.) and antihypertensive treatment (study V, see 2.3.) proved to have a significant effect on plasma ADMA. Thus, the evaluation of the above listed variables on plasma ADMA was also performed after excluding subjects with DM or/and antihypertensive treatment (Table 4).

In the pooled data, plasma ADMA levels were significantly lower ($p < 0.001$) in diabetic subjects compared to subjects without diabetes. The youngest subjects (lowest quartile of age distribution) had 42 % ($p < 0.0001$) higher plasma ADMA levels than the oldest subjects (highest quartile). Subjects with a normal weight (BMI $\leq$ 24 kg/m^2) had 33 % higher ADMA levels than overweight (BMI $\geq$ 30 kg/m^2) subjects ($p < 0.01$). The results were also similar after excluding subjects with DM or/and antihypertensive treatment. In addition, in the whole pooled population, subjects with arterial hypertension had 10 % lower ADMA levels than subjects without hypertension ($p < 0.05$).

In the pooled data, plasma ADMA was not significantly associated with age, gender, BMI, BP, smoking or plasma cholesterol and triglyceride levels.

Table 3. Concentrations of plasma arginine and its methylated derivatives in different studies (I-V) and their subgroups. Values shown as mean±SD.

Study	Subgroups	N	Arginine and its methylated derivatives (µmol/l)			
			Arginine	ADMA	LMMA	SDMA
I	Controls	47	118±31	0.43±0.12	_	0.33±0.08
	BHT-group	16	107±31	0.59±0.13	_	0.33±0.06
	FH-group	14	135±25	0.44±0.19	_	0.34±0.12
II	ND patients	65	150±25	0.34±0.16	_	0.90±0.51
	Diabetic patients	86	149±30	0.29±0.15	_	0.79±0.41
III	Placebo-group	14	_	0.58±0.16	0.10±0.04	0.52±0.27
	Simvastatin-group	15	_	0.57±0.13	0.09±0.06	0.46±0.18
	Atorvastatin-group	15	_	0.58±0.10	0.10±0.06	0.50±0.17
IV	Healthy HC	34	_	0.47±0.13	_	0.34±0.07
V	PREHT-group	23	_	0.37±0.12	_	0.82±0.32
	ST1HT-group	29	_	0.32±0.08	_	0.70±0.15
	ST2HT-group	15	_	0.32±0.10	_	0.70±0.16

ABBREVIATIONS: BHT, borderline hypertensive; FH, familial hypercholesterolemia; ND, non-diabetic; HC, hypercholesterolemic; PREHT, prehypertension; ST1HT, stage 1 hypertension; ST2HT, stage 2 hypertension; ADMA, asymmetric dimethylarginine; LMMA, monomethylarginine; SDMA, symmetric dimethylarginine; -, not measured

Table 4. Plasma ADMA concentrations in the pooled data (studies I-V) before and after exclusion of subjects with diabetes or/and antihypertensive treatment. Values are mean±SD.

Study subgroup	Combined population			Combined selected population		
	N	ADMA (µmol/l)	*P-value	N	ADMA (µmol/l)	*P-value
Healthy	129	0.43±0.15		129	0.43±0.15	
Type 2 diabetes						
Yes	91	0.30±0.15	< 0.0001	31[†]	0.32±0.20	< 0.003
No	282	0.42±0.16		208[†]	0.41±0.15	
Age						
Young (≤ 44 / 36[‡] years)	94	0.47±0.15	< 0.0001	53[‡]	0.46±0.15	< 0.0001
Elderly (≥ 65 / 56[‡] years)	93	0.33±0.15		51[‡]	0.36±0.14	
BMI						
Normal weight (≤ 24 / 24[‡] kg/m^2)	92	0.45±0.16	< 0.0001	52[‡]	0.44±0.14	< 0.05
Overweight (≥ 30 / 28[‡] kg/m^2)	94	0.34±0.15		51[‡]	0.37±0.16	
Hypertension						
Yes	175	0.37±0.17	< 0.05	36[‡]	0.37±0.14	NS
No	170	0.41±0.16		164[‡]	0.42±0.15	
Hypercholesterolemia						
Yes (TC ≥ 5.0 mmol/l)	183	0.39±0.16	NS	97[‡]	0.39±0.14	NS
No (TC < 5.0 mmol/l)	190	0.40±0.17		111[‡]	0.43±0.15	
Smoking						
Yes	54	0.33±0.12	NS	35[‡]	0.37±0.15	NS
No	268	0.37±0.16		162[‡]	0.42±0.12	
All subjects	373	0.39±0.17		373	0.39±0.17	

*STATISTICS: Analysis of variance between subgroups. **ABBREVIATIONS:** NS, non significant; BMI, body mass index; ADMA, asymmetric dimethylarginine; TC, total cholesterol. **DEFINITIONS:** Healthy, no hypertension, no diabetes, no drugs; age and BMI groups were formed according to lower and upper quartiles; hypertension, systolic ≥ 160 or diastolic BP ≥ 95 mmHg, or both, or having antihypertensive treatment. **SELECTIONS:** [†] excluded subjects on antihypertensive treatment, [‡] excluded subjects on antihypertensive treatment and subjects with type 2 diabetes

2. Plasma ADMA in relation to carotid artery IMT and indices of vascular function

2.1. ADMA and carotid artery intima-media-thickness

In the first study, plasma ADMA concentrations were observed to be associated with carotic artery IMT (r = 0.25, p < 0.05) in the study group that include subjects with untreated BHT, FH and healthy controls. Furthermore, subjects with high ADMA levels (0.58 µmol/l) had significantly higher carotid IMT than others (0.67 ± 0.14 vs. 0.60 ± 0.10 mm, p = 0.03).

In our previous case-control, with an average of seven years follow-up, high ADMA was a potent predictor of acute coronary events in non-smoking middle-aged men (Valkonen et al. 2001). To strengthen our earlier findings, we also tested the association between plasma ADMA concentration and carotid artery IMT in 154 middle-aged men, including smokers and non-smokers. In that population, plasma ADMA concentration did not associate significantly with carotid IMT at baseline or at the seven-year follow-up but was instead significantly associated with change in carotid IMT over follow-up (r = 0.18, p = 0.023, unpublished data).

2.2. ADMA and myocardial blood flow

In the first study, the relationship between myocardial blood flow and ADMA was evaluated. Plasma ADMA concentration correlated inversely with blood flow induced by dipyridamole when the data of all subgroups (untreated BHT, FH and healthy controls) was combined (r = -0.22, p < 0.05). To study the association of very high ADMA levels on flow variables, we compared those subjects belonging to the highest quartile of ADMA distribution (n = 15, ADMA > 0.58 µmol/l) to others. In this analysis,

subjects with high ADMA levels had significantly lower dipyridamole flow (2.60 ± 1.12 vs. 3.63 ± 1.65 ml/min/g, p = 0.03) than rest of the subjects. A similar association between high ADMA levels and low dipyridamole flow was also observed in healthy control subjects (high ADMA n = 5 vs. others n = 42, dipyridamole flow: 2.35 ± 1.24 vs. 3.85 ± 1.66, p = 0.05). In addition, plasma ADMA concentration was a significant determinant of dipyridamole induced blood flow also after adjustment of other risk factors (age and BMI) using multivariate stepwise regression analysis.

2.3. ADMA and blood pressure

Subjects with untreated BHT had higher systolic and diastolic BP values (137 ± 22 and 82 ± 10 mmHg) than subjects with FH (122 ± 8 and 63 ± 10 mmHg) or healthy controls (114 ± 12 and 63 ± 8 mmHg) (study I). They also had significantly higher plasma ADMA concentrations than the FH-group (0.59 ± 0.13 vs. 0.44 ± 0.19 µmol/l, p < 0.05) or control group (0.43 ± 0.12 µmol/l, p < 0.05). Furthermore, we compared subjects in the highest quartile of ADMA distribution (n = 15, ADMA > 0.58 µmol/l) to others and found that high ADMA concentration did not associate with higher BP-values.

Moreover, in another study including type 2 diabetic subjects (n = 86) and age and gender matched controls (n = 65), no association was observed between plasma ADMA and recorded BP values. Removal of subjects on current antihypertensive treatment did not affect the result. In fact, type 2 diabetic subjects had lower plasma ADMA concentrations than the controls despite significantly higher systolic (161 ± 21 vs. 152 ± 19 mmHg, p < 0.01) and mean (112 ± 11 vs. 108 ± 12, p < 0.05) BP values.

In study IV, there were thirty-four healthy subjects who did not have any antihypertensive treatment. In these subjects, similar to above mentioned findings, no association was found between

plasma ADMA concentrations and systolic or diastolic BP values. Plasma ADMA concentrations were comparable in normotensive and hypertensive subjects. However, there were 10 abstainers among the study subjects who had significantly lower plasma ADMA concentrations than the others (0.42 ± 0.11 vs. 0.50 ± 0.13 µmol/l, $p = 0.04$). In the abstainers, plasma ADMA associated significantly with systolic ($r = 0.60$, $p = 0.005$) and with diastolic BP ($r = 0.53$, $p = 0.02$).

In the last part (study V) of this work, 77 middle-aged men with elevated BP were examined. In this group, plasma ADMA concentrations again did not associate with systolic or diastolic BP values recorded by casual measurements. Furthermore, during 24-hour continuous monitoring, no correlation was observed between plasma arginine derivatives and BP values either in day-time or night-time recordings. Difference between day- and night-time BP values was not related to plasma ADMA concentrations. In a dynamic exercise test, plasma ADMA was not related to the BP response during the test. Plasma ADMA concentrations were comparable in all three defined BP groups (PREHT, ST1HT, ST2HT, see methods). Moreover, a lack of direct association between ADMA and BP was also found in a subgroup analysis in subjects not receiving antihypertensive treatment. To study whether higher ADMA levels, i.e., above a certain threshold value, would affect BP readings we compared subjects in the highest quartile of ADMA distribution ($n = 17$, ADMA > 0.40 µmol/l) to the others ($n = 53$). Both casual and 24-hour BP measurements were comparable in both ADMA groups. However, subjects receiving antihypertensive treatment had lower plasma ADMA concentrations than non-treated subjects (0.30 ± 0.08 and 0.36 ± 0.11 µmol/l, respectively, $p = 0.04$).

In the combined group, data collected from all studies (I-V) (Table 4), hypertensive subjects had slightly but non-significantly lower ADMA values than normotensive subjects. Also, in correlation analysis no direct association between plasma ADMA concentration and BP was found in this combined group.

2.4. ADMA, plasma nitrate and other hemodynamic values

In study V, SVRI and PWV were correlated with systolic ($r = 0.42$, $p = 0.001$ and $r = 0.25$, $p < 0.05$) and diastolic BP ($r = 0.34$, $p < 0.01$ and $r = 0.26$, $p = 0.05$). Subjects on antihypertensive treatment had significantly higher SVRI than others (3196 ± 544 and 2851 ± 481 dyn·s/cm^5·m^2, respectively; $p < 0.02$). Arginine derivatives or plasma NO_3 did not directly associate with recorded SVRI, PWV or CO values in the whole study group or in different subgroups as mentioned above. No association was observed between plasma ADMA and NO_3 or BP and NO_3 either. These analyses were also done separately for smokers and non-smokers.

3. ADMA in relation to atherosclerosis risk factors

3.1. ADMA and its determinants in patients with type 2 diabetes

The diabetic patients in study II had lower plasma ADMA levels than non-diabetic control subjects (0.29 ± 0.15 vs. 0.34 ± 0.16 µmol/l, $p < 0.03$). Plasma L-Arginine concentrations in diabetic patients and control subjects were comparable (149.09 ± 30.30 and 149.96 ± 25.30 µmol/l, respectively; $p = 0.67$), but plasma SDMA concentrations tended to be non-significantly lower in diabetic patients compared to non-diabetic controls (0.79 ± 0.41 and 0.92 ± 0.51 µmol/l, respectively; $p = 0.13$).

Twenty-seven diabetic patients were treated with insulin, either as a single therapy or combined with oral drug therapy. Average levels of GHbA$_1$c in the insulin treated patients were significantly higher than in patients treated with diet and oral drug therapy (8.9 ± 1.8 and 8.0 ± 1.5 mmol/l, respectively; $p = 0.02$). In the diabetic subjects, plasma ADMA concentrations were inversely

correlated with $GHbA_1c$ (r = -0.28, p = 0.01), an indicator of glycemic control. The patients also had significantly elevated GFR compared to controls (98 ± 23 and 90 ± 19 ml/min/1.73 m^2, respectively; p = 0.002). There was a significant negative correlation between GFR and ADMA levels (r = -0.29, p = 0.012) in patients with diabetes mellitus, but the GFR was not associated with the $GHbA_1c$.

In the diabetic subjects, the only significant predictor of plasma ADMA was GFR (r = -0.32, p = 0.008) in a multivariate analysis (r^2 = 0.13). In all subjects, predictors of ADMA were $GHbA_1c$ (r = -0.19, p = 0.03) and GFR (r = -0.19, p = 0.02, r^2 = 0.12). The other explanatory variables in the multivariate model were age, smoking, MAP, TC and TG.

3.2. Relationship between plasma ADMA and dyslipidemia and the impact of statin therapy

Plasma ADMA concentration did not correlate significantly with TC, LDL cholesterol or HDL cholesterol in any of the studies. Moreover, high-dose simvastatin (80 mg/day) and atorvastatin (40 mg/day) treatment did not lower plasma concentrations of measured arginine derivatives, despite very significant TC and LDL cholesterol lowering effect in study III (Table 5).

In studies I and IV, we found a weak correlation (r = 0.24, p = 0.03 and r = 0.26, p = 0.04, respectively) between plasma ADMA concentration and TG. To study the association of high ADMA levels on TG, we compared the subjects in the highest quintile of ADMA distribution (n = 15, ADMA > 0.58 µmol/l, in study I and n = 17, ADMA > 0.55 µmol/l, in study IV) to others. Subjects with high ADMA levels had significantly higher plasma TG concentrations than the others (1.79 ± 1.02 vs. 1.17 ± 0.56 mmol/l, p = 0.04, in study I and 1.93 ± 1.28 vs. 1.27 ± 0.79 mmol/l, p = 0.01, in study IV). High plasma TG also turned out to be a significant determinant of high ADMA after adjusting for other

factors in a multivariate regression model. In the other studies (II, III, V), there was no observed association between plasma ADMA and TG concentrations.

Table 5. Impact of high-dose statin therapy on plasma arginine derivative and lipid changes (%) during follow-up.

Variable	Simvastatin 80 mg/day N=15		Atorvastatin 40 mg/day N=15		Placebo N=14	
	Change (%)	* P-value	Change (%)	* P-value	Change (%)	* P-value
Total cholesterol	-37	< 0.00001	-36	< 0.00001	+3	NS
LDL cholesterol	-54	< 0.00001	-49	< 0.00001	+5	NS
HDL cholesterol	+4	NS	+4	NS	0	NS
Triglycerides	-29	< 0.01	-33	< 0.01	0	NS
ADMA	+4	NS	+5	NS	-3	NS
LMMA	-6	NS	-14	NS	+10	NS
SDMA	-3	NS	-1	NS	-8	NS

*STATISTICS: Analysis of variance**;** NS, not significant. **ABBREVIATIONS**: HDL, high density lipoprotein; LDL, low density lipoprotein; ADMA, asymmetric dimethylarginine; LMMA, monomethylarginine; SDMA, symmetric dimethylarginine.

4. ADMA in relation to dietary factors

Table 6 summarises the results from a univariate correlation analysis between ADMA, SDMA and various dietary components.

In study IV, plasma ADMA concentrations were inversely correlated with carbohydrate–derived energy intake (r = -0.33, p = 0.007) and there was a weak correlation between plasma ADMA concentration and the amount of alcohol intake (r = 0.26, p = 0.04). Moreover, there was a significant

correlation between plasma ADMA and total dietary energy intake (r = 0.26, p = 0.04). Plasma ADMA concentration did not associate with the other dietary components.

In forward stepwise multiple regression analysis (r^2 = 0.20, p < 0.002), low amounts of energy received from carbohydrates (r = -0.31, p = 0.009) and high plasma TG (r = 0.30, p = 0.01) were the significant predictors of high plasma ADMA levels. The other explanatory variables in this multivariate model were total energy intake, TC and amount of alcohol intake.

Table 6. Associations between plasma ADMA, SDMA and various dietary components.

Dietary component	ADMA		SDMA	
	R	*P-value	R	*P-value
Total energy intake	0.26	**0.04**	0.08	NS
Energy derived from fat	0.16	NS	-0.03	NS
Energy derived from protein	0.22	NS	-0.14	NS
Energy derived from carbohydrate	-0.35	**0.004**	-0.02	NS
Energy derived from PUFA	0.07	NS	-0.22	NS
Energy derived from MUFA	0.09	NS	-0.04	NS
Energy derived from SAFA	0.09	NS	0.02	NS
Dietary E-vitamin intake	0.20	NS	0.08	NS
Dietary D-vitamin intake	0.18	NS	-0.05	NS
Dietary C-vitamin intake	0.10	NS	0.15	NS
Dietary cholesterol intake	0.13	NS	0.009	NS
Amount of alcohol intake	0.26	**0.04**	-0.02	NS

*STATISTICS: Pearson`s correlation analysis. **ABBREVIATIONS:** ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SAFA, saturated fatty acids.

DISCUSSION

1. Study subjects

The relationship between plasma ADMA and several risk factors of atherosclerosis, carotid IMT and hemodynamic parameters was evaluated in five clinical studies (see Table 2). Three series consisted of both men and women (II, III, IV) and two series only of men (I, V). Therefore, the results from these studies (I, V) can not be generalized to women. The study subjects had a wide age range from 19 to 78 years of age. Participants consisted of both healthy subjects as well as subjects with findings or diseases reflecting endothelial dysfunction. Thus, the heterogeneity of study subjects enabled us to widely compare plasma ADMA in various groups of subjects. On the other hand, different patient selection criteria, determination methods, and study designs used in different substudies made it difficult to compare different study groups (Table 4). Furthermore, several study subjects were, for example, on antihypertensives while others were not, and the criteria for hypertension also varied somewhat between studies. Since the GFR was measured only in study II, we had a possibility to study renal function only in this trial and not in others.

2. Methodological consideration

2.1. Measurement of plasma argine derivatives

Plasma ADMA levels may be determined by employing a variety of methods including HPLC with UV or fluoresencence detection after a derivatization step (Böger et al. 1998b, Böger et al. 2000), capillary

electrophoresis with laser-induced fluorescence detection (Causse et al. 2000) and HPLC tandem mass spectrometry (Meyer et al. 1997) (see Table 1).

In the present study, HPLC and OPA derivatization and tandem mass spectrometry were used to measure plasma ADMA. Possibly, due to differences in methodology, the results regarding plasma ADMA levels have had a wide range in published studies (see Table 1). Therefore, it is very difficult to determine a normal range for plasma ADMA. The wide range of variability in plasma ADMA levels can lead to the situation where "high" levels in subjects with vascular disease in one study may be lower than the "normal" levels in healthy subjects in another study (Fujiwara et al. 2000, Kielstein et al. 2003).

Recently, it has been suggested that plasma ADMA may be an important predictor for vascular disease and events. Thus, an ADMA measurement could be a useful tool for clinical medicine in the future. However, careful standardization of methodology must be carried out before taking plasma ADMA measurements during clinical use. As well, it is currently unknown whether or not ADMA concentrations measured from plasma give relevant information concerning cellular ADMA metabolism. ADMA is generated within cells (Fickling et al. 1993, Ueno et al. 1992) and there is evidence that cellular levels of ADMA alter with pathophysiology (Azuma et al. 1995). The biological role of circulating ADMA is unclear and we do not know whether plasma concentration associates for example with ADMA concentrations in endothelial cells. It has been argued that typical plasma ADMA levels found in healthy (0.30 - 2.77 µmol/l) or many disease states (up to 7.3 µmol/l, see Table 1) are too low to be biologically active. However, experimental evidence suggests that despite the theoretical concerns, even very low concentrations of methylarginines exert profound effects. An experimental human study showed that at a time when circulating concentrations of ADMA are in the order of 2 µmol/l, substantial cardiovascular effects were evident and persistent (Achan et al. 2003). It is also known that intracellular concentrations of ADMA are higher than the overspill concentrations found in

plasma (Fickling et al. 1993, MacAllister et al. 1994). These findings suggest that low micromolar changes in plasma ADMA concentrations may reflect biological importance and, therefore, it may be relevant to measure plasma ADMA concentrations

2.2. IMT in evaluating early atherosclerosis

There is a wide variation in the methods and the arterial site of IMT measurement (Ebrahim et al. 1999). In the present study, IMT was measured from the common carotid artery alone, which is considered to be more reliable and reproducible than measurements from external and internal carotid arteries (Crouse and Thompson 1993). Increased IMT of the common carotid artery measured with ultrasound is a structural marker of subclinical atherosclerosis and correlates with traditional vascular risk factors (Howard et al. 1993, Sharrett et al. 1994, Bonithon-Kopp et al. 1996) and the severity and extent of coronary artery disease (Craven et al. 1990, Wofford et al. 1991). Moreover, it predicts the likelihood of cardiovascular events (Salonen et al. 1983, Bots et al. 1997, O'Leary et al. 1999).

2.3. PET in evaluating myocardial perfusion and coronary function

Exact endothelial function exposing methods were not used in this study. However, we studied dipyridamole-induced myocardial vasodilation, which is partly endothelium dependent (Buus et al. 2001). PET with ^{15}O-labeled water allows noninvasive quantification of myocardial blood flow at baseline and during pharmacologically induced hyperemia to assess the coronary vasodilator reserve. Intravenous dipyridamole is commonly used for the induction of hyperemia. Dipyridamole increases interstitial adenosine concentration in vascular smooth muscle cells and leads to relaxation of coronary resistance vessels. PET is used relatively widespread and its reproducibility of baseline and hyperemic

myocardial blood flow measurements has been tested (Nagamachi et al. 1996). Kaufmann et al. (1999) demonstrated that mean difference between two baselines was 13 % ± 11 % and between two hyperemic myocardial blood flows 10 % ± 14 %. Another study showed that the lowest intraindividual day-to-day coefficient variation was 15 %, both at rest and during adenosine. Repeated measurements of myocardial blood flow and coronary vasodilator reserve during the same study session were not significantly different, supporting the validity of the technique (Johansson et al. 2004).

2.4. Blood pressure measurements

BP was recorded by casual measurements as well as by 24-hour ambulatory BP monitorings. Most of the casual recordings were performed by calculating the mean of two measurements at one session. This kind of procedure is not ideal because casual measurements are susceptible to misinterpretation due to stress and other circumstances. In the present study however, BP values from casual and ambulatory measurements correlated significantly, suggesting the reliability of casual measurements. In this study, the casual recordings were performed extremely carefully and were very similar compared to ambulatory measurements, reflecting the reliability of casual measurements. Taken together, ambulatory monitoring or thoroughly recorded casual measurements improve the reliability of BP values and should be carried out in this kind of study design.

2.5. Other hemodynamic measurements

To measure CO, the oxygen Fick method is widely accepted as a "gold standard" but it also involves some inaccuracies (Hillis et al. 1985, Mahutte et al. 1994). The most popular method of bedside

measurement of CO is thermodilution using a pulmonary artery catheter. However, since this is an invasive method, its use is mostly restricted to critically ill patients. For subjects who require a hemodynamic evaluation and in whom pulmonary artery catheter application is not essential or possible, an alternative method to thermodilution has been used. In the 1970s, Tishchenko (Tishchenko 1973) developed an impedance method, known as whole-body impedance cardiography. The method, used in the present study is based on the Tischenko (Tishchenko 1973) SV equation with a correction factor for tetrapolar registration and also includes a correction of SV by hematocrit and BMI. Previously, it has been demonstrated that the whole body impedance cardiography reliably measures CO, and has an excellent agreement with the invasive thermodilution and direct oxygen Fick methods for measuring CO in subjects without cardiac shunts and valvular lesions. Also, its repeatability is excellent (Kööbi et al. 1997a, Kööbi et al. 1997b, Kööbi et al. 2003).

According to recent studies, pulse pressure and other parameters reflecting arterial stiffness are associated with an increased cardiovascular risk (Lehmann et al. 1998, Amar et al. 2001). The ratio of SV to pulse pressure as well as PWV have been used as crude non-invasive measures of arterial stiffness (Amar et al. 2001). In the present study, PWV was measured using the whole-body impedance cardiography method, which is in good agreement with measurements obtained with the Doppler ultrasound or invasive methods, respectively. Also, the repeatability and reproducibility of impedance-based PWV measurements is excellent (Kööbi et al. 2003).

3. Evaluation of results

3.1. Plasma arginine derivatives in different studies (I-V) and subgroups

In this study, the results of ADMA levels in different studies and their subgroups are summarised in Table 3 and 4. Plasma ADMA levels in healthy subjects averaged 0.43 ± 0.15 μmol/l (range 0.15 to 0.87 μmol/l). These values are similar to those reported in healthy controls in some previous studies (Meyer et al. 1997, Miyazaki et al. 1999), but slightly higher than in several reports (MacAllister et al. 1996b, Anderstam et al. 1997, Pi et al. 2000, Vishwanathan et al. 2000) and lower than in others (Böger et al. 1998b, Kielstein et al. 1999, Surdacki et al. 1999, Abbasi et al. 2001, Lundman et al. 2001, Kielstein et al. 2003). Mean concentrations of plasma LMMA were similar to one study (Meyer et al. 1997), higher than in another study (Vishwanathan et al. 2000) and lower than in one report (Pi et al. 2000). Respectively, plasma SDMA levels in healthy subjects were lower than in some previous studies (Böger et al. 1997b, Böger et al. 1998b, Kielstein et al. 1999, Kielstein et al. 2003) but higher than in some other reports (Pi et al. 2000, Vishwanathan et al. 2000).

As mentioned above, the most important reason for different levels of arginine derivatives may be the differences in methodology. Another explanation for different plasma ADMA levels may be a different genetic background. In fact, two mutations in DDAH enzymes have recently been discovered (Jones et al. 2003, Valkonen et al. unpublished). However, these mutations seem to be rare and, therefore, they are unlikely to explain observed plasma ADMA differences in published studies. Also, dietary factors may explain different levels of plasma ADMA.

The youngest subjects had significantly higher plasma ADMA levels than the oldest ones. This is line with the finding of a previous study (Böger et al. 1997b). In contrast, some studies have found opposite results (Miyazaki et al. 1999, Kielstein et al. 2003). Thus, the association between plasma

ADMA and ageing remains unclear. The oldest subjects had higher SDMA levels than the youngest ones. Elderly subjects may have lower GFR compared to young subjects despite normal plasma creatine levels.

3.2. Plasma ADMA in relation to carotid IMT and indices of vascular function

In this study, we widely evaluated whether plasma arginine derivatives relate to carotid IMT, vascular reactivity as measured by dipyridamole induced hyperemic myocardial flow, BP and other hemodynamic parameters.

ADMA in relation to carotid IMT. Previously, an association between elevated plasma ADMA and increased carotid IMT has been observed in healthy subjects (Miyazaki et al. 1999). In this work (study I), plasma ADMA concentration was associated with carotid IMT in all studied subjects and in addition, subjects in the highest quartile of ADMA distribution had significantly higher IMT compared to others. These findings suggest that high ADMA is also related to subclinical atherosclerosis in Finnish young adults. In our previous study population (Valkonen et al. 2001), the change in carotid IMT over a seven-year follow-up was significantly associated with plasma ADMA (unpublished) and at the same time, high ADMA was a significant predictor for cardiovascular events in non-smoking men. Together these findings suggest that plasma ADMA is related to IMT, a structural marker of subclinical atherosclerosis.

ADMA in relation to myocardial perfusion and coronary reactivity. Myocardial vasodilator capacity and impaired endothelium-dependent brachial artery vasodilation have been found to have an association with the concentrations of plasma ADMA (Böger et al. 1998b). This work (study I) demonstrated an inverse correlation between plasma ADMA concentration and dipyridamole induced vasodilatory function in young males, independent of BP elevation and hypercholesterolemia.

Furthermore, borderline hypertensive young men in the highest quartile of ADMA distribution had lower myocardial blood flow during dipyridamole infusion compared to others. A similar association between high ADMA levels and low blood flow during dipyridamole infusion was also observed in healthy control subjects. In addition, in a multivariate analysis plasma ADMA was a significant determinant of dipyridamole induced blood flow in all subjects. Together these findings suggest that high ADMA concentration is related to reduced hyperemic myocardial blood flow responses in healthy subjects. The association between high ADMA levels and low dipyridamole flow was observed not only in borderline hypertensive subjects but also in healthy control subjects, suggesting that ADMA level may be related to myocardial hyperemic responses also under normal physiologic conditions.

In the present study, high plasma ADMA concentration was associated with reduced myocardial flow responses in hypertensive subjects who also had significantly higher carotid IMT compared to normotensive subjects. Also, a relationship between reduced myocardial flow response and carotid IMT was observed, suggesting that both high ADMA and reduced flow response may be explained by advanced subclinical atherosclerosis seen as increased IMT.

Plasma ADMA in relation to BP. In some previous studies, a significant association between plasma ADMA and BP has been observed (Miyazaki et al. 1999, Surdacki et al. 1999). High plasma ADMA may lead to diminished NO bioavailability and thereby, increased vascular resistance and elevated BP (Miyazaki et al. 1999). The present study did not find any direct association between BP and biologically active arginine derivatives in young or old subjects. No association was found even when the analysis was restricted to subjects in the highest quartile of ADMA distribution. In addition, plasma ADMA was not related to BP responses in a dynamic exercise test either. Diabetic subjects had significantly higher systolic BP values than the control subjects but their plasma ADMA levels were even lower than in control subjects.

Some significant associations between ADMA and BP were observed in certain subgroups. In subjects avoiding alcohol (study IV), plasma ADMA correlated with systolic and diastolic blood BP. Despite a distinctive observation, this finding must be taken critically because the number of subjects avoiding alcohol was small (n = 10). Another significant association between ADMA and BP was recorded in study I, in which ADMA levels were significantly elevated in BHT subjects compared to controls (0.59 ± 0.13 vs. 0.43 ± 0.12 µmol/l, p < 0.001). Plasma ADMA concentrations in this group were relatively higher compared, for example, to the threshold values in subjects of other studies (0.58 vs. 0.40 (study 5) vs. 0.35 (study 2) µmol/l). Thus, it is possible that only significantly elevated plasma ADMA levels are significant determinants of BP. Such high plasma ADMA values may be related for example, to dysfunctional ADMA degrading enzymes, DDAH-I and -II, due to specific genetic mutations (Jones et al. 2003, Valkonen et al. unpublished). Furthermore, plasma ADMA levels may not be associated with ADMA levels in tissues where ADMA may have a direct and more potent regulatory role. Recently, it was demonstrated that shear stress under physiologic conditions caused by hypertension for example, stimulates ADMA release from vascular endothelial cells suggesting that elevated ADMA is a result of endothelial dysfunction (Osanai et al. 2003). Thus, it may be hypothesised that elevated plasma ADMA could be a marker of subclinical atherosclerosis so that hypertension per se may lead to vascular disease and damage presented for example, as increased carotid IMT. This will also affect vascular reactivity and further elevate plasma ADMA levels as seen in borderline hypertensive men in this study.

Interestingly, subjects on antihypertensive treatment had lower plasma ADMA concentrations than non-treated subjects (0.30 ± 0.08 and 0.36 ± 0.11 µmol/l, respectively, p = 0.04). It can be assumed that antihypertensive treatment may lower plasma ADMA levels. This idea is supported by observations in previous studies where at least ACE inhibitors (Delles et al. 2002, Ito et al. 2002) and

angiotensin II AT 1 receptor blockers (Delles et al. 2002) decreased plasma ADMA levels. However, in this study the number of subjects on ACE inhibitors was small and none of subjects used angiotensin II AT receptor blockers. Despite antihypertensive treatment, these patients were still clearly hypertensive. Thus, ADMA levels in these patients may have been lowered to compensate for inappropriately high BP. One can suggest that ADMA may have a dual role in the regulation of BP. As suggested earlier, high ADMA levels due to genetic reasons, for example, may cause elevated BP levels in some persons, while in others ADMA levels are possibly lower due to physiological up-regulation of DDAH enzymes due to hypertension.

Plasma ADMA in relation to other indices of vascular function. Previously, it has been demonstrated that parameters reflecting arterial stiffness are associated with increased cardiovascular risk (Lehmann et al. 1998, Amar et al. 2001). This study demonstrated that SVRI and PWV (describing vasodilatory capacity) were significantly associated with BP. Recently, Achan et al. (2003) demonstrated in an experimental clinical study that intravenously dosed ADMA increased systemic vascular resistance and lowered CO in healthy male volunteers. As well, Kielstein et al. (2004) demonstrated a decrease in effective renal plasma flow and an increase in renovascular resistance in healthy subjects in a similar experimental trial. These findings suggest that ADMA in high doses is a biologically active regulator of vascular tone and thus a possible cardiovascular risk factor. Contrary to those findings, the present study did not find any association between plasma ADMA concentrations and SVRI or PWV, suggesting that plasma ADMA in a physiological setting is not a major determinant of increased vascular resistance in hypertensive subjects.

All the findings of previous studies and the present study suggest that initially, significantly increased plasma ADMA may have a causative role in endothelial dysfunction but in a normal physiological setting, ADMA seems to be a marker of vascular disease rather than a cause of it.

3.3. ADMA in relation to plasma nitrate and hemodynamics

Study V demonstrated a well known (Shepherd et al. 1995) association between BP and vascular resistance. Since ADMA is an endogenous competitive inhibitor of the NO synthase (Leiper and Vallance 1999) and can modulate endogenous vasodilator NO (Palmer et al. 1987) production, it was also assumed that plasma ADMA and nitrate (metabolite of NO) are associated with BP and other hemodynamic regulations in general. However, this study indicated no apparent association between plasma ADMA or nitrate and hemodynamic regulation in our study group. The main cause for diminished bioavailability of NO in hypertension is the action of reactive oxygen species (Kerr et al. 1999, Bouloumie et al. 1997, Somers et al. 2000). However, most of the evidence from some clinical studies (Dominiczak and Bohr 1995) but not all (Laurent et al. 1990, Cockcroft et al. 1994) indicate that there is also a deficiency in the release of NO by endothelium in hypertension. In fact, some previous studies have demonstrated reduced NO production in hypertensive subjects compared to normotensive controls measured by decreased levels of urinary nitrate (Forte et al. 1997, Bode-Böger et al. 2000). Since NO is short-lived *in vivo*, direct measurement of NO production is difficult (Forte et al. 1997). Although, plasma and urinary nitrate measurements have been used to estimate NO production (Forte et al. 1997), these assays may be confounded especially by dietary nitrates. In the present study, standardized diets were not used, which may, in part, explain the lack of association between measured plasma nitrate concentrations and hemodynamic parameters. NO inhaled via tobacco smoke is another source of nitrate (National Academy of Sciences Committee on Nitrate and Alternative Curing Agents in Food. The health effects of nitrate, nitrite and N-nitroso compounds 1981). However, in the present study, plasma nitrate levels did not associate with BP or vascular resistance even after smokers were excluded from analyses.

3.4. ADMA and its determinants in subjects with type 2 diabetes

This is the only published study that thoroughly considers how plasma ADMA concentration is related to renal function and glycemic control in subjects with type 2 diabetes. This study demonstrated that patients type 2 diabetes lasting over nine years had lowered plasma ADMA levels and elevated GFR compared to non-diabetic control subjects. A noteworthy observation also was that plasma ADMA concentration correlated inversely with GFR. Hyperfiltration is a consistently observed phenomenon in early diabetic nephropathy (Wirta et al. 1996, Chaiken et al. 1998, Chiarelli et al. 2000). Previously, it has also been demonstrated that some methylarginines are in part eliminated by renal excretion (Vallance et al. 1992) and are known to accumulate in patients with chronic renal failure (Vallance et al. 1992, Kielstein et al. 2002). Furthermore, in hypertensive children a significant and inverse association with levels of plasma ADMA and GFR has been published (Goonasekera et al. 1997). In addition, it has been shown that plasma homocysteine levels are inversely related to GFR, indicating an increased clearance of homocysteine in patients with even slightly increased filtration rates. Based on these observations, it was hypothesised that low ADMA in type 2 diabetic subjects is at least partly explained by the increased GFR measured in these patients. This hypothesis could have been tested by measuring urine ADMA levels, but that was not possible in this study.

The results of the present study were conflicting with the results of a study published by Abbasi et al. (2001) who reported that type 2 diabetic subjects have elevated plasma ADMA levels. However, it must be noted that they did not report the renal function in their diabetic subjects. It is possible that their diabetic subjects had recent diabetes mellitus without hyperfiltration or that their renal function and filtration rate had already decreased. Secondly, the study design was different compared to the present study. In their study, no patients with type 2 diabetes had received any pharmacologic treatment for type 2 diabetes within the past 4 weeks before the study, possibly explaining their poor glycemic

control, whereas in the present study, all diabetic subjects had oral drug therapy, insulin therapy or combined oral drug and insulin therapy. It is fully uncertain whether the pharmacologic treatment affected plasma ADMA levels in diabetic subjects in the present study. However, it has been demonstrated that metformin lowers plasma ADMA levels in patients with type 2 diabetes (Asagami et al. 2002). Thus, pharmacolocic treatment may partly explain the different results of these two studies.

Dysregulation of the enzyme DDAH may be responsible for the increase in plasma ADMA concentrations in diabetic subjects in a study published by Abbasi et al. (2001). This theory was supported in an animal model in which hyperglycemia elevated ADMA by impairing DDAH activity in vascular smooth muscle and endothelium (Lin et al. 2002). However, in the present study, low plasma ADMA correlated with a poor glycemic control and with increased filtration rates in diabetic subjects. Against the hypothesis that these associations were inter-related, no direct relation between glycemic control and GFR was observed in the present study. Based on the findings of previous studies (Abbasi et al. 2001, Lin et al. 2002), it is unlikely that dysregulation of the DDAH could explain the association between low ADMA and a poor glycemic control. Instead, poor glycemic control may also directly influence NO synthesis and thus be related to hyperfiltration and renal hyperperfusion as shown in early type 1 diabetes (Mattar et al. 1996, Soper et al. 1998). Another mechanism could be that long lasting hyperglycemia may lower ADMA directly by decreasing the production of ADMA or increasing the metabolism of ADMA by an as yet unknown mechanism.

3.5. ADMA, dyslipidemia, dietary factors and statins

ADMA and dyslipidemias. Some previous animal models (Bode-Böger et al. 1996, Böger et al. 1998a, Böger et al. 2000) and human studies (Böger et al. 1998b, Eid et al. 2003) have found an association between plasma ADMA and hypercholesterolemia. However, a number of later studies

have failed to show any association between concentrations of circulating cholesterol levels and ADMA (Miyazaki et al. 1999, Cardinale 2001, Stühlinger et al. 2002). In line with the latter findings, the present study did not find any association between plasma arginine derivatives and TC, LDL or HDL cholesterol.

Previously, some studies (Lundman et al. 2001, Stühlinger et al. 2002) but not all (Miyazaki et al. 1999) have demonstrated a relation between plasma ADMA and TG. In the present study, plasma ADMA did not associate with TG in three studies (II, IIII, V). However, in other studies (I, IV), plasma ADMA correlated with TG, which was even a significant determinant of ADMA in a multivariate model.

Recently, Fard et al. (2000) demonstrated in subjects with type 2 diabetes that a high-fat meal, 5 hours after ingestion, acutely increased plasma levels of TG, decreased LDL cholesterol levels but had no effect on TC. These changes occurred with increased plasma ADMA levels and reduced brachial artery flow responses. Similarly, Ong et al. (1999) demonstrated that a high-fat meal significantly increased plasma TG levels and reduced brachial artery flow whereas a high intake of dietary carbohydrates had an acute beneficial effect on flow responses. In the present study, the subjects fasted 12 hours before blood tests, which is a common practice. However, it can be speculated that dietary composition a day before blood tests could affect levels of plasma lipids and ADMA and thereby affect the association between plasma ADMA and TG as well as the association between ADMA and cholesterol. The differences in methodology may also explain differences in the association between plasma ADMA and cholesterol levels.

Impact of statins on plasma ADMA levels. A possible association between ADMA and hypercholesterolemia has been previously observed (Böger et al. 1998b, Eid et al. 2003). In addition, Laufs et al. (1998) demonstrated that simvastatin reverses, in a dose-dependent manner, the inhibitory effect of oxidized LDL on LMMA inhibitable NO production. Thus, our hypothesis in the present

study was that the lowering of plasma cholesterol levels would also result in a reduction of circulating ADMA and possibly LMMA levels. However, this study demonstrated that both simvastatin or atorvastatin in high doses had no influence on arginine derivatives despite aggressive reduction in LDL cholesterol levels of 54 % and 49 %, respectively. Eid et al. (2003) demonstrated a similar finding in hypercholesterolemic men without overt cardiovascular disease. Treatment with 40 mg/day of pravastatin for 8 weeks had no effect on the levels of plasma ADMA or L-arginine/ADMA ratio despite a significant reduction in total and LDL cholesterol. Furthermore, Jiang et al. (2004) reported that simvastatin protects from the vascular endothelium damages induced by LDL or oxidized LDL but has no effect on serum ADMA levels in an animal model (Kielstein et al. 2003). Therefore, it can be suggested that although statins can improve endothelial dysfunction by increasing NO production, it occurs by mechanisms other than diminishing endogenous inhibitors of NO. However, in a recent study Janatuinen et al. (2003), observed that although 40 mg/day of pravastatin did not affect plasma ADMA levels, the ADMA concentration modulated the therapeutic effect of pravastatin. They investigated the effects of 6 months treatment with pravastatin versus a placebo on myocardial blood flow. Basically, pravastatin did not significantly improve myocardial blood flow but subjects with ADMA below the median concentration responded to pravastatin with a significant 35 % increase in myocardial blood flow. Subjects with an ADMA concentration above the median did not benefit. It is plausible that coronary reactivity was improved in the pravastatin-treated subjects with low ADMA due to increased NO release, while high ADMA levels probably efficiently inhibited NO-related effects of pravastatin on coronary vasodilatory function.

Association of ADMA with dietary factors. Study IV demonstrated that a high amount of energy received from carbohydrates is strongly associated with low plasma ADMA concentrations. Plasma ADMA also seemed to be higher in alcohol drinkers than abstainers. Dietary carbohydrates have been linked not only with beneficial flow responses but also with fewer new coronary lesions

(Blankenhorn et al. 1990). Based on the observations of the present study and results received from previous studies (Ong et al. 1999, Fard et al. 2000), it seems that there is an interplay between plasma ADMA, energy intake and plasma TG. A high-carbohydrate diet may have beneficial effects by lowering ADMA levels.

FUTURE DIRECTIONS

Data from previous studies and the present research suggest that an increased plasma ADMA concentration may be a marker of atherosclerosis related vascular disease. Thus in the future, an ADMA measurement could be a useful tool for clinical medicine in estimating a humans` risk for disease. However, the usefulness of ADMA assays must first be demonstrated with fully standardized methods before taking plasma ADMA measurements in the clinical use. Genetic variables may have a strong influence on cellular metabolism. Thus, genetic diagnostics related ADMA metabolism may in the future be a more clinically relevant application than plasma based ADMA assays.

SUMMARY AND CONCLUSIONS

The major findings and conclusions were:

1. Plasma ADMA was related to carotid artery IMT, a structural marker of subclinical atherosclerosis. High ADMA concentration was related to reduced hyperemic myocardial blood flow responses in healthy subjects. Plasma ADMA levels were not directly associated with BP. However, high ADMA level may affect BP.

2. Patients with type 2 diabetes had lowered plasma ADMA levels, which was at least partly explained by the increased GFR in these patients. Although endothelial dysfunction is related to early diabetes, it can be suggested that risk factors other than ADMA contribute to endothelial dysfunction in type 2 diabetic patients with increased GFR. However, normal ADMA in diabetes does not rule out endothelial dysfunction because of increased GFR.

3. Statin therapy in high doses had no influence on arginine derivatives despite aggressive reduction in LDL cholesterol levels. However, dietary carbohydrates were associated with low plasma ADMA concentrations and may therefore lower plasma ADMA. ADMA seemed to be higher in alcohol drinkers than in abstainers.

This study found no association between plasma ADMA and classical risk factors for atherosclerosis such as hypercholesterolemia, aging, gender and hypertension. The present study suggests that increased plasma ADMA may be a result of vascular disease rather than a cause for it. However, it is quite possible that elevated plasma ADMA may regulate NO balance and vascular reactivity and thus be a risk factor for further deteriorating vascular disease. Fuelling development of vascular disease

plasma ADMA could be a novel marker of vascular status. However, fast and validated methods are needed for possible clinical use and possibilities for thorough renal function evaluation are required.

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