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PIRJO VALTONEN

Asymmetric Dimethylarginine

Assay Methodology and Serum Levels in Non-Pregnant and Pregnant Women

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium 1, Kuopio University Hospital, on Friday 11th December 2009, at 12 noon

Eastern Finland Laboratory Centre
Institute of Clinical Medicine
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ABSTRACT

Asymmetric dimethylarginine (ADMA) is a naturally occurring methylated amino acid which is released into the circulation during protein metabolism. Elevated ADMA levels reduce the formation of nitric oxide (NO) by inhibiting nitric oxide synthase and are associated with endothelial dysfunction. The female sex hormone, estrogen, is known to have an effect on ADMA concentration by regulating the expression of ADMA degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH). Earlier it has been observed that the ADMA concentration is elevated in women with complicated pregnancies, such as pre-eclampsia.

In the present series of studies, the effects of hormonal cyclic changes on the levels of ADMA and its related compounds were investigated in healthy non-pregnant women with and without hormonal contraceptive use. Another aim was to examine if there was an association between ADMA levels and flow-mediated dilatation and factors that are known to be involved during normal pregnancy, such as hypercholesterolemia and changes in the immune system.

In order to measure the concentrations of ADMA, symmetric dimethylarginine (SDMA), L-homoarginine and L-arginine in human serum and plasma, the HPLC method developed by Teerlink et al. (2002) was optimized and it proved to have good recovery, excellent linearity, low detection limits and good separation efficiency of the analytes. ADMA and its related compound concentrations were measured from two study populations; The Cardiovascular Risk in Young Finns Study and The Complicated Pregnancy Study. Brachial artery flow-mediated dilatation (FMD) was measured by ultrasound.

It was found that the ADMA concentration was dependent on hormonal status of women without oral contraceptive (OC) use. The use of oral contraceptives decreased ADMA and SDMA concentrations as compared with non-OC users but there was no variation in the ADMA concentration during menstrual cycle phases in women with OC-use. Furthermore, it was found that ADMA, SDMA and L-arginine concentrations declined and L-homoarginine concentrations were elevated in normal pregnancy in comparison with the levels in non-pregnant females. During normal pregnancy, serum levels of ADMA and SDMA were not associated with hypercholesterolemia or circulating cytokines, IL-6 and TNF-α, or C-reactive protein. FMD was enhanced in normal pregnancy but it was not associated with ADMA levels.

In summary, the present studies provide new information about ADMA and related compounds regarding pregnancy related phenomenon: endothelial function, immune system upregulation and increased lipid concentration during normal pregnancy. In this thesis, it was found that the ADMA concentration varied during menstrual cycle. Thus if women of fertile age are used in studies, the menstrual cycle phase and not only their possible use of hormonal contraception should be taken into account. Furthermore, also the type of contraception is important. This applies also to CRP measurements where the use of OC has been shown to increase the circulating CRP concentration. On the contrary, endothelial function measured as FMD seemed to be unaffected by the menstrual cycle phase. These results may be useful when ADMA concentrations from normal pregnancy are compared with the results from complicated pregnancies.

National Library of Medicine Classification: WQ 244, QU 60

Medical Subject Headings: Humans; Female; Pregnancy; Arginine/blood; C-Reactive Protein; Cytokines; Homoarginine/blood; Interleukin-6/blood; Endothelium, Vascular; Menstrual Cycle; Nitric Oxide/blood; Tumor Necrosis Factor-alpha; Vasodilation

To my Family

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Kuopio, November 2009

Pirjo Valtonen

ABBREVIATIONS

AccQ	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
ADMA	N^G,N^G-dimethyl-L-arginine, asymmetric dimethylarginine
CE	capillary electrophoresis
CV	coeffient of variation
DDAH	dimethylarginine dimethylaminohydrolase
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FMD	flow-mediated dilatation
GC	gas chromatography
GDM	gestational diabetes mellitus
GFR	glomerular filtration rate
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
hsCRP	high sensitive C-reactive protein
IL-6	interleukin-6
L-Arg	L-arginine
LC	liquid chromatography
LDL	low-density lipoprotein
L-NMMA	N^G-monomethyl-L-arginine
MRE11	meiotic recombination 11
MS	mass spectrometry
NDA	naphthalene-2,3-dicarboxaldehyde
NO	nitric oxide
NOS	nitric oxide synthase
OC	oral contraceptives
OPA	ortho-phthaldialdehyde
PCOS	polycystic ovary syndrome
PRMT	S-adenosylmethionine:protein arginine methyltransferase
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SEM	standard error of the mean
SD	standard deviation
SDMA	$N^G, N^{G'}$-dimethyl-L-arginine, symmetric dimethylarginine
TNF-α	tumor necrosis factor-α

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-V).

I Valtonen P, Karppi J, Nyyssönen K, Valkonen V-P, Halonen T, Punnonen K. Comparison of HPLC method and commercial ELISA assay for asymmetric dimethylarginine (ADMA) determination in human serum. J Chromatogr B. 2005;828:97-102.

II Valtonen P, Punnonen K, Saarelainen H, Heiskanen N, Raitakari OT, Juonala M, Viikari JSA, Alfthan G, Kähönen M, Laaksonen R, Lyyra-Laitinen T, Laitinen T, Heinonen S. ADMA concentration changes across the menstrual cycle and during oral contraceptive use. The Cardiovascular Risk in Young Finns Study. Revised version submitted.

III Saarelainen H, Valtonen P, Punnonen K, Laitinen T, Raitakari OT, Juonala M, Heiskanen N, Lyyra-Laitinen T, Viikari JSA, Vanninen E, Heinonen S. Subtle changes in ADMA and L-arginine concentrations in normal pregnancies are unlikely to account for pregnancy-related increased flow-mediated dilatation. Clin Physiol Funct Imaging. 2008;28:120-124.

IV Valtonen P, Laitinen T, Lyyra-Laitinen T, Raitakari OT, Juonala M, Viikari JSA, Heiskanen N, Vanninen E, Punnonen K, Heinonen S. Serum L-homoarginine concentration is elevated during normal pregnancy and is related to flow-mediated vasodilatation. Circ J. 2008;72:1879-1884.

V Valtonen P, Punnonen K, Saarelainen H, Heiskanen N, Raitakari OT, Viikari JSA, Lyyra-Laitinen T, Laitinen T, Heinonen S. Maternal serum ADMA is not associated with proinflammatory cytokines or C-reactive protein during normal pregnancy. Cytokine. 2009;46:216-221.

CONTENTS

1 INTRODUCTION

There is abundant evidence that endothelial cells play a crucial role in the maintenance of vascular tone by producing several vasoactive agents. One of the major endothelium-derived vasoactive mediators is nitric oxide (NO). Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase (NOS) since it can compete with the binding of the natural substrate L-arginine (Vallance et al. 1992). ADMA is a naturally occurring methylated amino acid and a post-translationally modified form of arginine that is generated in all cells during the process of protein turnover. It is eliminated from the body by a combination of renal excretion and metabolism by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). This enzyme plays a critical role in the regulation of the cellular and tissue levels of ADMA (Ogawa et al. 1987). Thus, the inhibition of DDAH leads to accumulation of ADMA within cells (MacAllister et al. 1996). Several studies have demonstrated that ADMA is a useful marker of endothelial dysfunction and elevated concentration of ADMA has been reported in a number of diseases such as hypercholesterolemia (Böger et al. 1998), hypertension (Goonasekera et al. 1997), diabetes (Päivä et al. 2003), renal failure (Vallance et al. 1992), pre-eclampsia (Savvidou et al. 2003) and chronic heart failure (Usui et al. 1998). The elevated ADMA concentration was also associated with an increased risk of adverse cardiovascular events and atherosclerosis (Miyazaki et al. 1999; Valkonen et al. 2001; Mittermeyer et al. 2006; Krzyzanowska et al. 2007b).

There are a variety of methods for determining plasma concentration of ADMA and these have been used to examine the association between various disease states and endothelial function (Cooke 2000; Perticone et al. 2005). The most widely used methods for the determination of ADMA are based on chromatographic analysis by high-performance liquid chromatography (HPLC) with fluorescence detection (Pettersson et al. 1997; Teerlink et al. 2002; Marra et al. 2003; Heresztyn et al. 2004; Zhang and Kaye 2004). These techniques permit the simultaneous determination of ADMA, its isomer symmetric dimethylarginine (SDMA) and L-arginine. Recently, methods based on mass spectrometry (MS) have also been developed (reviewed in Martens-Lobenhoffer and Bode-Böger 2007). Furthermore, a commercially available

enzyme-linked immunosorbent assay (ELISA) for ADMA (Schulze et al. 2004) with high sample throughput has been developed but it is not able to assay L-arginine or SDMA simultaneously with ADMA.

Normal pregnancy is associated with enhanced vasodilatation, since there are many vasodilatory systems, such as the increased NO production (reviewed in Valdes et al. 2009). In addition, the circulating plasma level of ADMA decreases during normal pregnancy (Holden et al. 1998; Pettersson et al. 1998; Ellis et al. 2001; Savvidou et al. 2003) but is often elevated in endothelial dysfunction and pregnancy complications, such as preeclampsia (Fickling et al. 1993; Holden et al. 1998; Savvidou et al. 2003). In the present series of studies, the aim was to examine if there is an association of ADMA or related compounds with factors that are involved in normal pregnancy and may be detrimental to health under non-pregnant circumstances, such as hypercholesterolemia and changes in the immune system. First, the HPLC method developed by Teerlink et al. (2002) was optimized and evaluated against a new commercially available ELISA assay. Secondly, the effect of menstrual cycle phase and oral contraceptive (OC) use on dimethylarginine and L-arginine concentrations was evaluated. The concentrations of ADMA, SDMA, L-homoarginine and L-arginine were measured by HPLC in non-pregnant and pregnant women. In addition, the relationship between ADMA and flow-mediated dilatation (FMD), hypercholesterolemia, and inflammation markers was examined in non-pregnant and pregnant state.

2 REVIEW OF THE LITERATURE

2.1 L-arginine-nitric oxide pathway

The L-arginine-nitric oxide pathway is involved in the regulation of a wide variety of physiological functions including endothelium-dependent vasodilation, platelet aggregation, neurotransmission, immune defence and apoptosis (Moncada and Higgs 1993). Nitric oxide is synthesized from the amino acid L-arginine by the action of nitric oxide synthases (Figure 1), a family of enzymes with endothelial, neuronal, and inducible isoforms (Palmer et al. 1988; Furchgott 1996). Synthesis of NO can be blocked by ADMA, which is able to inhibit all isoforms of NOS (Vallance et al. 1992; MacAllister et al. 1994). Additionally, N^G-monomethyl-L-arginine (L-NMMA) produces near-identical effects on ADMA, but its concentration in plasma is about ten-fold lower (Furchgott 1996; Matsuoka et al. 1997; Cardounel and Zweier 2002). SDMA cannot directly inhibit NOS, but it is able to indirectly reduce intracellular L-arginine availability and limit NO synthesis by competing with L-arginine, ADMA and L-NMMA for cellular transport by one of the cationic amino-acid transporters (y+ transporter).

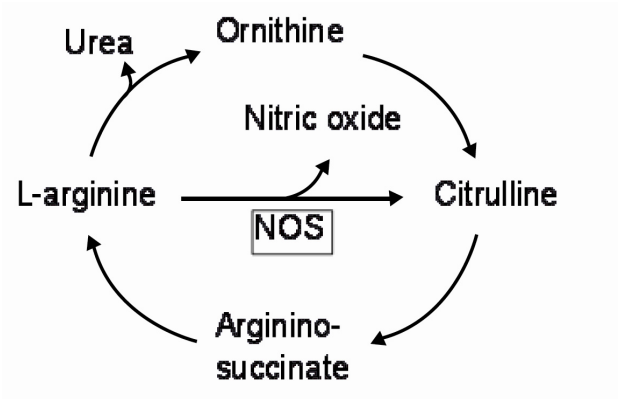

Figure 1. Nitric oxide production by nitric oxide synthase (NOS) in L-arginine-nitric oxide pathway.

2.2 Metabolism and clearance of dimethylarginines

The plasma concentration of ADMA is a complex result of many parallel processes occurring in a variety of organs (Figure 2). ADMA is formed by proteolysis of proteins with arginine residues which are methylated by protein arginine methyltransferases (PRMTs). Two other endogenous methylarginines are also synthesized by PRMT: L-NMMA and SDMA, an isomer of ADMA. The structures of ADMA and related compounds are depicted in Figure 3.

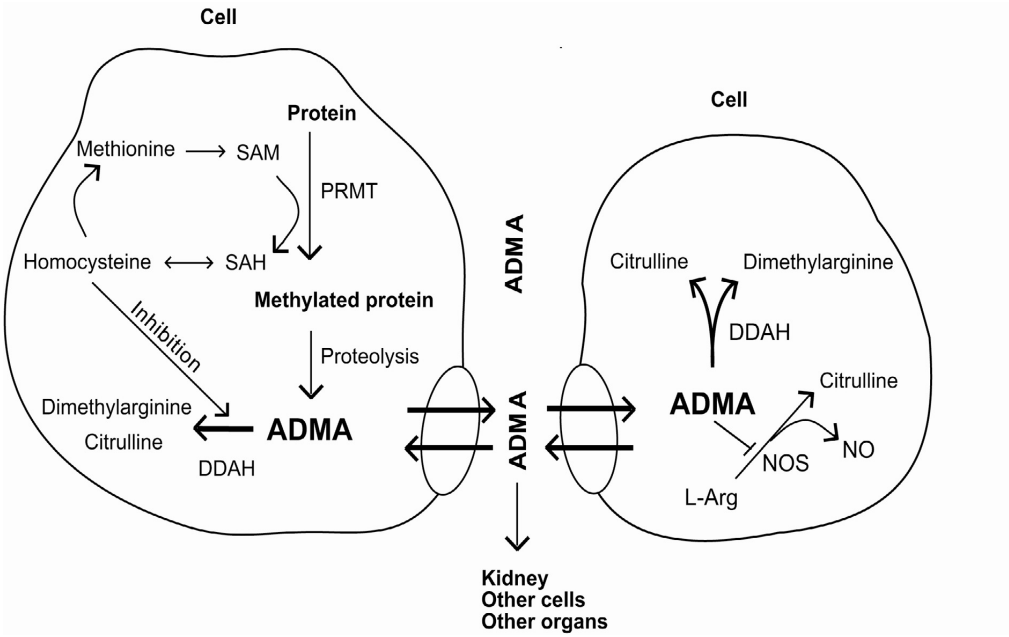

Figure 2. Biochemical pathways for generation of ADMA and its degradation by enzyme dimethylarginine dimethylaminohydrolase (DDAH) in cells.

ADMA, asymmetric dimethylarginine; L-Arg, L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; PRMT, protein arginine methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

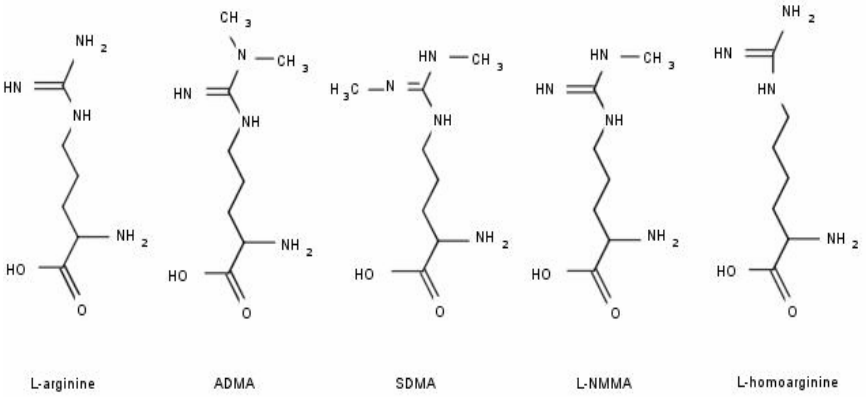

Figure 3. L-arginine, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), N^G-monomethyl-L-arginine (L-NMMA) and L-homoarginine structures.

Although the overall physiological roles of PRMTs are not clear, one of their functions appears to be regulation of mRNA processing and maturation by modulating the activity of RNA-binding proteins. However, it has been shown that PRMT-1 catalyzes the formation of ADMA and L-NMMA, whereas PRMT-2 leads to the formation of SDMA (Gary and Clarke 1998). A recent proteomic analysis has identified more than 200 proteins that are putatively arginine-methylated (Boisvert et al. 2003). Many of these proteins, such as histones, RNA binding proteins, cytokine receptors, and MRE11, play a regulatory role in important cellular processes such as transcription, RNA processing, signal transduction, and DNA repair (reviewed in Bedford and Richard 2005). Even though the methylation of arginine residues on proteins seems to be ubiquitous, the degree of arginine methylation is organ specific and elevated dimethylarginine residues have been shown to be present in brain, liver, lung, spleen, kidney and small intestine (Nakajima et al. 1971).

Protein degradation represents the major source of intracellular and plasma arginine and methylarginines. Proteolysis usually results in complete degradation of proteins to free amino acids, which in turn can be used for *de novo* protein synthesis. Post-translationally modified amino acids cannot be used for protein synthesis and must be excreted or metabolized. Recently, one demethylating enzyme has been found that can

deaminate monomethylated arginine residues but it does not seem to be able to hydrolyse ADMA (Cuthbert et al. 2004). Another study has found that proteins with methylated arginine residues are degraded rapidly (Teerlink 2005a). It was reported that a homogenate of rat kidney rapidly released free ADMA in an in vitro incubation.

Asymmetric dimethylarginine is continuously released into the extracellular space after its release from proteins during physiological protein turnover. In healthy humans, its accumulation in the body is prevented by both renal excretion, and metabolic degradation. Most ADMA (and L-NMMA) is hydrolyzed by an intracellular enzyme called dimethylarginine dimethylaminohydrolase into citrulline and dimethylamine and only a small proportion of ADMA is excreted into urine through the kidneys (Ogawa et al. 1989). On the contrary, DDAH does not exhibit activity towards SDMA. The Michaelis-Menten constant of DDAH for ADMA is 0.18 mM which is much higher than the intracellular concentrations of ADMA (Ogawa et al. 1989). This ensures that ADMA is efficiently degraded as the enzyme is functioning in the linear part of the substrate-velocity curve. It is not known what percentage of the endogenously formed ADMA is directly metabolized in the cells where it has been formed, and how much is released from the cells to other tissues to be metabolized or excreted. There are two DDAH isoforms (DDAH-1 and DDAH-2) with different tissue distributions (Leiper et al. 1999). DDAH-1 is mainly expressed in the kidney and liver, which are also the major sites of ADMA metabolism (Nijveldt et al. 2003a and 2003b). DDAH-2 is particularly abundant in the vascular endothelium, heart, placenta and kidney (Tran et al. 2000). Interestingly, it has been recently demonstrated that the lungs may be a major source of ADMA (Bulau et al. 2007). In studies conducted in rats, a very high uptake of ADMA by the liver was found (Nijveldt et al. 2003a), which can be considered as evidence for the important role of the liver in regulating the systemic ADMA concentration. It has also been demonstrated that DDAH is co-localized with NOS in several anatomical sites (Tojo et al. 1997). Thus, the substrate specificity of DDAH and its co-localization with NOS have provided the basis for the concept that regulation of intracellular ADMA levels may occur via DDAH, which in turn might regulate NOS activity.

Changes in renal excretory function or changes in DDAH activity may lead to elevated ADMA levels in several cardiovascular and metabolic diseases. Although increased NO synthesis due to upregulation of DDAH expression or activity is usually desired in cardiovascular diseases, it may have undesirable effects in tumors. In these situations, overexpression of both DDAH isoforms has been demonstrated to enhance tumor growth and promote tumor angiogenesis (Kostourou et al. 2002). These observations suggest that neither very high nor very low NO and ADMA levels are desirable, and NO and ADMA concentrations must be kept between certain limits in plasma and normal tissues.

2.3 Analysis methods of ADMA

The measurement of ADMA concentrations in plasma has been a focus of interest since its association with NO metabolism was discovered in 1992 (Vallance et al. 1992). To date, a broad range of methods for dimethylarginine assay has been published (Vishwanathan et al. 2000; Wahbi et al. 2001; Teerlink et al. 2002; Marra et al. 2003; Tsikas et al. 2003; Schulze et al. 2004; Trapp et al. 2004; Xu et al. 2004; Horowitz and Heresztyn 2006). However, there are large discrepancies in the dimethylarginine values reported with these methods which may be due to different sample purification steps and the different methods used for the analysis (Table 1).

2.3.1 Chromatographic methods

Most methods that are intended to measure ADMA are based on HPLC with sensitive fluorescent detection (Pettersson et al. 1997; Teerlink et al. 2002; Marra et al. 2003; Heresztyn et al. 2004; Zhang and Kaye 2004). The methods allow chromatographic separation of these two structurally very similar, but functionally completely different isomers, ADMA and SDMA. The HPLC methods are hampered by the laborious sample preparation necessary to detect the small amounts of analyte present in human plasma and serum, and protein precipitation or solid phase extraction is normally used (Bode-Böger et al. 1996; Anderstam et al. 1997). Protein precipitation can be performed with 5-sulfosalicylic acid (Anderstam et al. 1997; Chen et al. 1997) or ethanol (Zhang and Kaye 2004). The samples need to be diluted with water or buffer to

avoid clogging of proteins in the extraction column. These columns can be either silica based or equipped with a polymeric stationary phase. Stationary phases are usually modified with weak carboxylic acid or strong cation exchange resins (Bode-Böger et al. 1996; Pettersson et al. 1997; Teerlink et al. 2002). Recoveries for L-arginine are crucial, and protocols need to be optimized in this respect (Teerlink et al. 2002; Heresztyn et al. 2004). Furthermore, only volatile compounds can be used for analyte elution from the extraction column, because the solvent is removed by evaporation. Sample cleanup by solid phase extraction is labour intensive, but the procedure can be fully automated (de Jong and Teerlink 2006).

Table 1. Summary of the mean basal plasma or serum ADMA, SDMA and L-arginine concentrations in normal control subjects obtained with different methods.

Method	ADMA (μM)	SDMA (μM)	L-Arg (μM)	n	Reference
HPLC (OPA)	1.25 ± 0.11	0.71 ± 0.09	83 ± 5	47	Böger 1997
HPLC (OPA)	0.58 ± 0.02	0.56 ± 0.02		10	Pettersson 1997
HPLC (OPA)	0.51 ± 0.01			116	Miyazaki 1999
HPLC (OPA)	1.3 ± 0.2	0.7 ± 0.2	60 ± 6	24	Chan 2000
HPLC (OPA)	0.51 ± 0.16	0.39 ± 0.12	140 ± 29	150	Valkonen 2001
HPLC (isotope)	0.61 ± 0.13	0.41 ± 0.12	118 ± 18	9	Wahbi 2001
HPLC (OPA)	1.4 ± 0.7	0.64 ± 0.41	59 ± 9	16	Kielstein 2002
HPLC (OPA)	0.47 ± 0.03	0.50 ± 0.03		17	Mittermayer 2002
HPLC (OPA)	0.42 ± 0.06	0.47 ± 0.08	94 ± 26	53	Teerlink 2002
HPLC (OPA)	0.43 ± 0.12	0.33 ± 0.08	118 ± 31	47	Päivä 2002
HPLC (NDA)	0.38 ± 1.3	1.32 ± 2.7		50	Marra 2003
HPLC (OPA)	0.93	0.27	85	20	Eid 2003
HPLC	0.66 ± 0.04	0.49 ± 0.03	76 ± 4	26	Fleck 2003
HPLC	0.41 ± 0.06		81 ± 23	NM	Maeda 2003
HPLC (OPA)	0.30 ± 0.05	0.34 ± 0.06	61 ± 19	7	Pi 2000
HPLC (OPA)	0.50 ± 0.06			726	Teerlink 2005b
HPLC (AccQ)	0.50 ± 0.09			157	Horowitz 2006
CE	0.34 ± 0.02		85 ± 6	5	Caussé 2000
CE	0.42 ± 0.02		27 ± 4	NM	Trapp 2004
LC-MS	0.12 ± 0.05	0.16 ± 0.07	63 ± 24	20	Vishwanathan 2000
LC-MS	0.48 ± 0.07	0.41 ± 0.05	72 ± 17	40	Huang 2004
LC-MS/MS	1.41 ± 0.05			25	Selley 2003
LC-MS/MS	0.35 ± 0.01	0.51 ± 0.01		14	Xu 2004
LC-MS/MS	0.46	0.68		42	Kirchherr 2005
LC-MS/MS	0.37 ± 0.06	0.45 ± 0.06	60 ± 18	14	Martens-L. 2006
GC-MS/MS	0.39 ± 0.06			12	Tsikas 2003
GC-MS	0.60 ± 0.08			10	Albsmeier 2004
ELISA	0.72 ± 0.16			10	Schulze 2004
ELISA	0.69 ± 0.20			500	Schulze 2005

Results are expressed as mean ± standard deviation (SD). NM = not mentioned in the article.

AccQ, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; CE, capillary electrophoresis; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; HPLC, high performance liquid chromatography; LC, liquid chromatorgraphy; MS, mass spectrometry; NDA, naphthalene-2,3-dicarboxaldehyde; OPA, ortho-phthaldialdehyde.

In most cases, purified samples are usually derivatized using o-phthaldialdehyde (OPA) reagent before injection onto the HPLC column, although underivatized dimethylarginines have also been quantified in plasma using ultraviolet detection at 200 nm (MacAllister et al. 1996). Other derivatization reagents that have been successfully used for the dimethylarginines are naphthalene-2,3-dicarboxaldehyde (NDA) (Marra et al. 2003), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ) (Anderstam et al. 1997; Heresztyn et al. 2004), 4-fluoro-7-nitro-2,1,3-benzoxadiazole (Nonaka et al. 2005) and phenyl isothiocyanate (Ueno et al. 1992). The major advantages of the most widely used OPA are its rapid reaction at room temperature and at +4ºC as well as the fact that OPA itself is non-fluorescent, leading to comparatively clean chromatograms. Both 2-mercaptoethanol and 3-mercaptopropionic acid can be used as the thiol reagents needed in the OPA derivatization reaction. Fluorescence detection of OPA is performed at excitation and emission wavelengths of 340 and 455 nm, respectively.

Inclusion of an internal standard is necessary for sample purification steps and derivatization reactions before chromatographic separation of the analytes. An optimal internal standard should not be present in biological samples, although it is not always possible to meet this requirement. This may lead to systematic errors in the quantification, since the total concentration of the internal standard, i.e. endogenous plus added, is unknown and varying within individual samples. L-homoarginine and L-NMMA are the most widely used internal standards in dimethylarginine HPLC analysis (Chan et al. 2000; Pi et al. 2000; Böger et al. 2001; Teerlink et al. 2002). Although L-NMMA is an endogenous methylated amino acid which is catalyzed by PRMT-1 in the same reaction where ADMA is formed during protein degradation, its plasma concentration is more than tenfold lower than that of ADMA (Vallance et al. 1992). Only N^{ω}-propyl-L-arginine, which is also used as an internal standard (Marra et al. 2003), does not occur endogenously. A recently published study has used a new non-endogenous internal standard, monoethylarginine, in an HPLC assay with OPA-derivatization. It was claimed that monoethylarginine should replace the endogenous internal standard L-NMMA (Blackwell et al. 2009).

The choice of the column, mobile phase composition and pH, and column temperature are all critical for the chromatographic separation of ADMA, SDMA and

L-arginine. Chromatographic separation is usually performed by reversed-phase chromatography using isocratic or gradient elution. Phenyl and C18 columns are commonly used in HPLC based assays of dimethylarginines. In many studies, octadecyl silane C18 or C20 columns have been used and the running buffer has been phosphate or acetate buffer combined with methanol as the solvent component (Teerlink et al. 2002; Heresztyn et al. 2004; Zhang and Kaye 2004). Additionally, tetrahydrofuran has been used in the mobile phase and its inclusion can be crucial in achieving a successful separation (Pettersson et al. 1997; Chu et al. 2003; Eid et al. 2003; Jiang et al. 2004). In phenyl-based columns, the mobile phase most often is citric acid with methanol (Kielstein et al. 1999; Böger et al. 2000b). The pH of the buffers in mobile phase is in the range of 6.0-7.1 with the column kept at room temperature or higher (27-42ºC), during the analysis. The total running time has varied from 35 min to 78 min. In these methods, detection limit was 0.025-0.1 μM for ADMA and SDMA with coefficient variation (CV) of less than 5-7% being achieved.

Recently, new mass spectrometry (MS) -based methods have been described for L-arginine, ADMA and SDMA (Vishwanathan et al. 2000; Huang et al. 2004; Xu et al. 2004; Kirchherr and Kühn-Velten 2005; Schwedhelm et al. 2005; Martens-Lobenhoffer and Bode-Böger 2006). In addition, it is also possible to measure L-citrulline, which is involved in L-arginine-nitric oxide pathway, in the same run (Martens-Lobenhoffer and Bode-Böger 2003). Mass spectrometry increases the selectivity of the procedure because analytes are identified due to their characteristic molecular mass-to-charge (m/z) ratio, by their fragmentation pattern, as well as by their retention times in HPLC. Due to of the superior selectivity of MS methods, sample preparation can be reduced to protein precipitation for plasma and dilution for urine samples. Commercially available or synthesized isotope-labeled L-arginine or ADMA analogues were used as internal standards. L-arginine and dimethylarginines were separated in their underivatized (Vishwanathan et al. 2000; Huang et al. 2004; Kirchherr and Kühn-Velten 2005) or derivatized states (Schwedhelm et al. 2005; Martens-Lobenhoffer and Bode-Böger 2006) and quantification was carried out by electrospray (Vishwanathan et al. 2000; Kirchherr and Kühn-Velten 2005; Martens-Lobenhoffer and Bode-Böger 2006) or atmospheric pressure chemical ionization (Huang et al. 2004) techniques. At their best,

mass spectrometry based methods provided a high sample throughput with short analysis times (4 min) with sharp peaks in the chromatograms (Schwedhelm et al. 2005).

In gas chromatography (GC)-based MS methods, it is not possible to analyze polar amino acids, such as L-arginine or dimethylarginines, unless they are derivatized (Tsikas et al. 2003; Albsmeier et al. 2004). Plasma sample cleanup has been performed by ultrafiltration or by protein precipitation, and the derivatization has consisted of esterification and pentafluoropropionic anhydride conversion. For L-arginine, there is a commercially available internal standard [$^{15}N_2$]-Arg but for ADMA, internal standard has to be synthesized. Both GC-MS and GC-MS/MS methods have used Optima-17 capillary column separation and negative-ion chemical ionization detection (Tsikas et al. 2003; Albsmeier et al. 2004). The methods were reported to be accurate and stable with no interference from endogenous substances being observed in the chromatograms.

2.3.2 Immunological methods

A newly developed ELISA test is based on competitive enzyme linked immunoassay with polyclonal antibodies. This assay allows the measurement of ADMA in human plasma or serum (Schulze et al. 2004). Acylation is needed for sample preparation before the ELISA assay, and only a small sample volume (20 µl) is required for the test. The amount of antibody bound to the plate well is determined by the reaction of tetramethylbenzidine with the horseradish peroxidise that is coupled to the secondary antibody. The intensity of the developing colour is inversely proportional to the amount of ADMA in the sample and measured by reading the optical density of the wells at 450 nm in a microtiter plate reader. This ELISA test has been validated by comparing it with the LC-MS/MS technique and the correlation was good (R=0.984, n=29). Cross-reactivities with other L-arginine analogues present in human plasma and serum have been found to be negligible (L-NMMA 1.0%, SDMA 1.2%, L-arginine <0.02%). This ELISA test has a linear range between 0.1 and 3 µM for ADMA in human serum and plasma.

2.4 ADMA in health and disease

2.4.1 Biological variation in ADMA plasma concentration

ADMA and SDMA concentrations are tightly controlled in normal healthy population. It has been demonstrated that ADMA has a very narrow concentration distribution in plasma (Teerlink 2005b). In normal healthy individuals, the concentrations are usually low (0.40-0.77 μM, n=238) (Hov et al. 2007), and at the most in the low micromolar range in diseased states. Blackwell et al. (2007) have recently shown that ADMA and SDMA exhibit low intra-individual biological variation (7.4% and 5.8%, respectively) when measured once a week for 20 weeks. Inter-individual variation for ADMA and SDMA was 9.6% and 14.7%, respectively. They also found that plasma ADMA and SDMA concentrations were normally distributed. Furthermore, it has been noted that ADMA concentration increases linearly with age (Miyazaki et al. 1999; Schulze et al. 2005; Marliss et al. 2006) and this finding may suggest that there are larger variations in ADMA concentration if the study group exhibits a broad age range. On the other hand, large population based studies have revealed conflicting results about whether there are differences between genders or age groups in the ADMA concentrations (Schulze et al. 2005; Blackwell et al. 2007; Teerlink 2007).

2.4.2 Function of ADMA

ADMA is able to inhibit vascular NO production by inhibiting all three isoforms of NOS within the concentration range found in patients with vascular diseases (Böger 2004). It has been observed that acute systemic administration of ADMA to healthy men elicits a transient fall in heart rate and cardiac output and increases vascular resistance (Achan et al. 2003). Smith et al. (2005) have examined the mechanisms by which low concentrations of ADMA produce adverse effects on the cardiovascular system. They treated human endothelial cells from coronary artery with high ADMA concentrations and measured expression of the genes involved in cell cycle regulation, cell proliferation, DNA repair, regulation of transcription and metabolism. Significant changes in genes expression of more than 50 genes were found in the endothelial cells. Interestingly, with pathophysiological concentrations of ADMA (2 μM), the change

was seen in endothelial cell gene expression even in the presence of very high L-arginine concentrations (300 μM). These results were confirmed in an in vivo study with DDAH-1 heterozygous knockout mice.

2.4.3 ADMA and endothelial function

In recent years, endothelial function has been estimated widely by the non-invasive brachial artery flow-mediated dilatation technique. Endothelium plays a role in the vascular tonus control through NO generated by NOS. Normally NO increases blood flow by acting as a signal resulting in vasodilatation. Endothelial dysfunction is characterized by several pathological conditions, such as altered anti-coagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth and dysregulation of vascular remodelling. Recent genetic and chemical-biological approaches provide compelling evidence that loss of DDAH-1 function as studied in Ddah1$^{+/-}$ mice resulted in increased ADMA concentrations and thereby disrupted vascular NO signalling (Leiper et al. 2007). Endothelial dysfunction may also be caused by decreased availability of its substrate, L-arginine, or cofactors, or a change in cellular signalling (Endemann and Schiffrin 2004; Xiao et al 2009). However, ADMA is considered as a key factor contributing to endothelial dysfunction and in several clinical studies an increased ADMA concentration has been associated with endothelial dysfunction in patients with hypertension, hypercholesterolemia and heart failure (Böger et al. 1998; Usui et al. 1998; Surdacki et al. 1999). In the Cardiovascular Risk in Young Finns Study, it has also been demonstrated that at the normal population level elevated plasma ADMA concentrations are associated with decreased brachial FMD (Juonala et al. 2007). It is also evident that endothelial dysfunction is closely associated with oxidative stress and one proposed mechanism is that reactive oxygen species increase the comsumption of NO (Guzik et al. 2000; Kalinowski and Malinski 2004). Recently, it has also been suggested that ADMA in addition to inhibiting NO modulation, directly induces oxidative stress (Böger et al. 2000b). Increased oxidative stress is suggested to disminish DDAH activity which leads to accumulation of ADMA (Ito et al. 1999, Palm et al. 2007).

2.4.4 ADMA and cardiovascular diseases

Asymmetric dimethylarginine is now considered to be an important biomarker in the assessment of cardiovascular risk (reviewed in Siroen et al. 2006b; Böger et al. 2009). Several studies have shown that plasma levels of ADMA are increased in patients with vascular disease, or with risk factors for vascular disease (Böger et al. 1998; Valkonen et al. 2001; Lu et al. 2003; Cooke 2004). NO is the most potent endogenous vasodilator and thus any impairment of NO synthesis or bioactivity may increase the risk of vascular disease. When the NOS pathway becomes dysregulated, its vasoprotective functions are lost, and the NOS pathway may even contribute to vascular pathophysiology. In mice, a chronic infusion of ADMA has induced atherosclerosis (Suda et al. 2004). In humans, the ADMA concentration in plasma correlates with markers of subclinical atherosclerosis, such as carotid artery intima media thickness (Zoccali et al. 2002). Derangements of the NOS pathway may be categorized as reductions in the NO half-life, altered sensitivities to NO, as well as changes in NOS expression or NOS activity. NO also acts as an endogenous inhibitor of platelet aggregation (Azuma et al. 1986; Radomski et al. 1987). Furthermore, NO inhibits the adhesion of monocytes and leucocytes in the healthy vascular endothelium (Kubes et al. 1994, Tsao et al. 1994) – an effect that once disturbed precedes the migration of inflammatory cells into the vascular wall at sites that later become plaques. NO also inhibits the proliferation of vascular smooth muscle cells (Garg and Hassid 1989) and reduces the vascular release of superoxide radicals (Violi et al. 1999) that are involved in inflammatory and cytotoxic processes, and it also inhibits low-density lipoprotein (LDL) oxidation (Hogg et al. 1993).

Endogenous inhibitors of NOS are responsible for endothelial vasodilator dysfunction in individuals with coronary and peripheral arterial disease, as well as those with risk factors, such as hypercholesterolemia, hypertension, hyperhomocysteinemia and insulin resistance. In experimental animal models, ADMA levels start to increase very rapidly after the induction of dietary hypercholesterolemia. Even though at that time, no overt atherosclerotic lesions can be found macroscopically (Bode-Böger et al. 1996). Similarly, elevated ADMA plasma levels have been observed in clinically healthy human subjects with isolated hypercholesterolemia and other cardiovascular

risk factors (Böger et al. 1998; Vlamidirova-Kitova et al. 2008). Several animal and clinical studies have also demonstrated a strong association between plasma total homocysteine, plasma ADMA, and endothelial dysfunction (Böger et al. 2000a; Böger et al. 2001; Yoo and Lee 2001; Holven et al. 2003; Selley 2003; Stühlinger et al. 2003; Sydow et al. 2003). It has been suggested that ADMA may be a mediator of the atherogenic effects of homocysteine (Holven et al. 2002). These data suggest that ADMA is an early marker of the initial stages of atherogenesis, and thus may be useful in devising the primary prevention to assess a patient's total cardiovascular risk and to supplement the information generated by traditional risk factors.

2.4.5 ADMA in other diseases

Some examples of ADMA concentrations in different diseases are collected in Table 2. More detailed information about some diseases is discussed below. In seriously ill patients, the level of methylated arginines may be considerably elevated by the unfavourable combination of an increased production of dimethylarginines as a result of increased protein turnover, e.g. catabolic state, synthesis of acute phase proteins, and decreased elimination as a result of impaired renal and hepatic clearance capacities (Nijveldt et al. 2003c). Additionally, elevated plasma ADMA levels are known to be associated with cardiovascular complications such as stroke, congestive heart failure and peripheral arterial disease (Böger et al. 1997; Usui et al. 1998; Yoo and Lee 2001). Furthermore, in patients with cardiovascular disease, end-state renal disease, or some other serious illnesses, elevated plasma ADMA concentrations independently predict progression of atherosclerosis, future vascular events and/or overall mortality (Valkonen et al. 2001; Zoccali et al. 2001; Lu et al. 2003; Aucella et al. 2009).

The kidneys play an important role in the elimination of dimethylarginines from the body and a significant elevation of the plasma ADMA levels is observed in patients with end-stage renal failure (Vallance et al. 1992; Zoggali et al. 2001; Billecke et al. 2009). Elevated ADMA levels and endothelial dysfunction may in part be responsible for the highly elevated cardiovascular morbidity and mortality in patients with chronic renal failure (Kielstein et al. 1999). Additionally, in patients with end-state renal disease, ADMA concentrations have emerged as the second strongest predictor of all-

cause mortality after age, outweighing other established risk factors such as hypertension, diabetes, hypercholesterolemia and smoking (Zoggali et al. 2001; Mallamaci et al. 2005).

Table 2. Mean plasma or serum ADMA concentrations in different disease states.

Disease state	ADMA (μM)		Number of	Reference
	Controls	Cases	controls+cases	
Coronary heart disease	0.47 ± 0.12	0.66 ± 0.17	139 + 70	Valkonen et al. 2001
Coronary heart disease	0.44 ± 0.09	0.47 ± 0.12	46 + 51	Lu et al. 2003
Chronic kidney disease	0.61 ± 0.13	1.04 ± 0.17	9 + 13	Wahbi et al. 2001
Chronic kidney disease	1.4 ± 0.7	4.2 ± 0.9	16 + 44	Kielstein et al. 2002
Hypercholesterolemia	1.03 ± 0.09	2.17 ± 0.09	31 + 49	Böger at al. 1998
Hypercholesterolemia	1.3 ± 0.2	2.1 ± 0.2	18 + 24	Chan et al. 2000
Liver cirrhosis	0.58 ± 0.05	1.12 ± 0.08	7 + 20	Lluch et al. 2004
Acute liver failure	0.37 ± 0.02	1.75 ± 0.3	10 + 10	Mookerjee et al. 2007
End-stage renal disease	0.42 ± 0.02	0.82 ± 0.03	16 + 18	Billecke et al. 2009
Cerebral infarction	0.93 ± 0.32	1.46 ± 0.77	35 + 27	Yoo et al. 2001
Recurrent cerebral infarction	0.93 ± 0.32	2.28 ± 1.63	35 + 25	Yoo et al. 2001
Hypertension	0.43 ± 0.12	0.59 ± 0.13	47 + 16	Päivä et al. 2002
Hypertension	0.42 ± 0.02	0.60 ± 0.04	16 + 18	Billecke et al. 2009
Diabetes type 1	0.40 ± 0.06 #	0.46 ± 0.08 #	175 + 397	Lajer et al. 2008
Diabetes type 1	0.43 §	0.50 §	50 + 20	Cighetti et al. 2009
Pre-eclampsia	0.81	2.7	43 + 10	Savvidou et al. 2003
Polycystic ovary syndrome	0.652 ± 0.040	0.746 ± 0.025	30 + 160	Charitidou et al. 2008
Polycystic ovary syndrome	0.09 ± 0.02	0.17 ± 0.02	22 + 44	Ozgyrtas et al. 2008

Results are expressed as mean ± SD.
Controls have diabetes without microalbuminuria and cases have diabetes with nephropathy.
§ Concentration is expressed as median.

The liver is also a critical organ in regulating plasma ADMA concentration and dysfunction of the liver may disturb normal ADMA metabolism (Nijveldt et al. 2003a; Siroen et al. 2005; Mookerjee et al. 2007). In patients undergoing liver transplantation, the preoperative ADMA concentrations in plasma were highly elevated, but decreased significantly after the operation (Siroen et al. 2004). Additionally, in patients with acute

rejection, ADMA levels were higher than those in nonrejectors. ADMA may also be of significance in the pathophysiology of liver cirrhosis, since it has been shown that ADMA concentrations are elevated in patients with alcoholic cirrhosis (Lluch et al. 2004 and 2006). However, the exact mechanism of how ADMA is involved in the pathophysiology of liver cirrhosis is not known.

There is also evidence that the lung can generate a significant amount of ADMA and therefore it may directly contribute to interstitial and circulating ADMA concentration (Bulau et al. 2007). In recent studies, dysregulated arginine methylation has been shown to contribute to the pathogenesis of several chronic pulmonary diseases such as pulmonary arterial hypertension and pulmonary fibrosis (Zakrzewicz and Eickelberg 2009).

In diabetic patients, there have been controversial results of studies showing elevated, normal or even decreased circulating ADMA concentration (Päivä et al. 2003; Krzyzanowska et al. 2007a; Lajer et al 2008; Cighetti et al. 2009), although it has been recently reported that ADMA levels above the median may predict an increased risk of fatal and non-fatal cardiovascular events in type 1 diabetic patients with nephropathy (Lajer et al. 2008). Furthermore, Krzyzanowska et al. (2007b) have shown that an elevated ADMA concentration was associated with an increased risk of cardiovascular events also in type 2 diabetic patients with albuminuria. It has been also suggested that ADMA plays an important role as a risk marker in insulin resistance (Chan and Chan 2002; Sydow et al. 2005), and pharmacological agents that improve insulin sensitivity are able to lower the plasma ADMA concentration (Stühlinger et al. 2002; Wakino et al. 2005).

2.5 Reproduction and ADMA

2.5.1 Effect of menstrual cycle changes on ADMA

Giusti et al. (2002) evaluated the concentrations of circulating NO throughout the menstrual cycle in healthy women and they found that the NO concentration significantly declined from the early follicular phase (day 7) towards the luteal phase (day 21). It is known that exogenous estrogen has a lowering effect on circulating

ADMA levels (Holden et al. 2003) and that the plasma ADMA concentration correlated with age in women but not in men (Hov et al. 2007). Cevik et al. (2006) measured the ADMA concentrations at a low menstrual estrogenic stage and at ovarian hyperstimulation state when the estrogen concentration was 20-fold higher. They found that ADMA was lower in women with high plasma estrogen levels than that in women with low estrogen levels. Furthermore, there is evidence that the use of oral contraceptives reduced significantly the ADMA concentration and the delivery method of OC was important since transdermally administered estrogen had no effect on the ADMA concentration (Verhoeven et al. 2006 and 2007).

Recently, it has been found that the ADMA concentration is significantly higher in women above 45 years of age in comparison with women younger than that age (Hov et al. 2007) and postmenopausal hormone therapy has been reported to reduce the ADMA concentration (Post et al. 2003; Teerlink et al. 2003; Verhoeven et al. 2006).

The polycystic ovary syndrome (PCOS) is the most common hormonal disorder causing menstrual abnormalities among women of reproductive age and is a leading cause of infertility (Diamanti-Kandarakis and Panidis 2006). It is characterized by disturbances in gonadotropin secretion, steroidogenesis and increased resistance to insulin. Women with PCOS have an increased risk of metabolic and cardiovascular morbidity (Dahlgren et al. 1992) and PCOS is also associated with endothelial dysfunction (Paradisi et al. 2001). An elevated ADMA concentration has been detected in women with PCOS in comparison with healthy control women (Charitidou et al. 2008; Ozgurtas et al. 2008). In these PCOS studies, the ADMA concentration was significantly decreased by natural or synthetic estrogen treatment combined with anti-androgen therapy. Estrogen treatment may decrease the serum ADMA concentration in PCOS patients by several mechanisms, such as stimulating NO production, increasing ADMA degradation by up-regulation of DDAH activity, and having a protective effect on DDAH activity against oxidative stress (Charitidou et al. 2008).

2.5.2 ADMA and pregnancy

2.5.2.1 ADMA in normal pregnancies

Normal pregnancy is characterized by enhanced vasodilatation mainly mediated by endothelial NOS and the increased production of NO (Faber-Swensson et al. 2004). Additionally, there are several changes occurring during pregnancy e.g. the appearance of hypercholesterolemia, insulin resistance and an upregulation of the immune system which may in normal non-pregnant conditions be detrimental to health (Aagaard-Tillery et al. 2006; Saarelainen et al. 2006; Zavalza-Gomez et al. 2008). Hypercholesterolemia during pregnancy may be associated with the increased production of estrogen (Kvasnicka et al. 1997) and the maintenance of adequate supply of nutrients to mother and fetus. Furthermore, the immune system plays a vital role in pregnancy and cytokines are involved in both the maintenance of pregnancy and the onset of normal labor (Elenkov et al. 1999). In normal pregnancy, ADMA and SDMA levels are decreased in comparison with the levels found in non-pregnant females (Holden et al. 1998; Pettersson et al. 1998; Ellis et al. 2001; Savvidou et al. 2003). The reduced circulating ADMA concentration may be due to increased DDAH activity induced by estrogen, increased renal clearance or hemodilution typical of normal pregnancy. A very recent study investigated the relationship between maternal risk factors, neonatal demographic features and ADMA concentration and reported that the most significant factor affecting umbilical vein ADMA concentrations seemed to be perinatal hypoxia (Kul et al. 2009).

2.5.2.2 ADMA in complicated pregnancies

Gestational diabetes mellitus (GDM) develops in 3-6% of all pregnant women being characterized by a pronounced decrease in insulin sensitivity leading to higher plasma insulin levels and abnormalities in glucose tolerance during pregnancy (Alwan et al. 2009). Women with previous GDM have a high risk of developing impaired glucose tolerance or manifest type 2 diabetes mellitus in the future (Kim et al. 2002). A recent study by Telejko et al. (2009) has shown that there was no significant difference in the ADMA concentration between healthy pregnant controls and pregnant women with

GDM. Nonetheless it has been previously demonstrated that high glucose concentrations can induce an impairment of DDAH activity, thereby slowing ADMA degradation (Lin et al. 2002). In an earlier study, it was revealed that high ADMA concentrations after delivery are associated with deterioration in glucose tolerance in women with previous GDM. However in GDM pregnancies, circulating ADMA concentrations decreased after a median follow-up of 2.75 years after delivery (Mittermayer et al. 2007).

Preeclampsia is considered to be one of the most serious complications occurring in pregnancy being responsible for 5-8% of all gestation complications (Lain and Roberts 2002). The exact pathophysiology leading to preeclampsia is unknown, but it has been suggested that the invading trophoblast leads to a hypoperfused placenta which then releases factors into the maternal circulation eventually leading to maternal endothelial dysfunction (Pijnenborg et al. 1983; Rajagopal et al. 2003). It has been shown that plasma ADMA concentrations can become elevated in preeclamptic pregnancies (Fickling et al. 1993). In addition, it has been suggested that the high molar ratio of ADMA/SDMA is evidence of an impairment of DDAH activity (Savvidou et al. 2003) and furthermore, that it is the reduced L-arginine concentrations, rather than an increased ADMA level, which contributes to the development of preeclampsia (Kim et al. 2006). L-arginine levels have been found to be significantly lower in preeclamptic women in comparison with normal pregnant women, but there were no significant differences in ADMA levels between normal and preeclamptic women (Kim et al. 2006). However, there are differences in both ADMA and L-arginine levels between normal and preeclamptic pregnancies, and these may be attributable to several reasons such as differences in blood pressure, renal function, ethnic groups and sample analysis techniques (Petersson et al. 1998; Savvidou et al. 2003; Kim et al. 2006; Siroen et al. 2006a). ADMA concentrations have also been measured from fetal samples and only SDMA concentration was higher in the preeclampsia group compared to the controls. It was reported that the median ADMA concentration was three times higher in the fetal circulation than in the maternal plasma, but there was no difference between the preeclampsia group and the control group (Braekke et al. 2009).

3 AIMS OF THE STUDY

In the present series of studies, the aim was to study if there is an association between ADMA and flow-mediated dilatation or with factors that are involved in normal pregnancy, such as hypercholesterolemia and changes in the immune system.

The specific aims of the studies were as follows:

I To devise and validate a suitable analysis method based on previously published HPLC methods for L-arginine, ADMA and SDMA concentrations. The performance of the ADMA ELISA assay was also evaluated.

II To investigate the effect of hormonal cyclic changes on levels of ADMA and its related compounds in healthy non-pregnant women with and without hormonal contraceptive use.

III To investigate the relationship between ADMA and flow-mediated dilatation in normal pregnancy.

IV To measure the concentration of L-homoarginine concentration in non-pregnant women and in normal pregnancy. To investigate whether the ADMA concentration is related to the level of L-homoarginine.

V To study whether there is an association between serum levels of ADMA and proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) and C-reactive protein in normal pregnancy.

4 SUBJECTS AND METHODS

4.1 Study subjects

Blood samples for ADMA HPLC method validation (I) were collected from healthy volunteers. Samples (n=55) for ADMA HPLC and ELISA method comparison were collected from the Research Institute of Public Health, University of Kuopio.

4.1.1 Subjects from The Cardiovascular Risk in Young Finns Study (II - V)

Non-pregnant women and women with normal pregnancy were derived from The Cardiovascular Risk in Young Finns Study (Åkerblom et al. 1985; Raitakari et al. 2008) which is an ongoing 5-center follow-up study of risk factors of atherosclerosis in Finnish children and adolescents. The participants were randomly chosen from a national population register, as previously described (Raitakari et al. 2008). At the beginning of the study in 1980, there were 3 596 participants. In the follow-up study in 2001, 2 283 young adults were studied in the age range from 24 to 39 years (Juonala et al. 2004). The menstrual cycle phase at the sampling moment and the use of oral contraceptives were inquired from the participants. Menstrual cycle was divided into four different phases; early follicular phase (1-7 days from the beginning of menstruation), late follicular phase (8-14 days from the beginning of menstruation), early luteal phase (15-19 days from the beginning of menstruation) and late luteal phase (20-40 days from the beginning of menstruation). Contraceptive types were divided into 4 categories; constant estrogen dose with low progestin dose (n=110), constant estrogen dose with high progestin dose (n=120), progestin alone (n=10) and combination preparation with changing doses (n=68). All subjects provided written informed consent before participating in the study. The local ethics committee in Turku University approved The Cardiovascular Risk in Young Finns Study. Summary of study subjects in different studies are collected to Table 3.

4.1.2 Subjects from The Complicated Pregnancy Study (IV - V)

Subjects from The Complicated Pregnancy Study were recruited from Kuopio University Hospital area and there were women with normal and complicated

pregnancies with the followup organized through 2004-2007 from the third trimester of pregnancy to the postpartum period (4-7 months). Age of the studied subjects was ranging from 21 to 39 years. There were subjects with normal uncomplicated pregnancies who were studied once in the third trimester and 79% of them were also seen at 3 months postpartum. These groups were used to enhance the information obtained from The Cardiovascular Risk in Young Finns Study on what would happen to the concentration of ADMA and its related compounds a few months after delivery. The Complicated Pregnancy Study was approved by the Research Ethics Committee of the Kuopio University Hospital. Summary of study subjects in different studies are collected to Table 3.

Table 3. Summary of study subjects in different studies.

Subjects	**Number of participants**	**Original publication**
Subjects from The Cardiovascular Risk in Young Finns Study		
Non-pregnant women		
without OC use	761	II
with OC use	318	II
Non-pregnant women	61	III-V
Pregnant women		
1. trimester (≤ 14 weeks)	13	III-V
2. trimester (15–27 weeks)	22	III-V
3. trimester (≥ 28 weeks)	23	III-V
Subjects from The Complicated Pregnancy Study in Kuopio		
Pregnant women		
3. trimester (≥ 28 weeks)	19	IV-V
after delivery	15	IV-V

OC, oral contraceptives.

4.2 Analysis methods

4.2.1 Blood samples and basic measurements of study subjects

Venous blood samples were drawn for laboratory assays after a 12-h overnight fast. The samples were centrifuged at 2000g for 10 min and the serum or plasma was separated and stored frozen at -70°C until analysis. The height, weight and waist circumference of the subjects were measured, and body mass index (BMI) was calculated (weight/height^2). Glomerular filtration rate (GFR) was calculated by the Cockcroft-Gault formula (Cockcroft and Gault 1976). Blood pressure was measured with a sphygmomanometer. In The Cardiovascular Risk in Young Finns Study, the basic measurements of analytes in clinical chemistry (lipids, glucose, creatinine and high sensitive C-reactive protein (hsCRP)) were conducted in Turku. In The Complicated Pregnancy Study, the basic measurements of analytes in clinical chemistry (lipids, glucose, creatinine and hsCRP) were undertaken in Kuopio University Hospital. All cytokine measurements were analyzed in Kuopio University Hospital. ADMA measurements in studies I and III-V were done in Kuopio and in study II they were analyzed in National Institute of Health and Welfare in Helsinki.

4.2.2 ADMA HPLC methods (I-V)

In studies I and III-V, the analysis method for ADMA, SDMA, L-arginine and L-homoarginine levels in human serum or plasma was set up according to the method described by Teerlink et al. (2002) with minor modifications and it is based on HPLC technology and fluorescence detection. HPLC analysis was carried out on a Merck Hitachi liquid chromatography system (Hitachi, Tokyo, Japan) consisting of a gradient pump (D-6200), an autosampler (AS-4000) and a fluorescence detector (F1000). Data acquisition and analysis were performed using D-7000 HPLC System Manager software (Hitachi, Tokyo, Japan).

In comparison to the original ADMA method (Teerlink et al. 2002), our HPLC method had longer total run time (38 vs. 30 min) and lower acetonitrile concentration (35 vs 50 vol.%) for column washing after elution of the last analyte. The injection volume was twice as great (40 vs 20 µl) and the autosampler derivatized every sample

just before each HPLC run instead of derivatization of all purified samples at the same time.

One-point calibration was used and the standard contained 21 µM L-arginine (Calbiochem, Merck Biosciences, Darmstadt, Germany), 2 µM L-homoarginine (Fluka, Buchs, Switzerland).), 3 µM ADMA (N^G,N^G-dimethyl-L-arginine, Sigma, St. Louis, MO, USA) and 2 µM SDMA (N^G, $N^{G'}$ -dimethyl-L-arginine, Calbiochem, Merck Biosciences, Darmstadt, Germany). L-NMMA (N^G-monomethyl-L-arginine, Fluka, Buchs, Switzerland) was used as an internal standard. A plasma pool (58.6 ± 4.3 µM for L-arginine, 1.2 ± 0.03 µM for L-homoarginine, 0.643 ± 0.029 µM for ADMA and 0.654 ± 0.028 µM for SDMA) was used as the quality control.

Prior to analysis, the standards, quality controls and samples were extracted on Oasis MCX solid phase extraction cartridges (Waters, Milford, MA, USA). Briefly, standards, quality controls and samples (200 µl) were mixed with 100 µl internal standard L-NMMA (6 µM) and 700 µl phosphate buffered saline, pH 7.2 and then applied onto the columns. The columns were washed with 1 ml of 100 mM hydrochlorid acid and 1 ml of methanol. Dimethylarginines were eluted with 1 ml ammonia-water-methanol (10:40:50, v/v). The eluents were dried under nitrogen (+55 °C) and dissolved in 100 µl ion exchanged water (Milli Q, Millipore, Billerica, MA, USA) for HPLC analysis.

Standards, quality controls and samples (100 µl) were incubated for 2 min with the 100 µl OPA reagent (1 mg/ml OPA in borate buffer, pH 9.5, containing 0.1 vol.% 3-mercaptopropionic acid) before automatic injection (40 µl) into the HPLC. The OPA-derivatives of ADMA and internal standard were separated on Symmetry C18 column (4.6 x 150 mm, 5 µm, Waters, Milford, MA, USA) with the fluorescence monitor set at λ^{ex} = 340 nm and λ^{em} = 455 nm. The column temperature was kept at +30 °C. Standards, quality controls and samples were eluted from the column with 50 mM K-phosphate buffer, pH 6.5 and 8.7 vol.% acetonitrile, at a flow rate of 1.1 ml/min. After elution of the last analyte, the column was washed with a stronger solvent (35 vol.% acetonitrile from 24 to 29 min). After washing, the column was equilibrated for 8 min with separation buffer, resulting in a total run time of 38 min. L-arginine was analyzed with the same method but in that case, the injection volume was 10 µl and the total run

time was shorter (33 min). The retention times were as follows: L-arginine (10.52 ± 0.13 min), internal standard, L-NMMA, (14.87 ± 0.22 min), L-homoarginine (16.85 ± 0.25 min), ADMA (19.76 ± 0.29 min) and SDMA (20.89 ± 0.30 min).

In study II, the HPLC method (Juonala et al. 2007) was also modified from the assay described by Teerlink et al. (2002). In this method, proteins were precipitated before solid phase extraction as distinct from the method used in other studies. The intra- and interassay CVs of the plasma pool for ADMA, SDMA, L-homoarginine and L-arginine are shown in Table 4.

Table 4. Characteristics of ADMA and related compounds analysis methods.

Analyte	Source of reagents	Principle of assay	Coefficient of variation	
			Intra-assay	Interassay
Method used in studies I, III-V				
ADMA	In-house	HPLC	2.5%	4.2%
SDMA			5.6%	3.7%
L-arginine			2.5%	2.8%
L-homoarginine			1.4%	2.9%
Method used in study II				
ADMA	In-house	HPLC	7.5%	12.9%
SDMA			5.7%	10.6%
L-arginine			6.5%	12.1%
Method used in study I				
ADMA	DLD Diagnostika GmbH, Germany	ELISA	19%	9-14%

ADMA, asymmetric dimethylarginine; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; SDMA, symmetric dimethylarginine.

4.2.3 ADMA ELISA assay (I)

Sample preparation for ELISA assay was done according to the instructions of the manufacturer (ADMA-ELISA kit, DLD Diagnostika GmbH, Hamburg, Germany). The ADMA-ELISA kit consists of a split-type reaction plate (12 x 8) coated with ADMA,

six standards (0-5 μM), rabbit anti-ADMA antiserum, goat anti-rabbit-IgG-peroxidase conjugate, tetramethylbenzidine substrate solution, stop solution and wash buffer. Acylation was conducted in the 96-well reaction plate supplied with the kit. Standards, controls and samples (20 μl) were mixed with 25 μl acylation buffer and 25 μl equalizing reagent. Subsequently 25 μl acylation reagent was added and the reaction plate was incubated for 30 min at room temperature on an orbital shaker. Diluted equalizing reagent (100 μl) was added and the incubation was continued for 45 min prior to the assay. In ELISA assay, aliquots (50 μl) of the acylated standards, controls or samples were processed according to the instructions of the kit manufacturer. The absorbances were measured with a microplate reader (Tecan SPECTRAFluor, Tecan Group Ltd., Maennedorf, Switzerland) using a wavelength of 450 nm (reference wavelength 620 nm). All samples, controls and standards were analyzed in duplicate.

4.2.4 Measurement of flow-mediated dilatation (II -IV)

Brachial artery flow-mediated dilatation was measured by ultrasound according to the guidelines (Corretti et al. 2002) and as described earlier (Juonala et al. 2004). Ultrasound studies were performed using Sequoia 512 mainframes (Acuson, CA, USA) with 14.0 MHz linear array transducers. The segment of the brachial artery above the antecubital crease was imaged in the longitudinal plane at rest and during reactive hyperemia, induced by a sphygmomanometer cuff, which was placed around the forearm, inflated to a pressure of 250 mmHg and deflated after 4.5 minutes. End-diastolic (incident with the R-wave) arterial diameter was measured at rest (baseline) and at 40, 60 and 80 s after cuff release from 5 s ultrasound image sets. The vessel diameter response in scans after reactive hyperemia was expressed both as the absolute change in diameter (FMD) and as the percentage relative to the resting scan (FMD%). The three month between-visit CV was 3.2% and 26.0% for brachial artery diameter and for FMD measurements, respectively.

4.2.5 Measurement of serum lipid, glucose and creatinine concentrations (II - V)

Lipid analyses from samples of The Cardiovascular Risk in Young Finns Study were performed by standard enzymatic methods (Olympus System Reagents, Olympus

Diagnostica GmbH, Hamburg, Germany) in a clinical chemistry analyser (AU400; Olympus Optical Ltd, Mishima, Japan) (Juonala et al. 2004). Glucose concentrations and creatinine concentrations were analyzed by standard methods (Olympus Diagnostica GmbH, Hamburg, Germany).

Lipid analyses from samples of The Complicated Pregnancy Study in Kuopio were performed with Konelab 60i Clinical Chemistry Analyzer (Thermo Electron Co, Finland). The triglyceride concentration was determined by enzymatic, photometric assay (Konelab TRIGLYCERIDES kit, Thermo Electron Co, Finland) and the total serum cholesterol concentration was analyzed by enzymatic, photometric assay (Konelab CHOLESTEROL kit, Thermo Electron Co, Finland). High-density lipoprotein (HDL)-cholesterol and low-density lipoprotein (LDL)-cholesterol were determined by a direct, enzymatic, photometric method (Konelab HDL-CHOLESTEROL and Konelab LDL-CHOLESTEROL kits, Thermo Electron Co, Finland). Glucose and creatinine concentrations were analyzed by standard methods (Konelab 60i Clinical Chemistry Analyzer, Thermo Electron Co, Finland).

4.2.6 Measurement of proinflammatory cytokines (V)

All serum IL-6 and TNF-α concentrations were measured with a commercially available ELISA assay according to the protocol supplied by the manufacturer (Quantikine HS Human IL-6 and Quantikine HS Human TNF-α/TNFSF1A Immunossay Kits, R&D Systems, Minneapolis, USA). The working range was 0.156–10 pg/ml for IL-6 and 0.5–32 pg/ml for TNF-α. Calibrators for both IL-6 and TNF-α assays were analyzed in duplicate but the samples were assayed as single measurements. The absorbances in ELISA tests were measured at a wavelength of 490 nm using a microplate reader (Tecan SPECTRAFluor, Tecan Group Ltd., Maennedorf, Switzerland). The TNF-α/IL-6 ratio was calculated and used as an index of Th1/Th2 response. The detection limits and CVs of the methods are shown in Table 5.

4.2.7 Measurement of high sensitive C-reactive protein (II, V)

Samples from The Cardiovascular Risk in Young Finns Study were analyzed by latex immunoturbidometric assay (CRP-UL, Wako Chemicals GmbH, Neuss, Germany) by an automated analyzer (Olympus AU400, Tokyo, Japan).

Samples from Complicated Pregnancy Study in Kuopio were analyzed by a kinetic immunoturbidometric method. Serum samples were analyzed by IMMAGE- automated analyzer using Beckman Coulter High Sensitivity C-Reactive Protein reagents (Beckman-Coulter, Fullerton, CA, USA). The detection limits and CVs of the methods are shown in Table 5.

Table 5. Characteristics of hsCRP, IL-6 and TNF-α analysis methods.

Analyte (study)	Source of reagents	Principle of assay	Coefficient of variation		Detection limit
			Intra-assay	Interassay	
hsCRP (study A)	Wako Chemicals GmbH, Germany	Kinetic immuno-turbidometric		3.3% (1.52 mg/l)	0.06 mg/l
hsCRP (study B)	Beckman-Coulter, USA	Kinetic immuno-turbidometric		13.5% (0.8 mg/l)	0.2 mg/l
IL-6	R&D Systems, USA	ELISA	6.5%	14%	0.156 pg/ml
TNF-α (study A+B)	R&D Systems, USA	ELISA	7.4%	15%	0.5 pg/ml

Study A: The Cardiovascular Risk in Young Finns Study (Juonala et al. 2006).
Study B: Complicated Pregnancy Study in Kuopio.

ELISA, enzyme-linked immunosorbent assay; hsCRP, high sensitive C-reactive protein; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.

4.3 Statistical analyses (studies I – V)

In statistical analysis, results were presented as the mean ± standard deviation (SD), mean ± standard error of the mean (SEM) or as median values. Comparisons between groups were performed with the Kruskal-Wallis test and the non-parametric Mann-Whitney *U* test with Bonferroni correction (III - V) or one-way ANOVA test and the *t*-test analysis (II). The correlations were calculated by Pearson or Spearman correlation

coefficient test. Skewness was assessed using Kolmogorov-Smirnov test. Log-transformed values were used for L-homoarginine, CRP and GFR because of skewed distribution. Linear regression analysis was used for evaluation of association between studied groups. Multiple-regression analysis was used to evaluate the independent correlations of studied parameters. A computer software program (SPSS 11.5 and 15.0 for Windows; SPSS Inc., Chicago, IL, USA) was used to analyze the data. A probability level of < 0.05 was considered statistically significant.

5 RESULTS

5.1 ADMA HPLC and ELISA assays

5.1.1 Performance of the HPLC assay

The performance of the HPLC method published by Teerlink et al. (2002) was good after minor modifications in our laboratory as described thoroughly in study I. ADMA and SDMA peaks had good separation efficiency under isocratic conditions. The representative chromatograms of standard and sample of HPLC method are presented in the original publication (I). The linearity of the calibration curve of the HPLC method was determined at 7-8 concentrations and it was excellent (Table 3). The lower limit of detection and the lower limit of quantitation calculations were based on a signal/noise ratio of 3 and a signal/noise ratio of 10, respectively. The performance of the HPLC assay is shown in Table 6.

Table 6. The linearity, the detection limits of the validated ADMA HPLC method and the mean recoveries of analytes.

Analyte	Concentration range (μM)	Correlation coefficient (r)	Lower limit of detection (μM)	Lower limit of quantitation (μM)	Mean recovery (%)
ADMA	0.1 - 7.5	0.999	0.1	0.3	95
SDMA	0.1 - 7.5	0.999	0.1	0.3	95
L-homoArg	0.1 - 12.9	0.997	0.1	0.3	100
L-arginine	1.5 - 200	0.999	3	10	113

ADMA, asymmetric dimethylarginine; L-homoArg, L-homoarginine; SDMA, symmetric dimethylarginine.

There were no significant differences between the ADMA (0.5364 ± 0.015 μM, CV=2.7%) or SDMA (0.402 ± 0.010 μM, CV=2.6%) concentrations assayed from 5 different Vacutainer tube types with and without anticoagulant. In addition in study I, mean concentration measurements of plasma (0.607 ± 0.064 μM) versus serum (0.603 ± 0.072 μM) samples gave similar results for ADMA (n=15). ADMA and SDMA

concentrations in EDTA-plasma did not change after four freezing and thawing cycles (0.479 ± 0.011 μM, CV=2.4% and 0.462 ± 0.003 μM, CV=0.7%, respectively). The ADMA concentrations were stable in EDTA-plasma stored at -20 °C for at least 7 months (mean concentration 0.661 ± 0.008 μM, CV=1.2%).

5.1.2 Performance of the ELISA assay and method comparison

Three ADMA-ELISA kits were used, and standards, kit controls and samples were analyzed in duplicate and quality controls were analyzed in every plate. The mean concentrations of the kit control 1 was 0.410 ± 0.037 μM and it fitted to the target range values (0.24-0.45 μM) in three analyzed plates, whereas the mean concentration of the kit control 2 was 1.174 ± 0.165 μM and two results of three did not fit to the target range values (0.6-1 μM). The intra-assay CV of the plasma pool (0.436 ± 0.083 μM, n=10) was 19% in the ADMA-ELISA assay. The interassay CVs of ADMA (3 different plates) was 9% and 14% for kit controls 1 and 2, respectively.

The ELISA assay was less precise (Table 4) with mean level of the results being higher than that of the present HPLC method. In addition, the correlation was poor between these assays over the studied concentration range (0.39 to 0.909 μM, R^2=0.0972) as shown in the original publication (I).

5.2 ADMA and menstrual cycle phases in women with and without hormonal contraceptive use

Information was available in the menstrual cycle phase of 1079 women with or without OC use. The effect of menstrual cycle phases on dimethylarginine and L-arginine concentrations was evaluated by dividing the cycle into four phases and comparing the concentrations of studied analytes between these phases (Table 7). In non-OC users, ADMA, L-arginine and ADMA/SDMA ratio were significantly decreased in the luteal phase compared to the follicular phase of the menstrual cycle ($P = 0.017$, $P = 0.002$ and $P < 0.001$, respectively). The use of OC seemed to abolish the differences found in ADMA and ADMA related parameters in non-OC users between menstrual cycle phases and no significant changes were found.

The use of estrogen containing pills was associated with decreased plasma ADMA ($P < 0.001$), SDMA ($P = 0.002$) concentrations and ADMA/SDMA ratio ($P < 0.001$) as compared with the non-OC users. On the other hand, L-arginine concentrations ($P < 0.001$) and L-arginine/ADMA ratio ($P < 0.001$) were higher in the OC-users than in the non-OC users. Progestin-only contraceptive pills did not affect plasma ADMA concentrations.

Table 7. The mean concentrations of ADMA, SDMA, L-arginine, ADMA related ratios and hsCRP of non-OC users and OC-users in different menstrual cycle phases.

Analyte	**Early follicular phase (μM)**	**Late follicular phase (μM)**	**Early luteal phase (μM)**	**Late luteal phase (μM)**
Non-OC users	(n=159)	(n=187)	(n=116)	(n=213)
ADMA	0.642 ± 0.153	0.630 ± 0.136	0.605 ± 0.130	0.610 ± 0.157
SDMA	0.382 ± 0.102	0.389 ± 0.106	0.394 ± 0.097	0.382 ± 0.106
L-arginine	114.8 ± 35.8	115.9 ± 34.5	104.9 ± 30.2	108.7 ± 33.9
L-Arg/ADMA	180.5 ± 43.4	186.9 ± 53.3	174.3 ± 39.6	181.5 ± 50.9
ADMA/SDMA	1.72 ± 0.25	1.67 ± 0.30	1.57 ± 0.27	1.63 ± 0.33
hsCRP	0.65	0.57	0.52	0.70
OC-users	(n=70)	(n=80)	(n=51)	(n=107)
ADMA	0.551 ± 0.145	0.533 ± 0.131	0.532 ± 0.116	0.542 ± 0.123
SDMA	0.351 ± 0.094	0.377 ± 0.100	0.383 ± 0.091	0.363 ± 0.077
L-arginine	126.6 ± 45.7	127.6 ± 40.4	132.6 ± 42.2	130.1 ± 41.2
L-Arg/ADMA	231.3 ± 65.0	243.7 ± 68.1	252.9 ± 71.8	246.2 ± 77.2
ADMA/SDMA	1.59 ± 0.23	1.44 ± 0.25	1.42 ± 0.27	1.51 ± 0.23
hsCRP	1.34	1.50	2.06	2.22

Results are expressed as mean ± SD. hsCRP is expressed as median.

ADMA, asymmetric dimethylarginine; hsCRP, high sensitive C-reactive protein; L-Arg, L-arginine; OC, oral contraceptives; SDMA, symmetric dimethylarginine.

5.3 Dimethylarginine, L-arginine and L-homoarginine concentrations in non-pregnant and pregnant states

Concentrations of studied analytes from the original publications have been gathered in Table 8. Pregnant women had significantly lower ADMA, SDMA and L-arginine concentrations than the corresponding values in non-pregnant women (Figure 4). However, there were no significant differences in the concentrations of ADMA and SDMA between three trimesters. The L-arginine concentration was lowest in the first trimester and then increased up to term, but three months after delivery the concentration was still somewhat lower than that detected in non-pregnant women. On the contrary, the L-homoarginine concentrations were 2-fold higher in the third trimester of pregnancy compared to in non-pregnant women. After delivery, the L-homoarginine concentration normalized back to the level of non-pregnant women within three months. Interestingly, the ADMA and SDMA concentrations appeared to be significantly higher after delivery compared to the concentrations during pregnancy.

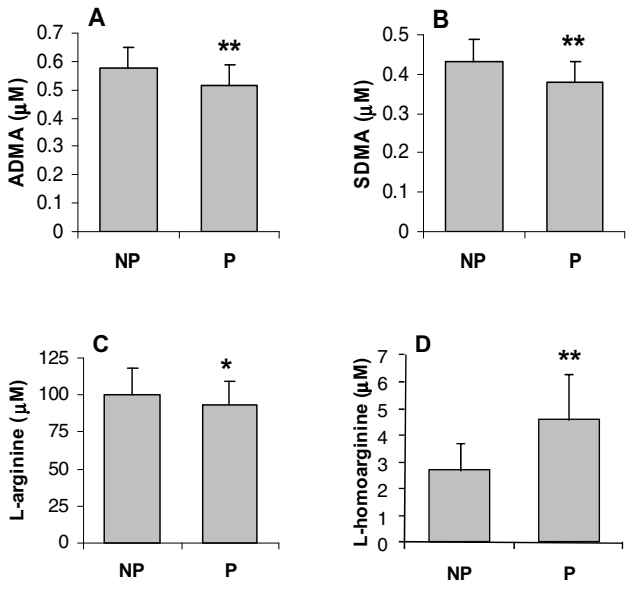

Figure 4. Serum asymmetric dimethylarginine (ADMA) (A), symmetric dimethylarginine (SDMA) (B), L-arginine (C) and L-homoarginine (D) concentrations in non-pregnant (NP) and pregnant females (P) in the The Cardiovascular Risk in Young Finns Study. Results are expressed as mean ± SD. *P < 0.05, **P < 0.001 in Mann-Whitney *U* test.

Glomerular filtration rate was clearly elevated in pregnant women compared to non-pregnant controls and dramatically decreased after delivery to a value even below that found in the non-pregnant women (107.7 ± 22.1 vs. 137.1 ± 34.1, P = 0.012). This dramatic decrease in GFR after delivery may explain the post-partum increase in both ADMA and SDMA levels. Since SDMA is more dependent on urinary excretion than ADMA, then the levels of plasma SDMA may increase even more than the corresponding values of ADMA.

Significantly different ADMA/SDMA and L-Arg/ADMA ratios were recorded during the non-pregnant and pregnant state as well as after delivery. The lowest ADMA/SDMA and L-Arg/ADMA ratios were observed three months after delivery (Table 9).

During pregnancy, ADMA was positively correlated with gestation week (P < 0.01) and SDMA (P < 0.001). The association between ADMA and SDMA was also present after delivery (P < 0.05). L-homoarginine concentration correlated positively with gestational age (P = 0.001) but not with the dimethylarginines or L-arginine.

Table 8. Summary of mean concentrations of ADMA, SDMA, L-homoarginine and L-arginine in different studies.

Study subjects	ADMA (μM)	SDMA (μM)	L-homoArg (μM)	L-Arg (μM)	n	Original publication
Non-pregnant women						
without OC	0.624 ± 0.149	0.388 ± 0.106	NM	111.1 ± 35.3	761	II
with OC	0.541 ± 0.129	0.368 ± 0.092	NM	129.2 ± 41.7	318	II
Non-pregnant women	0.577 ± 0.071	0.430 ± 0.058	2.7 ± 1.1	100.6 ± 17.1	61	III-V
Pregnant women						
1. trimester	0.513 ± 0.082	0.394 ± 0.061	3.1 ± 1.4	87.1 ± 11.5	13	III-V
2. trimester	0.518 ± 0.076	0.351 ± 0.033	4.8 ± 1.7	97.2 ± 17.2	22	III-V
3. trimester	0.543 ± 0.060	0.434 ± 0.073	5.0 ± 2.2	96.2 ± 19.3	42	III-V
after delivery	0.664 ± 0.098	0.568 ± 0.137	2.2 ± 0.8	90.9 ± 16.5	15	IV-V

Results are expressed as mean ± SD, NM = not measured.

ADMA, asymmetric dimethylarginine; L-Arg, L-arginine; L-homoArg, L-homoarginine; OC, oral contraceptives; SDMA, symmetric dimethylarginine.

Table 9. Summary of mean L-arginine/ADMA and ADMA/SDMA ratios in different studies.

Study subjects	L-arginine/ ADMA ratio	ADMA/SDMA ratio	n	Original publication
Non-pregnant women				
without OC use	181.5 ± 48.2	1.65 ± 0.30	761	II
with OC use	243.3 ± 71.4	1.49 ± 0.25	318	II
Non-pregnant women	176.1 ± 33.3	1.36 ± 0.19	61	III-V
Pregnant women				
1. trimester	174.7 ± 43.7	1.31 ± 0.15	13	III-V
2. trimester	190.2 ± 38.2	1.48 ± 0.20	22	III-V
3. trimester	178.6 ± 38.7	1.27 ± 0.16	42	III-V
after delivery	138.8 ± 30.8	1.18 ± 0.17	15	IV-V

Results are expressed as mean ± SD.

ADMA, asymmetric dimethylarginine; OC, oral contraceptives; SDMA, symmetric dimethylarginine.

5.4 ADMA and endothelial function

Endothelial function was assessed by brachial artery flow-mediated dilatation (FMD). There were no significant changes in FMD or FMD% during the different phases of the menstrual cycle in non-OC users or OC-users. In non-OC users, brachial artery diameter remained unchanged during the menstrual cycle, whereas it was significantly decreased in the luteal phase ($P = 0.013$) as compared with the follicular phase in OC-users. In the early follicular phase, there was a significant negative correlation between ADMA levels and FMD ($r = -0.275$, $P = 0.001$) and FMD% ($r = -0.280$, $P < 0.001$) and also between SDMA levels and FMD ($r = -0.186$, $P = 0.022$) and FMD% ($r = -0.202$, $P < 0.013$) in non-OC users. Whereas in OC-users, there were no correlations between ADMA concentrations and FMD during the different menstrual cycle phases.

When examining all non-pregnant women, both OC and non-OC-users exhibited a significant negative correlation between ADMA concentrations and FMD ($r = -0.101$, $P = 0.007$ and $r = -0.139$, $P = 0.016$, respectively) and FMD% ($r = -0.088$, $P = 0.019$ and $r = -0.142$, $P = 0.013$, respectively). Accordingly in non-users, the negative correlation of SDMA concentration with FMD or FMD% was significant ($P = 0.003$ and $P = 0.006$, respectively).

The brachial artery diameter was slightly higher during pregnancy compared with non-pregnant state (3.17 ± 0.31 vs 3.06 ± 0.32, respectively), although the difference was not statistically significant. Also FMD and FMD% tended to increase during pregnancy and FMD was statistically different between three trimesters and in non-pregnant groups (P = 0.008). However, the difference in the FMD% did not reach statistical significance between the trimesters and the non-pregnant state groups (P = 0.067). The concentrations of ADMA or SDMA did not correlate with FMD parameters during pregnancy. L-arginine had a weak negative correlation with FMD in non-pregnant women. L-homoarginine correlated positively with FMD and brachial artery diameter (P = 0.022 and P = 0.006, respectively) during pregnancy but not in non-pregnant women.

5.5 ADMA and inflammation markers

The mean serum concentrations of hsCRP and cytokines are presented in Table 10. There were significant changes in inflammation markers hsCRP and IL-6 concentrations during pregnancy but not in TNF-α concentration. The hsCRP concentration increased during the second and the third trimester in comparison with the non-pregnant state (P = 0.016 and P = 0.001, respectively). In addition, IL-6 was significantly elevated during the third trimester compared with the levels found in non-pregnant women (P = 0.029). No correlation was found between the concentrations of dimethylarginines or L-arginine and the measured inflammation markers.

Table 10. Summary of mean serum hsCRP and cytokine concentrations in study V.

Study subjects	hsCRP (mg/l)	IL-6 (pg/ml)	TNF-α (pg/ml)	n
Non-pregnant women	3.0 ± 5.3 (1.2)	1.77 ± 1.73 (1.33)	3.42 ± 5.56 (2.38)	61
Pregnant women				
1. trimester	3.6 ± 4.1 (2.4)	1.46 ± 0.88 (1.42)	2.19 ± 0.69 (2.20)	13
2. trimester	4.4 ± 4.1 (2.9)	1.70 ± 1.36 (1.22)	4.05 ± 7.10 (2.17)	22
3. trimester	4.5 ± 4.1 (2.9)	2.05 ± 1.22 (1.75)	2.29 ± 0.86 (2.10)	42
after delivery	2.6 ± 2.1 (2.4)	1.47 ± 1.26 (1.28)	1.97 ± 0.70 (1.78)	15

Results are expressed as mean ± SD (median).
hsCRP, high sensitive C-reactive protein; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.

The TNF-α/IL-6 ratio is considered to be an index of Th1/Th2 immune response. In our study, the median TNF-α/IL-6 ratio decreased during pregnancy and started to increase towards the baseline after delivery (Figure 5). The CRP concentration was 2.6-fold higher in OC-users than in non-OC users but its concentration did not fluctuate between menstrual cycle phases.

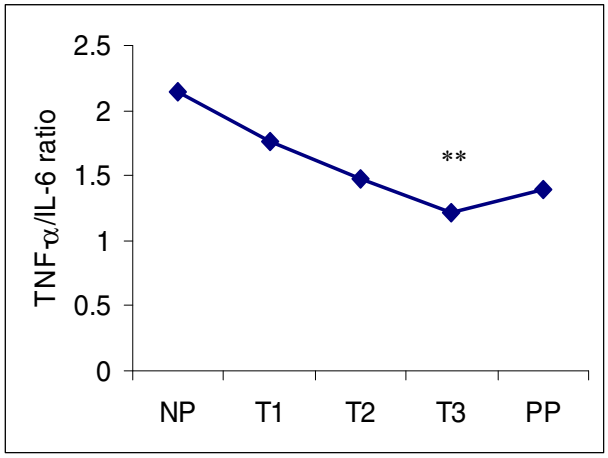

Figure 5. Serum tumor necrosis factor-α/interleukin-6 (TNF-α/IL-6) ratio (median) in non-pregnant females (NP), in three trimesters of normal pregnancy (T1-T3) and post partum period (PP). **Significant difference (P = 0.001) was observed in TNF-α/IL-6 ratio between non-pregnant women and women in the third trimester of pregnancy.

6 DISCUSSION

6.1 Evaluation of the main results

The purpose of the present study was devise an ADMA HPLC assay procedure and to measure the concentration of endogenous NOS inhibitor ADMA, SDMA, L-homoarginine and L-arginine in non-pregnant state and in normal pregnancy. We also examined whether pregnancy related changes in the maternal body e.g. hyperlipidemia, enhanced endothelium-dependent vasodilatation and changes in immune system would be associated with concentrations of ADMA and ADMA related compounds. Since the non-pregnant control group consisted of women at fertile age, we were interested in whether menstrual cycle and the use of oral contraception would have any effect on the ADMA concentration such that it would need to be taken into account in the planning of future studies.

In the present study, we observed that the circulating ADMA concentration varied across the menstrual cycle in young adult women who were not using hormonal contraception. The concentrations of ADMA and L-arginine were significantly lower in the luteal phase compared to the follicular phase of the menstrual cycle. In the normal menstrual cycle, the estrogen concentration starts to increase towards the end of the follicular phase, peaking during the surge of luteinizing hormone and follicle-stimulating hormone. In addition, the estrogen levels are high during most of the luteal phase. The high endogenous estrogen concentration or estrogen and progesterone together possibly evoked a decline in the ADMA concentration. High estrogen levels may exert an inhibitory action on ADMA accumulation by increasing the production of NO by stimulation of the enzyme NOS (Binko and Majewski 1998), upregulation of the activity of DDAH and the induction of ADMA degradation (Kimoto et al. 1995), and protecting DDAH from oxidative stress which may have an inhibitory effect on its enzyme activity (Ito et al. 1999). In women using hormonal contraceptives, there was no significant variation in the plasma ADMA concentration across the menstrual cycle but the ADMA concentrations were significantly lower in women on estrogen containing pills in comparison with those women not using OC. Interestingly, the levels of SDMA, the isomer of ADMA, did not exhibit any variations across the menstrual cycle. This

may be due to the fact that SDMA is not as sensitive to hormonal changes because it is excreted into urine (Kakimoto and Akazawa 1970) and is not eliminated enzymatically by DDAH. In the present study, we found that progesterone alone pills did not lower the ADMA concentration. Thus one can speculate that the decreasing effect on ADMA was associated with fluctuations in the estrogen concentration.

Hashimoto et al. (1995) evaluated endothelial function by measuring FMD during different menstrual cycle phases and they found that FMD was associated with a change in the serum estrogen levels. However in previous studies, the variation of FMD during the menstrual cycle in women not using OC was not statistically significant in all reports but the number of participants in these studies was rather low (Hashimoto et al. 1995; Kawano et al. 1996, Williams et al. 2001). In accordance with earlier studies, we did not find any significant variation in FMD parameters between the different menstrual cycle phases in women not using OC.

The concentrations of ADMA, SDMA and L-arginine were significantly decreased during pregnancy as compared with the corresponding levels in non-pregnant controls. This is in accordance with some (Fickling et al. 1993; Holden et al. 1998; Maeda et al. 2003), but not all (Siroen et al. 2006a), previous studies. In the study of Siroen et al. (2006a), the decrease in the ADMA level was observed but it was not statistically significant because the number of participants in this study was rather low. The decreased maternal ADMA concentration may be due to hemodilution and increased renal clearance typical of normal pregnancy. In addition, high estrogen levels, which are common in normal pregnancy, may inhibit ADMA accumulation.

Maternal hypercholesterolemia and an elevated triglyceride concentration typically occur in normal pregnancies (Saarelainen et al. 2006) and they are believed to be due to increased levels of sex steroids and the increased need for adequate supply of nutrients to the mother and growing fetus (Chiang et al. 1995). Additionally, reduced total peripheral resistance and increases of uterine and placental blood flows during pregnancy are fundamental to normal fetal development because an increased blood circulation is needed to provide sufficient nutrients and oxygen supplies to the growing fetus. The enhanced FMD during pregnancy is thought to be caused by increased NO synthesis and general vasodilatation (Cockell and Poston 1997). In a recent study with

non-pregnant hypercholesterolemia patients, ADMA was negatively associated with FMD (Vladimirova-Kitova et al. 2008). However, we did not find correlation between ADMA and FMD regardless of hypercholesterolemic state during normal pregnancy.

The immune system plays a vital role in pregnancy and cytokines are involved in both the maintenance of pregnancy and the onset of normal labor (Elenkov et al. 1999). The concentrations of proinflammatory marker, hsCRP, and proinflammatory cytokine, IL-6, were increased during the last trimester of pregnancy in comparison with those in non-pregnant women. The TNF-α concentration remained unchanged during pregnancy. This is in line with earlier studies (Ellis et al. 2001; Sacks et al. 2004; Sharma et al. 2007; Aris et al. 2008). Sacks et al. (2004) reported that CRP concentration starts to rise as early as gestational week 4 and this suggests that a mild systemic inflammation state is present during early pregnancy. Similar to the endogenously increased estrogen level encountered during pregnancy, oral contraceptives and postmenopausal hormone therapy have been shown to increase the concentration of CRP (Kluft et al. 2002; Raitakari et al. 2005; Viikari et al. 2007; Haarala et al. 2009). In the present study, we found that there was a positive correlation between CRP and IL-6 both in pregnant and non-pregnant women. This finding is in accordance with the report that IL-6 can stimulate the production of an acute phase protein i.e. CRP, in the liver (Castell et al. 1988). Krzyzanowska et al. (2007b) suggested that the enhanced inflammation might be associated with elevated plasma ADMA levels. However, we did not find any association between the concentrations ADMA and CRP, IL-6 or TNF-α. Thus the decreased ADMA concentration may not be directly influenced by these proinflammatory markers during normal pregnancy.

One of the novel findings of the present study was that the concentrations of an endogenous amino acid, L-homoarginine, increased during normal pregnancy and there was a positive correlation between serum L-homoarginine concentrations and FMD. Previous studies have shown that L-homoarginine is a vasodilator (Bhardwaj and Moore 1989) and can act as a substrate for NOS in a similar manner as L-arginine (Hecker et al. 1991; Chen and Sanders 1993; Hrabak et al. 1994). However, it is not possible to conclude that L-homoarginine is directly involved in regulating endothelial function in normal pregnancy because of the cross-sectional study design. We did not find any

correlation between levels of L-homoarginine and ADMA, SDMA or L-arginine. In this respect, it is not likely that L-homoarginine is a key regulator of the circulating ADMA concentration. Nevertheless, these findings indicate that L-homoarginine has a biological function in pregnancy although the significance of this phenomenon is still unclear. Since L-homoarginine is widely used as an internal standard in ADMA studies by HPLC methods, its use as an internal standard should be avoided if there are pregnant subjects included in the study.

6.2 Validity of the study: Subjects and methodological considerations

Our study has some limitations. There were subjects from different study populations. First, there were subjects from The Cardiovascular Risk in Young Finns Study, which is a retrospective non-longitudinal study and has the flaws of a small cross-sectional study. The size of this second group was large and rather evenly distributed in the three trimesters of pregnant women and in the four menstrual cycle phases of non-pregnant women. However, it would have been better if it had been possible to follow the same woman through the three trimesters of her pregnancy or over several menstrual cycles instead of having only one measurement per pregnant or non-pregnant woman. In addition, the measurement of hormonal concentrations in non-pregnant women, especially the estrogen concentration, would have provided more information for the last study where we evaluated the effect of menstrual cycle on ADMA concentration. Another limitation in this study group was that information was unavailable about the course of the pregnancy of the study participants and whether were there any complications during the late pregnancy. Additionally in cross-sectional studies, confounding factors may not be equally distributed between the groups being compared and this kind of unequal distribution may bias the results and lead to subsequent misinterpretations. Thus causality and temporal sequences cannot be defined. Secondly, the subject group from Complicated Pregnancy Study in Kuopio was rather small but it did provide the opportunity to study the same mothers once during pregnancy and three months after delivery. However, all of the women who attended the first examination during their pregnancy were not able to come to the re-examination after delivery.

The analysis of ADMA and SDMA is a challenging task because the concentrations are in the submicromolar range, whereas the levels of most other amino acids are two to three orders of magnitude higher. To date, most published methods for ADMA analysis have been HPLC assays and they have been suitable for the simultaneous determination of L-arginine, ADMA and SDMA. More recently, methods based on mass spectrometry have also been developed but these assays require expensive instrumentation and highly skilled personnel, which are not available in every laboratory. However, there has been a large variation between ADMA concentrations reported from different measuring methods (Table 1) which complicates comparisons between results carried out by different research groups. Thus there is a need for commercially available standard reference material with traceability against which the calibration of different methods could be reliably checked.

Our ADMA assay had good recovery, excellent linearity, low detection limits and good separation efficiency of the analytes. One disadvantage with the present method was that the total run time was rather long (38 min), and thus the capacity of the method was limited to approximately 24 samples per day. Recently, it has been shown that it is possible to decrease the analysis time in the chromatography by using a silica-based monolithic C18 column instead of the normal C18 column because its permeable pore structure makes it possible to use much higher flow rates (de Jong and Teerlink 2006). In addition, this improvement does not decrease the high separation efficiency of the peaks, and the backpressure is much lower in comparison with conventional silica columns. Other ways to decrease the analysis time are to use smaller particles in the column, to decrease the length of the column or to use higher flow rates. ADMA ELISA is a commercially available assay which has a larger capacity but it is able to measure only one analyte at a time and its precision was worse than that achieved with the HPLC method. The intra-assay CV was exceedingly high in the ELISA assay; this may be due to the difficulties of pipetting the precipitated sample onto the ELISA plate after acylation. Later, Siroká et al. (2006) noted that ADMA ELISA was comparable with their HPLC method and its reproducibility was good. However, Horowitz and Heresztyn (2006) stated that in their opinion ELISA method was not as selective and accurate as HPLC and MS-based methods for ADMA measurements. Since ADMA and

SDMA have demonstrated low intra-individual variation (Blackwell et al. 2007) it is extremely important that the precision of the assay is good to obtain statistically reliably results.

The assessment of FMD from brachial artery is a non-invasive way of evaluating endothelial function. Although the method is not complicated, it does require a highly experienced investigator to use the ultrasound equipment and the study subjects should be carefully prepared prior to assessment. One limitation of this technique is its low long-term reproducibility which might limit the interpretation of the study results. However in the Cardiovascular Risk in Young Finns Study (Juonala et al. 2007), the between-visit CVs for FMD parameter measurements were in accordance with previous studies (Lind et al. 2000; De Roos et al. 2003).

In cytokine measurements, one limitation is that we used maternal systemic blood samples and cytokines are often released locally in tissues and exert their effects on the cells adjacent to the release site. However, in vivo samples of placental tissue are difficult to obtain from women with normal pregnancies.

6.3 Clinical significance of the study

There are only a few large scale studies where the plasma ADMA concentration has been measured. Teerlink (2007) has reported a 95% reference interval for ADMA (0.39 -0.63 μM, HPLC study) in a large population based cohort study (n=2311; age 50-74 yrs). Schulze et al. (2005) studied 500 subjects with the ELISA method and their 95% reference interval was 0.36–1.17 μM. Both studies reported weak but significant positive correlations with ADMA levels and age and gender. Thus there is a clear need for population reference intervals for different gender and different age groups, in addition to women undergoing normal pregnancies, for L-arginine and methylated derivatives. An HPLC method was devised for the ADMA measurements which is sufficiently accurate and precise for reference interval measurements. In addition, it is important to have low inter- and intra-assay variation for measured analytes because even very minute increases in the ADMA concentration may be important; for example a recent clinical study measured the ADMA pre-operatively and after the surgery (Maas et al. 2007). It was demonstrated that each 0.1 μM increase in ADMA was associated

with a 33% elevated risk of complications. In the Gothenburg Study of women (n=880), it was noted that an 0.15 μM increase in the ADMA concentration was associated with a 30% increase in risk for fatal and non-fatal myocardial infarction and stroke with the relative risk factor being 1.75 in women who had ADMA above 0.71 μM (Leong et al. 2008). However, it must be noted that a single result from a study subject may be of limited diagnostic value.

In summary, the present studies provide new information about ADMA and related compounds regarding pregnancy related phenomenon: endothelial function, immune system upregulation and increased lipid concentration during normal pregnancy. In this thesis, it was found that the ADMA concentration is dependent on hormonal status of the women. Thus if women at fertile age are enrolled in studies, the menstrual cycle phase and not only their possible use of hormonal contraception should be taken into account. Furthermore, also the type of contraception is important. This applies also to CRP measurements where the use of OC seems to increase the circulating CRP concentration. On the contrary, endothelial function measured as FMD seemed to be unaffected by the menstrual cycle phase. These results may be useful when ADMA concentrations from normal pregnancies are compared with the results from complicated pregnancies. In the future, there is a clear need for a golden standard with tracebility for ADMA measurements so that results from different study groups could be comparable with each other. However, at the moment, ADMA measurements are of limited importance in clinical use on their own but they may be a useful risk marker and predictor of outcome when combined with other biochemical measurements i.e. renal clearance, markers of inflammation and endothelial function.

7 CONCLUSIONS

The present HPLC assay for ADMA was optimized and it proved to be sensitive and selective. The linearity was excellent and the variations within and between assays were low. In addition, the detection limits for ADMA and SDMA were low enough to detect the small concentrations typically seen in serum and plasma of the normal healthy population. The HPLC method is more precise than the current ELISA method and it has the advantage of permitting the simultaneous determination of ADMA, SDMA, L-homoarginine and L-arginine.

In women not using hormonal contraception, there were significant changes in the plasma ADMA and L-arginine concentration during the normal menstrual cycle. The use of OC decreased ADMA and SDMA concentrations as compared with non-OC users. However, there were no significant changes found in ADMA, SDMA, L-arginine concentrations during the various phases of the menstrual cycle in women with OC-use. FMD, a marker of endothelial function, was not affected by hormonal cyclic changes of menstrual cycle and it can be measured at any phase of the cycle without causing erroneous results whereas in ADMA measurements the menstrual cycle phase of the woman may interfere with the assay result.

ADMA, SDMA and L-arginine concentrations were significantly decreased during normal pregnancy. Marked hypercholesterolemia and enhanced FMD which is common during pregnancy were not associated with serum ADMA levels.

The L-homoarginine concentration is significantly increased in normal pregnancy in comparison with the corresponding value in non-pregnant women or the concentrations after delivery. Interestingly, the L-homoarginine concentration was associated with FMD but not with serum levels of ADMA or L-arginine in normal pregnancy. L-homoarginine cannot be used as an internal standard in the ADMA assay if pregnant women are included in the study population because it may cause erroneous results.

The concentrations of proinflammatory cytokine, IL-6, and inflammation marker, CRP, were significantly increased during the third trimester of the normal pregnancy. No association was found between levels of dimethylarginines and CRP or IL-6 and TNF-α. This study indicates that the maternal ADMA and SDMA serum concentrations may be decreased due to hemodilution and more extensive renal clearance. It seems that ADMA and its related compounds undergo metabolism independent from changes in the serum levels of CRP or the studied cytokines in normal pregnancy.

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