The clinical significance of asymmetric dimethylarginine

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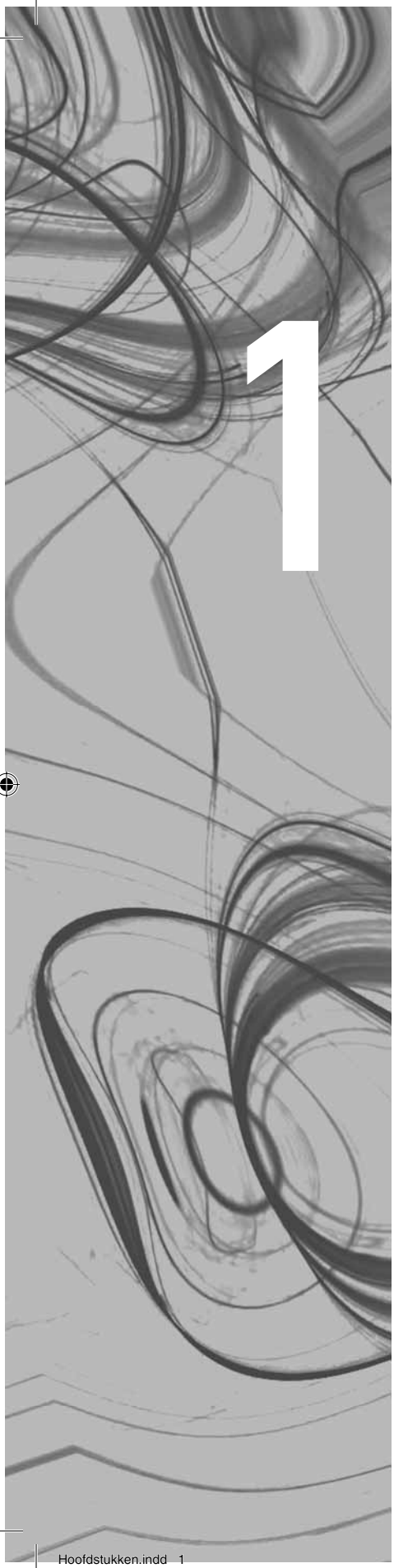

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General introduction and Outline of the thesis

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GENERAL INTRODUCTION

INTRODUCTION

In humans, the gaseous molecule nitric oxide (NO) is formed by three isoforms of NO synthase.[1] NO synthase uses arginine as a substrate to produce NO which plays a pivotal role in biology by exerting a wide variety of regulatory functions of the pulmonary, cardiovascular, immune, gastrointestinal, and neurological systems. Production of NO by the endothelial derived NO synthase (eNOS) is important for preservation of organ blood flow by regulating vascular tone, influencing the interaction of white blood cells and platelets with the endothelium, and limiting the development of neointimal hyperplasia by inducing apoptosis of vascular smooth muscle cells.[2] In addition, the inducible isoform of NO synthase (iNOS) is able to produce large amounts of NO during inflammation. Since NO is a free radical gas, it plays a crucial role in host defense by acting as a cytotoxic agent.[3] NO derived from the neuronal isoform (nNOS) is mainly important in relaxation of smooth muscle cells, but also plays a role in behavioural inhibition.[4,5]

The synthesis of NO is selectively inhibited by guanidino-substituted analogues of arginine, including monomethylarginine (MMA) and asymmetric dimethylarginine (ADMA).[6,7] Symmetric dimethylarginine (SDMA), the inactive stereoisomer of ADMA, does not directly inhibit the enzyme NO synthase but is able to interfere with NO synthesis by competing with arginine, MMA, and ADMA for cellular transport across cationic amino acid transporters (CAT) of system y^+.[8] Since physiological concentrations of ADMA are approximately tenfold higher than that of MMA, ADMA can be regarded as the predominant endogenous inhibitor of NO biosynthesis.[9]

Modification of ADMA concentrations has been shown to change vascular NO production and thereby vascular tone and systemic vascular resistance. Therefore, it has been suggested that elevated ADMA levels may explain the 'arginine paradox';[10] the observation that, although the arginine plasma levels fully saturate the enzyme NO synthase (physiologic arginine plasma levels in humans: 90-110 µmol/L, K_m NO synthase: 3 µmol/L), supplementation with exogenous arginine improves NO production and NO mediated functions. The study by Hornig and coworkers[11] is

illustrative for this paradox; supplementation of arginine in patients with chronic heart failure having increased plasma levels of ADMA resulted in endothelium dependent vasodilation, whereas arginine supplementation in patients without chronic heart failure and with normal plasma levels of ADMA did not affect the endothelium dependent vasodilation. Thus, arginine administration may help to normalise the arginine/ADMA ratio when disturbed. On the other hand, the effect of arginine supplementation may be less pronounced in patients without a disturbed arginine/ADMA ratio.

Since the discovery in 1992 that ADMA plays a regulatory role in the arginine-NO pathway by inhibiting the enzyme NO synthase,[7] many researchers have focussed on ADMA, especially with respect to cardiovascular disease. ADMA concentrations are increased in a number of clinical conditions, including many cardiovascular disorders,[12-19] critical illness,[20] and dysfunction of ADMA-eliminating organs like the liver[20-23] and the kidney.[9,24-26] There has been a growing number of publications concerning ADMA during the last two decades and nowadays ADMA is regarded as a novel cardiovascular risk factor.[27] The following sections will discuss the origin and fate of dimethylarginines and will particularly focus on the organs involved in the elimination of ADMA and on the significance of ADMA in different clinical conditions.

PRODUCTION AND ELIMINATION OF METHYLARGININES

Synthesis of methylarginines

Methylarginines are synthesised by post-translational modification, involving addition of methyl groups from the donor methionine to arginine residues in proteins by enzymes called protein arginine methyltransferases (PRMTs). These methylated proteins are predominantly found in the nucleus and play a role in RNA processing and transcriptional control.[28] ADMA is formed by PRMT type 1 and SDMA is formed by PRMT type 2. Both methyltransferase types are able to form MMA.[29]

Methylarginines are released into the cytosol when these proteins are hydrolysed, thereby being an obligatory product of protein turnover. Thus, the amount of ADMA generated is dependent on the extent of arginine methylation in proteins and on the rate of protein turnover. It should be noted that plasma ADMA concentrations

probably represent cellular spill-over and therefore weakly mirror intracellular concentrations.

Eliminatory pathways of methylarginines

The kidney plays an important role in the elimination of dimethylarginines from the body by excreting ADMA and SDMA into the urine.[30,31] There is an additional eliminatory pathway for ADMA, namely the conversion of ADMA by DDAH into citrulline and dimethylamine.[32] The enzyme DDAH is widely distributed in the rat and the human body but is particularly present in pancreas, spleen, liver, kidney, and endothelium.[33,34] It has been estimated that humans generate approximately 300 µmol of ADMA per day, of which more than 80% is metabolised by DDAH.[35] Inhibition of DDAH causes vasoconstriction in vascular segments that is reversed by arginine, indicating that regulation of intracellular ADMA by DDAH affects NO synthase activity.[36] In addition, overexpression of human DDAH in transgenic mice results in a reduction of plasma ADMA levels with a concomitant increase in tissue NO synthase activity, providing strong evidence for an important role of endogenous ADMA in regulating NO synthase activity.[37]

There are two isoforms of DDAH: type 1 to be found in tissues expressing nNOS and type 2 predominantly located in tissues expressing eNOS.[38] The activity of both type 1 and 2 DDAH depends on regulatory mechanisms that are not fully understood. A reduced activity of DDAH after incubation of endothelial cells with TNF-α or oxidised low-density lipoprotein (ox-LDL) has been reported.[39] Oxidative stress by *S*-nitrosylation inactivates DDAH,[40] which provides probably an important homeostatic mechanism whereby high levels of NO upregulate the levels of ADMA, thereby limiting further NO generation. High glucose levels and homocysteine have also been demonstrated to impair activity of DDAH, thereby causing ADMA accumulation.[41,42]

Interestingly, daviditin A, a xanthone compound with antioxidant properties, has been shown to increase activity of DDAH in endothelial cells damaged by lysophosphatidylcholine.[43] In addition, in endothelial cells DDAH expression is increased by retinoic acid, a vitamin A derivate which has some beneficial effects on the cardiovascular system.[44] Other recent studies provided evidence for upregulation of DDAH expression by thiazolidinediones,[45,46] and stimulation of DDAH activity by the sulfhydryl antioxidant pyrrolidine dithiocarbamate,[47] and estrogens.[48] The ADMA lowering effect of estrogens has been confirmed in placebo-controlled clinical studies

on oral hormone replacement therapy in postmenopausal women.[49,50] Metformin and angiotensin-converting enzyme inhibitors have also been shown to reduce ADMA concentrations, but their working mechanisms have not been elucidated.[13,51]

It may be concluded that the regulation of gene expression and acitivity of DDAH is not yet fully understood, but considering the differential expression of DDAH in different organs, it can be hypothesised that NO synthesis may be differentially regulated in different vascular beds in order to modulate vascular tone during various pathophysiological processes.

RENAL HANDLING OF DIMETHYLARGININES

Studies on the role of the kidney in the metabolism of dimethylarginines

In the past, several groups have demonstrated that the kidney of both animals and humans is capable of excreting both ADMA and SDMA.[31,52,53] McDermott and coworkers[53] revealed that urinary excretion was the main elimination route for SDMA in rabbits, whereas ADMA was partly eliminated by other metabolic pathways. In addition, Al Banchaabouchi and coworkers[52] investigated the relationship between plasma levels and urinary concentrations of ADMA and SDMA. They calculated clearances of dimethylarginines and fractional excretion rates (clearance of dimethylarginines divided by the clearance of creatinine times 100%) in healthy humans. They found similar fractional excretion rates for ADMA and SDMA (68% and 71%, respectively). Moreover, it was calculated that approximately 30% of both dimethylarginines was reabsorbed. The limitation of this study is the fact that no arteriovenous concentration differences were measured and therefore no net renal extraction could be determined, thereby making it impossible to calculate true reabsorption rates. The role of the kidney as ADMA eliminating organ has been confirmed in another study with healthy humans,[54] where plasma concentrations of dimethylarginines were determined in both arterial and renal venous blood in 20 fasting patients with normal renal function. Renal extraction of ADMA, as a measure of ADMA elimination, was calculated as the arteriovenous concentration difference divided by the arterial concentration times 100%. The main result was a significant net renal extraction for both dimethylarginines. Interestingly, for ADMA, a higher net renal extraction was found when compared with SDMA (16% and 11%, p=0.001,

respectively). In addition, arterial SDMA concentration, but not ADMA concentration, was significantly correlated to arterial creatinine concentration (r=0.6, p=0.005). In addition, the presence of a higher renal extraction of ADMA strongly suggested the presence of a catabolic pathway for ADMA in the kidney.

The role of the kidney was further explored in metabolic studies in rats.[55] A significant net uptake of both ADMA and SDMA by the rat kidney has been found with fractional extraction rates of 35% and 31%, respectively. Furthermore, strong evidence was obtained for a differential renal handling of the two dimethylarginines. It was also found that the elimination of ADMA by the rat kidney could not be explained by urinary excretion, because urinary concentration of unchanged ADMA was negligible. This finding points to a high metabolic turnover of ADMA in the kidney which is fully responsible for the observed net renal uptake of ADMA.

In contrast to the rat kidney, human kidneys are capable of excreting unchanged ADMA.[30,54] Thus, there also seems to be a difference in the handling of dimethylarginines between humans and rats. Ogawa and coworkers[56] investigated the metabolic fate of ADMA and SDMA isotopically in the rat and, interestingly, they found that only 4.6% of injected ADMA was found in the first 12-h urine as unchanged ADMA, compared with 17.8% for SDMA. Furthermore, they showed that both dimethylarginines are metabolised by a pathway forming the corresponding α-ketoacid analogues and the oxidatively decarboxylated products of the α-ketoacids in addition to N-acetyl conjugates, and that these metabolites were mainly found in urine. The potential presence of these metabolites, which would not have been detected by the actual high-performance liquid chromatography (HPLC) methods for determination of dimethylarginines, may explain the controversy between the data obtained from rat and human experiments. Their study provided strong evidence for the existence of an additional pathway for the elimination of ADMA, leading to the formation of citrulline and related amino acids. This pathway seemed to be the main route for ADMA elimination, as most ADMA derived radioactivity was found in tissues instead of urine. Later this catalytic pathway was recognised both in rats and humans and proven to be degradation by the enzyme DDAH.[57,58]

Theoretically, a reduced activity of DDAH may be responsible for elevated ADMA concentrations.[36] DDAH activity is influenced by factors as oxidative stress and inflammation. In an *in vitro* model of human umbilical vein endothelial cells, a reduced activity of DDAH was found after exposure to ox-LDL and TNF-α.[39] *In vivo*,

confirmation of the potential role of ox-LDL was obtained by the occurrence of high ADMA levels in hypercholesterolemia, making ADMA a potential risk factor for atherosclerosis.[14,39] However, no *in vivo* data were present on the role of TNF-α and inflammation on the metabolism of dimethylarginines. Therefore, the effect of inflammation induced by lipopolysaccharide (LPS), as a natural mediator in the cascade of inflammatory mediators, was studied. In rats treated with endotoxin, a significantly lower ADMA concentration was found, suggesting an increased metabolic turnover of ADMA during severe inflammation.[55] Interestingly, the increased metabolic turnover of ADMA was not accompanied by an increased renal elimination of ADMA as both renal fractional extraction rate and net renal uptake were significantly lower in LPS treated rats. In contrast to the decreased ADMA levels, SDMA levels were higher in endotoxin treated rats and the increase of SDMA was accompanied by a reduced renal fractional extraction and a reduced net uptake by the kidney. As creatinine levels were also significantly higher in LPS treated rats, an impaired renal clearance of SDMA could underlie the rise in SDMA levels. Thus, the kidney does not seem not to be responsible for this ADMA lowering effect of endotoxemia. A potential explanation for the lower plasma concentration of ADMA might be increased uptake by the y^+ transporter during endotoxemia. Cationic amino acids such as arginine, ornithine, and lysine are also transported into endothelial cells by this y^+ pump. Closs and coworker[8] investigated transport of dimethylarginines by CAT and found that both ADMA and SDMA were transported across this y^+ carrier. In rats, it has been shown that the expression of CAT was significantly increased in lung, heart, and kidney by LPS injection.[59] Clinical conditions associated with severe endotoxemia include sepsis and multiple organ failure. These conditions are characterised by overproduction of NO due to inducible NO synthase activity. One of the biological questions that has to be answered is: what is the potential role of ADMA during inflammation and infection? It has been speculated that ADMA could possibly serve as a 'brake' on the action of iNOS, and inhibit overwhelming NO synthesis.[60]

Clinical studies on dimethylarginines and renal dysfunction

The recognition of ADMA as potential player in diverse cardiovascular diseases paralleled the discovery of elevated levels of dimethylarginines in patients with end-stage renal disease. From then, a lot of research on the role of the kidney has

evolved. First in 1992, Vallance and coworkers[9] reported elevated levels of ADMA in patients with renal failure. Kielstein and coworkers[25] showed that ADMA was higher in dialysis patients with clinically manifest atherosclerosis than in those without atherosclerotic disease. It was suggested that elevated levels of dimethylarginines may be responsible for the hypertension seen in patients with end-stage renal disease. In patients with end-stage renal disease ADMA was independently associated with intima-media thickness (IMT) of the carotid artery, and predicted the progression of intimal thickening during 1 year of follow-up.[61] Recently, an independent association between plasma ADMA concentration and carotid IMT was also described in patients with mild to moderate renal failure.[62]

Zoccali and coworkers[26] studied the relation between cardiovascular risk factors and plasma ADMA concentration in a cohort of 225 haemodialysis patients, and found that plasma ADMA is a strong and independent risk factor of overall mortality and cardiovascular outcome. In another study in patients with end-stage renal disease, the same investigators revealed that raised plasma concentration of ADMA was associated to left ventricular dysfunction and left ventricular hypertrophy; important risk factors for mortality in these patients.[63] Furthermore, in a recent study on cardiovascular risk stratification in patients with end-stage renal disease, it was demonstrated that ADMA significantly adds predictive value to all-cause and cardiovascular mortality in dialysis patients.[64] Interestingly, in patients with chronic renal failure also a sharp rise of SDMA, the stereoisomer of ADMA, has been reported.[24] Although SDMA has no direct inhibitory activity towards the enzyme NO synthase, Fleck and coworkers[24] pointed out the potential importance of SDMA and concluded in their study in a large population of renal failure patients that not only ADMA levels, but also SDMA levels were likely responsible for hypertension, possibly by competition for reabsorption between SDMA and arginine in the kidney.

Recently, it was demonstrated that plasma ADMA is inversely related to glomerular filtration rate in patients with mild to advanced chronic kidney disease.[65] In these patients, ADMA represented a strong and independent risk marker for progression to end-stage renal disease and mortality. Another recent study showed that ADMA levels above median in patients with nondiabetic kidney diseases and mild to moderate renal failure were associated with a faster progression of the kidney disease.[66] The findings of the above mentioned studies indicate a potential role for

ADMA in diagnostic and therapeutic strategies aimed at detection and treatment of atherosclerotic complications in patients with renal disease.

HEPATIC HANDLING OF DIMETHYLARGININES

Studies on the role of the liver in the metabolism of dimethylarginines

The first report suggesting a role for the liver in the metabolism of ADMA was published in 1977 by Carnegie and coworkers.[30] They studied urinary excretion of methylarginines in human disease and showed an increased excretion rate of ADMA in patients with liver disease compared with healthy adults. Unfortunately, ADMA plasma concentrations were not measured. Therefore, no definite conclusions on the liver as ADMA clearing organ can be drawn from these results. Later, it was revealed that hepatocytes abundantly express CAT[59] and also contain large amounts of the ADMA degrading enzyme DDAH.[33,34] These findings strongly suggested a role for the liver in the metabolism of dimethylarginines. This hypothesis was confirmed by an organ balance study in rats in which net organ fluxes and fractional extraction rates of dimethylarginines across the liver and kidney were determined by measuring arteriovenous concentration differences and blood flow using radiolabeled microspheres.[67] The main finding of this study was a high uptake of ADMA by the liver, while the concentration of SDMA was barely affected. The exact contribution of the human liver in the metabolism of dimethylarginines is currently unknown.

Clinical studies on ADMA and hepatic dysfunction

Theoretically, dysfunction of the liver may disturb dimethylarginine metabolism. Indeed, in critically ill patients, hepatic dysfunction has proven to be the most prominent determinant of ADMA plasma concentration.[20] Moreover, in these patients, ADMA ranked as the strongest predictor of ICU mortality. Furthermore, increased plasma concentrations of ADMA have been measured in the postoperative course after major hepatectomy compared with patients undergoing colorectal surgery.[22] Additional research on the clinical significance of the liver as ADMA clearing organ has been performed in patients eligible for liver transplantation. Martín-Sanz and coworkers[68] showed that methylated arginine derivates are produced in human livers during the cold ischemia period of the graft and that a longer ischemia time caused

significantly greater concentrations of these inhibitors in the preservation solution. Of particular interest was the significant relationship between the extent of NO synthase inhibition and early liver graft function, suggesting that the concentration of methylarginines in the graft preservation solution may be used as a predictor of early liver graft function. In another study, it has been revealed that plasma concentrations of ADMA and the oxidative stress marker 15(S)-8-*iso*-$PGF_{2\alpha}$ were increased in patients with end-stage liver disease.[23]

Dimethylarginines may also be of siginificance in the pathophysiology of liver cirrhosis, a condition characterised by endothelial dysfunction (i.e. NO deficiency) in the intrahepatic circulation and, paradoxically, overproduction of NO by the splanchnic circulation. Since Lluch and coworkers[21] showed elevated plasma concentrations of nitrate and nitrite and ADMA in patients with decompensated alcoholic cirrhosis (Child-Pugh $\geq$ 8), they suggested that ADMA might oppose the peripheral vasodilation caused by excessive systemic NO production during liver cirrhosis. Furthermore, the development of renal failure in patients with severe liver disease is characterised by renal hypoperfusion. The kidney is highly vulnerable to accumulation of ADMA because renal blood flow and glomerular filtration are both dependent on basal NO synthesis. Therefore, a causal role for ADMA in the development of the hepatorenal syndrome has been suggested.[69]

CRITICALL ILLNESS

ADMA levels in ICU patients with multiple organ failure

Highly raised ADMA levels are present in critically ill patients with multiple organ dysfunction.[20] This is most likely caused by the unfavourable combination of increased protein turnover (hypercatabolic state and synthesis of acute phase proteins) and a decreased elimination of ADMA when hepatic and renal dysfunction are present. In a cross-sectional study of 52 critically ill patients with clinical evidence of dysfunction of more than two organs, plasma ADMA concentration was independently related to the presence of hepatic and renal failure.[20] Moreover, in a logistic regression model, plasma ADMA ranked as the first and strongest predictor for outcome, with a 17-fold increased risk for ICU death in patients who were in the highest quartile for ADMA.

The most likely mechanism by which ADMA increases the risk of adverse outcome in critically ill patients is inhibition of the constitutively expressed eNOS. NO produced by eNOS is important for preservation of organ blood flow by regulating vascular tone and influencing the interaction of white blood cells and platelets with the endothelium. In addition, NO is involved in host defense by acting as a cytotoxic agent. During inflammation, iNOS is able to produce large amounts of NO. Overproduction of NO may aggravate tissue damage and may cause systemic vasodilation with therapeutically refractory hypotension and coagulation disorders as seen in septic shock. Assuming that inhibition of these adverse effects has therapeutic potential, the pharmacological NO synthase inhibitor MMA has been given to septic patients. However, the results of this study revealed increased mortality rates in patients receiving monomethylarginine.[70] In several human and animal studies reporting adverse effects of NO synthase inhibition, the inhibitors were non-selective; inhibiting both eNOS and iNOS. ADMA is also a non-selective inhibitor of NO synthase and is endogenously produced. Therefore, when plasma levels of ADMA increase, interference with physiological functions of NO may be expected.

PREECLAMPSIA

There is ample evidence that impaired endothelial function (i.e. NO deficiency) is involved in the etiology of preeclampsia. Endothelial derived NO regulates physiological changes during normal pregnancy, including vasodilation and an increase in circulating blood volume. The last decennium, several investigators have focused on ADMA as a potential causative factor in the development of preeclampsia. ADMA reduces bioavailability of NO and is thought to impair the physiological adaptation process during pregnancy, thereby causing preeclampsia and the ‘hemolysis, elevated liver enzymes, and low platelets’ (HELLP) syndrome. The first report showing increased plasma concentrations of ADMA in females with preeclampsia in comparison with healthy pregnant women during the third trimester was published in 1993 by Fickling and coworkers.[71] They also revealed that ADMA levels decreased during normal pregnancy compared with nonpregnant females. These findings have been confirmed in other studies.[72-75] Changes in renal function during normal pregnancy and during preeclampsia may explain the difference in

ADMA concentration between these patient groups, but the exact cause of elevated ADMA concentrations during preeclampsia has still not been elucidated.
Interestingly, the placenta contains the ADMA degrading enzyme DDAH.[38,76] Therefore, placental dysfunction has been suggested as an initiator in the development of preeclampsia.[75] Maeda and coworkers[77] have reported significantly higher ADMA concentrations in umbilical venous blood compared with maternal concentrations at term, suggesting an important role of the placenta in placental transport/metabolism of ADMA. Unfortunately, ADMA concentrations and DDAH activity within the placenta were not determined in this study.

THE ROLE OF ADMA IN CARDIOVASCULAR DISEASE

Hypercholesterolemia

Cholesterol feeding of rabbits increases their plasma ADMA concentration.[78-80] These animals develop atherosclerosis and endothelial dysfunction and it has been shown that the production of NO is inversely associated with the plasma concentration of ADMA.[78] Also in monkeys with diet-induced hypercholesterolemia, plasma concentrations of ADMA were elevated and inversely associated with endothelial function.[81] Dietary arginine can reduce the progression of atherosclerosis and improve endothelium-dependent vasodilation in cholesterol-fed rabbits.[79] Overall, these animal experiments provide evidence that inhibition of NO synthase by elevated ADMA concentrations plays a role in cholesterol-induced atherosclerosis.
In humans, a positive association between cholesterol and ADMA has been observed as well. In a group of asymptomatic hypercholesterolemic subjects, the mean plasma ADMA concentration was approximately two-fold higher than in age-matched normocholesterolemic subjects and in the combined groups, ADMA was positively associated with LDL cholesterol.[14] Notably, other studies have observed no significant association between ADMA and LDL cholesterol[45,82] and in most intervention studies, plasma ADMA concentrations were not influenced by aggressive lowering of LDL cholesterol by statin treatment.[15,82-84] Despite these negative results, it was shown that the effect of pravastatin treatment was modified by ADMA, with low baseline concentrations of ADMA being predictive of a significant improvement in adenosine-induced myocardial blood flow.[84]

ox-LDL in the vascular wall plays a key role in the process of atherogenesis and results from *in vitro* experiments suggest that oxidation of LDL, rather than its cholesterol content per se, may be responsible for the ADMA increasing effect of LDL. Incubation of endothelial cells with ox-LDL has been shown to lead to increased levels of ADMA in the culture medium, due to a reduction of DDAH activity.[39] The lectin-like oxidised LDL receptor-1 (LOX-1) is a major receptor for ox-LDL in endothelial cells and binding of ox-LDL to LOX-1 increases the intracellular generation of reactive oxygen species. Interestingly, it has been reported that incubation of endothelial cells with ADMA results in upregulation of LOX-1.[85] It is noteworthy that ADMA itself may also increase oxidative stress by uncoupling of endothelial NO synthase, resulting in a shift from NO production to superoxide production.[85,86] From these data, it seems that ADMA and ox-LDL are part of a vicious cycle, in which oxidative stress induced by uptake of ox-LDL leads to inhibition of DDAH, and the ensuing increased ADMA concentration further enhances oxidative stress and induces LOX-1 expression, thereby resulting in the augmented uptake of ox-LDL.

Hyperhomocysteinemia

The metabolic pathways of homocysteine and ADMA are strongly intertwined and emerging evidence suggests that ADMA is a key mediator of the link between hyperhomocysteinemia and endothelial dysfunction.[87] During the process of arginine methylation, S-adenosylmethionine (SAM), which serves as methyl-group donor, is converted into S-adenosylhomocysteine (SAH), which is subsequently hydrolysed to homocysteine. Because ADMA contains two methyl groups, its synthesis is accompanied by the generation of two equivalents of homocysteine. It should be noted, however, that synthesis of ADMA is only a minor source of homocysteine.

Homocysteine affects the metabolism of ADMA in several ways. First, homocysteine, by inducing disulfide exchange reactions, can disrupt protein folding.[88] This may accelerate protein degradation, resulting in an increased release of free ADMA from methylated proteins. Second, as already mentioned, the enzymatic activity of DDAH, which is responsible for the breakdown of ADMA, is inhibited by homocysteine.[42] Finally, it is important to note that the enzymatic hydrolysis of SAH to homocysteine is reversible, with equilibrium dynamics that strongly favor SAH synthesis rather than hydrolysis. In situations where homocysteine is elevated, intracellular SAH levels

may thus increase. Because SAH is a potent inhibitor of transmethylation reactions, its accumulation may lead to hypomethylation of macromolecules.[89] That increased SAH levels also inhibit the methylation of arginine residues in proteins is supported by the observation that the production of ADMA by endothelial cells is reduced upon incubation with SAH.[90]

Oral methionine loading induces acute hyperhomocysteinemia, which is associated with impairment of vascular endothelial function.[91] Several studies have reported a significant increase of plasma ADMA concentrations after a methionine-loading test,[17,92,93] suggesting that inhibition of NO synthase by increased ADMA concentrations contributes to the acute impairment of endothelial function.

Lowering of elevated homocysteine concentrations is easily accomplished by treatment with B vitamins, of which folic acid is especially effective. There is a single report showing that ADMA concentrations decreased in parallel with homocysteine concentrations during folic acid treatment,[16] but several other studies have shown no effect of treatment with combined B vitamins on ADMA concentrations.[94-97]

A significant positive association between basal plasma concentrations of homocysteine and ADMA has been reported in subjects with hyperhomocysteinemia[16] and patients with peripheral arterial disease,[17] incipient primary renal disease,[98] and stroke.[19] This association seems not to be restricted to specific patient groups, because a moderate but highly significant positive association between plasma concentrations of ADMA and log-transformed homocysteine in a large population-based cohort study has been observed recently.[18]

Diabetes mellitus

There is accumulating evidence that ADMA plays a role in insulin resistance.[99,100] In a study by Miyazaki and coworkers[101] in subjects without symptoms of coronary or peripheral artery disease, age, mean arterial blood pressure, and insulin resistance were the main determinants of plasma ADMA. Stühlinger and coworkers[45] also demonstrated a positive relationship between insulin resistance and plasma ADMA concentration in healthy, nondiabetic subjects. In addition, pharmacological agents that improve insulin sensitivity, such as rosiglitazone and metformin, have been shown to lower plasma ADMA concentrations.[13,45,46]

In rats, streptozotocin-induced type 2 diabetes results in elevated ADMA levels.[41,102] Treatment of these animals with insulin results in normalisation of ADMA, suggesting that the elevation of ADMA is closely related to glycemic control.[103] In diabetic rats, aortic DDAH activity was significantly reduced and negatively associated with ADMA levels in plasma.[41] It has been shown that the reduction of DDAH activity in human endothelial cells exposed to high glucose conditions can be reversed by co-incubation with a superoxide-dismutase conjugate.[41] Hence, glucose-induced oxidative stress leading to impairment of DDAH seems a likely mechanism for accumulation of ADMA.

Data on plasma levels of ADMA in subjects with diabetes are inconclusive. In patients with poorly controlled type 2 diabetes, increased plasma concentrations of ADMA were found.[12,13] However, Päivä and coworkers[104] reported decreased ADMA concentrations in type 2 diabetes patients, which was ascribed to renal hyperfiltration. Increased concentrations of ADMA have also been reported in women with gestational diabetes[105] and in subjects with type 1 diabetes.[15] In a large cohort of patients with longstanding type 1 diabetes, plasma ADMA concentrations were in the normal range. However, ADMA was significantly higher in patients with diabetic nephropathy compared with those with normoalbuminuria.[106] Although there is strong evidence for a relation between insulin sensitivity and ADMA, the association between ADMA and diabetes per se may be confounded by renal function and diabetes-associated vascular pathology.

The effect of ADMA on cardiac physiology

Proteome analysis of the canine myocardium has revealed that regulation of NO synthase activity by ADMA plays an important role in local distribution of blood flow within the left ventricular myocardium.[107] Specifically, in areas of low flow, expression of the ADMA degrading enzyme DDAH was more pronounced and ADMA was reduced to only 25% of the ADMA content in high-flow areas. The strong reduction of ADMA in the presence of identical levels of NO synthase strongly suggests enhanced NO formation in low-flow areas. Recent work of Osanai and coworkers[108] demonstrated that the release of ADMA by vascular endothelial cells was increased after shear stress $\leq$ 15 dyne/cm^2, which is comparable to mechanical forces on the arterial wall under physiological conditions, but was unchanged by shear stress at 25 dyne/cm^2. In addition, the activity of DDAH was enhanced by shear stress at 25

dyne/cm^2, but was not affected by shear stress ≤ 15 dyne/cm^2. Since eNOS is also stimulated by shear stress, ADMA and NO synthase activity might antagonistically regulate production of NO in the systemic circulation. In transgenic mice expressing human DDAH, plasma ADMA was 2-fold reduced and tissue NO synthase activity was increased.[37] Compared to wild-type control animals, systemic vascular resistance and blood pressure were decreased. Interestingly, basal heart rate was increased by 10%, but this was balanced by a 10% reduction in stroke volume, resulting in unaltered cardiac output.[37] Conversely, studies in humans have shown that systemic infusion of ADMA in healthy subjects results in an immediate increase of systemic vascular resistance and blood pressure and a decreased heart rate and cardiac output.[35,109] Not only resting, but also excercise-induced stimulation of cardiac output was strongly attenuated after infusion of ADMA, suggesting a possible role for ADMA in the pathophysiology of heart failure and reduced exercise tolerance.[35] Indeed, increased plasma levels of ADMA have been observed in animal models of congestive heart failure.[110,111] It has also been reported that plasma concentrations of ADMA are elevated in patients with heart failure[112] and coronary syndrome X.[113] Finally, ADMA was significantly associated with left ventricular dysfunction and hypertrophy in hemodialysis patients.[63]

Prospective studies

The results of prospective clinical studies provide the most compelling evidence for a role of ADMA in the development of cardiovascular disease. In patients with end-stage renal disease, ADMA and age were the strongest predictors of cardiovascular events and total mortality, after correction for other traditional and novel risk factors.[26] In a Finnish study with a nested case-control design, high concentrations of ADMA were associated with an increased risk of acute coronary events among non-smoking middle-aged men, especially in men with previous coronary heart disease.[114] In a third study, conducted in patients with stable angina pectoris, high plasma concentrations of ADMA predicted adverse cardiovascular events after percutaneous coronary intervention.[115] Also in patients with idiopathic pulmonary arterial hypertension ADMA was an independent predictor of mortality.[116] Furthermore, in a large prospective study, high levels of baseline ADMA independently predicted future cardiovascular events in patients with coronary artery disease.[117] Finally, in a prospective study with critically ill patients, ADMA turned out to be the strongest

predictor of ICU death with a 17-fold increased risk in mortality for patients in the highest ADMA quartile.[20]

In all these studies the association between ADMA and risk was studied by multivariate analysis, including other risk factors and confounding variables, in support of the contention that ADMA is an independent cardiovascular risk factor.[118,119] However, most of these studies were performed in specific patient groups and should therefore be interpreted with some caution. To definitively establish the position of ADMA in the hierarchy of classic and novel cardiovascular risk factors, the results of large prospective studies in the general population are urgently needed.

OUTLINE OF THE THESIS

Although the uptake of ADMA by the liver has been shown in rats, the role of the human liver in handling dimethylarginines has not yet received attention. Therefore, we studied patients undergoing hepatic surgery and calculated net organ fluxes and fractional extraction rates of dimethylarginines by determining arteriovenous concentration differences across the liver and measuring liver blood flow using perioperative Doppler ultrasound techniques. The results of this study are presented and discussed in **Chapter 2**.

After confirming the crucial role of the human liver in the elimination of ADMA in Chapter 2, the clinical significance of ADMA in patients undergoing liver transplantation was studied. In patients with hepatic failure who are eligible for liver transplantation, increased ADMA levels may be expected and after a successful transplantation procedure, hepatic function improves and ADMA levels may decrease. In **Chapter 3**, this hypothesis is studied by investigating the course of ADMA levels before and after liver transplantation. This chapter also discusses the potential of ADMA as a marker of liver allograft rejection.

ADMA may also be of relevance in the pathophysiology of liver cirrhosis because this disease is characterised by endothelial dysfunction and NO deficiency in the intrahepatic circulation. Therefore, in **Chapter 4**, the role of dimethylarginines in patients suffering from liver cirrhosis receiving transjugular intrahepatic portosystemic shunt is described.

After studying the role of the liver as important ADMA clearing organ in different clinical settings, the link between ADMA concentrations and glucose regulation in critically ill patients was investigated. The rationale for performing this study was the combination of two recent findings; 1: tight glucose regulation by insulin administration has been shown to reduce morbidity and mortality in a large population of critically ill patients and 2: ADMA levels are highly raised in critically ill patients with multiple organ dysfunction and plasma ADMA ranked as the first and strongest predictor for outcome. **Chapter 5** describes a study in which ADMA levels were measured in critically ill patients receiving either conventional or intensive insulin treatment to determine whether modulation of ADMA concentration by insulin could explain the beneficial effects of intensive insulin therapy.

Considering the fact that ADMA reduces NO production and thereby possibly impairs the physiological adaptation process during pregnancy, studies have been performed to investigate whether ADMA is involved in the development of preeclampsia. Elevated plasma levels of ADMA have been reported in preeclamptic patients, but the exact cause of these high ADMA values has still not been elucidated. Placental dysfunction has been suggested as an initiator in the development of preeclampsia since it contains the ADMA degrading enzyme DDAH. Therefore, in **Chapter 6**, the role of the ADMA degrading enzyme DDAH within the placenta is studied in patients with preeclampsia.

A summary of the investigated topics in this thesis as well as conclusive remarks are described in **Chapter 7**.

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