All about ADMA

A novel risk factor is discovered
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1. ADMA – what is it?

ADMA is the abbreviation for asymmetric dimethylarginine.

This is an endogenous molecule which can be detected in human blood and urine. It shows structural homology to the amino acid L-arginine, and it acts as an inhibitor of nitric oxide (NO) synthesis.

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NO is synthesized from the amino acid precursor L-arginine. This reaction is made possible by an enzyme named nitric oxide synthase (NO synthase). NO is one of the most prominent small molecule mediators in the human body, and it plays an important role in many physiological functions as described further below. In 1992, Patrick Vallance and co-workers from London were the first to describe substances that show structural homology to L-arginine, but differ from it in that they contain one or two methyl groups act as inhibitors of NO synthesis [1]. These substances which have accordingly been named mono- (containing one methyl group) or di-methylarginines (containing two methyl groups) are present endogenously in human plasma and urine. Vallance and colleagues reported that asymmetric dimethylarginine was the one member of this group of substances that is present in sufficiently high concentrations to inhibit NO synthesis. Indeed, they showed that after its isolation from human urine, ADMA induced a significant and concentration-dependent inhibition of NO production by isolated human cells in vitro [2]. By contrast to ADMA, its structural isomer symmetric dimethylarginine (SDMA) had no effect on NO production (Figure 1).
Experimental studies in various laboratories around the globe have since shown that ADMA inhibits NO production in vitro within a concentration range that can be measured in plasma of patients with cardiovascular or metabolic diseases [3-5]. In cultured human macrophages (which express the inducible isoform of NO synthase) ADMA inhibits NO production in a concentration-dependent manner [2]. Moreover, experiments with isolated, purified, cloned isoforms of NO synthase in vitro [6] as well as clinical studies in patients with varying plasma concentrations of this substance also demonstrated that ADMA concentration-dependently inhibits NO production (Figure 2) [7-9].

Figure 2. The ratio of the concentrations of L-arginine and ADMA determines the activity of NO synthase in vivo and thereby influences vascular function.

a. In healthy individuals, L-arginine by far outweighs ADMA, resulting in an active NO modulated vascular tone and structure.

b. In patients with elevated ADMA levels, NO synthase is blocked by ADMA, and NO dependent vasodilation and the manifold inhibitory effects of NO on cell-cell interactions, cell proliferation, and on free radical reactions in the blood vessel are impaired. This is called endothelial dysfunction.
2. How is ADMA synthesized in the body?

Dimethylarginines are formed during proteolysis of methylated proteins. Protein methylation is a ubiquitously present mechanism of post-translational modification of proteins. It results in a modification of the tertiary structure and the function of proteins.

This process is catalyzed by a group of enzymes named S-adenosylmethionine protein N-methyltransferases (protein methylases I and II) [10]. The complex name of these enzymes suggests their molecular function: They transfer one or more methyl groups from the methyl group donor S-adenosylmethionine to L-arginine residues within proteins or polypeptides. Accordingly, depending on the number of transferred methyl groups, \( \text{NG} \)-monomethyl-L-arginine and \( \text{NG} \)-dimethyl-L-arginine (asymmetric dimethylarginine) are formed by the activity of protein methylase I, and \( \text{NG} \)-monomethyl-L-arginine and \( \text{NG} \)-dimethyl-L-arginine (symmetric dimethylarginine) are formed by the activity of protein methylase II. Free circulating ADMA and SDMA are then released after degradation of such methylated protein residues.

Methyl groups contained in dimethylarginines are derived from the ubiquitously available methyl group donor S-adenosylmethionine, an intermediate in the metabolism of homocysteine. This is experimentally proven: When cultured human endothelial cells are incubated with radioactively labelled S-[\(^{14}\text{C}\)]-adenosylmethionine, part of this radioactivity can be detected within newly synthesized ADMA (Figure 3) [11]. Interestingly, this finding may provide an explanation to the mechanism by which homocysteine impairs endothelial function in animals and humans.

Figure 3.
When cultured human endothelial cells are incubated with \( (^{14}\text{C}-\text{methyl}) \) labelled S-adenosylmethionine (SAM; an intermediate product of homocysteine metabolism), the radioactively labelled methyl group is being transferred onto ADMA.

The graph shows the chromatographic separation of a cell culture supernatant with subsequent scintillation counting of HPLC fractions after incubation with \( ^{14}\text{C} \) SAM. This experiment demonstrates the existence of a metabolic link between the homocysteine and ADMA pathways (from [11] with kind permission of the publishers).
3. What is the pathophysiological role of ADMA?

Cardiovascular diseases are the major cause of death in North America and in the Western European countries.

Traditional cardiovascular risk factors like hypercholesterolemia, hypertension, smoking, and diabetes mellitus can explain up to 80% of coronary events occurring in the population of these countries. In some patient groups with extraordinarily high coronary event rates, like hemodialysis patients, an even larger portion of cardiovascular events remains unexplained by traditional risk factors [12].

Intense research into the molecular and cellular mechanisms underlying atherogenesis has led to the understanding that the vascular endothelium plays a crucial role for early functional changes in the vascular wall which finally initiate and promote the atherogenic process.

There are numerous experimental data showing that the vascular endothelium plays a central role in the maintenance of physiological vascular tone and vascular structure [13]. One of the major mediators that are released by healthy endothelial cells is nitric oxide (NO) [14]. NO is formed by the enzyme NO synthase from the amino acid precursor L-arginine. NO is involved in a vast number of regulatory processes within the cardiovascular system. Its potent vasodilatory effect is most widely known, and this is the one that has led to the discovery, in the early 1980’s, of an „endothelium-derived relaxing factor (EDRF)” by Robert Furchgott and co-workers [15] (Robert Furchgott received the Nobel Prize for Medicine and Physiology in 1998 for this discovery) (Fig. 4).

Besides its potent vasodilatory effects, NO also acts as an endogenous inhibitor of platelet aggregation. Furthermore, NO inhibits the adhesion of monocytes and leukocytes at the healthy vascular endothelium – an effect that, once disturbed, precedes the immigration of inflammatory cells into the vascular wall at sites that later become plaques. It inhibits the

![Figure 4. The ground-breaking experiment by Robert Furchgott and co-workers from the year 1980, for which he was awarded the Nobel Prize 18 years later, for the first time demonstrated that endothelial cells secrete a soluble factor (which was later identified as nitric oxide (NO)) which causes vasodilation. Stimulation of the left arterial segment (of which the endothelium is left intact) with acetylcholine causes NO release, which in turn causes relaxation of the right arterial segment of which the endothelium was mechanically rubbed off. Direct stimulation of the right arterial segment with acetylcholine would cause vasoconstriction due to direct action of acetylcholine on vascular smooth muscle cells.]
proliferation of vascular smooth muscle cells – this might be of great importance during the development of restenosis after angioplasty. Moreover, NO reduces the vascular release of superoxide radicals \( (O_2^-) \), radicals that are involved in inflammatory and cytotoxic processes, and it inhibits LDL oxidation. These numerous salutary actions of NO in the vascular system have led to its name as an »endogenous anti-atherogenic molecule« (Figure 5).
What is the importance of ADMA in this context?

Under experimental conditions that lead to suboptimal L-arginine concentrations or to a relative deficiency of essential co-factors for NO synthase, the activity of this enzyme is “uncoupled”. This means that the oxidation of L-arginine to NO is not complete [17-19]. Normally, five electrons are being transferred in two steps of a coupled reduction-oxidation reaction by the two domains of NO synthase from molecular oxygen to L-arginine, resulting in the release of L-citrulline and NO (Figure 6a).

Under suboptimal conditions like those mentioned above, the electron flow within the two domains of NO synthase is disturbed, and molecular oxygen acts as an electron acceptor. This makes NO synthase a superoxide (O2-) radical-producing enzyme (Figure 6b).

Interestingly, cultured human endothelial cells produce O2- in the presence of ADMA. This led to the hypothesis that ADMA may interrupt the NO-producing activity of NO synthase and “uncouple” the enzyme, which results in a “switch” of the enzymatic activity from NO to O2-.

This in turn will lead to activation of redox-sensitive transcription factors, to subsequent upregulation of endothelial adhesion molecules and, thereby, to increased adhesiveness of monocytes to the vascular lining – an early step in the initiation and progression of atherosclerosis (Figure 7) [21].

Under experimental conditions, the expression of adhesion molecules is indeed upregulated and leukocyte adhesion is increased in cultured human endothelial cells in the presence of high ADMA concentrations. A similar phenomenon can be observed when monocytes are isolated from peripheral blood of patients with cardiovascular risk factors and are co-incubated with cultured human endothelial cells: Monocytes from hypercholesterolemic patients adhere more strongly to the endothelium than monocytes from normocholesterolemic controls. In this context it is interesting to note that monocyte hyperadhesiveness in hypercholesterolemic subjects can be normalized by supplemental L-arginine [22]. This also points in favour of a competitive displacement of endogenous L-arginine (by ADMA) as the cause of these pathophysiological changes (Figure 8).
Figure 7. Adhesion of monocytes to cultured human endothelial cells under “normal” cell culture conditions (a) as well as in the presence of ADMA (b). ADMA induces oxidative stress within the endothelial cells - probably via the mechanisms described in detail in the text. This causes upregulation of endothelial adhesion molecules. Monocyte adhesion is regarded as the major initial step leading to the development of atherosclerotic plaques, which are understood to be locations of local vascular inflammation.

Figure 8. Adhesion of monocytes that were isolated from peripheral venous blood of hypercholesterolemic patients or normocholesterolemic controls to cultured human endothelial cells ex vivo. During daily intake of supplemental L-arginine monocyte adhesion is reduced, as L-arginine can diminish the effects of elevated ADMA on the endothelium (Data from [22]).
In summary, current understanding is that ADMA is an important endogenous regulator of NO synthesis. An elevation of ADMA plasma levels was found in the context of various pathophysiological events that contrast with the influence that NO has on these functions (Figure 9). Thus, elevated ADMA may contribute to the induction and promotion of atherosclerotic vascular disease.

**Figure 9**
When ADMA is present in elevated concentrations, it can block the physiologically relevant functions of NO that have led to its role as an endogenous anti-atherogenic molecule (compare to Figure 5). Indeed, ADMA has been shown to impair endothelium-dependent, NO-mediated vasodilation, to enhance endothelial interaction with circulating blood cells (mainly platelets and monocytes), to induce the growth of vascular smooth muscle cells, and to promote pro-oxidant events in the vascular wall. These effects may promote the development of atherosclerosis.
4. How is the concentration of ADMA in human blood regulated?

The biosynthesis of ADMA occurs during methylation of protein residues, which release unbound ADMA upon their proteolytic degradation during physiological protein turnover. Thus, ADMA is formed in the cytoplasm of cells; it can then be released into the extracellular space and into blood plasma.

Human endothelial cells are capable of synthesizing ADMA. Accordingly, there is evidence to assume that ADMA acts as an autocrine regulator of endothelial NO synthase activity (i.e., within the same cell in which it is formed – by contrast to hormones, which act upon cells different from those in which they are formed). In the presence of native or oxidized LDL cholesterol ADMA release is significantly increased [11]. Elevated ADMA levels may thus be responsible for part of the detrimental action of LDL cholesterol on endothelial cell function (Figure 9).

Both isomers, ADMA and SDMA, are being excreted via the urine. In their first report on ADMA as an endogenous inhibitor of NO synthesis [2], Vallance and co-workers already showed that ADMA levels are significantly elevated in patients with end-stage renal disease. In subsequent studies several groups of researchers independently of each other confirmed the observation that the levels of ADMA and SDMA are elevated in chronic renal failure. In most of the studies, SDMA levels showed a stronger tendency to increase than ADMA levels [23], suggesting that ADMA may be excreted by different metabolic pathways, whereas renal excretion is the only way of elimination for SDMA (Figure 10).

Indeed, ADMA, but not SDMA, is metabolized by an enzyme named dimethylarginine dimethylaminohydrolase (DDAH) to yield L-citrulline and dimethylamine [24]. Pharmacological inhibition of DDAH causes a concentration-dependent constriction of isolated arterial segments in vitro which can be restored by excess L-arginine [25]. This latter finding indicates most specifically that the regulation of intracellular ADMA levels achieved by changes in DDAH activity can lead to changes in NO production.

DDAH activity, which mediates the metabolic degradation of ADMA, appears to underlie complex regulatory mechanisms which have not yet been fully elucidated. Oxidative stress leads to a reduced DDAH activity. This was shown not only in cultured endothelial cells, but also in tissue homogenates from aorta, kidneys and liver of hypercholesterolemic rabbits [26]. Homocysteine, a known cardiovascular risk factor, increases ADMA concentration, which was put down to a
redox-induced downregulation of DDAH activity by homocysteine [27] or, alternatively, by increased methylation of L-arginine residues and subsequently increased release of ADMA [11].

Taken together, these data allow to conclude that ADMA is formed during protein methylation and is continuously released into the extracellular space after its release from proteins during physiological protein turnover. Its accumulation in the body is prevented in healthy humans by renal excretion on the one hand, and by metabolic degradation by DDAH on the other hand. Changes in renal excretory function or changes in DDAH activity, like they can be induced by cardiovascular risk factors, lead to elevated ADMA levels in various cardiovascular and metabolic diseases.
5. Is there a role for ADMA in the regulation of physiological functions of the vascular system?

For many years there have been large discrepancies between different analytical techniques – in their majority chemical techniques like high-performance liquid chromatography (HPLC) or capillary chromatography – that have resulted in large variabilities and poor comparability of data generated in different laboratories. This has hampered our understanding of the physiological and pathophysiological role of ADMA for organ function. It has been four years now that more reliable analytical techniques have been introduced that have resulted in better reproducibilities and that are also more sensitive and specific for ADMA. Among these is liquid chromatography – (tandem) mass spectrometry (LC-MS/MS), a highly sophisticated and expensive analytical technology that is only available in few highly specialized clinical chemical laboratories, but which allows highly specific and sensitive determination of ADMA, SDMA, and L-arginine [28]. Besides this method, which can be regarded as the current analytical gold standard, a few well validated HPLC methods exist which have an excellent reproducibility [29].

Compared to these methods, the development of a sensitive and specific immunoassay (ELISA) for ADMA was a major step forward to a more widespread use of ADMA in large clinical studies [30]. This ELISA also allowed us to analyze large enough cohorts of healthy subjects across all age groups to determine age- and sex-specific reference levels for ADMA [31].

ADMA levels assessed with the help of these modern analytical techniques usually fall into the range from 0.4 to 0.7 µmol/L. Comparative measurements showed very good correlation of ADMA levels determined with ELISA and LC-MS/MS; respectively [28].

Data from numerous studies showed that ADMA levels are elevated by about 2-3-fold versus healthy controls. However, as L-arginine concentration still is in large excess (usually 60 – 140 µmol/l), the question may be asked whether the relatively small absolute changes in ADMA levels in disease states are sufficient to induce major changes in NO generation and, thereby, NO-dependent vascular function.

A number of experimental and clinical studies have addressed this question in recent years. First, experimental studies in cultured endothelial cells showed that the intracellular concentration of ADMA exceeds the one in the extracellular fluid by about ten-fold [11]. A group of researchers from the United States elegantly proved that incubation of endothelial cells with ADMA results in sufficiently large intracellular changes to not only significantly impair L-arginine/ADMA ratio, but also NO generation [32].

More evidence was provided by two independent groups of researchers from Europe who demonstrated that intravenous infusion of ADMA (that resulted in plasma levels in the range of 2-5 µmol/l, i.e. considerably supra-physiological) significantly increased systemic vascular resistance, impaired tissue blood flow, and increased blood
Finally, genetically modified animal models support these findings: A mouse that overexpresses the human DDAH-1 gene, i.e. the enzyme that degrades ADMA, is characterized by a reduction of circulating ADMA concentration by only 20%, but significantly decreased systemic blood pressure and peripheral vascular resistance, and is protected from vascular damage [37].

By contrast, mice in which the gene encoding for DDAH-1 was knocked out show ADMA plasma levels that are elevated by some 20%, resulting in impaired endothelial function and increased pulmonary artery pressure [38].

Taken together, these novel data certainly underscore the hypothesis that the relatively small absolute differences that have been reported in clinical trials on ADMA and cardiovascular morbidity and mortality when cases and controls were compared are sufficient to cause relevant changes in NO generation, hemodynamic variables, and thus may possibly determine vascular health or disease.
6. Which diseases are associated with elevated ADMA blood levels?

Elevated ADMA concentrations have been measured in numerous cardiovascular and metabolic diseases, e.g. in coronary artery disease, congestive heart failure, peripheral arterial occlusive disease, hypercholesterolemia, hypertension, diabetes mellitus, and others (Table 1).

Recently a close correlation was reported between ADMA and the presence of insulin resistance. This finding may point to a possible involvement of ADMA in the pathophysiology of the metabolic syndrome.

Erectile dysfunction is yet another disease which has been linked to a functional defect in the NO pathway. NO is an essential messenger which causes dilatation of penile erectile tissue, resulting in an erection. This occurs only when psychogenic sexual stimulation is present, which causes local NO release from penile nitrinergic nerve endings. In the presence of elevated ADMA levels, NO synthase is unable to adequately respond to sexual stimuli with NO production, and the erection is impaired or lacking – erectile dysfunction occurs. Substances like phosphodiesterase V inhibitors (sildenafil and similar compounds) inhibit the degradation of cyclic GMP, the second messenger molecule that mediates many of the biological actions of NO (Figure 12).

The diseases listed in Table 1 that have been shown to be associated with elevated ADMA levels are all characterized by endothelial dysfunction. In some of them, determination of ADMA levels has been shown to be of prognostic importance for predicting the incidence of major cardiovascular events (as detailed below).

Table 1

<table>
<thead>
<tr>
<th>Disease</th>
<th>x-fold increase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>chronic renal failure</td>
<td>2-7-fold</td>
<td>2, 39, 40</td>
</tr>
<tr>
<td>hypertension</td>
<td>2-fold</td>
<td>41</td>
</tr>
<tr>
<td>childhood hypertension</td>
<td>2.3-fold</td>
<td>42</td>
</tr>
<tr>
<td>pregnancy-induced hypertension</td>
<td>2-fold</td>
<td>43</td>
</tr>
<tr>
<td>pre-eclampsia</td>
<td>2.5-fold</td>
<td>49</td>
</tr>
<tr>
<td>pulmonary hypertension</td>
<td>2-fold</td>
<td>50-56</td>
</tr>
<tr>
<td>coronary artery disease</td>
<td>2-fold</td>
<td>55</td>
</tr>
<tr>
<td>peripheral arterial disease</td>
<td>2-fold</td>
<td>56</td>
</tr>
<tr>
<td>stroke</td>
<td>2-fold</td>
<td>56</td>
</tr>
<tr>
<td>hypercholesterolemia</td>
<td>2-fold</td>
<td>56</td>
</tr>
<tr>
<td>hyperhomocysteinemia</td>
<td>2-3-fold</td>
<td>57, 58</td>
</tr>
<tr>
<td>congestive heart failure</td>
<td>2-fold</td>
<td>59</td>
</tr>
</tbody>
</table>

Data represent fold differences in ADMA plasma or serum concentrations between patients and healthy controls. A selection of diseases is represented for which there are data from clinical studies in human subjects.

Figure 12.

NO physiologically induces the formation of the intracellular second messenger, cyclic GMP. This causes relaxation of vascular smooth muscle cells and is therefore the cause for hypotension and penile erection. In the presence of elevated ADMA levels, these functions are impaire. PDE V inhibitors inhibit the degradation of cyclic GMP and thereby can amplify the actions of NO (which, however, are reduced in the presence of ADMA).
ADMA in coronary artery disease

ADMA has been measured in a series of prospective and nested case-control studies of patients with coronary artery disease. Patients who at the time of coronary angioplasty had elevated ADMA levels were at an about threefold elevated risk of experiencing major adverse cardiovascular events relating to restenosis [52].

Large studies in patients with stable coronary artery disease like one by Schnabel and co-workers in which ADMA was determined by the ADMA-ELISA revealed that patients with ADMA in the top percentiles of the distribution had a significantly elevated risk of experiencing severe cardiovascular events [53]. In another study patients with acute coronary syndrome had slightly, but significantly higher ADMA plasma levels than patients with stable coronary artery disease [54]. Notably, in this study an additional blood sample was drawn at six weeks after the acute coronary event in patients with acute coronary syndrome. Patients whose ADMA levels had remained elevated at this time point were at increased risk of developing another acute coronary event during the subsequent 12 months, whereas patients whose ADMA had dropped to the levels of patients with stable coronary artery disease remained free of subsequent events [Figure 13].

These data suggest that ADMA is a risk marker for coronary artery disease that may provide information beyond currently established risk indicators.

Figure 13. ADMA plasma levels as a function of the number of traditional risk factors present (a). With increasing ADMA concentration the probability increases that a given patient suffers from coronary artery disease (b; data from [51]).
Patients with chronic renal failure were the first group of patients in whom elevated ADMA levels were shown to be present (Figure 15). Meanwhile, this finding has been confirmed in numerous studies [23].

The elevation of ADMA levels in patients with end-stage renal failure is partly caused by deficient elimination of ADMA during hemodialysis [28]. Elevated ADMA levels contribute to endothelial dysfunction present in this disease; they may in part be responsible for the highly elevated cardiovascular morbidity and mortality in chronic renal failure [39].

Determination of ADMA allows to draw prognostic conclusions in this patient group: In a prospective clinical study involving 224 patients undergoing regular hemodialysis treatment, patients whose
ADMA levels initially were in the highest quartile had the highest total mortality and the highest rate of major cardiovascular events during 3 years of follow-up. As an example, total mortality during 33 months of follow-up was 72% higher in patients with ADMA above the median (2.52 µmol/l), and by more than two-fold in patients with ADMA levels above the 75th percentile (3.85 µmol/l) (Figure 16) [40].

Despite the high elevation of ADMA levels in patients with chronic renal failure as compared to healthy subjects, quantification of ADMA allows to further differentiate between renal failure patients with high or low cardiovascular risk, and therapeutic consequences may accordingly be drawn from this information.

**ADMA in patients with peripheral arterial occlusive disease**

In patients with peripheral arterial occlusive disease ADMA levels are increasingly elevated depending upon the clinical stage of the disease: By comparison with age-matched subjects with normal vascular function, patients with intermittent claudication (Leriche-Fontaine stage II) had a two-fold elevation, patients with rest pain (Leriche-Fontaine stage III) had a three-fold elevation, and patients with peripheral necrosis and gangrene (Leriche-Fontaine stage IV) had a 3.5-fold elevation of circulating ADMA levels (Figure 17) [7].

In the same study, a stage-dependent reduction of urinary NO metabolite excretion was reported, which – like clinical symptoms of intermittent claudication – is reversible by administration of L-arginine [60].

| Selection of published clinical studies in which elevated ADMA levels were reported in patients with chronic renal failure. |
|---|---|---|---|
| Controls | Patients | x-fold increase | Reference |
| 0.6 | 4.3 | 7.5 | 61 |
| 0.4 | 0.9 | 2.5 | 62 |
| 0.4 | 0.7 | 1.9 | 39 |
| 1.0 | 6.0 | 6.0 | 63 |
| 0.4 | 0.8 | 2.0 | 64 |
| 0.7 | 1.1 | 1.6 | 65 |
| 0.6 | 1.0 | 1.7 | 66 |
| 1.4 | 4.2 | 3.0 | 66 |

Data represent ADMA concentration in plasma or serum in µmol/l in healthy controls and in patients with end-stage renal failure, respectively.
ADMA in chronic heart failure

Many patients with chronic heart failure show higher ADMA levels compared with healthy people [57,58].

In a study involving 198 patients with end-stage renal failure, left ventricular function was measured via echocardiography and correlated with ADMA concentration. A significant relationship between ADMA levels and left ventricular mass and an inverse correlation between ADMA and left ventricular ejection fraction was found [67], indicating a possible involvement of ADMA in hypertension-related myocardial remodeling.

ADMA in hypertensive patients

In patients with essential hypertension ADMA levels have been shown to be two-fold higher than those in normotensive controls (Figure 18). In parallel, urinary excretion of NO metabolites (nitrate) is significantly decreased in hypertensive patients [41].

Experimental studies have also demonstrated that intra-arterial infusion of ADMA causes local vasoconstriction in the corresponding circulation [68]. Moreover, intravenous infusion of ADMA in doses which lead to circulating levels of about 2 µmol/l, total peripheral resistance is significantly increased [33, 34].

These studies confirm previous data from animal experiments and suggest a pathophysiological role for ADMA in the regulation of normal blood
pressure, and a pathophysiological role for this molecule in hypertension. This view is also supported by other studies which demonstrated a correlation between ADMA levels and the presence of left ventricular hypertrophy.

**ADMA in diabetic patients**

ADMA may play an important role as a risk marker in the pathogenesis of diabetes mellitus and its cardiovascular complications. In a series of studies from different investigators it was repeatedly reported that patients with type II diabetes have significantly elevated ADMA levels [69-71]. Interestingly, even a single high-fat meal can acutely increase ADMA levels in type II diabetic patients [70]. Molecular mechanisms underlying the elevation of ADMA plasma levels in diabetes mellitus may be oxidative stress-induced dysregulation of the enzyme that inactivates ADMA, dimethylarginine dimethylaminohydrolase (DDAH) [72] or angiotensin II-dependent mechanisms that are also being discussed [73].

Experimental models of diabetes mellitus in animals have confirmed the relationship between ADMA and this metabolic disease: Rats that have been made diabetic by the injection of streptozotocin have significantly elevated ADMA levels as compared to control rats [72, 74].

Yet another, clinically relevant observation stems from therapeutic intervention studies. Patients with impaired glucose tolerance show significantly higher ADMA levels even before the onset of clinically overt diabetes [71]. Pharmacological restoration of insulin sensitivity with rosiglitazone leads to a reduction of ADMA levels [71], and metformin treatment has been shown to exert a similar effect [75].

<table>
<thead>
<tr>
<th>Type or stage of diabetes</th>
<th>Species</th>
<th>x-fold increase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes mellitus type 2</td>
<td>Human</td>
<td>1.3</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>2.3</td>
<td>70</td>
</tr>
<tr>
<td>Streptozotocin-induced diabetes</td>
<td>Rat</td>
<td>2.6</td>
<td>74</td>
</tr>
<tr>
<td>High-fat meal (acute)</td>
<td>Human</td>
<td>2.4</td>
<td>70</td>
</tr>
<tr>
<td>Insulin resistance</td>
<td>Human</td>
<td>1.9</td>
<td>75</td>
</tr>
</tbody>
</table>

**ADMA in lipid disorders**

One of the earliest clinical findings on ADMA was its elevation in plasma of clinically asymptomatic hypercholesterolemic subjects (Figure 19) [8].

In these patients, ADMA was found to be elevated by about two-fold as compared to age-matched normocholesterolemic controls. Importantly, this elevation was found in the absence of overt cardiovascular disease, suggesting that the increase in ADMA levels occurs early during the pathogenesis of atherosclerosis and may contribute to its progression. This is also supported by the finding that endothelial dysfunction in

Figure 19. ADMA plasma concentration in hypercholesterolemic patients and agematched normocholesterolemic controls (from [8] with kind permission of the publishers).
hypercholesterolemia is reversed by supplemental L-arginine. Similarly, hypertriglyceridemia leads to elevation of circulating ADMA levels and may induce endothelial dysfunction via this mechanism [76].

ADMA in pre-eclampsia

Pre-eclampsia is currently believed to be a consequence of impaired endothelium-dependent regulation of uterine and systemic maternal blood flow. These mechanisms have helped to explain the occurrence of the three major clinical symptoms of pre-eclampsia, hypertension, edema, and proteinuria, as well as the risk of intrauterine growth retardation and misconception, which are believed to be the results of impaired placental perfusion.

Pregnant women developing pre-eclampsia have been shown to be characterized by endothelial dysfunction, whereas healthy pregnant women have an intact endothelial function.

In several studies from Western European countries, elevated ADMA concentration was identified to be a risk marker for pre-eclampsia [44-48]. Thus, quantification of ADMA may be a diagnostic marker for women at risk of developing pre-eclampsia.

ADMA and erectile dysfunction

In many patients with erectile dysfunction the L-arginine – NO – cGMP pathway is disturbed [77]. This biochemical defect is often caused by concomitant cardiovascular disease or the presence of cardiovascular risk factors. The molecular relationship between a defect in the NO pathway and the presence of erectile dysfunction forms the background for therapeutic intervention with phosphodiesterase inhibitors like sildenafil (Viagra®), which cause increased cGMP levels and thereby mimic the functional effects of NO.

In recent years several studies have been performed which showed that erectile dysfunction can be restored by supplementation with L-arginine in a subset of patients [78, 79]. Response rates to L-arginine treatment are better when urinary excretion rates of NO metabolites are measured and found to be low before this intervention [79] – which indicates the presence of a biochemical defect in the NO pathway like it can be caused by ADMA.

Recent clinical studies showed that patients with erectile dysfunction on the basis of a concomitant coronary artery disease or diabetes mellitus not only have elevated ADMA concentration, but also decreased L-arginine levels. Thus, the ratio of L-arginine to ADMA is highly unfavourable in these patients. By contrast, in patients with traumatically caused erectile dysfunction no

| Selection of published studies that showed the existence of a relationship between elevated ADMA levels and pre-eclampsia |
|---|---|---|---|
| healthy pregnancy | Pregnant women with pre-eclampsia | x-fold increase | Reference |
| 0.4 | 0.5 | 1.3 | 33 |
| 0.5 | 1.2 | 2.4 | 34 |
| 0.8 | 2.4 | 3.0 | 35 |
| 0.5 | 0.7 | 1.4 | 62 |
| 0.4 | 0.6 | 1.5 | 63 |

Data reflect ADMA concentration [μmol/l] in plasma or serum in healthy pregnant women and in pre-eclamptic patients.
elevation of ADMA was found in comparison to healthy controls [80].

Quantification of ADMA in plasma or serum of patients with erectile dysfunction may therefore be helpful in discriminating the pathogenesis of erectile dysfunction and in directing patients towards an optimized and individualized therapy.

**ADMA and the outcome during intensive care unit treatment**

In a prospective clinical trial investigators from the Netherlands were able to demonstrate that ADMA is a powerful predictor for the survival of patients during treatment in a multidisciplinary intensive care unit:

52 patients were included in this study who were being treated in the ICU unit for failure of at least two organ systems. ADMA levels were measured, as well as numerous other biomarkers for organ function. ADMA was the most powerful predictor of ICU unit survival in multivariate analysis [81].

ADMA levels were significantly higher at the beginning of the study in those patients who died during ICU treatment than in those who survived. Patients with ADMA levels in the highest quartile had a 17-fold elevated relative risk of not surviving ICU treatment. A large portion of the patients included in this study were patients who had experienced a complication during surgical intervention, i.e. with no explicit preexisting cardiovascular disease.

The same authors were able to show that circulating ADMA concentration is controlled in large part by renal and hepatic function [81, 82]. This finding is of particular importance, as dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that inactivates ADMA, is highly expressed in liver and kidney. Therefore, this enzyme may play an important role in the regulation of ADMA levels in several disease states.

An elevation of ADMA concentration in patients with end-stage liver failure was also found by other investigators [83].
7. By which methods can ADMA be measured?

The first methods that were set up to measure ADMA were based on high-performance liquid chromatography (HPLC). These methods allowed chromatographic separation of the two structurally very similar, but functionally highly different isomers, ADMA and SDMA (Figure 20).

Methods based on chromatographic separation of ADMA and SDMA do have the disadvantage, however, that laborious sample preparation is necessary to detect the minute amounts present in human plasma and serum, which needs large financial and personnel resources.

Therefore, these methods have only been available in few specialized laboratories and they were not useful for clinical routine diagnostic use because these methods only allowed the quantification of a small number of samples per day. The same holds true for more recently developed methods based on mass spectrometry (i.e., LC-MS, LC-MS/MS, or GC-MS).

Recently a novel, easy to use enzyme immunoassay (ELISA) has been developed and validated [30]. This assay allows any clinical laboratory in which a standard ELISA plate reader is available to measure ADMA in human plasma and serum. With the help of this assay, the necessary investment in terms of time, cost, and personnel has been drastically reduced, and large series of samples can now be measured in relatively short periods of time in clinical routine.

The ADMA-ELISA, which is protected by a patent, has been validated by comparing it with state-of-the-art LC-MS and GC-MS techniques. An extraordinarily good correlation of data was found (by comparison with LC-MS/MS, \( R = 0.984; p < 0.001; N = 29 \); Figure 21).
Cross-reactivities with other L-arginine analogues present in plasma and serum has been found to be negligible (L-NMMA, 1.0%; SDMA, 1.2%; L-arginine <0.02%). The ELISA has a linear range between 0.1 and 3 µmol/l in human serum and plasma and thus covers the whole range of physiological and pathophysiological concentrations. The assay has also been validated for experimental purposes for use in mouse and rat plasma as well as in cell culture supernatants.

A list of references to studies that were performed with the ADMA-ELISA can be found at the end of this brochure on page 33; an updated version can be requested from www.dld-diagnostika.de or www.germediq.de.

More and updated information on ADMA can also be found at www.allaboutADMA.com.

Large clinical chemical laboratories that have a high throughput of samples may want to use another analytical method based on LC-MS/MS. The ADMA LC-MS/MS assay distributed by DLD Diagnostika is a ready-to-use kit containing all the components necessary to perform ADMA analyses by LC-MS/MS. Among others, derivatisation reagents and stable isotope-labelled internal standard are contained. The assay is based upon a validated and published LC-MS/MS high throughput method [84] that reduces the necessity of chromatographic separation of analytes to a minimum, thereby allowing sample run times below 2 min. For further standardisation and convenience, the kit was adapted to a 96-well plate format, allowing a maximum of comparability and reproducibility. This method is also currently being used in large epidemiological and interventional clinical trials; more information will be posted on www.allaboutADMA.com when available.

Interestingly, Robin samples are also available upon request, allowing to easily meet the goals of quality control in the clinical laboratory.

By comparison of ADMA-ELISA and ADMA-LC-MS/MS; which of these methods is better suitable for what purpose? The ADMA-ELISA is suitable for laboratories that are equipped with a standard ELISA plate reader; any laboratory technician will be able to perform ADMA assays based upon this method. It provides rapid, reliable, and conveniently affordable measurement of ADMA. The LC-MS/MS assay is a more sophisticated assay which, however, is suitable for many large clinical laboratories that have LC-MS/MS equipment at their availability anyway. The ADMA-LC-MS/MS assay kit allows easy method set-up without losing much time by method development; it contains quality controls, stable-isotope labelled internal standards, and provides Round Robin samples incl. certificates upon request. Moreover, the LC-MS/MS assay generates data on L-arginine and SDMA in the same sample run; therefore, this method is also useful when two or all three of these arginine variants are to be measured.

Figure 21. Correlation analysis between ADMA levels measured by a novel ELISA and by LC-MS/MS. The correlation was found to be linear and excellent over the whole range of (patho-)physiologically relevant concentrations. This confirms the validity of measurements using the ADMA ELISA [30].
8. What is the clinical relevance of measuring ADMA levels?

**Figure 22.**
ADMA concentrations in cholesterol-fed rabbits over time. Immediately after the onset of a high cholesterol diet ADMA levels start to increase, long before overt atherosclerotic plaques are detectable (from [85] with kind permission of the publishers).

**ADMA is a marker of endothelial dysfunction**

In experimental animals ADMA levels start to increase very rapidly after the induction of dietary hypercholesterolemia. At that time, no overt atherosclerotic lesions can be found macroscopically (Figure 22).

Similarly, in clinically healthy human subjects with isolated hypercholesterolemia and other cardiovascular risk factors elevated ADMA plasma levels have been observed (cf. Figure 19) [8].

These data suggest that ADMA is an early marker of the initial stages of atherogenesis, which may be useful in the setting of primary prevention to assess a patient’s total cardiovascular risk beyond the information generated by traditional risk factors.

In cholesterol-fed rabbits elevated ADMA levels are correlated with the extent of intimal thickening in the carotid artery, which is regarded as a useful surrogate marker for the progression of atherosclerosis in this animal model (Figure 23) [86].

**Figure 23.**
Relationship between ADMA levels and intima/media thickness of the carotid artery in cholesterol-fed rabbits. The statistical relationship between both parameters is closer than that between intima/media thickness and serum cholesterol in this animal model (from [86] with kind permission of the publishers).

Intima-media thickness in the carotid artery as measured by ultrasonography has been shown to be related to the progression of atherosclerosis in humans as well. In a clinical study in patients with end-stage renal failure, a statistically significant relationship between ADMA levels and carotid artery intima-media thickness was observed (Figure 24); in that study, ADMA was the prognostic factor that had the highest predictive power for intimal thickening among all factors tested [87].

Elevated ADMA levels are associated with reduced systemic NO production. The latter can be assessed as reduced urinary excretion of the
stable NO metabolites, nitrite and nitrate, in urine, and an impaired endothelium-dependent vasodilation [7, 8].

Taken together, these studies strongly suggest that ADMA is a marker for endothelial dysfunction in humans.

The observation of endothelial dysfunction in a given patient is regarded by many cardiologists as an indicator of an elevated cardiovascular risk for major adverse cardiovascular events or death. This conclusion has been drawn from several prospective clinical studies which have reported that patients with endothelial dysfunction (either measured as vasoconstriction in response to intraarterial infusion of acetylcholine or as impaired flow-induced vasodilation in the brachial artery) have a significantly elevated risk of experiencing major adverse cardiovascular events or death as compared to patients with functionally intact endothelium (either determined as vasodilation in response to intraarterial acetylcholine or as flow-induced vasodilation within the range of a healthy control group) in the last few years [88, 89].

As ADMA directly impairs the physiological, NO-dependent functions of the endothelial lining - as described in detail above - , its primary pathophysiological mechanism of action is different from all other known risk factors like hypertension (pressure overload of the arterial wall, reduced arterial elasticity), hypercholesterolemia (uptake of (oxidized) LDL into the intimal layer, generation of foam cells and local inflammation), smoking (induction and potentiation of oxidative damage of cellular structures within the arterial wall), etc. Accordingly, it can be expected that the deleterious effects of ADMA are independent of other risk factors and add to their effects.

Figure 24.
Relationship between ADMA levels and intima/media thickness of the carotid artery in hemodialysis patients. In this study ADMA was the serum marker which provided the best prognosis for the degree of progression of intimal thickening during 12 months of followup (from [87] with kind permission of the publishers).
Beyond the established relationship between elevated ADMA concentration and endothelial dysfunction that was described above, several studies have directly addressed the possible relationship between elevated ADMA levels and the incidence of major adverse cardiovascular events - even more, several groups of researchers observed an association between elevated ADMA concentration and death of any cause.

Miyazaki and co-workers [88] measured plasma ADMA levels in 116 clinically healthy humans who had no overt signs of coronary or peripheral arterial disease. They found a significant relationship between ADMA concentration and age, mean arterial blood pressure, and glucose tolerance. In a multivariate regression analysis, a significant relationship between ADMA and intima-media thickness of the carotid artery was found. From this study the authors concluded that ADMA is a marker for cardiovascular disease.

In a prospective clinical study ADMA plasma levels and numerous other traditional and emerging cardiovascular risk markers were determined in 225 patients with end-stage renal failure undergoing regular hemodialysis treatment [40]. After a median follow-up of 33.4 months, during which all major adverse cardiovascular events and fatalities were recorded and evaluated by an independent committee, 120 major cardiovascular events (fatal and non-fatal) and 83 deaths (53 deaths from cardiovascular causes) were detected. In a multivariate Cox regression analysis, only ADMA and age emerged as significant, independent predictors of the incidence of major adverse cardiovascular events and death of any cause. Patients whose initial ADMA plasma concentration had been above the 75th percentile had a 3-fold elevation of the risk of experiencing a major adverse cardiovascular event as compared to patients whose ADMA had initially been below the median (Figure 25).

Another group of investigators from the Netherlands studied the survival of patients undergoing intensive care unit treatment, and aimed to identify novel risk factors for survival during ICU treatment [81]. Among all biochemical markers of organ function and disease risk that were measured in this study, ADMA was the factor with the highest predictive power. Patients with elevated ADMA levels had a 17-fold increased risk of fatality during ICU treatment.

Currently numerous case-control studies and prospective clinical trials are being undertaken throughout the world including a wide variety of patient populations that will contribute further to our understanding of the role of ADMA as an independent risk factor for cardiovascular disease.
and mortality. The data generated in these studies will enhance our ability to determine the role of ADMA as a novel risk factor, and to explore its diagnostic role in different diseases.

Figure 26 gives an overview of currently available data from prospective clinical studies on the relationship of ADMA and cardiovascular events. This overview clearly shows that ADMA is an independent predictor of major cardiovascular morbidity and mortality, equally in patients at high, medium, or low absolute risk.

If one understands cardiovascular disease as a continuum ranging from the first appearance of cardiovascular risk factors in clinically asymptomatic patients, along preclinical stages of atherosclerosis, to stable coronary artery disease, and further via plaque rupture and acute coronary syndrome to myocardial infarction, remodelling in the post-infarction period, and on to chronic heart failure and cardiovascular death, Figure 27 helps to understand in which stages of this disease continuum ADMA has been found to exert a clinically relevant diagnostic role.

**Figure 26.** Summary of prospective clinical trials assessing the relationship of ADMA with severe cardiovascular events and mortality. The vertical line marks the level of diagnostic neutrality; symbols on the right depict the extent of increase in disease risk in patients with elevated ADMA vs. the respective reference group, and the vertical lines represent the 95% confidence intervals.

**Figure 27.** Cardiovascular disease can be seen as a cardiovascular continuum. Starting with cardiovascular risk factors in clinically asymptomatic persons (lower left), asymptomatic atherosclerosis proceeds into clinically overt coronary artery disease, which, after being stable for a while, may cause acute coronary syndrome or myocardial infarction upon plaque rupture. Scarring after myocardial infarction results in myocardial remodelling with incipient reduction of cardiac contractility, manifest chronic heart failure, and end-stage cardiac failure and death – if not an acute coronary event has ended lethally before. The figure demonstrates in which phases of this continuum there are prospectively generated data for ADMA as a risk predictor available.
10. Elevated ADMA - what are the therapeutic consequences?

According to our increasing understanding of the pathophysiological role of ADMA for the development of cardiovascular diseases during the last years, this molecule has become a novel goal for therapeutic intervention. This is all the more true, as ADMA has been shown to induce endothelial dysfunction not only in patients with cardiovascular or metabolic diseases, but in the elderly in general [91].

The most obvious option to antagonize the deleterious effects of ADMA on the endothelium is dietary supplementation with L-arginine. ADMA competes with L-arginine for binding at the NO synthase, and the inhibitory action of ADMA on this enzyme’s activity can be reversed by L-arginine [8, 22, 92]. Thus, the ratio of the concentrations of L-arginine versus ADMA determines NO synthase activity, as depicted in Figures 2a and 2b.

This means that elevated ADMA levels cause a relative, functional “L-arginine deficiency” even in the presence of normal plasma levels within the normal range. Targeted dietary supplementation resulting in elevation of L-arginine’s plasma levels will normalize the L-arginine-to-ADMA ratio in the presence of high ADMA levels. Normally, sufficient provision of L-arginine cannot be maintained by modification of the usual Western diet alone in patients with “L-arginine deficiency diseases” (that are characterized by impaired NO-dependent vascular function). Regular intake of dietary L-arginine supplements is necessary in most patients to reach this therapeutic goal.

The ability of exogenous L-arginine to enhance vascular function, vascular structure, and clinical course of cardiovascular diseases has been proven in a multitude of experimental and clinical studies [60, 92-95]. These studies showed that L-arginine not only improves endothelial function in patient populations characterized by elevated ADMA levels, but also reduces clinical symptoms of overt cardiovascular disease [60, 92-95].

By using L-arginine, a specific preventive strategy is available for patients for whom an increased cardiovascular risk has been determined by measuring an elevated ADMA level. The clinical effectiveness of this preventive strategy is currently being investigated in ongoing clinical trials. However, this strategy is not based on pharmacotherapeutic intervention in the classical sense, but it is a nutritional strategy which aims at maintaining physiological functions of endogenous NO and which, therefore, has its place in very early stages (i.e., in primary prevention).

When elevated ADMA levels have been found in patients that already suffer from overt cardiovascular disease, dietary supplementation with L-arginine may also have a role in secondary prevention. There are a few early clinical studies that have now shown that statins, a drug class primarily used for lowering LDL cholesterol, exert many of their beneficial effects also by improving endothelial function. These effects have been shown to vary according to a patient’s ADMA concentration.
Experimental data have proven that statins upregulate the gene expression of endothelial NO synthase. In patients with elevated ADMA levels, NO synthase will not exert its expected function because its activity is blocked [96, 97]. Indeed, a randomized clinical study has demonstrated for the first time that in patients with elevated ADMA concentration, statins only enhance endothelium-dependent vasodilation when they are administered concomitantly with dietary L-arginine [98].

Treatment with angiotensin converting enzyme inhibitors or angiotensin receptor blockers has been shown to lead to a small, but significant reduction of circulating ADMA levels. However, the clinical relevance of this finding for these drugs’ therapeutic effects is still unclear [99, 100]. It can be speculated that combination with supplemental L-arginine might also increase the beneficial effects on the vascular endothelium for these classes of drugs. Other groups of drugs that have also been shown to reduce circulating ADMA levels are the drugs used for treating type II diabetic patients, metformin and rosiglitazone [71, 75] and estrogens [101]. Substances exerting their main action by interfering with ADMA metabolism have not yet been discovered.
11. ADMA: a brief summary

Asymmetric dimethylarginine (ADMA) is a naturally occurring component of human blood plasma. It is formed as a metabolic by-product of continuous protein turnover in all cells of the body. More than one decade ago ADMA was first reported to exert biological effects by inhibiting NO synthesis. Starting with this initial finding, the pathophysiological role of ADMA has subsequently been elucidated in more detail by the collaborative efforts of many groups of researchers throughout the world. Many researchers today agree that ADMA may play a prominent role in the pathogenesis and in the progression of cardiovascular diseases - specifically atherosclerosis.

The quantification of ADMA levels in serum or plasma of a given patient therefore provides evidence beyond information gained by traditional risk markers, which help in a more profound risk analysis for the patient - and, therefore, a more specific therapeutical approach. With the availability of the competitive ADMA-ELISA a simple and rapid, yet specific, sensitive, and fully validated method is now available for diagnostic assessment of ADMA concentration that is feasible in virtually any laboratory throughout the world.

Additional and updated information on ADMA and the ADMA-ELISA can be found in the internet at www.allaboutADMA.com and www.dld-diagnostika.de.

**Figure 28.** Selection of clinical conditions that have been reported to be associated with elevated ADMA concentration and for which there is data available supporting a role for ADMA in the pathophysiology of the disease.
12. Published studies which used the ADMA-ELISA


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Asymmetric dimethyl-arginine and coronary artery calcification in young adults entering middle age: the CARDIA Study Eur J Cardiovasc Prev Rehabil. 2007; 14:222-229

Asymmetric dimethylarginine and reduced nitric oxide bioavailability in young Black African men Hypertension 2007; 49: 873-877


13. Korish AA, Arafah MM. 
Catechin combined with vitamins C and E ameliorates insulin resistance (IR) and atherosclerotic changes in aged rats with chronic renal failure (CRF) 

The administration of estrogens, combined with anti-androgens, has beneficial effects on the hormonal features and asymmetric dimethyl-arginine levels, in women with the polycystic ovary syndrome 
Atherosclerosis 2008; 196: 958-965

Effect of simvastatin on plasma asymmetric dimethylarginine concentration in patients with chronic kidney disease 
J Nephrol 2008; 21: 38-44

Role of asymmetric dimethylarginine for angiotensin II-induced target organ damage in mice 

17. Siroka R, Trefil L, Rajdl D, Racek J, Cibulka R. 
Asymmetric dimethylarginine – comparison HPLC and ELISA method (Letter to the Editor) J. Chromatogr. 2007; B850: 586-587

Disturbed angionesis in systemic scleroris: high levels of soluble endoglin 
Rheumatology 2008: in press


Serum asymmetric dimethylarginine levels among Turks: association with metabolic syndrome in women and tendency to decrease in smokers 
Türk Kardiyol Dern Ars (Arch Turk Soc Cardiol) 2008; 36: 7-13

Asymmetric Dimethylarginine and Cardiac Allograft Vasculopathy Progression: Modulation by Sirolimus Transplantation 2008; 85: 827-833


13. Reviews about ADMA


14. References cited in the text


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99. Delles C, Schneider MP, John S, Gekle M, Schmieder RE. Angiotensin converting enzyme inhibition and angiotensin II AT1-receptor blockade reduce the levels of asymmetrical N(G), N(G)-dimethylarginine in human essential hypertension. Am. J. Hypertens. 2002; 15: 590-593


