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Review

# An overview of plasma concentrations of asymmetric dimethylarginine (ADMA) in health and disease and in clinical studies: Methodological considerations☆

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**Abstract**

Recent studies among patients including those with known coronary disease demonstrate that small elevations in asymmetric dimethylarginine (ADMA) concentrations in plasma are predictive of adverse outcomes. The precision of current methodologies for quantitation of ADMA such as HPLC, MS and ELISA is discussed with respect to many reports which appear to over-estimate ADMA levels and quote broad concentration ranges. While plasma ADMA concentrations tend to increase with age, the mean for a healthy population is between 0.4 and 0.6 μM. ADMA levels may fluctuate in normal subjects, and this needs to be considered in light of the relatively small differences in ADMA concentration between healthy normal subjects and patients.


*Keywords:* Reviews; ADMA; HPLC; ELISA; Mass spectrometry; Clinical studies

## Contents



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## 1. Introduction

Over the last 15 years, $N^G$,$N^G$-dimethyl-L-arginine or asymmetric dimethylarginine (ADMA) has emerged as the principal endogenous inhibitor of nitric oxide synthase (NOS) [1]. A variety of methods for determining ADMA concentrations in plasma have been used to examine the association between various disease states and endothelial function, and have identified elevation of ADMA to be associated with endothelial dysfunction [2,3]. Furthermore, elevation of ADMA levels in plasma has been used as a biochemical correlate of adverse outcomes in cardiovascular, renal and metabolic disease states [4–7].

Methods for the determination of ADMA have proliferated. The most widely used assays are based on solid-phase extraction (SPE) of basic plasma components, followed by derivatization and chromatographic analysis by HPLC with fluorescence detection [8–12]. This technique allows simultaneous determination of ADMA, its structural isomer $N^G$,$N^{G\prime}$-dimethyl-L-arginine, i.e. symmetric dimethylarginine (SDMA) and L-arginine. In recent years, it has become apparent that there are discrepancies in ADMA concentrations reported by different investigator groups, both for healthy subjects and patients [13,14].

Methods based on mass spectrometry (MS) such as GC–MS, GC–MS–MS, LC–MS and LC–MS–MS [15–21] have been developed more recently and enable more specific quantification of compounds of interest. However, these assays require considerably more expensive instrumentation that may be out of reach for some laboratories. An ELISA method has recently been developed as a rapid tool for determining ADMA in plasma and serum [22].

This overview will address issues related to the various methodologies currently available for the quantitation of ADMA in plasma. In particular, we will discuss: (1) what is a 'true' value for ADMA in a healthy individual; (2) possible reasons for differences in reported ADMA concentrations in healthy subject groups; and (3) implication of assay imprecision and inaccuracy on results of studies comparing normal populations and disease states, as well as prospective clinical trials.

In addition to presenting an overview of published data, we will include recent findings from our laboratory investigating sources of variability in ADMA concentration within a normal population.

## 2. ADMA concentration in plasma: what is "normal"?

In 1992, Vallance et al. [1] reported for the first time that plasma levels of ADMA were elevated approximately 8-fold in patients with chronic renal failure (during the pre-dialysis period) relative to normal subjects. The mean ADMA concentration in plasma from six healthy volunteers aged 22–48 years was approximately 0.6 μM calculated from a 1:1 ratio of ADMA and SDMA. Table 1 summarizes the results of these [1] investigations and a selection of more recent methodological and clinical studies reporting levels of ADMA in healthy subjects. Mean ADMA concentrations in plasma or serum of healthy individuals reported by different groups of investigators vary from approximately 0.12 to 4.0 μM. Furthermore, whilst most studies suggest that the heterogeneity of the normal population expressed as the relative standard deviation (R.S.D.) of the mean is 10–25%, several studies show a far greater scatter of values [5,23–32]. The implication of these findings is that not all current assays are accurate or precise enough for the determination of ADMA in plasma.

Three studies listed in Table 1 determined circulating ADMA levels by HPLC in more than 100 subjects. Teerlink [33] reported a narrow concentration range of 0.35–0.89 μM ADMA in 726 subjects (mean 0.50 μM) in a general population. This agrees with the work of Miyazaki et al. [34] who reported a range of 0.30–0.82 μM ADMA in 116 individuals (mean 0.51 μM); and with current data, which showed a distribution of 0.27–0.73 μM ADMA (mean 0.50 μM) in 157 'normal' individuals. A fourth major study measuring ADMA by ELISA [35] in 500 subjects, found ADMA in plasma ranging from 0.36 to 1.17 μM (mean 0.69 μM). Studies utilising mass spectrometry (Table 1) report ADMA levels between 0.12 and 1.34 μM, albeit for smaller numbers of subjects, with the majority of these studies suggesting a mean ADMA level between 0.4 and 0.6 μM. It also appears, from the data summarised, that the variability in ADMA concentrations for a group of healthy individuals is less than 25% of the mean.

Observations of other investigators support the conclusion that levels of ADMA in plasma occur within a much narrower concentration range than has been reported in a number of clinical studies. Martens-Lobenhoffer et al. [14] recently reviewed a number of studies citing values for arginine, ADMA and SDMA in plasma and serum of healthy volunteers and concluded that a "true" value of ADMA lies within the range 0.3–0.5 μM. In addition, a review of assay methodology by Schwedhelm [36] reports mean ADMA values in normal human populations between 0.12 and 0.76 μM.

### 2.1. Variability associated with assay methodology

HPLC is a sensitive and well established technique for detecting components in complex matrices. Fluorescent derivatization of plasma extracts with *ortho*-phthaldialdehyde (OPA) has been the most widely used method for measuring ADMA in plasma. Differences in extraction procedure and chromatographic separation may account for some of the variation in mean concentrations of ADMA reported in the plasma of normal subjects (0.30–4.02 μM, Table 1). Kielstein et al. [37] have also noted the variation in ADMA levels between laboratories using different analytical methods. Techniques utilizing the specificity of MS report mean values in normals between 0.12 and 1.34 μM ADMA. The majority of MS-based assays however, suggest the mean value for ADMA in 'normal' plasma lies within the range 0.36–0.60 μM, which agrees with data from some of the investigations performed with HPLC [8,9,33,34,38]. Concentrations of ADMA determined by ELISA are marginally higher than this range (0.59–0.82 μM). The data summarised in Table 1 suggest that the three techniques of HPLC, MS and ELISA show different degrees of variability in determining ADMA concentration

Table 1
Reported mean values and ranges of plasma or serum concentrations of ADMA in healthy subjects in various published studies

| Assay methodology | ADMA (μM) | R.S.D. (%) | *N* | Age (years) | Authors |
|---|---|---|---|---|---|
| HPLC/UV | ~0.60 | N.A. | 6 | 22–48[a] | Vallance et al. [1] |
| HPLC/UV | 0.36 | 75[b] | 9 | 24 ± 12 | MacAllister et al. [47] |
| HPLC/FL/AccQ | 0.36 | 22 | 7 | N.A. | Anderstam et al. [48] |
| HPLC/FL/AccQ | 0.44 | 18 | 12 | 33 ± 10 | Heresztyn et al. [9] |
| HPLC/FL/AccQ | 0.50 | 17 | 157 | 52 ± 19 | Present study |
| HPLC/FL/OPA | 0.58 | 11[b] | 10 | 40 ± 6 | Pettersson et al. [10] |
| HPLC/FL/OPA | 1.03 | 49[b] | 31 | 45 ± 14 | Böger et al. [23] |
| HPLC/FL/OPA | 1.0 | 61[b] | 37 | 68 ± 7 | Kielstein et al. [24] |
| HPLC/FL/OPA | 0.51 | 21[b] | 116 | 52 ± 11 | Miyazaki et al. [34] |
| HPLC/FL/OPA | 1.1 | 64 | 11 | 41 ± 9 | Surdacki et al. [25] |
| HPLC/FL/OPA | 0.30 | 17 | 7 | 25–35[a] | Pi et al. [49] |
| HPLC/FL/OPA | 0.69 | 25[b] | 18 | 54 ± 8 | Abbasi et al. [50] |
| HPLC/FL/OPA | 0.73 | 39[b] | 22 | 47 ± 8 | Fleck et al. [26] |
| HPLC/FL/OPA | 0.93 | 34 | 35 | 73 ± 9 | Yoo et al. [27] |
| HPLC/FL/OPA | 0.42 | 14 | 53 | 20–40[a] | Teerlink et al. [8] |
| HPLC/FL/OPA | 0.43 | 28 | 47 | 35 ± 4 | Päivä et al. [51] |
| HPLC/FL/OPA | 2.38 | 16[b] | 12 | 69 ± 7 | Kielstein et al. [52] |
| HPLC/FL/OPA | 0.37 | 16 | 16 | 58 ± 12 | Hori et al. [38] |
| HPLC/FL/OPA | 0.76 | 15 | 35 | ~48 | Zhang et al. [11] |
| HPLC/FL/OPA | 1.57 | 54 | 48 | 55 ± 7 | Bae et al. [28] |
| HPLC/FL/OPA | 4.02 | 44 | 20 | 22 ± 2 | Cakir et al. [29] |
| HPLC/FL/OPA | 0.40 | 23 | 8 | 45 ± 6 | Perticone et al. [2] |
| HPLC/FL/OPA | 0.50 | 12 | 726 | 64 ± 7 | Teerlink [33] |
| HPLC/FL/NDA | 0.38–1.3[a] | N.A. | 50 | 48 ± 5 | Marra et al. [12] |
| CE/LIF | 0.34 | 14[b] | 5 | N.A. | Caussé et al. [53] |
| GC–MS | 0.60 | 13 | 10 | 30 ± 6 | Albsmeier et al. [16] |
| GC–MS–MS | 0.39 | 15 | 12 | ~32 | Tsikas et al. [15] |
| LC–MS | 0.48 | 15 | 40 | 15–35[a] | Huang et al. [19] |
| LC–MS | 0.36 | 19 | 47 | 20–56[a] | Martens-Lobenhoffer et al. [17] |
| LC–MS–MS | 0.12 | 37 | 20 | N.A. | Vishwanathan et al. [30] |
| LC–MS–MS | 1.34 | 19 | 25 | 47 ± 10 | Selley [45] |
| LC–MS–MS | 0.55 | 25 | 22 | N.A. | Schwedhelm et al. [18] |
| LC–MS–MS | 0.46 | N.A. | 42 | 43 | Kirchherr et al. [20] |
| LC–MS–MS | 0.37 | 16 | 14 | 22–32[a] | Martens-Lobenhoffer et al. [21] |
| LC–MS–MS | 0.45 | 16 | 24 | 48 ± 11 | Elesber et al. [54] |
| ELISA | 0.65 | 20 | 10 | N.A. | Schultze et al. [22] |
| ELISA | 0.69 | 29 | 500 | 41 ± 14 | Schultze et al. [35] |
| ELISA | 0.59 | 39 | 40 | 63 ± 8 | Krempl et al. [31] |
| ELISA | 0.82 | 35 | 31 | 31 ± 10 | Široká et al. [32] |
| ELISA | 0.72 | 15 | 11 | N.A. | Martens-Lobenhoffer et al. [42] |
| ELISA | 0.67 | 30[b] | 25 | 48 ± 5 | Dooley et al. [55] |

N.A., not available; FL, fluorescent detection; AccQ, AccQ-Fluor derivatization; LIF, laser-induced fluorescence; OPA, *ortho*-phthaldialdehyde derivatization; NDA, naphthalene-2,3-dicarboxaldehyde derivatization.
[a] Data range, mean value not provided.
[b] R.S.D. was calculated from S.E.M. and *N*-values in studies where S.E.M. quoted.

not only within the technique but also relative to one another. These respective assay techniques will be discussed further in Section 3. Other physiological bases for variability within a normal population will be discussed in the following sections.

### 2.2. ADMA variation between individuals

The mean ages of the normal subject groups in Table 1 vary considerably as many of these studies utilised age-matched controls to study a patient population. However, age per se is likely to be a minor determinant of variability in plasma/serum ADMA concentration. In a group of 157 healthy adults (71 males, 86 females), ADMA concentrations determined by HPLC (present study) correlated with age (Pearson $r = 0.44$, $P < 0.0001$). ADMA concentrations increased linearly from $0.43 \pm 0.07$ μM for young adults (20–30 years) to $0.54 \pm 0.09$ μM (70–80 years). The overall range in age of these subjects was 20–87 years with a R.S.D. of 17% relative to the mean ADMA concentration of 0.50 μM. Increase in ADMA concentration with respect to age has also been observed in other studies [34,35,38,39]. Teerlink [33] reported a R.S.D. of only 12% for ADMA in 726 patients within a range of 50–75 years. Therefore, some of the variability in mean ADMA values observed in Table 1 may be attributed to the age of the subjects studied, and a larger variation in ADMA concentration relative to the mean can be expected if the study group exhibits a broad age range. A possible explanation for the relationship between ADMA and age may be increased protein turnover, reflecting

decreased sensitivity to insulin [39] although impaired clearance of ADMA or decreased DDAH activity can not be excluded.

In a large study of 500 subjects, Schulze et al. [35] found that ADMA levels determined by ELISA increased with age in both men and women, and the increase in ADMA seemed to be accentuated in postmenopausal females. Below 50 years of age, women had lower ADMA levels (mean ± S.D.) than men (0.62 ± 0.17 μM versus 0.69 ± 0.19 μM; $P = 0.001$). However, above 50 years of age ADMA levels in the plasma of women were higher than in men (0.80 ± 0.22 μM versus 0.73 ± 0.20 μM; $P = 0.036$). Marliss et al. [39] also found gender-related differences in an elderly group (mean age 69 years) of 16 individuals (approximately 0.8 μM in females, 1.05 μM in males). In the present study (Table 1), we did not find a difference in ADMA concentration between females and males (0.49 ± 0.08 μM in 86 females versus 0.50 ± 0.09 μM in 71 males). Furthermore, there was no significant difference for plasma ADMA levels in women and men in the age group below 50 years (0.47 ± 0.08 μM in 44 females, 0.46 ± 0.08 μM in 25 males) or the age group above 50 years (0.52 ± 0.07 μM in 42 females, 0.53 ± 0.08 μM in 46 males).

### 2.3. ADMA variability within the individual

An issue which has received little attention in previous studies is the variation in ADMA (as well as SDMA and arginine) concentrations within individuals. We recently measured ADMA in seven healthy subjects (three males, four females) at weekly intervals for 4 weeks. Blood was collected at the same time each week and the heparinised plasma prepared and stored until all samples were analysed in the same batch. A previously published method [9] was used with modifications as follows. A smaller volume of plasma (0.15 ml) was extracted to allow arginine to be quantitated from the same extract as ADMA and SDMA. Samples and standards were spiked with a lesser quantity of internal standard (0.06 ml of 5 μg/ml $N^G$-monomethyl-L-arginine, i.e. NMMA), 0.2 ml of 10% 5-sulfosalicylic acid and made up to 1.6 ml with distilled water prior to SPE extraction. The SPE eluates were dried under nitrogen gas, reconstituted in 0.15 ml distilled water and derivatized with AccQ-Fluor. ADMA and SDMA (20 μl injection) were analysed with the column and mobile phase described previously [9] except for the column temperature, which was kept constant at 40 °C. The gradient program was as follows: solvent A-solvent B (v/v) = 94:6 (16 min), 86:14 (16.4–29 min), 10:90 (29.5–33 min), return to 94:6 at 33.5 min with a total run time of 42 min. Recovery of ADMA and SDMA in plasma was 95 ± 1% ($n = 5$) and 94 ± 3.8% ($n = 5$), respectively. Arginine was quantitated from a 2 μl injection of the same derivatized extract with a column temperature of 30 °C and the following gradient program: solvent A-solvent B (v/v) = 95:5 (19.5 min), 84:16 (20–27 min), 10:90 (27.5–30.5 min), return to 95:5 at 31 min, and a total run time of 38 min. Arginine recovery from plasma was 84 ± 4.2% ($n = 5$).

Fig. 1 illustrates the variation in concentration of ADMA within seven individuals. All plasma samples were analysed in

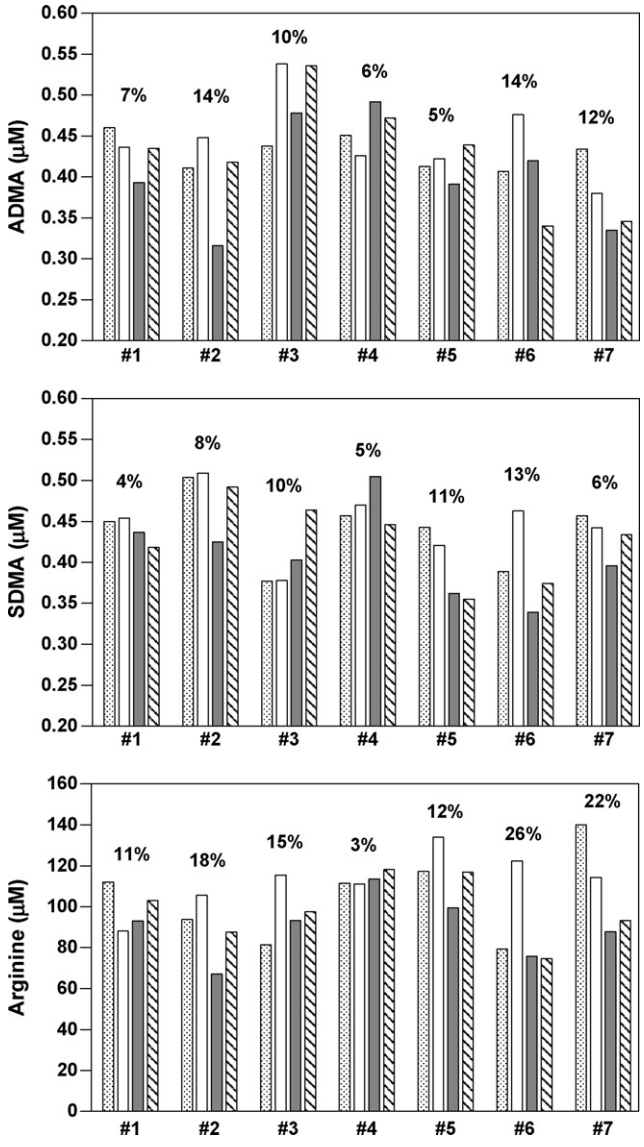


Fig. 1. ADMA, SDMA and arginine concentration (μM) in plasma from seven healthy individuals at 1 week intervals for 4 weeks, measured by HPLC [9]; numbers over the columns indicate R.S.D. values. Subjects #1, #3, #4 were males; (dot bar, week 1; open bar, week 2; shaded bar, week 3; striped bar, week 4).

replicates of four. The intra-assay variability ($n = 4$) for ADMA was <3.5%, whilst the R.S.D. for the seven subjects varied from 5 to 14% over the 4 week period. Interestingly, ADMA levels in some individuals changed by approximately 0.1 μM (e.g., subject #2, 0.13 μM from week 2 to 3; subject #3, 0.1 μM from week 1 to 2; and subject #6, 0.08 μM from week 3 to 4). The variations in ADMA concentration tended to be associated with parallel changes in SDMA and arginine concentration, suggesting a common mechanism such as periodic variability in rates of protein catabolism. The intra-assay precision of this method for SDMA and arginine was <5% ($n = 4$). A better understanding of the causes of such variations in ADMA concentrations is mandatory before changes in individual subject values are utilised for monitoring of individual cardiovascular risk.

Table 2
Impact of intra-group variability on outcome measures in clinical studies. Differential ADMA is the percent difference in mean ADMA concentration between the subgroup with adverse outcomes and those without adverse outcomes

| Patient group | *N* | Differential ADMA (%) | Adverse outcome | Authors |
|---|---|---|---|---|
| Previous coronary event | 70 | +19 | Acute coronary events | Valkonen et al. [4] |
| Haemodialysis | 225 | +34[a] | Mortality | Zoccali et al. [5] |
| Stable angina/PCI | 153 | +22 | Acute coronary events | Lu et al. [6] |
| Organ failure | 52 | +42[b] | Mortality | Nijveldt et al. [56] |
| Unstable angina | 36 | +15 | Acute coronary events | Krempl et al. [31] |
| CAD | 1872 | +11[a] | Mortality/AMI | Schnabel et al. [7] |

*N*, number of patients studied; PCI, percutaneous coronary intervention; CAD, coronary artery disease; AMI, acute myocardial infarction.
[a] ADMA values provided were expressed as median, not mean.
[b] This value is based on the highest quartile vs. mean of the whole group, relative risk = 17.2 (mean data for patients with adverse and favourable outcomes not available).

### *2.4. Implication of elevated ADMA concentration in cardiovascular disease*

Elevated ADMA concentrations in plasma have been described in a wide variety of cardiovascular (and non-cardiovascular) disease states. Tables 2 and 3 illustrate the reported magnitude of the differences in ADMA concentrations between normal subjects and various pathological states, as these are relevant to the suitability of some of the current methodologies. Relatively small increases in ADMA levels within patient groups in studies with event-driven endpoints (Table 2), may be associated with significant morbidity and/or mortality. The mean differences observed between the subgroups with adverse outcomes and the patients without adverse events are of the same order as the R.S.D. values for normal populations shown in Table 1.

Of particular interest is a large study [7] of 1872 patients with known coronary disease that demonstrated that while ADMA concentrations were significantly elevated in patients who subsequently died, the extent of elevation relative to the mean of the group who survived was only 11%. These results suggest that measurement of ADMA levels is unlikely to provide prognostic data relevant to individuals but may be a useful indicator of therapeutic efficacy in certain patient groups. It also emphasises the need for precise and accurate methodology for measuring ADMA and a consensus of opinion on what is a 'normal' basal value of ADMA for a healthy subject group.

The need for precise and accurate measurement of ADMA in plasma is further illustrated by the data in Table 3 which depicts a number of studies comparing ADMA concentrations between patients with cardiovascular disease states and age-matched normal subjects. ADMA concentrations were elevated 2- to 6-fold compared to controls in seven studies of chronic renal failure. Some of the studies of hypertension and hypercholesterolaemia report 2-fold elevations in the patient group. While these observations might reflect differences in treatment status of patients, it is more likely that differences in assay specificity and accuracy have contributed to the variations seen in the plasma concentration of ADMA in these different patient groups.

## 3. Comparison of available methodologies

### *3.1. HPLC methodology*

HPLC in combination with SPE remains one of the most accessible techniques for determining ADMA levels in plasma and other biological matrices. Tables 1 and 3 suggest that some HPLC assays over-estimate ADMA levels in the plasma of healthy and patient groups, and the variability of ADMA concentrations is greater than can be attributed to age, gender or daily variations. The reasons for this may be: (1) the SPE procedures utilized may not be sufficiently optimized to eliminate interfering plasma components; (2) the liquid chromatography of the derivatized extracts is not selective enough to separate ADMA from SDMA and other potentially interfering endogenous plasma components. Choice of column, mobile phase composition and pH, type of derivatizing reagent and column temperature are all critical in altering the chromatography of the methylarginines, internal standard and endogenous plasma components. In our assay development, raising or lowering the pH of the buffered mobile phase by 0.5 of a pH unit resulted in a respective increase or decrease in the retention times of the dimethylarginines. Raising the column temperature from 30 to 40 °C in the assay using AccQ-Fluor derivatives [9] decreased the total run time and altered the retention times of ADMA and SDMA relative to endogenous components in animal plasma. Chromatography of OPA derivatives on a phenyl column with a phosphate buffered mobile phase (see below) separated SDMA and ADMA very well (14.5 and 17.5 min respectively), however, the internal standard NMMA and arginine in particular were less well resolved from endogenous plasma components under these conditions. ADMA and SDMA derivatives (OPA and AccQ-Fluor) elute from C18 columns in the opposite order to phenyl columns which has been noted previously [40].

A number of published methods [8,9,11,12] provide extensive detail of validation data including illustrations of chromatograms of plasma extracts. There are a number of options in the choice of extraction cartridges and chromatographic conditions for the determination of ADMA in biological samples. Most assays include a sample extraction step by SPE with cation exchange cartridges, while Zhang et al. [11] use cold ethanol

Table 3
Summary of data involving comparisons of ADMA concentrations in plasma or serum between normal subjects and patients with coronary risk factors (hypertension and hypercholesterolaemia) and chronic renal failure

| Patient group | ADMA (μM, mean ± S.D.) | | Authors |
|---|---|---|---|
| | Patients | Normals | |
| Chronic renal failure | 0.90 ± 0.25[a] | 0.36 ± 0.27[a] | MacAllister et al. [47] |
| Chronic renal failure | 0.70 ± 0.27 | 0.36 ± 0.08 | Anderstam et al. [48] |
| Chronic renal failure | 1.81 ± 0.82 | 0.52 ± 0.21 | Tsikas et al. [40] |
| Chronic renal failure | 6.0 ± 3.3[a] | 1.0 ± 0.61[a] | Kielstein et al. [24] |
| Chronic renal failure | 2.52 ± 1.14 | 0.95 ± 0.39 | Zoccali et al. [5][b] |
| Chronic renal failure | 0.70 ± 0.12 | 0.37 ± 0.06 | Martens-Lobenhoffer et al. [42] |
| Chronic renal failure | 1.81 ± 0.57 | 0.82 ± 0.29 | Široká et al. [32] |
| Hypercholesterolaemia | 2.17 ± 1.05[a] | 1.03 ± 0.5[a] | Böger et al. [23] |
| Hypercholesterolaemia | 0.44 ± 0.19 | 0.43 ± 0.12 | Päivä et al. [51] |
| Hypercholesterolaemia | 0.46 ± 0.07 | 0.37 ± 0.06 | Martens-Lobenhoffer et al. [42] |
| Hypertension | 2.4 ± 1.1 | 1.1 ± 0.7 | Surdacki et al. [25] |
| Hypertension | 0.59 ± 0.13 | 0.43 ± 0.12 | Päivä et al. [51] |
| Hypertension | 0.59 ± 0.14 | 0.40 ± 0.09 | Perticone et al. [2] |

[a] S.D. was calculated from S.E.M. and *N* in studies where S.E.M. quoted.
[b] Median values published.

cleanup prior to derivatization in order to quantitate a range of arginine metabolites. Whilst OPA derivatives produced with 2-mercaptoethanol are unstable, the assays mentioned above [8,9,11,12] utilise derivatives with improved stability such as OPA with 3-mercaptopropionic acid [8,11] or naphthalene-2,3-dicarboxaldehyde (NDA) [12], and hold the derivatized extracts in a refrigerated autosampler compartment to minimize degradation. The assay utilising AccQ-Fluor reagent [9] produces fluorescent derivatives stable at room temperature for 1 week. Chromatographic separation in these assays is carried out on C18 [8,9,11] or phenyl [12] columns with a combination of buffer and solvent with run times varying from 30 to 45 min. These assays quantitate ADMA at >85% recovery with separation of ADMA and SDMA varying from satisfactory [8,11] to excellent [9,12]. While these assays [8,9,11,12] show good precision and sensitivity for measuring ADMA in plasma, two of these methods [11,12] report higher ADMA (and SDMA) concentrations in normal subjects than the MS methods [15–20] and HPLC studies on large numbers of subjects [33,34].

In the present study, we compared the quantitation of ADMA and SDMA by derivatization with OPA or AccQ-Fluor in seven human and animal plasma samples. Following SPE with SCX, the eluates were dried under nitrogen gas, reconstituted in water and derivatized with these fluorescent reagents. The AccQ-Fluor derivatives were analyzed with the modified HPLC conditions described above [9]. The OPA derivatives were prepared by the procedure of Teerlink et al. [8] and separated on Alltima Phenyl 5 μm (250 mm × 4.6 mm, Alltech Associates Pty Ltd., Australia). Mobile phase A consisted of 20% (v/v) methanol in 0.05 M phosphate buffer, pH 6.9; mobile phase B was 60% (v/v) methanol in 0.05 M phosphate buffer, pH 6.9. The OPA derivatives (20 μl) were separated at a column temperature of 30 °C by the following gradient: solvent A-solvent B (v/v) = 95:5 (18.5 min), 20:80 (19–22 min), return to 95:5 at 22.5 min. The concentrations determined for ADMA (0.76 ± 0.19 μM by AccQ-Fluor versus 0.76 ± 0.22 μM by OPA, $P = 0.9$) and SDMA (0.51 ± 0.29 μM by AccQ-Fluor versus 0.46 ± 0.24 μM by OPA, $P = 0.2$) were compared by paired *t*-test. Based on the peak areas of ADMA the assays were of similar sensitivity. Therefore, ADMA values obtained by HPLC using one of these fluorescent reagents can be verified with the second reagent on a different column.

### 3.2. ELISA

#### 3.2.1. ELISA comparison with GC–MS, HPLC and LC–MS: currently available data

An ELISA assay for the analysis of ADMA in plasma and serum has recently become commercially available (DLD Diagnostika GmbH, Hamburg, Germany). This technique is based on the principle of competitive immunoassay and allows relatively large numbers of samples to be analysed more quickly than by HPLC including SPE cleanup. It is however, only selective for ADMA, therefore SDMA and arginine need to be measured by other techniques if these data are required.

The first published validation by Schulze et al. [22] reported intra- and inter-assay R.S.D. of 7.5 and 10.3% respectively, for repeated analysis of control sera at normal concentration ranges. They found a 1:1 correlation between data obtained for nine human serum samples with the ELISA kit and GC–MS. A comparison between ELISA and LC–MS–MS performed on 29 serum samples demonstrated a strong linear relationship between these two methods however, the ELISA technique overestimated [41] the quantity of ADMA in the samples by approximately 15%. This was confirmed by Martens-Lobenhoffer et al. [42] who also found that ELISA produced higher values than their LC–MS assay. ADMA concentrations determined by ELISA were almost twice the value of those obtained by LC–MS in unspiked plasma of healthy individuals and three patient groups. This finding was attributed to interference of sample matrix with the ELISA kit [42].

Valtonen et al. [43] recently compared ADMA concentrations determined in 55 authentic (i.e., unspiked) serum samples with the ELISA method and a modified HPLC method based on

the assay of Teerlink et al. [8]. Derivatization of the extracted samples with OPA was performed by the autosampler just before injection to minimise degradation of the fluorescent derivative. Valtonen et al. [43] concluded that there was a poor correlation between the two assays ($r^2 = 0.0972$, $P = 0.796$), however, they also reported a high intra-assay R.S.D. of 19% with the ELISA method which may have contributed to some of the variability observed. In view of the increasing interest in the determination of ADMA in clinical research, more comparisons of methods would be useful [41].

### 3.2.2. ELISA comparison with HPLC: present data

We recently determined the concentration of ADMA in 60 samples of plasma and serum with the ELISA assay and HPLC (see Section 2.3). Plasma/serum was collected from a range of subjects including 15 healthy individuals, 17 renal patients, two pulmonary hypertensive patients, two obese subjects and 10 elderly subjects over 65 years of age. Samples were analysed in duplicate by ELISA along with the controls and standards included in the kits. Absorbance was measured at 450 nm with reference wavelength 655 nm. The lowest standard (A, 0 μM ADMA) was assigned a concentration of 0.001 so the ADMA values could be log-transformed, followed by a non-linear fit of the data with a sigmoidal dose-response equation (GraphPad Prism). The analytical recovery of ADMA spiked in plasma at 0.5 μM was 89% for the ELISA technique and intra-assay precision was 3% ($n = 4$).

Concentrations of ADMA determined by HPLC ranged from 0.3 to 1.0 μM (Fig. 2A). Whilst there was a linear relationship ($r^2 = 0.69$) between the values of ADMA determined by ELISA and HPLC over the concentration range found in normal subjects and patients, the ELISA assay appeared to over-estimate ADMA concentrations relative to HPLC. A Bland–Altman [44] plot (Fig. 2B) of the difference between the two assays (ELISA–HPLC values) versus their mean confirms that ELISA over-estimates the concentration of ADMA in plasma and serum. The bias calculated by GraphPad Prism over the range of averaged concentrations was 0.13 μM. However, it is apparent that the bias is not constant over this range, and that the difference between the two assays increases as ADMA concentration increases. Furthermore, the 95% limits of agreement encompass a range of approximately 0.47 μM. These results are consistent with previously reported data [41–43] which suggest that the ELISA method is not as selective and accurate as HPLC and MS-based assays for measuring ADMA concentrations in different groups of subjects.

Our study also included six plasma samples from a healthy individual, spiked with incremental concentrations of ADMA from 1.4 to 2.7 μM to assess the accuracy of the ELISA kit at these unusually high levels, as performed by Schulze et al. [22]. These data are not presented as they are outside the range of ADMA levels found in normal and patient samples in this laboratory, however, the linear correlation improved from $r^2 = 0.69$ to 0.93 when these values were included. It is apparent that these higher concentrations weight the fit of the regression line towards the value of one and do not accurately assess the correlation between the two assays at physiologically relevant

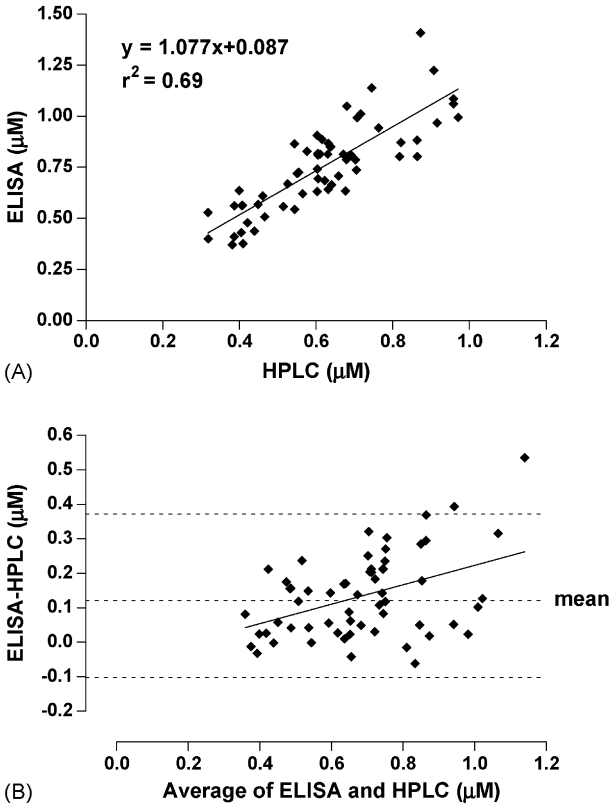


Fig. 2. Comparison of ADMA (μM) levels determined by HPLC and ELISA in 60 biological samples (14 serum, 46 plasma) in the present study. (A) Linear regression plot of ADMA values determined by the two assays; (B) Bland–Altman plot of difference in ADMA concentration between the two methods vs. average of the values obtained. Solid line is the regression line; dotted lines denote mean and 95% limits of agreement.

concentrations of ADMA in plasma and serum. Valtonen et al. [43] also compared HPLC with ELISA in 55 patients within a relatively narrow concentration range of 0.39–0.91 μM ADMA which may have contributed to their finding of a poor correlation between these two techniques.

### 3.3. Effect of blood anticoagulation: serum or plasma

Early ADMA assays measured ADMA in heparin-plasma, however, different laboratories now report values from EDTA-plasma and serum. In order to determine whether different blood anticoagulation methods influence the quantitation of ADMA, blood was collected from five individuals (three controls, two renal patients) in three different vacutainer tubes (VACUETTE® Lithium Heparin tube, EDTA K3 tube and Serum Sep. Clot Activator tube from Greiner Bio-One, Austria) to compare the levels of ADMA in heparin-plasma, EDTA-plasma and serum respectively by HPLC and ELISA. Comparing heparin-plasma and serum, no significant difference by paired *t*-test was observed in ADMA values (mean ± S.D.) obtained by HPLC ($0.57 \pm 0.10$ μM versus $0.56 \pm 0.10$ μM) and ELISA ($0.73 \pm 0.17$ μM versus $0.72 \pm 0.16$ μM). However, values of ADMA determined in EDTA-plasma were significantly lower than those in heparin-plasma by HPLC ($0.52 \pm 0.10$ μM; $P < 0.005$) and ELISA ($0.65 \pm 0.17$ μM; $P < 0.05$). Quantitation

of SDMA did not appear to be influenced by the type of blood collection tube, but levels of arginine in EDTA-plasma ($71 \pm 35$ μM) were lower than those obtained in heparin-plasma ($87 \pm 36$ μM) and serum ($99 \pm 40$ μM), $P < 0.05$. The volume of EDTA solution in the collection tubes was approximately 0.02 ml and did not alter the significance of these results. Teerlink [8] did not find any difference between EDTA-plasma and heparin-plasma for ADMA, SDMA or arginine by HPLC determination, however, he found that arginine concentrations were 60% higher in serum compared to heparin-plasma. It is therefore recommended that the blood collection procedure is standardized for the determination of ADMA, SDMA and arginine to avoid influencing assay results.

### 3.4. Mass spectrometry

GC–MS [16], GC–MS–MS [15], LC–MS [17,19] and LC–MS–MS [18,20,21] methods for ADMA developed in the last 3 years have provided data that agree with those from HPLC assays. Mass spectrometric methods have the advantage of specificity thereby overcoming the problem of matrix interference. These assays suggest that ADMA levels in healthy individuals are at the lower end of the concentration range shown in Table 1. Only two of the methods [30,45] reported mean ADMA values for healthy individuals outside of the range of 0.36–0.60 μM. In a recent inter-laboratory comparison [36] replicate human blood samples were analysed at basal and two spiked concentrations of ADMA by three HPLC assays, four GC–MS- and LC–MS-based methods and by the ELISA method. The results from this comparison were presented in a figure (Fig. 2 in [36]) from which it is apparent that not all the MS-based techniques agreed with one another. These results do, however, illustrate that a number of analytical techniques are currently available to quantitate ADMA with a greater degree of accuracy than has been shown in the past. It also demonstrates the value of inter-laboratory comparisons in determining levels of analytical accuracy in different laboratories and evaluating their quality control procedures.

The importance of precise and accurate assays for investigating the pathophysiological importance of ADMA is further illustrated by a study of patients admitted to hospital with stable angina [46]. ADMA plasma concentrations were measured in 80 patients by GC–MS–MS [15] and were found to correlate with the severity of coronary artery disease (CAD). ADMA levels in the plasma of patients with coronary stenoses involving three coronary vessels (CAD 3) showed a modest but significant increase in concentration compared to controls (CAD 0) i.e., $0.58 \pm 0.02$ μM versus $0.47 \pm 0.02$ μM, $P < 0.001$. Interestingly, in this study the concentration of ADMA in the CAD 0, CAD 2 and CAD 3 groups correlated inversely with the number of endothelial progenitor cells in the blood of these patient groups.

## 4. Concluding remarks

This overview summarises and discusses some of the reported data on ADMA levels in the plasma of healthy individuals and patients suffering from various diseases, and highlights the fact that there is considerable variation in the basal ADMA concentrations reported by different groups of investigators. It appears that the concentration of ADMA in the plasma of healthy subjects lies between 0.4 and 0.6 μM. Factors which influence variability in ADMA levels determined for a subject group include age and an inherent variation within individuals that has been described in this paper for the first time. However, the greatest determinant of variability in reported ADMA levels is assay methodology.

Mass spectrometry is the most specific analytical tool available to analysts measuring low levels of compounds in complex matrices, and this technique has provided reasonably consistent results for ADMA quantitation. A number of studies measuring ADMA in the plasma of healthy individuals by MS-based approaches confirm that levels lie within a narrow concentration distribution. HPLC assays for ADMA show the greatest disparity in measured values, however a number of validated methods have been published which quantitate ADMA in large numbers of subjects with a high degree of accuracy. There is a need for a more standardized approach with this technique since many laboratories have this instrumentation available to them. The ELISA technique provides a rapid tool for determining ADMA in plasma and serum, however, results presented in this paper and the results from other groups show that ELISA over-estimates ADMA levels compared to HPLC and LC–MS. The bias in the assay needs to be considered in light of the issues discussed in this paper.

There is considerable overlap between ADMA concentrations in healthy individuals and in patient groups and this further highlights the need for precise and accurate assays. A number of prospective studies have shown an association between relatively small elevations in ADMA levels (11–42%) in the patient group studied and adverse outcomes such as acute coronary events or death. Therefore, it is doubtful whether reliance on a single measurement of ADMA in the plasma of an individual will prove useful for risk indexation in patients.

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