Asymmetric Dimethylarginine Independently Predicts Fatal and Nonfatal Myocardial Infarction and Stroke in Women
24-Year Follow-Up of the Population Study of Women in Gothenburg

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Objective—Asymmetrical dimethylarginine (ADMA) reduces nitric oxide by inhibiting nitric oxide synthase is associated with cardiovascular disease (CVD). Our study examined the association of ADMA with CVD prospectively in a healthy population-based cohort of women.

Methods and Results—We measured baseline ADMA of 880 women in the Population Study of Women in Gothenburg using high-performance liquid chromatography. After adjustment for traditional risk factors, creatinine clearance, and homocysteine using Cox models, the HR (95% CI in parentheses) of CVD end points at 24 years for a 0.15 μmol/L (1 SD) increase in ADMA were: all-cause mortality 1.12 (0.96, 1.32), fatal CVD 1.30 (1.04, 1.62), total CVD events 1.29 (1.09, 1.53). The top quintile (ADMA ≥0.71 μmol/L) compared with the bottom four-fifths, conferred a cumulative risk 22 versus 14%, relative risk 1.75 (95% CI 1.18, 2.59) and population attributable risk 12.7% of total CVD events, and further identified individuals who are at higher than expected risk based on the SCORE and Framingham systems.

Conclusions—A 0.15 μmol/L increase in baseline ADMA levels is associated with approximately 30% increase in incident cardiovascular risk at 24 years in women after adjustment. ADMA levels ≥0.71 μmol/L enhances CVD risk assessment in women. (Arterioscler Thromb Vasc Biol 2008;28:961-967)

Key Words: asymmetrical dimethylarginine cardiovascular diseases myocardial infarction stroke women

A

symmetrical dimethylarginine (ADMA) has been proposed as a novel risk marker for cardiovascular disease 1-4 because of its possible role in endothelial dysfunction. 5-7 A process thought to be pivotal in the development of arteriosclerosis. 8 Both symmetrical dimethylarginine (SDMA) and ADMA are structural analogues of the amino acid L-arginine, the substrate for the synthesis of nitric oxide by the enzyme nitric oxide synthase. 9 ADMA is an endogenous competitive inhibitor of nitric oxide synthase. 1,5 Therefore, raised levels of ADMA lead to reduced nitric acid production and hence impair endothelial function.

Both ADMA and SDMA are excreted by the kidneys. Most of endogenous ADMA is broken down by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) into dimethylamine and citrulline, which are also excreted through the kidneys. Hence, ADMA, as well as SDMA, accumulates in patients with renal failure. 5 However, SDMA does not inhibit nitric oxide synthase, nor is it a substrate for DDAH. Furthermore, it has been thought that the effect of homocysteine on endothelial function may be mediated through ADMA 10,11 because of the fact that homocysteine may inhibit DDAH, leading to increased ADMA levels, and hence reduced nitric oxide production.

ADMA has been reported to be raised in the presence of other cardiovascular risk factors, as well as in several disease states. These cardiovascular risk factors include traditional risk factors such as blood pressure, 12-14 serum cholesterol, 6 serum triglycerides, 15 gestational diabetes, 16 insulin resistance, 17 smoking, and nontraditional risk markers such as homocysteine, 11,18,19 C-reactive protein, 20 and vascular cell adhesion molecule (VCAM). 21 Clinical conditions that have been associated with increased ADMA concentrations include peripheral vascular disease, 22 increased carotid intima media thickness, 20,21,23 chronic heart failure, 24 unstable angina, 25 stroke, 26 hyperthyroidism, 27 preeclampsia, 28 intensive care deaths, 29 and coronary artery calcium score. 30

At pathological levels found in end-stage renal failure, ADMA was found to be the second strongest independent predictor of mortality and cardiovascular events after age, and more powerful than other traditional risk factors and C-reactive protein. 31 ADMA has also been shown to predict cardiovascular events in patients with Type 2 diabetes 32 and

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961
peripheral arterial disease.\textsuperscript{33} ADMA, along with N-terminal prohormone brain natriuretic peptide, was also useful in cardiovascular risk stratification in patients with chronic heart failure.\textsuperscript{34}

There are now an increasing number of studies of ADMA in patients with preexisting coronary artery disease. One of the earliest is a prospective nested case-control study of middle-aged nonsmoking Finnish men which showed ADMA to be an independent predictor of coronary events over traditional risk factors, especially in those who had a history of coronary heart disease.\textsuperscript{35} In a Taiwanese population, ADMA levels measured before coronary angioplasty were also found to be an independent predictor of later cardiovascular events, even after allowing for traditional risk factors, homocysteine, C-reactive protein, left ventricular function, history of previous cardiovascular disease and medications.\textsuperscript{36}

The AtheroGene Study, a prospective cohort study on patients with coronary artery disease, showed that ADMA was related with increased CVD events.\textsuperscript{37} A multicenter German case-control study showed ADMA to be associated with increased coronary heart disease risk.\textsuperscript{38} Other more recently reported studies include the Ludwigshafen Risk and Cardiovascular Health Study showing the association of ADMA with all-cause and CVD mortality in an angiographic cohort of patients with coronary artery disease,\textsuperscript{39} and the population based Monitoring of Trends and Determinants in Cardiovascular Disease/Kooperative Gesundheitsforschung in der Region Augsburg study also showed that ADMA predicted future coronary events in nonsmoking healthy men using a nested case-control design.\textsuperscript{40}

Observational and case-control studies are regarded as less reliable indicators of a possible causal relationship between a risk marker and a disease process than prospective studies in which the marker clearly precedes the occurrence of the disease. There is also a paucity of studies examining the association of ADMA with incident CVD in healthy populations. In this study, we examine the role of ADMA in predicting cardiovascular risk and determine its possible relationship with homocysteine and other risk factors in a healthy population based cohort study of women.

\section*{Methods}

\textbf{Summary}

For detailed methods, please see the supplemental materials (available online at http://atvb.ahajournals.org). The study population comprised women recruited to the ongoing \textit{Kvinnostudier}, the Population Study of Women in Gothenburg which has been described previously.\textsuperscript{41–44} The stored serum samples used in this study were collected over a period of under 1 year at the baseline survey interview and examination in 1968/69. We determined ADMA levels using a validated high-performance liquid chromatography (HPLC) method.\textsuperscript{45} The coefficients of variation within-assay were 2.0 to 5.8\% and between-assay were 1.2 to 7.0\% for lower levels and 2.1 to 7.0\% for higher levels. Homocysteine was measured using the Abbot IMX assay in a previous study.\textsuperscript{46} We used Cox’s proportional hazards models to examine the association of ADMA as a continuous variable and time as the dependent variable with the following cardiovascular end-points at 24 years follow up: (1) All-cause mortality; (2) Myocardial infarction (MI) and stroke deaths; (3) Noncardiovascular deaths; (4) Myocardial infarction deaths; (5) Stroke deaths; (6) Myocardial infarction & stroke events (fatal & nonfatal); (7) Myocardial infarction events (fatal & nonfatal); and (8) Stroke events (fatal and nonfatal).

Analyses involving ADMA stratified by age were followed by multivariable models which consisted of sequentially adding further prespecified covariates. Model 1 adjusted for established cardiovascular risk factors commonly used in risk assessment (systolic and diastolic blood pressure, serum cholesterol and smoking). Model 2 further adjusted for traditional cardiovascular risk factors which are less established in terms of causality but nevertheless easily obtained and commonly available (serum triglycerides, plasma glucose, waist and hip circumference, and body mass index). Model 3 also included creatinine clearance, homocysteine, and vitamin B12. In the interest of having a cut point of clinical utility, we then considered the top quintile of the distribution of ADMA and presented the cumulative risk, relative risk, population attributable risk, and additional value to the Systematic Coronary Risk Evaluation (SCORE) and Framingham systems. The software used for the statistical analyses was Intercooled Stata 9.2 for Windows.

\section*{Results}

Of the total 1381 subjects in the original cohort, 998 had stored serum samples available for laboratory analysis. Of these, 28 were unsuitable for laboratory determination of ADMA levels and a further 82 were either duplicated or triplicated for 32 women. All duplicated samples were averaged, except for 3 which were excluded because of wide disagreements between ADMA or SDMA levels within the same individual. We excluded 5 women who had ADMA levels which were very far outside the normal distribution (\(>2.0\mu\text{mol/L}\)). The final number of women who had ADMA levels included in the statistical analyses for the present study was 880 (63.7\%). The baseline characteristics of the study population are summarized in Table 1. We found no significant differences in risk factor distributions between those who had stored samples available for ADMA determination and those without (supplemental Table I).

Spearman correlations of ADMA with other traditional and nontraditional risk factors generally showed weak univariate associations, but were statistically significant for age (\(r=0.23, \text{P}<0.001\)), cholesterol (\(r=0.11, \text{P}<0.001\)), smoking (\(r=0.07, \text{P}=0.02\)), triglycerides (\(r=0.12, \text{P}<0.001\)), waist circumference (\(r=0.15, \text{P}<0.001\)), hip circumference (\(r=0.07, \text{P}=0.03\)), BMI (\(r=0.12, \text{P}<0.001\)), and vitamin B12 (\(r=-0.24, \text{P}<0.001\)).

The hazard ratios (HR) of the various end points for a 0.15 \(\mu\text{mol/L}\) (1 standard deviation) increase in ADMA are listed in Table 2, determined using Cox proportional hazards models, sequentially adjusting for more traditional cardiovascular risk factors, creatinine clearance, homocysteine and its covariates, using time to event as the dependent variable. After adjustment (Model 3), there was a statistically significant increase in total cardiovascular deaths (myocardial infarction and stroke) HR 1.30 (95\% CI 1.04, 1.62), total fatal and nonfatal myocardial infarction and stroke HR 1.29 (95\% CI 1.09, 1.53), and total fatal and nonfatal myocardial infarction HR 1.33 (95\% CI 1.08, 1.63). This effect of ADMA was independent of traditional cardiovascular risk factors, creatinine clearance, and homocysteine. The magnitude of the increased risk conferred by ADMA was consistent and robust through the sequential adjustments during the multivariate analyses.
Table 1. Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Age Cohort at Baseline (Numbers Recruited at Baseline)</th>
<th>Age 38 Cohort (n=372)</th>
<th>Age 46 Cohort (n=431)</th>
<th>Age 50 Cohort (n=398)</th>
<th>Age 54 Cohort (n=180)</th>
<th>Total No. (n=1381)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With available samples, n (%)</td>
<td>182 (48.9%)</td>
<td>317 (73.5%)</td>
<td>282 (70.9%)</td>
<td>89 (49.4%)</td>
<td>880 (63.7%)</td>
</tr>
<tr>
<td>ADMA, μmol/L</td>
<td>0.59 (0.15)</td>
<td>0.60 (0.16)</td>
<td>0.64 (0.14)</td>
<td>0.65 (0.16)</td>
<td>0.62 (0.15)</td>
</tr>
<tr>
<td>SDMA, μmol/L</td>
<td>0.41 (0.14)</td>
<td>0.40 (0.10)</td>
<td>0.40 (0.10)</td>
<td>0.42 (0.13)</td>
<td>0.40 (0.12)</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>121 (15)</td>
<td>131 (20)</td>
<td>138 (22)</td>
<td>143 (24)</td>
<td>132 (21)</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>81 (9)</td>
<td>84 (10)</td>
<td>88 (11)</td>
<td>90 (12)</td>
<td>85 (11)</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>6.3 (0.9)</td>
<td>6.8 (1.5)</td>
<td>7.2 (1.1)</td>
<td>7.3 (1.1)</td>
<td>6.9 (1.3)</td>
</tr>
<tr>
<td>Smokers, %</td>
<td>45</td>
<td>44</td>
<td>36</td>
<td>37</td>
<td>41</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>3.9 (0.6)</td>
<td>4.1 (0.7)</td>
<td>4.2 (1.0)</td>
<td>4.3 (1.0)</td>
<td>4.1 (0.8)</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.1 (0.4)</td>
<td>1.2 (0.7)</td>
<td>1.3 (0.6)</td>
<td>1.4 (0.7)</td>
<td>1.2 (0.6)</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>71 (8)</td>
<td>72 (7)</td>
<td>75 (9)</td>
<td>75 (9)</td>
<td>73 (8)</td>
</tr>
<tr>
<td>Hip circumference, cm</td>
<td>98 (7)</td>
<td>99 (7)</td>
<td>100 (8)</td>
<td>100 (8)</td>
<td>99 (7)</td>
</tr>
<tr>
<td>BMI</td>
<td>23.1 (3.5)</td>
<td>23.5 (3.2)</td>
<td>24.8 (3.9)</td>
<td>24.6 (4.0)</td>
<td>23.9 (3.7)</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>10.8 (4.2)</td>
<td>11.6 (4.1)</td>
<td>11.7 (3.9)</td>
<td>13.7 (4.7)</td>
<td>11.7 (4.2)</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>73.6 (13.2)</td>
<td>75.2 (11.7)</td>
<td>76.2 (39.2)</td>
<td>77.9 (13.8)</td>
<td>75.5 (24.8)</td>
</tr>
<tr>
<td>Vitamin B12, pmol/L</td>
<td>407 (132)</td>
<td>396 (129)</td>
<td>395 (142)</td>
<td>396 (144)</td>
<td>398 (135)</td>
</tr>
<tr>
<td>Previous MI, n (%)</td>
<td>1 (0.5%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>1 (0.1%)</td>
</tr>
<tr>
<td>Previous stroke, n (%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>1 (0.6%)</td>
<td>1 (0.1%)</td>
</tr>
<tr>
<td>Known diabetics, n (%)</td>
<td>1 (0.5%)</td>
<td>0 (0.0%)</td>
<td>5 (1.7%)</td>
<td>1 (1.1%)</td>
<td>7 (0.8%)</td>
</tr>
<tr>
<td>Premenopausal, n (%)</td>
<td>180 (98.9%)</td>
<td>259 (81.7)</td>
<td>131 (44.9)</td>
<td>2 (2.3)</td>
<td>572 (65.0%)</td>
</tr>
</tbody>
</table>

Standard deviations in parentheses unless otherwise stated.

In the above models where we assumed a multiplicative effect of risk factors, we found no statistically significant interactions between ADMA and smoking status, diabetes (glucose ≥7.1 mmol/L), and obesity (BMI ≥30). There were also no statistically significant interactions found between raised ADMA (ADMA ≥0.71 μmol/L) with systolic and diastolic blood pressure, triglycerides, and total and HDL cholesterol. We also examined models of using ADMA as a higher order term, and also ADMA in categories and found no significant differences in likelihood ratio between these and the presented models with ADMA as a continuous variable.

To determine a possible cut point for clinical use, we assessed the cumulative risk of women in the top one-fifth of the distribution of ADMA levels (≥0.71 μmol/L) compared with those of the bottom four-fifths. Across the quintiles, the risk of incident fatal and nonfatal myocardial infarction and stroke increased only from the fourth quintile. Compared to the bottom fifth, the risk ratios across the quintiles were 1.0, 1.0, 0.7, 1.3, 1.4 (supplemental Table II). The ability of ADMA to discriminate fatal and nonfatal incident myocardial infarction & stroke by 24 years was examined using a logistic regression and the areas under the ROC curve for the various models are shown in supplemental Table III. The discriminative ability of ADMA ≥0.71 μmol/L for incident fatal & nonfatal myocardial infarction and strokes by 24 years was: sensitivity 30%, specificity 82%, positive predictive value 18% and negative predictive value 90%.

A higher cumulative risk of total fatal & nonfatal myocardial infarction & stroke was found for those in the top quintile of ADMA (22% versus 12%, P=0.004; Figure 1). The relative risk of ADMA (≥0.71 μmol/L) for incident fatal and nonfatal myocardial infarction & stroke was 1.75 (95% CI 1.18, 2.59). The population attributable risk of ADMA (≥0.71 μmol/L) for incident fatal & nonfatal myocardial infarction and stroke was 12.7% in this study. Table 3 compares the relative and population attributable risks of ADMA with other traditional and nontraditional risk factors.

For the purposes of cardiovascular risk assessment in healthy individuals, most clinicians use a combination of traditional risk factors. The Systematic COOrony Risk Evaluation (SCORE) model is the risk assessment system recommended by the European Third Joint Task Force for Cardiovascular Disease Prevention in Clinical Practice. As illustrated in Figure 2a, in those 836 women at low estimated risk (10 year risk of CVD death of <5% now or projected to age 60), those with levels of ADMA ≥0.71 μmol/L are at higher cumulative risk of incident MI and stroke by 24 years compared to those with ADMA <0.71 μmol/L (22% versus 12% risk of incident MI & stroke by 24 years, P=0.003). In fact, also illustrated in Figure 2 (a), the low-risk women with ADMA ≥0.71 μmol/L had a cardiovascular risk similar to the women who were identified as high-risk at baseline by the SCORE system by 24 years (22% versus 21%, P=0.68).

Similarly, in Figure 2 (b), ADMA ≥0.71 μmol/L also further stratified the 875 women identified by the Framingham risk score as low- and intermediate-risk (10 year risk of coronary...
heart disease (≥20%) into 2 significant risk categories (22% versus 12% risk of incident MI and stroke by 24 years, \(P = 0.003\)).

**Discussion**

Our study showed that a 0.15 \(\mu\)mol/L (1 SD) increase in ADMA levels was associated with an approximate 30% increase in incident myocardial infarction and stroke, both fatal and nonfatal, in women after 24 years of follow-up, allowing for time to event. The effect of ADMA was independent of cardiovascular risk factors commonly used for risk assessment. The association with risk remained robust when additionally adjusted for serum triglycerides, waist & hip circumference, body mass index, creatinine clearance, and homocysteine, with no statistically significant interactions between ADMA and these risk factors, supporting the independent effect of ADMA on cardiovascular risk.

The risk for all-cause mortality was increased by approximately 15% for each 0.15 \(\mu\)mol/L increase in ADMA levels. Although this did not reach statistical significance, the hazard ratio was consistent through the adjustments. The neutral effect of ADMA on the noncardiovascular causes of death implies that the lack of statistical significance of total mortality was attributable to the dilution of the increased risk

<table>
<thead>
<tr>
<th>End Points</th>
<th>Univariate</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-cause mortality (138 deaths)</td>
<td>1.14 (0.98, 1.32)</td>
<td>1.14 (0.97, 1.32)</td>
<td>1.13 (0.97, 1.32)</td>
<td>1.12 (0.96, 1.32)</td>
</tr>
<tr>
<td>MI and stroke deaths (52 deaths)</td>
<td>1.28 (1.04, 1.59)</td>
<td>1.29 (1.05, 1.60)</td>
<td>1.30 (1.05, 1.62)</td>
<td>1.30 (1.04, 1.62)</td>
</tr>
<tr>
<td>MI and deaths (24 deaths)</td>
<td>1.31 (0.97, 1.78)</td>
<td>1.35 (0.99, 1.83)</td>
<td>1.32 (0.94, 1.86)</td>
<td>1.30 (0.90, 1.88)</td>
</tr>
<tr>
<td>Stroke deaths (28 deaths)</td>
<td>1.26 (0.93, 1.70)</td>
<td>1.25 (0.93, 1.80)</td>
<td>1.27 (0.95, 1.70)</td>
<td>1.24 (0.92, 1.67)</td>
</tr>
</tbody>
</table>

Table 2. Hazard Ratios of the Various End Points for a 1 Standard Deviation (0.15 \(\mu\)mol/L) Increase in ADMA

All analyses stratified by the age cohorts (95% CI in parentheses).
of myocardial infarction and stroke deaths, rather than some “protective” effect of ADMA on other causes of death in this population.

The choice of an ADMA level of 0.71 μmol/L or more (the top quintile of distribution) gave a relative risk of future myocardial infarction and stroke events of 1.75 (95% CI 1.18, 2.59) and a population attributable risk of 12.7%. This may be a clinically useful cut point. Separation of the cumulative risk curves for incident myocardial infarction and stroke (Figure 1) was only seen at about 15 years. This finding may be consistent with the gradual nature of the atherosclerotic process in response to endothelial dysfunction.

Several limitations of the study were considered. Serum samples were not available in 36% of subjects. However, the cardiovascular risk factor profile of the missing subjects was indistinguishable from those included, rendering a systematic bias improbable. It is possible that their inclusion, by increasing the sample size, would have strengthened our findings. The age and history of thawing of the serum samples were also of concern, though we know from previous experiments that ADMA is stable after at least 4 freeze-thaw cycles. Any possible errors that may have arisen would have been systematic as the samples were collected within a short time span (1 year) and underwent exactly the same thawing and refreezing process and conditions. The range and distribution of ADMA levels were very similar to those in a previous study on middle-aged Finnish men, a population based study of ADMA and cardiovascular disease, and also similar to a recent study which sought to identify reference values for ADMA in healthy men. Although our study population was exclusively women, previous studies had shown no differences in ADMA levels between men and women.

The possibility of residual confounding by unmeasured risk factors such as C-reactive protein has also been considered. We considered this unlikely as the effect of ADMA remained robust even when adjusted for the established traditional and nontraditional risk factors in our models. Also, other studies have shown the effect of ADMA remains independent when adjusted for C-reactive protein as well as other risk factors.

In our models, the inclusion of ADMA did not yield a significant improvement in discrimination for incident cardiovascular events as a continuous variable using the C statistic. Nevertheless, this is also true of the established risk factors such as blood pressure and cholesterol. However, in cardiovascular risk estimation, the ability of the marker to reclassify subjects into other risk categories requiring a change in management may be more clinically important than assessments of discrimination using the areas under the ROC curve.

A particular strength of this study is its prospective nature, and to our knowledge, it is the first report of ADMA in association with cardiovascular risk in a healthy population based cohort of women using the entire cohort that had available serum samples. The robustness of the magnitude of the risk through the numerous adjustments for traditional and nontraditional risk factors also strengthens the association. More importantly, in terms of clinical practice, ADMA enhances cardiovascular risk assessment by further stratifying women who were not identified as high-risk by both the SCORE and Framingham systems, both of which are the predominant cardiovascular risk assessment systems in Europe and North America respectively. This may have important management implications as the high-risk category is
often used as a threshold for intensive lifestyle and possibly drug intervention. We did not consider the role of ADMA in those who were already identified as high-risk by both the SCORE and Framingham risk assessment system as those individuals are already targeted for intensive management in most guidelines.

In conclusion, ADMA is an independent predictor of fatal and nonfatal myocardial infarction and stroke in women even after allowing for the effects of traditional and nontraditional risk factors for cardiovascular disease. Our study also suggests that the effect of ADMA on cardiovascular disease operates independently of homocysteine and creatinine clearance. It is reasonable to now consider whether reducing ADMA levels would improve endothelial function and reduce cardiovascular disease risk.

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Disclosures

None.

References


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Table I. Comparison of selected characteristics of women with and without stored samples available for ADMA determination

<table>
<thead>
<tr>
<th></th>
<th>Women with stored samples for ADMA determination</th>
<th>Women without stored samples for ADMA determination</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD) (n=880)</td>
<td>Mean (SD) (n=501)</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>132 (21)</td>
<td>135 (23)</td>
<td>0.19</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>85 (11)</td>
<td>86 (12)</td>
<td>0.51</td>
</tr>
<tr>
<td>Serum Cholesterol (mmol/L)</td>
<td>6.9 (1.3)</td>
<td>6.8 (1.1)</td>
<td>0.08</td>
</tr>
<tr>
<td>Serum Triglycerides (mmol/L)</td>
<td>1.2 (0.6)</td>
<td>1.2 (0.5)</td>
<td>0.30</td>
</tr>
<tr>
<td>Homocysteine (μmol/L)</td>
<td>11.7 (4.2)</td>
<td>11.9 (5.3)</td>
<td>0.48</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>75.5 (24.8)</td>
<td>74.2 (15.4)</td>
<td>0.28</td>
</tr>
</tbody>
</table>
Table II. Relative risk of fatal and non-fatal incident myocardial infarction & stroke for ADMA across the quintiles compared to the bottom fifth

<table>
<thead>
<tr>
<th>ADMA Quintile</th>
<th>RR compared to Quintile 1</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (&lt;0.51 µmol/L)</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>2 (≥0.51 µmol/L)</td>
<td>1.0</td>
<td>0.5, 1.9</td>
</tr>
<tr>
<td>3 (≥0.57 µmol/L)</td>
<td>0.7</td>
<td>0.3, 1.5</td>
</tr>
<tr>
<td>4 (≥0.63 µmol/L)</td>
<td>1.3</td>
<td>0.7, 2.4</td>
</tr>
<tr>
<td>5 (≥0.71 µmol/L)</td>
<td>1.4</td>
<td>0.8, 2.6</td>
</tr>
</tbody>
</table>
Table III. The effect on the areas under the ROC curve (C-statistic) of incrementally adding the various variables used in our predictive models for incident fatal and non-fatal myocardial infarction & stroke by 24 years, in order of ease of clinical availability after the established risk factors.

<table>
<thead>
<tr>
<th>Model</th>
<th>C-statistic</th>
<th>P value (compared to previous model)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.59</td>
<td>-</td>
</tr>
<tr>
<td>Plus Blood pressure</td>
<td>0.60</td>
<td>0.27</td>
</tr>
<tr>
<td>Plus Cholesterol</td>
<td>0.61</td>
<td>0.19</td>
</tr>
<tr>
<td>Plus Smoking status</td>
<td>0.65</td>
<td>0.08</td>
</tr>
<tr>
<td>Plus Glucose</td>
<td>0.65</td>
<td>0.79</td>
</tr>
<tr>
<td>Plus Waist circumference</td>
<td>0.69</td>
<td>0.02</td>
</tr>
<tr>
<td>Plus Hip circumference</td>
<td>0.70</td>
<td>0.46</td>
</tr>
<tr>
<td>Plus Body mass index</td>
<td>0.71</td>
<td>0.59</td>
</tr>
<tr>
<td>Plus Triglycerides</td>
<td>0.71</td>
<td>0.91</td>
</tr>
<tr>
<td>Plus Homocysteine (incl. vitamin B12)</td>
<td>0.71</td>
<td>0.31</td>
</tr>
<tr>
<td>Plus ADMA (incl. GFR)</td>
<td>0.72</td>
<td>0.39</td>
</tr>
</tbody>
</table>
METHODS

Study Population

The study population comprised of women recruited to the ongoing *Kvinnostudien*, the Population Study of Women in Gothenburg which has been described previously\(^1\)-\(^4\). Briefly, the baseline survey of the study took place in 1968/69\(^1\). The participants were a representative sample of women in Gothenburg, Sweden aged 38, 46, 50, 54 and 60 at entry who were invited from local population registries based on specifically selected days of birth. Of the 1,622 women who were invited, 1,462 (90.1%) underwent the baseline screening survey in 1968/69. For this study, serum samples were not available for the 81 women in the age 60 cohort, making the size of the baseline cohort 1,381.

Serum samples

The stored serum samples used in this study were collected over a period of under 1 year at the baseline survey interview and examination in 1968/69. The fasting blood samples were allowed to clot for 2 to 3 hours after collection before being centrifuged at room temperature, and then stored at -20°C in polystyrene cups. The samples used in this study were stored for 28 years at -20°C, before being thawed on 2 separate occasions for other studies. The period between the first\(^5\) and second thaw\(^6\) was 2 years, while that between the second and third thaw (for the current study) was 5 years. After the first thaw, the samples have been stored at -80°C. The samples were transported frozen from Oslo, Norway to Dublin, Ireland packed in dry ice for ADMA determination. ADMA has been previously shown to be stable after 4 freezing and thawing cycles\(^7\).
Laboratory analyses

We determined ADMA levels using a validated high-performance liquid chromatography (HPLC) method\(^8\). ADMA was converted into fluorescent derivatives with ortho-phtaldialdehyde reagent containing 3-mercaptoproprionic acid. The analytes were separated by isocratic reverse-phased HPLC with fluorescence detection. Sample cleanup was performed by solid-phase extraction on polymeric cation-exchange columns using monomethylarginine (MMA) as the internal standard.

The HPLC assay used in the present study was also previously validated in our laboratory and the assay was found to be linear over the range 0.1 to 20 \(\mu\)mol/L for ADMA and SDMA which encompasses the normal biological range. In our previous validation study (unpublished), good recovery was achieved for all analytes – arginine (85%), ADMA (99%) and SDMA (97%). The coefficients of variation within-assay were 2.0-5.8% and between-assay were 1.2-7.0% for lower levels and 2.1-2.8% for higher levels. Homocysteine was measured using the Abbot IMX assay in a previous study\(^6\).

Statistical Methods

Follow up data (endpoints) at 24 years was used for this study because for the 24 year follow up, special consideration had been given to participation, representativeness after a long follow-up period, and comparison of mortality between participants and non-participants\(^4\). We used Cox’s proportion hazards models to examine the association of ADMA as a continuous variable and time as the dependent variable with the following cardiovascular end-points at 24 years follow up:

1. All-cause mortality
2. Myocardial infarction (MI) & stroke deaths
3. Non-cardiovascular deaths
4. Myocardial infarction deaths
5. Stroke deaths
6. Myocardial infarction & stroke events (fatal & non-fatal)
7. Myocardial infarction events (fatal & non-fatal)
8. Stroke events (fatal & non-fatal)

Myocardial infarction was defined when at least 2 of the following 3 criteria are fulfilled: central chest pain more than 15 minutes, electrocardiograph changes characteristic of myocardial infarction and elevation in serum transaminases. The myocardial infarction endpoint was defined through interview, and verified using the Swedish Myocardial Infarction Register and hospital records, death certificate for the fatal cases, and postal interview with verification as described above for the non-attenders. Similarly, the stroke endpoint was defined through interview with verification as described above, while the fatal stroke cases were defined through death certification and autopsy findings.

All analyses were stratified by the age cohorts, and accounted for survival time which was up to 24 years. Risk factor levels from the baseline survey were used and were examined as continuous variables. Non-smokers were defined as those who never smoked and ex-smokers. Blood pressure was measured in the sitting position. Creatinine clearance was calculated using the Cockroft & Gault equation.

Analyses involving ADMA stratified by age were followed by multivariable models which consisted of sequentially adding further pre-specified covariates. Model 1 adjusted for
established cardiovascular risk factors commonly used in risk assessment (systolic &
diastolic blood pressure, serum cholesterol and smoking). Model 2 further adjusted for
traditional cardiovascular risk factors which are less established in terms of causality but
nevertheless easily obtained and commonly available (serum triglycerides, plasma glucose,
waist & hip circumference and body mass index). Model 3 also included creatinine
clearance, homocysteine and vitamin B12.

Clinical Utility
In the interest of having a cut-point of clinical utility, we then considered the top quintile of
the distribution of ADMA. Discrimination was shown using areas under the ROC curve,
sensitivities, specificities and predictive values. The cumulative hazard of cardiovascular risk
of the group in the top one-fifth was compared to the group in the bottom four-fifths using
Nelson-Aalen analyses. We also examined the relative risk and population attributable risk
conferred by this level of ADMA compared with those of the traditional risk factors.
Furthermore, we examined whether this level of ADMA will enhance cardiovascular risk
assessment both using the Systematic COronary Risk Evaluation (SCORE) system\textsuperscript{9}, the risk
assessment system recommended by the European Third Joint Task Force for Cardiovascular
Disease Prevention in Clinical Practice\textsuperscript{10}, as well as the Framingham risk score as
recommended by the Third Adult Treatment Plan\textsuperscript{11} (ATPIII) of the National Cholesterol
Education Program. The software used for the statistical analyses was Intercooled Stata 9.2
for Windows.
ONLINE REFERENCES


