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Effect of metformin on arginine and dimethylarginines in patients with advanced type 2 diabetes: A post hoc analysis of a randomized trial

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Abstract
Aim: To study the effect of metformin on plasma levels of arginine, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA), indicators of the nitric oxide pathway.

Materials and Methods: In this post hoc analysis of the HOME trial, we analysed plasma levels of arginine, ADMA and SDMA during the 4.3-year follow-up (comparing the effects of metformin versus placebo on top of insulin therapy). Statistical analysis was performed with a mixed model approach, in which simultaneously constant treatment effects were estimated, as well as time-dependent treatment effects.

Results: We found that metformin compared with placebo did not affect ADMA or SDMA plasma levels but rapidly decreased arginine plasma levels and hence the arginine to ADMA ratio. The constant treatment effect on ADMA was 0.99 (95% CI 0.97, 1.00) relative to placebo and the time-dependent treatment effect was 1.00 (95% CI 1.00, 1.01). By contrast, the constant treatment effect on arginine was 0.86 (95% CI 0.84, 0.88), with only a minimal time-dependent change of 1.01 (95% CI 1.00, 1.01).

Conclusions: The potential benefits of metformin on endothelial function cannot be explained by a decrease in ADMA or by improved global arginine availability. The clinical significance of the decreased arginine plasma levels is not clear and can be harmful or beneficial, depending on the mechanism involved. However, a potential effect of metformin on the nitric oxide pathway is not restricted to the studied metabolites.

Keywords
cardiovascular disease, metformin, randomized trial, type 2 diabetes

1 | INTRODUCTION

Patients with type 2 diabetes (T2D) have a strongly increased cardiovascular (CV) risk, even in the presence of good glycaemic control.\textsuperscript{1} Therefore, prevention of CV disease is, apart from good glycaemic control, an important target of diabetes treatment. This is increasingly recognized as part of treatment guidelines and influences the choice of glucose-lowering drugs.

Metformin is the first drug of choice for the treatment of T2D. Metformin suppresses hepatic gluconeogenesis and thereby increases hepatic insulin sensitivity. In addition, metformin has gut-based working mechanisms that result in lowering glucose levels. Apart from its
effects on glucose metabolism, metformin is also associated with a reduction in CV mortality and morbidity. One of the mechanisms involved is improvement of endothelial dysfunction.

Endothelial dysfunction is an important contributor to CV disease. Metformin is able to improve endothelial function by several pathways. It may reduce oxidative stress and endothelial inflammation, including leucocyte adhesion. Another pathway in endothelial function is the regulation of vasomotor tone by endothelial-derived nitric oxide synthase (eNOS).

In T2D, the endothelium-dependent vasodilatation is impaired, resulting in a vasoconstrictive state. Plasma levels of arginine, the precursor of nitric oxide (NO), are decreased compared with non-diabetes patients and the function of the eNOS enzyme is compromised (uncoupled), resulting in the production of oxygen radicals. Metformin opposes the pathological vasoconstriction by causing NO-mediated vasodilatation and reduces nitoxidative stress.

Asymmetric dimethylarginine (ADMA) is the major endogenous inhibitor for NO synthesis, antagonizing the NO precursor arginine, and thereby blocking vasodilatation. Symmetric dimethylarginine (SDMA) does not antagonize NO directly, but may, together with ADMA, compete with transmembrane arginine transport (Figure 1) and thereby restrict intracellular uptake of arginine.

Both increased levels of ADMA and SDMA and a decreased arginine to ADMA ratio (AAR) are associated with CV disease and endothelial dysfunction. Arginine levels taken in isolation are not related to CV disease.

Pharmacological interventions that decrease ADMA or SDMA levels, or enhance the AAR, are expected to result in an increased bioavailability of NO and a decrease in oxidative stress. ADMA levels have been shown to determine the endothelial effects of statins. Also, arginine supplementation can be beneficial in selected populations, especially those with increased AAR because of elevated ADMA.

We already showed, in the cohort of the Hyperinsulinaemia: the Outcomes of its Metabolic Effects (HOME) study, a 4.3-year randomized controlled trial, that metformin versus placebo improved biomarkers of endothelial function. In the current study we evaluate, in the same cohort, post hoc, the effect of metformin on the NO-pathway by studying the arginine, ADMA and SDMA plasma levels. Our hypothesis was that metformin might favourably decrease ADMA or SDMA plasma levels or increase the AAR.

## Materials and Methods

Arginine, ADMA and SDMA plasma levels were analysed with ultra-performance liquid chromatography–tandem mass spectrometry. Blood samples were collected at baseline and after 4, 17, 30, 43 and 52 months, then stored at -80 °C until analysis.

The total coefficients of variation (CVs) were 5.6% and 3.1% for ADMA, 6.2% and 4.7% for SDMA and 6.0% and 1.9% for arginine at concentration levels of 0.4, 1.5, 0.4, 1.3, 59 and 212 μmol/L, respectively.

The effects of metformin versus placebo on plasma levels of arginine, ADMA, SDMA and the AAR were assessed by a mixed model for repeated measures, where treatment effect was simultaneously assessed by the interaction of metformin treatment effect with time and a constant treatment effect from the first visit and remaining constant until the end of the trial by assuming placebo effects at baseline. Time and treatment (metformin vs. placebo) were considered fixed effects and patient was a random factor.

Because of a non-normal distribution, analysis was performed using log-normalized values of arginine, ADMA, SDMA and the AAR. Results are reported as antilog values and hence the ratio of the geometric means of the placebo and treatment group.

## Results

The median baseline values of all patients for plasma arginine, ADMA and SDMA levels were 121 (106, 137), 0.52 (0.48, 0.57) and 0.43 (0.38, 0.50) μmol/L (IQR), respectively. The median baseline AAR was 234 (205, 261). Baseline values of these four values did not differ

### Table 1: Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n = 194)</th>
<th>Metformin (n = 196)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>59.0 (11.0)</td>
<td>63.5 (9.6)</td>
</tr>
<tr>
<td>Men/women</td>
<td>97/97</td>
<td>81/115</td>
</tr>
<tr>
<td>T2D duration, y</td>
<td>12.1 (7.9)</td>
<td>14.2 (8.7)</td>
</tr>
<tr>
<td>CV score</td>
<td>0.9 (1.3)</td>
<td>1.2 (1.4)</td>
</tr>
<tr>
<td>ADMA, μmol/L</td>
<td>0.52 [0.47, 0.57]</td>
<td>0.53 [0.49, 0.57]</td>
</tr>
<tr>
<td>SDMA, μmol/L</td>
<td>0.42 [0.39, 0.5]</td>
<td>0.44 [0.38, 0.51]</td>
</tr>
<tr>
<td>Arginine, μmol/L</td>
<td>121 [106, 137]</td>
<td>121 [106, 136]</td>
</tr>
<tr>
<td>Arginine/ADMA ratio</td>
<td>238 [211, 264]</td>
<td>230 [202, 256]</td>
</tr>
</tbody>
</table>

Note. Mean (standard deviation) or median [interquartile range].

Abbreviations: ADMA, asymmetric dimethylarginine; CV score, cardiovascular history (calculated as a sum score of prior events); SDMA, symmetric dimethylarginine; T2D, type 2 diabetes.
### Table 2: Mixed model fixed effects estimates

<table>
<thead>
<tr>
<th></th>
<th>Log (arginine)</th>
<th>Log (ADMA)</th>
<th>Log (SDMA)</th>
<th>Log (arginine/ADMA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time</strong></td>
<td>1.00 (0.99, 1.00)</td>
<td>1.00 (1.00, 1.01)</td>
<td>1.02 (1.02, 1.02)</td>
<td>1.00 (0.99, 1.00)</td>
</tr>
<tr>
<td><em>P</em></td>
<td>.86</td>
<td>.10</td>
<td>.00*</td>
<td>.29</td>
</tr>
<tr>
<td><strong>Trt</strong></td>
<td>0.86 (0.84, 0.88)</td>
<td>0.99 (0.97, 1.00)</td>
<td>1.03 (1.01, 1.05)</td>
<td>0.87 (0.85, 0.89)</td>
</tr>
<tr>
<td><em>P</em></td>
<td>.00*</td>
<td>.10</td>
<td>.01*</td>
<td>.00*</td>
</tr>
<tr>
<td><strong>Time:Trt</strong></td>
<td>1.01 (1.00, 1.02)</td>
<td>1.00 (1.00, 1.01)</td>
<td>1.00 (0.99, 1.01)</td>
<td>1.01 (1.00, 1.01)</td>
</tr>
<tr>
<td><em>P</em></td>
<td>.05*</td>
<td>.64</td>
<td>.66</td>
<td>.12</td>
</tr>
</tbody>
</table>

**Note.** Fixed effects estimates (95% confidence intervals). Antilogs are reported, ‘Time’ is change per year, ‘Trt’ is constant treatment effect from the first postbaseline visit to the end of the study and ‘Time: Trt’ is the change per year in the treatment group compared with the placebo group. Abbreviations: ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine.

*P < .05.

### Figure 2: Change over time of arginine, ADMA, SDMA and arginine/ADMA ratio, means with 95% confidence intervals. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine
between the groups, as shown in Table 1 Table 2. The changes over time for all four variables are shown in Figure 1.

The mixed model analysis shows no effect of metformin versus placebo on ADMA. Both the constant treatment effect from the first postbaseline visit until the end of the trial (0.99 [95% CI 0.97, 1.00], P = .1), as well as the time-treatment interaction (1.00 [95% CI 1.00, 1.01], P = .64), did not relevantly change Figure 2.

This pattern was similar for SDMA with a small constant treatment effect (1.03 [95% CI 1.01, 1.05], P = .01) and no time-treatment interaction (1.00 [95% CI 0.99, 1.01], P = .66).

By contrast, metformin versus placebo decreased arginine plasma levels (0.86 [95% CI 0.84, 0.88], P < .01) as a constant treatment effect, with only a marginal further increase (1.01 [95% CI 1.00, 1.02], P < .05) as a time-treatment interaction.

As a consequence, the AAR decreased accordingly (0.87 [95% CI 0.85, 0.89], P < .01) as a constant treatment effect with no additional time-treatment interaction (1.01 [95% CI 1.00, 1.01], P = .12).

As a sensitivity analysis, we adjusted for age, sex and CV history in the model. This did not change the results (data not shown).

4 | DISCUSSION

We found that metformin compared with placebo did not affect ADMA or SDMA plasma levels, but rapidly decreased arginine plasma levels and hence the AAR.

Metformin has been shown to decrease ADMA plasma levels in spontaneously hypertensive rats.\textsuperscript{15} Regarding the possible mechanism, it was shown that metformin did not influence the concentration of ADMA synthesizing enzymes or ADMA metabolizing enzymes. The authors concluded that a change in interorgan ADMA transport was the most probable explanation.

In human research, Asagami et al. found a reduction in ADMA plasma levels in patients who were treated with metformin for 3 months.\textsuperscript{16} However, their trial was designed to include patients with poor glycemic control and their findings could be explained by reduced glucotoxicity instead of a direct effect of metformin.

By contrast, two other trials in T2D patients showed no change in ADMA plasma levels. Lund et al. conducted a crossover trial on 96 insulin-naïve T2D patients with 4-month treatment periods with metformin.\textsuperscript{17} Sutkowska et al. randomized 25 patients with prediabetes between high- and low-dose metformin and found no change after 3 months.\textsuperscript{18} Our results confirm and strengthen these findings with a long-term placebo-controlled trial and extend them to a population of insulin-using T2D patients.

Our finding that metformin reduces arginine plasma levels is in accordance with recent literature. Irving et al. reported reduced arginine and citrulline plasma levels in 12 patients randomized to the combination of metformin and pioglitazone.\textsuperscript{19} Adam et al. confirmed these findings in 74 T2D patients treated with metformin in a metabolomics approach, in which 353 metabolites were assessed.\textsuperscript{20} In a randomized controlled trial, Hanff et al. found that metformin treatment in 10 muscular dystrophy patients resulted in a decrease of arginine plasma levels from 129 to 100 µmol/L, comparable with our results.\textsuperscript{21} In addition, Sutkowska et al. reported a non-significant decrease in arginine from a median of 104 to 82 µmol/L.\textsuperscript{18}

Although an increase in ADMA plasma level is considered harmful and a decrease in ADMA is considered beneficial, the clinical significance of the decrease in arginine plasma levels is more difficult to interpret.

At first glance a decreased arginine level and the resulting decrease in the AAR appears disadvantageous. It seems that there is less precursor for eNOS. However, arginine metabolism is complex, distributed over multiple organs and intracellular compartments, and is involved in several metabolic pathways.\textsuperscript{22} Therefore, the plasma compartment may not be representative of eNOS substrate capacity, first of all because of the so-called arginine paradox.\textsuperscript{23} The required arginine concentrations to saturate the NO synthase receptors are in the micromolar range, while both plasma and intracellular arginine concentrations greatly exceed these concentrations with millimolar ranges. Therefore, a decrease in arginine concentration is not expected to be rate-limiting for NO synthesis. However, short-term augmentation of the extracellular arginine concentrations results in increased NO production, suggesting that local arginine availability to the NO synthase may be partly dependent on extracellular concentrations.\textsuperscript{24}

The arginine plasma levels in our study, despite decreasing in the metformin-treated patients, are still comparatively high. Mean values above 100 micromol/l are high-normal compared with reference values for individuals without diabetes.\textsuperscript{25} Moreover, the supply of arginine for the endothelial NO synthase is not restricted to plasma-membrane transport from the extracellular space. It was shown that eNOS preferentially uses the intracellular arginine pool instead of the extracellular arginine pool.\textsuperscript{26} Even with complete inhibition of transmembrane transport, sufficient arginine is available from protein degradation and from citrulline to arginine recycling.\textsuperscript{27}

Tang et al. puts this further into perspective by showing that plasma levels of arginine taken in isolation were not correlated with CV morbidity.\textsuperscript{10} Only decreased arginine levels because of increased catabolization of arginine, with concomitant increased levels of ornithine and citrulline, were positively correlated with CV events. Also, citrulline taken apart, as a proxy of arginine metabolization, was positively correlated with CV morbidity.

Apart from modulating the bioavailability of NO, arginine is also associated with beta-cell function. As the pancreatic beta cell senses calorigenic nutrients, arginine turns out to be one of the more potent amino acid insulin secretagogues.\textsuperscript{28} However, supraphysiological doses are needed in beta-cell stimulation tests. Smaller increases of arginine levels (~50%) were not effective in stimulating insulin secretion.\textsuperscript{29} Also, insulin secretion was maintained in arginine-deficient states.\textsuperscript{30} Therefore, the small decrease in arginine plasma levels in the metformin group is not expected to result in changes in beta-cell function.

The two major catabolization pathways of arginine are metabolization by arginase (part of the urea cycle) or metabolism by NO synthase. Both routes will result in enhanced citrulline levels. In our
study we did not measure citrulline or ornithine plasma levels. However, it is a consistent finding in the literature that metformin does decrease both citrulline and arginine levels, of which citrulline is most profoundly decreased.\textsuperscript{18-21,31} This does not fit with a mechanism of increased catabolization of arginine.

Regarding possible mechanisms by which metformin may decrease arginine plasma levels, a more plausible mechanism is interference with enterocyte function. Although arginine is only partly dependent on intestinal absorption, its metabolic precursor, citrulline, is highly dependent on intact enterocyte function. Metformin is known to accumulate in the intestinal mucosa with up to 300 times greater concentrations than in plasma.\textsuperscript{32} Metformin may interfere with the synthesis of citrulline within the enterocyte and hence limit production of the major precursor of arginine.

A shift between the plasma and the intracellular compartment could theoretically be an alternative mechanism of the decrease in arginine plasma levels. Transmembrane transport of arginine is performed by cationic transporters (CATs), in particular CAT-1. Because arginine shares CAT-1 with ADMA and SDMA, there may be competitive inhibition or (trans-) stimulation. Metformin is a structural analogue of ADMA and may also interfere with cation transport. This concept was proposed by Bestermann, who suggested a competitive antagonism of metformin and ADMA.\textsuperscript{33} However, Strobel et al. showed that metformin, in contrast to ADMA, did not influence CAT-1-mediated arginine transport.\textsuperscript{34}

Another mechanism to explain decreased citrulline and arginine plasma levels may involve the reduction of NO production by inducible NO synthase.\textsuperscript{35} As NO production by endothelial NO synthase is considered beneficial in T2D, the induced NO production during low-grade inflammation is potentially harmful. Metformin is known to reduce inflammation and limit the inflammation-induced NO production.\textsuperscript{36} Because citrulline is formed during NO production, a metformin-induced decrease in inducible NO production will result in less citrulline production and hence lower citrulline levels. Although this is expected to result in preserved arginine levels inside the cell, it may lead to a lower arginine plasma level by way of reduced citrulline-arginine conversion in the kidney.

Regarding the beneficial effects of metformin on eNOS, potential mechanisms are not restricted to arginine transport or inhibition of ADMA. Endothelial NO synthase is dependent on several post-translational modifications, one of which is phosphorylation. Metformin is known to enhance AMPK and has been shown to increase the phosphorylation of endothelial NO synthase.\textsuperscript{37}

In short, metformin has been shown to improve endothelial function. Beneficial effects of metformin could be mediated by improving the endothelial NO pathway or by inhibition of the inducible NO pathway. Our current results from the HOME study, a long-term placebo-controlled randomized controlled trial, do not show a beneficial effect of metformin on ADMA levels, nor a beneficial effect on global arginine availability assessed from plasma arginine or the AAR.

Our results do, however, strengthen earlier observations that metformin reduces arginine plasma levels. The clinical importance of reduced arginine plasma levels remains to be debated and could be beneficial or harmful depending on the mechanism involved.

It could be viewed as potentially harmful if plasma arginine levels are regarded as rate-limiting for the function of eNOS. It could be viewed as potentially beneficial if the decrease is explained by a metformin-induced decrease in inflammatory activity (inducible NO).

To gain clarity on this issue, it would be interesting to perform a study of oral citrulline supplementation (as the precursor of arginine) combined with metformin in T2D patients. A similar study has been performed in patients with Becker muscular dystrophy.\textsuperscript{21} In this study, the metformin-induced decrease in arginine was prevented by the supplementation of citrulline.

This certainly strengthens the presumed enteral mechanism by which metformin results in citrate depletion. However, it does not disprove a potential simultaneous anti-inflammatory effect with a decrease in inducible NO.

Further research will be necessary to unravel the complex interplay between metformin and the endothelium. From a clinical perspective it is important to combine this with patient-relevant clinical outcomes, instead of only metabolic surrogates.

**AUTHOR CONTRIBUTIONS**

A.K. and C.D.A.S. designed the study. W.M.C.T. and P.L. analysed the data and performed the statistical analyses. W.M.C.T. drafted the manuscript. A.K. and C.D.A.S. reviewed the manuscript. C.G.S. performed the analysis of plasma levels of arginine, ADMA and SDMA. W.M.C.T., A.K. and P.L. are guarantors and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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**CONFLICT OF INTEREST**

The authors report no conflicts of interest.

**PEER REVIEW**

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**DATA AVAILABILITY STATEMENT**

Data is available upon reasonable request

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