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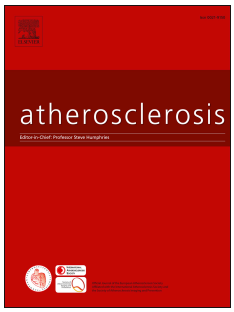

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PII: S0021-9150(17)31324-2

DOI: 10.1016/j.atherosclerosis.2017.10.004

Reference: ATH 15226

To appear in: *Atherosclerosis*

Received Date: 25 April 2017

Revised Date: 9 September 2017

Accepted Date: 5 October 2017

Please cite this article as: Radhakutty A, Mangelsdorf BL, Drake SM, Rowland A, Smith MD, Mangoni AA, Thompson CH, Burt MG, Opposing effects of rheumatoid arthritis and low dose prednisolone on arginine metabolomics, *Atherosclerosis* (2017), doi: 10.1016/j.atherosclerosis.2017.10.004.

Opposing effects of rheumatoid arthritis and low dose prednisolone on arginine metabolomics

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Key words: Glucocorticoid, asymmetric dimethyl arginine, mono methyl arginine, symmetric dimethyl arginine, endothelial function

Abbreviations: ADMA, asymmetric dimethyl arginine; MMA, mono methyl arginine; SDMA, symmetric dimethyl arginine; e-NOS, endothelial nitric oxide synthase; DDAH, dimethyl arginine dimethyl amino hydrolase.

Abstract

Background and aims: The effects of low dose prednisolone on circulating markers of endothelial function, the arginine metabolites asymmetric dimethyl arginine (ADMA), mono methyl arginine (MMA), and homoarginine, are uncertain. We assessed whether patients with rheumatoid arthritis have perturbations in arginine metabolite concentrations that are reversed by low dose prednisolone.

Methods: Eighteen rheumatoid arthritis patients who had not taken prednisolone for >6 months (non-glucocorticoid (GC) users), 18 patients taking continuous oral prednisolone (6.5±1.8 mg/day) for >6 months (GC users) and 20 healthy controls were studied. Fasting plasma concentrations of ADMA, MMA, and homoarginine were measured by ultra-performance liquid-chromatography. Baseline data from non-GC users were compared with healthy controls to assess the effect of rheumatoid arthritis. The change in arginine metabolites in non-GC users after 7 days of prednisolone (6 mg/day) was used to assess the acute effects of prednisolone. Baseline data from non-GC users were compared with GC users to assess the chronic effects of prednisolone.

Results: Non-GC users had higher ADMA (0.59±0.03 *vs.* 0.47±0.01 µM, p=0.004) and MMA concentrations (0.10±0.01 *vs.* 0.05±0.00 µM, p <0.001) than controls. The only change with acute prednisolone was a reduction in homoarginine (1.23±0.06 *vs.* 1.08±0.06 µM, p=0.04) versus baseline. GC users had lower concentrations of ADMA (0.51±0.02 *vs.* 0.59±0.03 µM, p=0.03) than non-GC users.

*Conclusions***:** Rheumatoid arthritis patients have higher concentrations of ADMA and MMA, inhibitors of endothelial function. Chronic, but not acute, prednisolone therapy is associated with a lower ADMA concentration, suggesting a salutary effect of long-term glucocorticoid treatment on endothelial function.

Introduction

Rheumatoid arthritis is associated with a 30-60% increased risk of cardiovascular events [1-6] and a 50% increased risk of death from cardiovascular disease [7]. Glucocorticoids are often prescribed to patients with rheumatoid arthritis, but there are concerns regarding potential adverse cardiovascular events in these patients already at high cardiovascular risk [8, 9]. While high dose glucocorticoids are associated with increased cardiovascular events, it is unclear whether lower doses (e.g., prednisolone <10 mg/day), commonly prescribed long-term, alter cardiovascular risk [10]. Some epidemiological studies have reported an increase in cardiovascular events with low dose prednisolone, while others have reported no effect [11, 12]. Furthermore, the sample size and duration of randomized-controlled studies of glucocorticoid therapy in patients with rheumatoid arthritis are insufficient to assess cardiovascular events [13, 14].

Endothelial dysfunction is a key event in the pathogenesis of atherosclerosis and develops early in the course of rheumatoid arthritis [15, 16]. A patient's vasodilatory response to hypoxia is often used to assess endothelial function. However, the effect of glucocorticoids on endothelial function assessed by this approach is uncertain. Endothelial function was reduced after an increase of glucocorticoid dose in hypopituitary patients [17] and in patients with IgA nephropathy prescribed glucocorticoids [18]. In contrast, glucocorticoids did not change endothelial function in healthy adults [19] or patients with rheumatoid arthritis [20]. Moreover, we recently reported that endothelial function is not affected by acute prednisolone, but is better in patients on long-term prednisolone [21, 22]. These contrasting findings suggest that the effects of glucocorticoids on endothelial function might differ depending on the patient group, the methods used to assess vasodilation, and the dose and duration of glucocorticoid treatment.

The measurement of circulating arginine metabolites is an alternative method to assess endothelial function and cardiovascular risk. Asymmetric dimethyl arginine (ADMA) is a competitive inhibitor of endothelial nitric oxide synthase (e-NOS), the enzyme that converts L-arginine to citrulline and releases nitric oxide. ADMA is positively associated with endothelial dysfunction [23] and cardiovascular mortality [24, 25]. Emerging evidence suggests that other arginine metabolites also influence cardiovascular risk. Mono methyl arginine (MMA), another inhibitor of e-NOS, and symmetric dimethyl arginine (SDMA), which reduces L-arginine bioavailability, are also associated with atherosclerosis and cardiovascular events [26-28]. L-arginine is also metabolized by arginase to ornithine and by arginine : glycine amidino transferase (AGAT) to homoarginine. Perturbations in these pathways have also been associated with vascular dysfunction and increased cardiovascular mortality [29, 30].

Increased ADMA concentrations in patients with rheumatoid arthritis have been linked to endothelial dysfunction and impaired endothelial repair [31, 32]. However, little is known about the effect of rheumatoid arthritis on other arginine metabolites. High dose glucocorticoids increased ADMA in patients with IgA nephropathy [18] and arginase activity in an animal model [33]. However, it is not clear whether the typical therapeutic glucocorticoid doses prescribed to patients with rheumatoid arthritis affect arginine metabolite concentrations.

We hypothesized that 1) patients with rheumatoid arthritis have alterations in arginine metabolism that will influence the effect of prednisolone on endothelial function and 2) the acute and chronic effects of prednisolone on arginine metabolism differ. Consequentially, the aims of this study were firstly to assess whether patients with rheumatoid arthritis have

perturbations in arginine metabolism and then to assess the acute and chronic effects of low dose prednisolone on arginine metabolism in patients with rheumatoid arthritis.

Patients and methods

Subjects and study design

Subjects with rheumatoid arthritis aged 50 years or older were recruited from the rheumatology outpatient clinic at Repatriation General Hospital, Adelaide, Australia and healthy controls from the general community. We studied 18 subjects who had not been administered any oral glucocorticoids for at least 6 months (non-GC users), 18 subjects taking a stable continuous oral prednisolone dose of 4-10 mg/day for at least 6 months (GC users) and 20 healthy controls with no history of inflammatory disease. The groups were matched for age, sex and renal function and subjects on oral hypoglycaemic agents and /or insulin were excluded from the study. First, we compared arginine metabolite concentrations in non-GC users and controls to assess the effect of rheumatoid arthritis on arginine metabolism. Secondly, non-GC users were studied before and after a 7 day course of oral prednisolone 6 mg daily to determine the acute effects of prednisolone. Finally, baseline data from non-GC users were compared with data from GC users to determine the chronic effects of prednisolone.

The study was approved by the Southern Adelaide Clinical Human Research Ethics Committee, Flinders Medical Centre, and all subjects provided written informed consent in accordance with the 1975 Declaration of Helsinki. The primary analyses of this study investigated the effect of prednisolone on clinical measures of vascular function and energy and substrate metabolism in the rheumatoid arthritis patients; these have previously been reported [21, 34].

Study protocol

Subjects attended the Endocrine Research Unit at Repatriation General Hospital at 0830 h after a 12 h overnight fast. All subjects took their regular medications in the morning prior to arrival, including prednisolone. Basic anthropometric measures were recorded. In each study participant, fasting blood samples were collected in EDTA tubes for measurement of 7 key components of arginine metabolism that are directly or indirectly involved in the regulation of endothelial function: arginine, homoarginine, citrulline, ornithine, ADMA, MMA and SDMA. Blood samples were centrifuged at 4,000 rpm at 4^0 Centigrade for 10 min and plasma frozen at -80^0 Centigrade until analysis.

Arginine metabolomics

Samples were prepared for analysis by solvent precipitation. 100 µL of sample was mixed with 400 µL of assay precipitating solution (0.1% formic acid in methanol), centrifuged for 5 min at 16,000 g, and a 400 µL aliquot of the resulting supernatant evaporated to dryness. Dried eluates were then reconstituted in 200 µL ammonium formate for liquid chromatography-mass spectrometry (LC-MS). Chromatographic separations were performed on a Waters ACQUITY™ T3 HSS C18 analytical column (150 mm × 2.1 mm,1.8 µm; Waters Corp., Milford, USA) using a Waters ACQUITY Ultra Performance LC™ system. Column elutant was monitored by mass spectrometry, performed on a Waters Quad-Time of Flight Premier™ quadrupole [35].

Other laboratory analysis

Serum creatinine was measured using Roche automated clinical chemistry analyser (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim, Germany) and estimated

glomerular filtration rate (eGFR) was measured using the Chronic Kidney Disease-Epidemiology collaboration equation (CKD-EPI equation). C-reactive protein (CRP) was measured using a Tinaquant immunoturbidimetric assay (Roche Diagnostics GMBH, Mannheim, Germany) on a Roche Modular Analyser (Hitachi High-Technologies Corporation, Tokyo, Japan). The between-run coefficient of variation was 3.6 % at a CRP of 3.9 mg/L and 2.3 % at a CRP of 49.5 mg/L.

Statistical analysis

Statistical analysis was performed using IBM SPSS version 20 for Windows (IBM, New York, USA). A *p*-value of <0.05 was considered statistically significant. Subject characteristics are presented as mean ± standard deviation if the distribution was Normal and median (interquartile range) if the distribution was not Normal. All other data are presented as mean ± standard error of mean. Subject characteristics in the three groups were compared using one-way analysis of variance. Non-GC users were compared to controls using unpaired *t*-tests if normally distributed or Mann-Whitney U tests if the distribution was not normal. Changes in variables in non-GC users after 7 days prednisolone were analysed using paired t-tests. Hereafter in the manuscript these results are reported as the acute effects of prednisolone. GC users were compared with baseline data from non-GC users using unpaired t-tests if normally distributed or Mann-Whitney U tests if the distribution was not normal. Differences between these two groups are reported in the manuscript as the chronic effects of prednisolone. In cross-sectional analyses, if a variable was significant in univariate analysis it was corrected for potential confounders using analysis of covariance.

The primary end point of this analysis was the difference in concentration of ADMA. A sample size of 18 per group in the cross-sectional study had 80 % power to detect a 0.07 µM

difference in ADMA assuming a standard deviation of 0.07. In the longitudinal study, a sample size of 18 per group had 80 % power to detect a 0.05 µM difference in ADMA assuming a standard deviation of 0.07.

Results

Subject characteristics

There were no significant differences in sex, age, body mass index, eGFR, smoking, history of hypertension, ischemic heart disease or diabetes between the three groups (Table 1). GC users were taking a mean prednisolone dose of 6.5 ± 1.8 mg/day, with a median duration of continuous prednisolone therapy of 48 (6-240) months. There was no significant difference in C-reactive protein (1.6 (0.5-7.6) *vs.* 2.4 (1.1-4.5) mg/L, p=0.44), or in the number of patients taking disease modifying anti-rheumatic drug use (11 *vs.* 9, p= 0.50) between GC and non-GC users.

Arginine metabolomics

Effect of rheumatoid arthritis

In univariate analyses, ADMA (0.59 ± 0.03 *vs.* 0.48 ± 0.01 µM, p=0.004), MMA (0.10 ± 0.01 *vs.* 0.05 ± 0.00, p <0.001), arginine (93.9 ± 4.8 *vs.* 75.0 ± 2.3 µM, p=0.001) and citrulline (37.1 ± 2.2 *vs.* 29.3 ± 1.1 µM, p=0.002) concentrations were higher in non-GC users than in controls. The higher concentrations of ADMA (p=0.008, Fig. 1A), MMA (p <0.001, Fig. 1B), arginine (94.3 ± 4.2 *vs.* 75.0 ± 4.2 µM, p=0.003) and citrulline (37.1 ± 1.4 *vs.* 28.7 ± 1.4 µM, p <0.001) in non-GC users remained significant after adjustment for age, sex, eGFR, smoking and cholesterol. There were no significant differences in SDMA (0.69 ± 0.06 *vs.* 0.56 ± 0.04, p=0.08), ornithine (52.3 ± 3.7 *vs.* 56.8 ± 3.3, p=0.37) and homoarginine (1.23 ± 0.06 *vs.* 1.08 ± 0.06 µM, p=0.08) concentrations between non-GC users and controls.

Acute effects of prednisolone

Homoarginine concentration was significantly lower (Δ -0.15 ± 0.07 µM, p=0.04) after 7 days prednisolone. There were no significant changes in ADMA (Δ -0.02 ± 0.02 µM, p=0.47), MMA (Δ -0.002 ± 0.003 µM, p=0.70), SDMA (Δ -0.08 ± 0.05 µM, p=0.14), arginine (Δ -5.2 ± 5.0 µM, p=0.31), citrulline (Δ +0.2 ± 1.6 µM, p=0.90) or ornithine (Δ +7.8 ± 4.0 µM, p=0.07) concentrations after acute prednisolone.

Chronic effect of prednisolone

In univariate analyses, GC users had lower concentrations of ADMA (0.51 ± 0.02 *vs.* 0.59 ± 0.03 µM, p=0.03) and SDMA (0.53 ± 0.03 *vs.* 0.69 ± 0.06, p=0.03) than non-GC users. The lower concentrations of ADMA (p=0.03, Fig. 2A), and SDMA (p=0.02, Fig. 2B) in GC users remained significant after adjustment for age, sex, eGFR, smoking cholesterol, CRP and disease modifying anti-rheumatic drug use. There were no significant differences in the concentrations of MMA (0.09 ± 0.00 *vs.* 0.10 ± 0.01 µM, p=0.12), arginine (86.3 ± 4.7 *vs.* 93.9 ± 4.8 µM, p=0.27), citrulline (33.6 ± 2.6 *vs.* 37.1 ± 2.2 µM, p=0.26), ornithine (59.9 ± 5.5 *vs.* 52.3 ± 3.7 µM, p=0.26) or homoarginine (1.16 ± 0.06 *vs.* 1.23 ± 0.06 µM, p=0.42) between GC and non-GC users.

Discussion

This study assessed the effects of rheumatoid arthritis on arginine metabolism and then the acute and chronic effects of low dose prednisolone on arginine metabolism in patients with rheumatoid arthritis. We demonstrated that patients with rheumatoid arthritis had higher concentrations of ADMA and MMA, endogenous inhibitors of eNOS, than healthy controls. Acute prednisolone treatment resulted in a small reduction in homoarginine, but there were no significant changes in other arginine metabolites. In contrast, rheumatoid arthritis patients

on chronic prednisolone treatment had significantly lower concentrations of ADMA and SDMA than patients not on prednisolone. These findings suggest that rheumatoid arthritis *per se* is associated with an increase in plasma concentrations of endogenous inhibitors of nitric oxide synthase, which are likely to contribute to endothelial dysfunction. The reduction in ADMA and SDMA with chronic, but not acute, prednisolone could provide a mechanism that explains why clinical measures of endothelial function improves with chronic, but not acute, prednisolone in this patient group [21, 22].

In this study, patients with rheumatoid arthritis had higher concentrations of ADMA and MMA than controls. The finding of increased ADMA in patients with rheumatoid arthritis is consistent with other studies [31, 32], in whom ADMA is associated with increased carotid intima media thickness and depleted endothelial progenitor cells [31, 36, 37]. This study extends these observations by demonstrating that MMA, another inhibitor of eNOS, is also increased in rheumatoid arthritis. ADMA and MMA are both degraded by dimethyl arginine dimethyl amino hydrolase (DDAH). DDAH activity is reduced in inflammatory states [38, 39]. Elevations of ADMA and MMA are a potential mechanism underlying endothelial dysfunction in patients with rheumatoid arthritis. SDMA was also increased by 19%, although this difference was not statistically significant. This finding may represent a type 2 error, given the relatively small sample size. Alternatively, SDMA is metabolized by different pathways to ADMA and MMA, and this could explain the discordant results [40].

Patients with rheumatoid arthritis also had higher plasma concentrations of arginine and citrulline. However, most of the plasma arginine arises from diet with only a small fraction synthesized from other amino acids [41], while citrulline is predominantly synthesized from glutamate in the small intestine [42]. Hence the increased arginine and citrulline

concentrations are likely to reflect increased protein catabolism in rheumatoid arthritis [43] and not increased eNOS activity. The concentrations of homoarginine and ornithine were similar in patients with rheumatoid arthritis and controls. These metabolic pathways have not been extensively studied in patients with rheumatoid arthritis, although one study also reported homoarginine is not different in patients with rheumatoid arthritis [44]. Our study suggests that changes in arginase and AGAT activity do not contribute to endothelial dysfunction in patients with rheumatoid arthritis.

The only significant change in arginine metabolites after acute low dose prednisolone consisted of a reduction in homoarginine concentration. Homoarginine is a weak substrate for nitric oxide synthase that has been negatively associated with cardiovascular morbidity and mortality in epidemiologic studies [29, 45]. However, the mechanism underlying this association is not well understood and the role of this metabolic pathway in rheumatoid arthritis is unclear [44]. There were no significant changes in inhibitors of eNOS or ornithine, a marker for arginase activity after acute prednisolone. This is consistent with studies reporting that acute low dose prednisolone does not affect endothelial function in patients with rheumatoid arthritis [20, 21].

In contrast to acute prednisolone and despite greater insulin resistance [21], patients with rheumatoid arthritis on chronic prednisolone treatment had lower ADMA and SDMA concentrations than patients with rheumatoid arthritis who were not taking prednisolone. Previous studies reporting the effects of glucocorticoids on ADMA have been discordant with lower serum ADMA concentrations in patients with Duchenne's muscular dystrophy treated with glucocorticoids [46], but an increase in ADMA, coupled with a reduction in flow-mediated vasodilatation, in patients with IgA nephropathy treated with high dose

glucocorticoids [18]. Moreover, TNF-alpha inhibitors were also shown to reduce ADMA-arginine ratio and improve vascular function in patients with rheumatoid arthritis in some [47], but not all [48], studies. We postulate that the effects of glucocorticoids on arginine metabolism are influenced by the glucocorticoid dose and underlying disease state. In patients with an active inflammatory disease, anti-inflammatory treatment is associated with a reduction in ADMA, possibly via increasing DDAH activity [38, 39]. The reduction in ADMA is consistent with better endothelial function in patients with rheumatoid arthritis prescribed chronic prednisolone.

This study does not provide direct insights on the cardiovascular effects of prednisolone. However, available epidemiologic data suggesting ADMA has an important physiologic role is strong; an increase in serum ADMA concentration of 0.1 µmol/L was associated with a 27 fold increase in relative risk of an acute coronary event [48]. A reduction in ADMA and SDMA, together with a higher fasting and postprandial reactive hyperaemia index [21, 22], suggests that chronic low dose prednisolone treatment in patients with rheumatoid arthritis may not worsen endothelial function. Given the lack of direct evidence of the cardiovascular effects of low dose prednisolone in literature, our study give some reassurance that long-term low dose prednisolone can be used to attenuate disease progression in this patient group without increasing cardiovascular risk.

We acknowledge the following limitations of this study. We have only assessed extracellular concentrations of arginine metabolites and must extrapolate these results to assess intracellular eNOS activity and vascular function. However, studies of enzyme kinetics have shown enhanced cellular uptake of methylarginines and increased NOS inhibition with elevated plasma concentrations [49]. Secondly, there was wide variability in the duration of

prednisolne treatment in GC users and this could have affected results. However, the small sample size precludes subgrouping GC users further based on duration of prednisolone use. Thirdly, other markers of endothelial dysfunction such as monocyte chemoattractant protein 1 (MCP1), vascular cell adhesion molecule 1 (VCAM 1), Selectins or interleukin 6 (IL6) were not measured. Fourthly, inherent in any cross-sectional study is the possibility that an unmeasured variable affected results. However, the groups were well matched for a number of key variables (Table 1). Finally, our findings cannot be translated to prednisolone doses of >10 mg/day.

In summary, patients with rheumatoid arthritis have higher concentrations of ADMA and MMA, inhibitors of eNOS that could contribute to the endothelial dysfunction associated with this disease. Acute and chronic prednisolone treatment have differing effects on arginine metabolomics. While acute prednisolone has little effect, chronic prednisolone reduces ADMA and SDMA concentrations. Reducing these elevated inhibitors of nitric oxide synthesis could explain why endothelial function is better in patients with rheumatoid arthritis prescribed prednisolone long-term.

Trial registration:

Australia New Zealand Clinical Trial Registry http://www.anzctr.org.au/; ACTRN12612000540819.

Conflict of interests

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Financial support

This work was supported by grants from Diabetes Australia Research Trust (grant number: Y12G BURM) and Foundation Daw Park. AR was supported by a National Health Medical Research Council, Australia Postgraduate Research Scholarship and MGB by a South Australian Health Practitioner Fellowship.

Author contributions

AR was responsible for study design, subject recruitment, data acquisition, data analysis and manuscript preparation. AR guarantees the integrity of the data and holds final responsibility for the published manuscript. BLM was responsible for data acquisition. SMD was responsible for data acquisition. AR2 was responsible for laboratory analysis and manuscript revision. MDS was responsible for subject recruitment and manuscript revision. AAM was responsible for study design, data analysis and manuscript revision. CHT was responsible for study design, supervision and manuscript revision. MGB was responsible for obtaining funding, study design, data analysis, supervision and manuscript revision. All authors have reviewed and approved the final version of the manuscript.

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Table 1: Subject characteristics.

	Controls (n=20)	Non-GC users (n=18)	GC users (n=18)	*p*-value
Female (n (%))	16 (80)	12 (67)	12 (67)	0.57
Age (years)	63 ± 6	64 ± 7	66 ± 7	0.24
BMI (kg/m^2)	28.6 ± 4.2	28.1 ± 5.2	27.9 ± 6.1	0.95
e-GFR (ml/min)	82 ± 17	87 ± 19	82 ± 13	0.61
Smoking (n, %))	0 (0)	2 (11)	1 (6)	0.32
Hypertension (n, (%))	3 (15)	5 (25)	4 (20)	0.63
Ischemic heart disease (n, (%))	0 (0)	1 (6)	1 (6)	0.56
Diabetes (n, (%))	0 (0)	1 (6)	1 (6)	0.56
Anti hypertensives (n)	2	5	3	0.58
Statins (n)	1	5	3	0.17

Data are mean ± standard deviation.

GC, glucocorticoid; n, number of subjects with a specified variable; BMI, body mass index; e-GFR, estimated glomerular filtration rate.

Fig.ure legends

Fig. 1: Arginine metabolism.

Simplified diagram showing the principal pathways of arginine metabolism and nitric oxide production: ADMA, asymmetric dimethyl arginine; MMA, mono methyl arginine; SDMA, symmetric dimethyl arginine; AGAT, arginine:glycine amidino transferase; ASS/ASL, arginosuccinate synthase/arginosuccinate lyase.

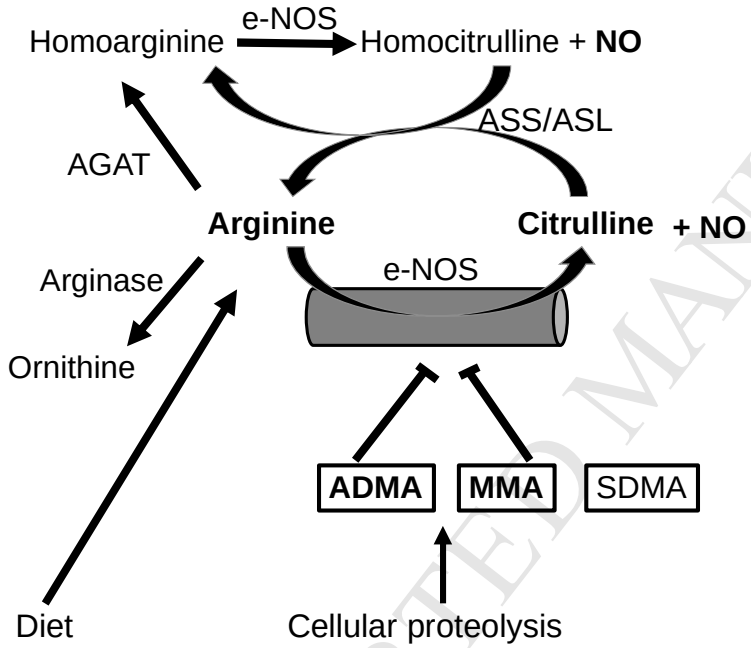
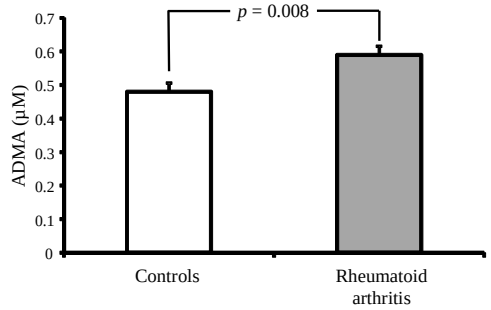


Fig. 2: Effect of rheumatoid arthritis on ADMA and MMA.

Plasma concentrations of (A) asymmetric dimethyl arginine (ADMA) and (B) monomethyl arginine (MMA) in 20 healthy controls (white bar) and in 18 patients with rheumatoid arthritis who were not taking prednisolone (grey bar). Results are mean ± standard error and are corrected for age, sex, eGFR, smoking and cholesterol.

A



B

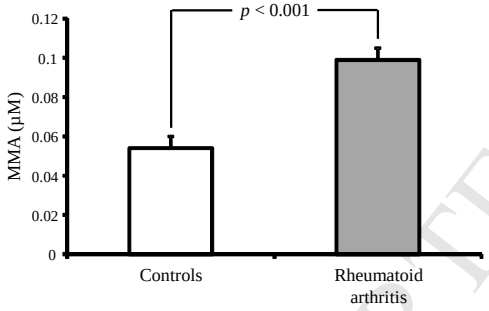
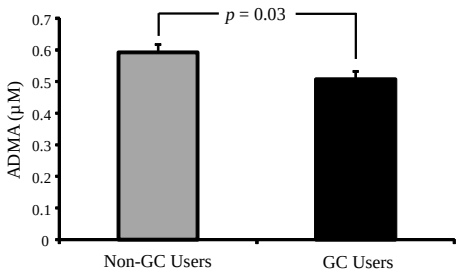


Fig. 3: Effect of long-term prednisolone on ADMA and SDMA.

Plasma concentrations of (A) asymmetric dimethyl arginine (ADMA) and (B) symmetric dimethyl arginine (SDMA) in 18 patients with rheumatoid arthritis who were not taking prednisolone (non-GC users, grey bar), and 18 patients with rheumatoid arthritis on chronic (>6 months) prednisolone (GC users, black bar). Results are mean ± standard error and are corrected for age, sex, eGFR, smoking cholesterol, CRP and disease modifying anti-rheumatic drug use.

A



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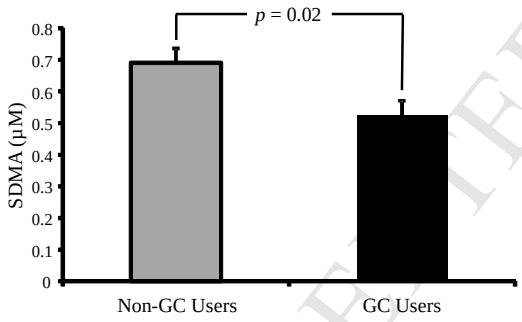

Highlights

1. ADMA, a marker of endothelial dysfunction, is increased in rheumatoid arthritis.
2. Acute prednisolone in rheumatoid arthritis reduces plasma homoarginine.
3. Long-term prednisolone is associated with lower ADMA.