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L-arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in plasma and synovial fluid of patients with knee osteoarthritis

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Background: The aim of this study was to investigate the involvement of the nitric oxide (NO) pathway in osteoarthritis (OA).





Material/Methods: The study groups consisted of 32 patients with knee OA and 31 healthy controls. In peripheral venous blood samples (from the OA patients and the controls) and in synovial fluid samples (from the OA patients), the concentrations of L-arginine (ARN), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) were evaluated. In plasma samples, thiobarbituric acid reactive substances (TBARS) were also measured.

Results: Plasma ARN concentrations were lower in the OA patients than in controls (53.55 ± 16.37 vs. 70.20 ± 25.68 $\mu\text{mol/l}$) ($P < 0.05$), while plasma ADMA concentrations were similar. Accordingly, the ARN/ADMA ratio was lower in the OA patients than in the control group (80.85 ± 29.58 vs. 110.51 ± 30.48 , $P < 0.05$). Plasma SDMA and TBARS concentrations were higher in the OA patients than in controls (0.69 ± 0.15 vs. 0.60 ± 0.10 $\mu\text{mol/l}$, $P < 0.05$ and 1.21 ± 0.29 vs. 0.55 ± 0.12 , respectively) ($P < 0.001$). In the OA patients, ADMA concentrations were significantly higher in the synovial fluid than in plasma (0.75 ± 0.09 vs. 0.69 ± 0.14 $\mu\text{mol/l}$, $P < 0.05$), as were ARN concentrations (76.96 ± 16.73 vs. 53.55 ± 16.73 $\mu\text{mol/l}$) ($P < 0.00001$).

Conclusions: These results indicate a poor availability of NO in the synovial fluid of the OA patients, which may contribute to the progression of OA. The decreased ARN/ADMA ratio and the increased SDMA and TBARS in the plasma of the OA patients suggest an impairment of endothelial function in these subjects.

Key words: **osteoarthritis • nitric oxide • asymmetric dimethylarginine**

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Background

NO, a molecule endowed with important anti-atherosclerotic properties, is synthesized by stereospecific oxidation of the terminal guanidine nitrogen of the amino acid L-arginine by the action of a group of enzymes known as nitric oxide synthases (NOSs) [1]. Synthesis of NO can be selectively inhibited by guanidino-substituted analogues of L-arginine (AR), which competitively block the active site of NOS. N^G, N^G-dimethyl L-arginine (asymmetric dimethylarginine, ADMA), an L-arginine analogue identified in human plasma and urine, is the major endogenous NOS inhibitor, inducing decreased NO bioavailability and endothelial dysfunction [2,3]. ADMA may be considered a reliable cardiovascular risk molecule [4–9]. SDMA (symmetric dimethylarginine), another methylated form of L-arginine, may indirectly interfere with NO synthesis and recently has been reported as an independent cardiovascular risk factor [10]. Reduced NO bioavailability and increased oxidative stress are closely connected. In fact, in presence of a reduced bioavailability of the substrate L-arginine or an increase in intracellular ADMA concentrations, an “uncoupling” of the endothelial NOS (eNOS) occurs, enhancing reactive oxygen species (ROS) generation [11]. ROS can inhibit dimethylarginine dimethylaminohydrolase (DDAH), the enzyme responsible for ADMA catabolism, thus increasing plasma ADMA levels [12,13]. Finally, ROS break down NO, reducing its bio-availability and producing peroxynitrite, which maintains the oxidant characteristics of superoxide. In this regard, ADMA may be considered a mediator of oxidative stress. NO is deeply involved in the pathogenesis of osteoarthritis (OA) [14]. In a recent paper, Yan et al. [15] provided evidence for an important function of eNOS in the regulation of chondrocyte proliferation and differentiation. Mice carrying a null mutation in the eNOS gene (animals deficient in eNOS) showed reduced bone growth with a marked decrease in the number of proliferating chondrocytes [15]. These effects were due to altered expression of various cell cycle proteins, such as the reduction of cyclin D1 protein (a protein required for maximal chondrocyte proliferation *in vivo* and *in vitro* expression in eNOS-deficient mice [15]. Oxidative stress also plays a major role in the articular environment and contributes to the development of osteoarthritis (OA) [16]. Excessive ROS production induces cellular and tissue structural functional changes, causing damage to the cartilage [17]. Henrotin et al. found elevated nitrated type II collagen (type II collagen is the basis for articular cartilage and hyaline cartilage and it makes up 50% of all proteins in cartilage and 90% of collagen articular cartilage) peptides in sera of patients with OA, suggesting the formation of ONOO⁻ in OA cartilage [18]. In patients with OA, oxidative stress can determine the rate of progression of the disease and modify the response to treatment [19]. Furthermore, oxidative stress in patients with knee OA has been recently associated to atherosclerosis and increased cardiovascular risk

Table 1. Exclusion criteria for patients with knee osteoarthritis and healthy controls enrolled in the study.

1. Lymphoproliferative disorders and other neoplasms
2. Arterial Hypertension
3. Metabolic syndrome
4. Dislipidemia
5. Diabetes
6. Renal pathology
7. CAD
8. CHD
9. Mental retardation
10. Depression treated with drugs
11. Pulmonary hypertension with COPD
12. Autoimmune diseases

[20,21]. It is noteworthy that both reduced NO bioavailability and increased oxidative stress play a major role in the pathogenesis and progression of OA and are also able to induce endothelial dysfunction and accelerated atherosclerosis. In patients with OA, the oxidative status has been fully investigated [20–23], but data on the functional activity of the NO pathway are lacking at present. Based on these premises, we thought it would be interesting to evaluate the concentrations of ARN, ADMA (the most potent endogenous NOS inhibitor, able to induce effective NO deficiency), and SDMA (another ARN analogue and an indirect inhibitor of NO synthesis) in the plasma and in the synovial fluid of patients with knee OA. As an index of oxidative stress, plasma concentrations of TBARS, a reliable biomarker of lipid peroxidation [24], were evaluated in the same patients.

Material and Methods

Patients

Between January 2011 and May 2012, we selected 32 patients with idiopathic OA of the knee who were scheduled to undergo a total knee arthroplasty (TKA) and had received no prior treatment for their OA. Thirty-one controls were also chosen from among healthy nurses and physicians at our institute who wanted to be enrolled. The members of the study and control groups were chosen according to specific exclusion criteria (Table 1), as each of these conditions is known to be involved in raising ADMA levels in peripheral blood. No patient in either the study or control group had taken NSAIDs or undergone hormone therapy. The baseline characteristics of the patients and healthy controls are listed in Table 2. Venous blood samples were stored in pre-chilled Vacutainers containing heparin, placed immediately on ice, and centrifuged for 10 min at 3000 g within 30 min of the sampling. Plasma samples were stored at –20°C until required. The blood samples of the OA patients were taken in the operating room, just

Table 2. Characteristics of patients with knee osteoarthritis (OA) and healthy controls (Controls) enrolled in the study.

	OA patients N=32	Controls N=31
Age (years)	65.2±5.5	64.2±6.3
Sex (male/female)	14/18	14/17
Non smokers	25 (78.1%)	23 (74.1%)

before the TKA was performed. At the same time, a 5-ml sample of synovial joint fluid was taken only from OA patients, according to the following method: after inflating a tourniquet to 350 mmHg to avoid blood contamination from the vessels, a skin incision was made, then a sample was aspirated through the capsule with a syringe and stored at -20°C prior to biochemical analysis. The protocol study was approved by our local Ethics Committee.

Biochemical analysis

Plasma and synovial fluid concentrations of ADMA, SDMA, and ARN were determined by the high performance liquid chromatography (HPLC) method of Teerlink et al. [25] with minor modifications. A Spectra System P2000 liquid chromatograph equipped with an injection valve (model 7125 Rheodyne) and a Waters 474 Scanning Fluorescence Detector was employed. The system was connected to a D-2000 chromato-integrator Hitachi-Merck. A Waters Symmetry C_{18} 3.5 μm (150×4.6 mm i.d.) coupled to a Waters Sentry Symmetry C_{18} guard column was operated at room temperature. The mobile phase was 9:91 (v/v) acetonitrile: 50 mM potassium phosphate buffer (K_2HPO_4 pH=6.50). The flow rate was 1.1 ml/min and the column effluent was monitored at excitation and emission wavelengths of 340 and 455 nm, respectively. Before HPLC analysis, samples (200 μl) were purified by solid phase extraction (SPE) on a Waters Oasis MCX SPE 1 ml, 100 mg cartridges after addition of N^G -monomethyl arginine as an internal standard. Extracted samples were derivatized with o-phthalaldehyde (OPA).

Plasma TBARS, a marker of oxidative stress, reflects plasma malondialdehyde concentrations resulting from lipid peroxidation [26,27]. TBARS were determined with the HPLC method of Seljeskog et al. [28] with minor modifications. A SpectraSystem P2000 liquid chromatograph equipped with an injection valve (model 7125 Rheodyne) and a WATERS 474 fluorescence detector was employed. The system was connected to a D-2000 chromato-integrator Hitachi-Merck. A Supelcosil C18 3.5 μm (150×4.6 mm ID) coupled to Supelcosil C18 guard column was operated at room temperature. The mobile phase was methanol and PBS 25 mM at pH 6.20 (25: 75 V:V). The flow rate was 1.0 ml/min and the column effluent was monitored at

excitation and emission wavelengths of 525 and 560 nm, respectively. A standard curve was made from 1,1,3,3-tetraethoxypropane (TEP), dissolved in methanol and diluted in sterile water at concentrations of 10.0, 5.0, 2.5, 1.25, 0.62, 0.31, 0.16 μM , and blank. TEP standards were heated at 50°C for 60 min and were stored at 4°C for a maximum of 1 week. Plasma samples or standard/blanks (50 μl) were mixed with perchloric acid (PCA) (0.1125 N, 150 μl) and thiobarbituric acid (TBA) (40 mM, 150 μl) in a 1.5-ml screw-cap Eppendorf tube, vortexed for 10 s and placed in a heating bath at 97°C for 60 min. After cooling at -20°C for 20 min, methanol (300 μl) and 20% trichloroacetic acid (TCA) (100 μl) were added to the suspension and mixed for 10 s. The samples were centrifuged at $13\,000 \times g$ for 6 min, then 100 μl of the supernatant was injected into the chromatograph.

Statistical analysis

The descriptive data are presented as mean values \pm standard deviation (SD). Differences between groups were compared using Student's t-test. A P value of <0.05 was considered statistically significant.

Results

As shown in Table 2, the OA patients and the controls were similar for age, sex, and smoking status. Plasma ARN concentrations were significantly lower in the OA patients than in the healthy controls (53.55 ± 16.37 vs. 70.20 ± 25.68 $\mu\text{mol/l}$, $P < 0.05$), while plasma SDMA concentrations were higher in the OA patients (0.69 ± 0.15 vs. 0.60 ± 0.10 $\mu\text{mol/l}$, $P < 0.05$) (Table 3). Plasma ADMA concentrations were not statistically different in the OA patients and in the controls (0.69 ± 0.14 vs. 0.64 ± 0.14 $\mu\text{mol/l}$, $P = \text{NS}$) (Table 2). The ARN/ADMA ratio was lower in the OA patient than in the control group (80.85 ± 29.58 vs. 110.51 ± 30.48 , $P < 0.05$) (Table 3). Plasma TBARS levels were significantly higher in the patient group compared to the control group (1.21 ± 0.29 vs. 0.55 ± 0.12 $\mu\text{mol/l}$, $P < 0.001$) (Table 3).

In the patients with OA, ARN concentrations were significantly higher in the synovial fluid than in plasma (76.96 ± 16.73 vs. 53.55 ± 16.73 $\mu\text{mol/l}$, $P < 0.00001$), as were ADMA concentrations (0.75 ± 0.09 vs. 0.69 ± 0.14 $\mu\text{mol/l}$, $P < 0.05$) and the ARN/ADMA ratio (103.37 ± 13.65 vs. 80.85 ± 29.58 , $P < 0.0005$) (Table 4). SDMA levels in the synovial fluid and in plasma were not significantly different ($P = \text{NS}$) (Table 4).

Discussion

Our results show lower levels of plasma ARN concentrations in the OA patients as compared to the controls while plasma

Table 3. Mean Values of arginine (ARN), asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), ARNA/ADMA ratio and malondialdehyde (MDA) in blood plasma of patients with knee osteoarthritis (OA) and healthy controls (Controls).

Blood Plasma	OA patients N=32	Controls N=31	P
ARN ($\mu\text{mol/l}$)	53.55 \pm 16.73 (Range: 21.67–98.25)	70.20 \pm 25.68 (Range: 33.08–173.0)	P<0.05
ADMA ($\mu\text{mol/l}$)	0.69 \pm 0.14 (Range: 0.49–0.93)	0.64 \pm 0.14 (Range: 0.51–0.81)	N.S.
SDMA ($\mu\text{mol/l}$)	0.69 \pm 0.15 (Range: 0.41–1.03)	0.60 \pm 0.10 (Range: 0.48–0.86)	P<0.05
ARN/ADMA	80.85 \pm 29.58 (Range: 34.68–166.5)	110.51 \pm 30.48 (Range: 50.62–213.0)	P<0.05
TBARS ($\mu\text{mol/l}$)	1.21 \pm 0.29 (Range: 0.65–1.60)	0.55 \pm 0.12 (Range: 0.39–0.95)	P<0.001

Table 4. Mean Values of arginine (ARN), asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA) and ARNA/ADMA ratio in synovial fluid (synov.f) and blood plasma of patients with knee osteoarthritis (OA).

	synov.f. N=32	Plasma N=31	P
ARN ($\mu\text{mol/l}$)	76.96 \pm 16.73 (Range: 68.00–91.53)	53.55 \pm 16.73 (Range: 21.67–98.25)	P<0.00001
ADMA ($\mu\text{mol/l}$)	0.75 \pm 0.09 (Range: 0.65–0.96)	0.69 \pm 0.14 (Range: 0.49–0.93)	P<0.05
SDMA ($\mu\text{mol/l}$)	0.66 \pm 0.10 (Range: 0.55–0.95)	0.69 \pm 0.15 (Range: 0.41–1.03)	N.S.
ARN/ADMA	103.37 \pm 13.65 (Range: 81.64–127.1)	80.85 \pm 29.58 (Range: 34.68–166.5)	P<0.0005

ADMA levels were similar. Therefore, the ARN/ADMA ratio was significantly reduced in the OA patients. On the contrary, in the patients with OA, ARN levels in the synovial fluid were significantly higher than those detected in plasma. The reasons for the opposite trend of ARN levels in the plasma and in the synovial fluid of the patients with OA are not clear. One explanation may be an intensive move of ARN from plasma to the synovial fluid to antagonize ADMA (increased in the synovial fluid of the OA patients) in the synthesis of NO in the arthritic joint of the patients. Another possible explanation is that the decrease of plasma ARN levels is directly correlated to joint inflammation. In fact, inflammatory processes may decrease L-arginine levels by increasing the activity of arginase, an enzyme that converts L-arginine into L-ornithine, and through a decrease in the L-arginine transport into endothelial cells mediated by the γ^+ -transporter system [29]. An unfavorable change in the ARN/ADMA ratio was demonstrated in the plasma of the OA patients of our study. It is known that an improved ARN/ADMA ratio indicates increased conversion of ARN to NO, while a decreased ARN/ADMA ratio indicates a diminished NO production by NOS [30,31]. Reduction of ADMA and l-arginine/ADMA ratio may be considered a predictor of endothelial dysfunction and enhanced atherosclerosis

[32]. In keeping with this view, in elderly subjects, the l-arginine/ADMA ratio was positively related to endothelium-dependent vasodilation in resistance arteries [33], while in patients with stable angina, lowering of the l-arginine/ADMA ratio was related to coronary artery disease (CAD) and the severity of lesions found by coronary artery angiography [34]. Thus, it is possible that our patients with OA may have been affected by some form of endothelial dysfunction, as indicated by the decreased ARN/ADMA ratio, even though they did not show any sign or symptom of cardiovascular disease. Another interesting result of our study was the increase of plasma SDMA concentrations found in the OA patients. This increase has no clear explanation, since the patients enrolled in this study had normal renal function. SDMA, which is reportedly mainly eliminated by renal excretion, is an established marker of renal function [35]. However, SDMA is also capable to inhibit both the intracellular uptake of L-arginine and renal tubular arginine absorption [36,37], thus indirectly inhibiting NO synthesis. Accordingly, SDMA should also be considered a cardiovascular risk marker [10,38]. Hence, in the OA patients of our study, both the decrease of ARN/ADMA ratio and the increase of plasma SDMA concentrations represent factors capable of inducing endothelial dysfunction and accelerated

atherosclerosis. Another factor able to enhance cardiovascular risk in the patients with OA was the increased plasma TBARS levels found in the patient group as compared to the control group (this study). TBARS, a reliable biomarker of lipid peroxidation, reportedly are strongly predictive of cardiovascular events in patients with stable artery coronary disease [24]. The association of oxidative stress with OA is well established [16,17,23,39]. Moreover, lipid peroxidation is a known mechanism of cellular injury that operates in the pathogenesis of OA [22]. In this vein, recent studies [20,21] have demonstrated elevated serum lipid hydroperoxide (LOOH, the primary end-products of the lipid peroxidation cascade) levels in patients with knee OA. In the same patients, decreased levels of serum paraoxonase-1 (PON1) were also demonstrated [20,21]. PON1 is an antioxidant enzyme able to hydrolyze lipid peroxidation products, thus decreasing their accumulation [40]. Reduced serum PON1 activity promotes atherosclerosis and coronary artery disease [41]. From these reports (increased oxidative stress and decreased serum PON1 activity) we can envisage an association between OA and cardiovascular risk. The results of our study strengthen this view. In fact, in our patients with knee OA, the presence of an enhanced oxidative stress was confirmed (increased TBARS levels) and pro-atherogenic modifications of ARN/ADMA ratio (decrease) and plasma SDMA (increase) were demonstrated. In all, these data indicate a propensity of patients with OA to develop endothelial dysfunction and atherosclerosis. In keeping with this view, many reports have shown an association between OA and enhanced cardiovascular comorbidity [42–46].

As regards the articular environment, in the patients with OA, ADMA concentration in the synovial fluid was increased as compared to values detected in plasma. We can hypothesize several sources of ADMA in the joint fluid: a type I synovial cell dysfunction, synovium vessel malfunction, collagen protein

degradation, or subchondral bone vessels defect. The increase of ADMA concentrations in the synovial fluid indicates an inhibition of the NO pathway in the joint, likely responsible for a decrease in chondrocyte proliferation and differentiation. Furthermore, the increase of ADMA is also able to enhance ROS generation in the articular environment, further promoting cartilage damage [17]. Therefore, the finding of increased ADMA levels in the synovial fluid could indicate a progression of the articular disease in the OA patients of this study.

Conclusions

We found a significant decrease of plasma ARN concentrations and consequently of the ARN/ADMA ratio in the OA patients. These findings, coupled with the increase of plasma SDMA and MDA concentrations, suggest the presence of an endothelial dysfunction in these patients. Accordingly, the patients with knee OA in our study might have an increased cardiovascular risk. Some limitations of this study should be noted. Firstly, the patient group size was small. In future studies, we will need to measure ARN, ADMA, and SDMA in a larger population of OA patients with homogeneous characteristics. Secondly, we infer the presence of an endothelial dysfunction in our patients only from measurements of circulating biomarkers. It could be productive to further investigate the endothelial function of OA patients with special clinical non-invasive methods such as brachial artery flow mediated dilation (FMD) [47]. In conclusion, the results of this study indicate that our patients with knee OA are likely affected by some form of endothelial dysfunction with increased cardiovascular risk. Our findings suggest that in clinical practice the cardiovascular function of patients with OA should be thoroughly investigated. Possible recognized cardiovascular abnormalities, such as arterial hypertension, should be effectively treated.

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