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## Characterization of nitrotyrosine as a biomarker for arthritis and joint injury

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**SUMMARY** (limit: 250 words); current word count: 237

*Objectives:* To characterize the utility of nitrotyrosine (NT) as a biomarker for arthritis and joint injury.

*Design:* Synovial fluid, plasma, and urine from patients diagnosed with osteoarthritis (OA), rheumatoid arthritis (RA), anterior cruciate ligament (ACL) injury, meniscus injury and pseudogout, and knee-healthy volunteers were analyzed for concentrations of NT, nitrate and nitrite (NO<sub>x</sub>), matrix metalloproteinase (MMP)-3, MMP-1, MMP-9, more than 40 chemokines and cytokines.

*Results:* In OA, plasma and synovial fluid NT were increased versus healthy volunteers. Synovial fluid to plasma NT ratios were elevated in OA patients. Synovial fluid from patients with ACL and meniscus injury and pseudogout had increased levels of NT ( $P < 0.001$ ). In these samples, NT levels significantly correlated with ARGS-aggrecan neoepitope generated by aggrecanase cleavage of aggrecan ( $P \leq 0.001$ ), cross-linked C-telopeptides of type II collagen ( $P < 0.001$ ), MMP-1 ( $P = 0.008$ ), and MMP-3 ( $P \leq 0.001$ ). In RA, plasma NT decreased following 6 months of anti-tumor necrosis factor (TNF) treatment. For every 1.1% change in  $\log_{10}$  NT, there was a 1.0% change in the  $\log_{10}$  disease activity scores (DAS28-3 CRP). Both predicted and observed DAS28-3 CRP showed a robust linear relationship with NT. RA plasma NT positively correlated with CRP, MMP-3 and interferon  $\gamma$ -induced protein 10.

*Conclusions:* NT may serve as a useful biomarker for arthritis and joint injury. In RA, NT is highly correlated with several biomarkers and clinical correlates of disease activity and responds to anti-TNF therapy.

Keywords: Nitric oxide, Nitrotyrosine, Biomarkers, Arthritis, Joint injury

## Introduction

Increased nitric oxide synthase (NOS) activity has been linked to joint injury and is associated with increased chondrocyte apoptosis, increased matrix metalloproteinase (MMP) activity and decreased extracellular matrix synthesis<sup>1</sup>. Osteoarthritis (OA) and rheumatoid arthritis (RA) show increased tissue staining for both inducible nitric oxide synthase (iNOS) and its downstream product, nitrotyrosine (NT)<sup>1-6</sup>. NT is a stable marker resulting from the generation of peroxynitrite, a powerful oxidant arising from the diffusion-limited reaction of nitric oxide (NO) with superoxide<sup>1</sup>. Although diet is a source of nitrate in vivo and should be considered when assessing this metabolite as a biomarker for NOS activity, numerous studies have used nitrate and nitrite (NOx) as a measure of NO production in arthritis<sup>7-12</sup>. Studies on RA showed increased levels of plasma and synovial fluid nitrite<sup>7-12</sup>, and synovial fluid NT<sup>13</sup> but have not demonstrated a therapeutic response of these biomarkers.

Recent work has shown increased nitrated type II collagen in serum from patients with OA and RA<sup>14</sup>, although this measure is specific to type II collagen and would not include synovial and other joint tissue targets for peroxynitrite. A biomarker, such as NT that is derived from multiple nitratively modified joint proteins rather than solely from type II collagen, may better reflect the extent of joint pathology and provide a more robust response to therapeutic intervention. In

the present investigation, we therefore determined NT and NOx in plasma, urine and synovial fluid samples from patients with joint injury, OA, pseudogout and RA, and related these levels to arthritis biomarkers and clinical correlates of disease activity.

## **Methods**

### *Human samples*

For all human samples, the protocol and informed consent documentations were reviewed and approved by Institutional Review Board(s) and/or Independent Ethics Committee(s) at each study center. Written informed consent was received from all eligible patients before procedures were initiated. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Hel<sup>s</sup>inki Declaration of 1975, as revised in 2000. Samples (plasma, synovial fluid, and urine) were obtained from the following cohorts.

### *Cohort 1. Human plasma samples*

Subjects with knee OA were all female and obese (body mass index [BMI]  $\geq 30$ ) with symptomatic OA as evidenced by frequent knee symptoms during the course of the year. These included pain, aching or stiffness on most days of the month, along with the frequent use of medication. Subjects with Kellgren

and Lawrence grades 2 or 3 of the signal knee (with either the same or less severe OA or no OA of the contralateral knee) were included in this OA cohort ( $n = 83$ ). The control group ( $n = 92$ ) had no evidence of knee OA in either knee (i.e., Kellgren and Lawrence grade 0 diagnosed by x-ray on anteroposterior view; infrequent knee pain, aching or stiffness the year prior to the study; or infrequent use of medication for treatment of knee pain).

### *Cohort 2. Matched human OA plasma and urine samples*

Matched plasma and urine samples were obtained from male and female patients ( $n = 20$ ) with painful knee OA, x-ray confirmed Kellgren and Lawrence grade 2 or 3, aged  $\geq 40$  years and a BMI  $\leq 35$ . Any evidence of inflammatory arthritis resulted in exclusion from the study. Patients had no non-steroidal anti-inflammatory drug use for 7 days prior to sample collection. Control subjects ( $n = 20$ ) were age- and gender-matched with the OA group and were confirmed by radiography to have no evidence of OA in the knees, hips or dominant hands.

### *Cohort 3. Human synovial fluid samples (OA, joint injury, pseudogout, controls)*

Because NT in plasma and urine could be derived from sources outside OA joints, we analyzed synovial fluid from OA and joint injury (anterior cruciate ligament [ACL] and/or meniscus rupture) patients who often develop OA following injury<sup>15</sup>. In addition, because increased synovial fluid levels of NT support a role for peroxynitrite-mediated joint tissue injury in OA and ACL

injury, we assessed its level in pseudogout, an acute inflammatory condition involving joint damage. Briefly, human synovial fluid samples ( $n = 382$ ) were aspirated without lavage from a cross-sectional convenience cohort with informed consent and approval of the Lund University research ethics committee. Diagnosis was made by arthroscopy, radiography, assessment of joint fluid and clinical examination. Diagnostic groups were knee-healthy references with no history of joint injury or joint pain ( $n = 10$ ); pseudogout (pyrophosphate crystal arthritis,  $n = 34$ ); joint injury sustained between less than 1 week and 20 years before sample acquisition (knee ACL rupture, with or without concomitant meniscus lesions,  $n = 136$ ) or isolated knee meniscus injury ( $n = 118$ ); and knee OA ( $n = 84$ ). Patients with pseudogout had radiographic OA corresponding to Kellgren and Lawrence grade 1 to 3, whereas patients with OA had radiographic OA corresponding to grade 2 or greater. Patients with joint injury generally showed mild to moderate cartilage damage on arthroscopic examination, with a few additionally having radiographic signs of OA corresponding to Kellgren and Lawrence grade 1 or 2. The samples of this cohort were partly identical with those used in previous studies<sup>16-19</sup>.

#### *Cohort 4. Matched human OA plasma and synovial fluid samples*

To better understand the relationship between synovial fluid and plasma NT in OA, we obtained matched plasma and synovial fluid samples from male and female knee OA patients ( $n = 40$ ;  $n = 20$  per gender) just prior to knee



replacement surgery from Clinomics BioSciences Inc. (Pittsfield, MA, USA). In addition, age- and gender-matched control samples ( $n = 40$ ) were purchased from Clinomics Biosciences, Inc.

#### *Cohort 5. RA plasma samples*

Plasma was collected from RA patients ( $n = 18$ ) before and after treatment with anti-tumor necrosis factor (TNF) biotherapeutics (etanercept, infliximab, or adalimumab) over a 6-month period. Control plasma samples ( $n = 21$ ) from an age-matched cohort of healthy volunteers were obtained from PrecisionMed (San Diego, CA, USA).

#### *Disease Activity Score of 28 Joints*

The disease activity score of 28 joints using C-reactive protein (DAS28-3 CRP) was the outcome measure used as an indicator of RA disease activity and response to treatment. It is the basis for several other RA measurement tools and is widely used as an indicator of RA disease activity and response to treatment<sup>20</sup>.

#### *3-NT and NOx Assays*

We used a newly described assay for total NT (protein-containing and protein-free) to measure NT levels in biological fluids (plasma, synovial fluid and

urine)<sup>21</sup>. Briefly, quantification of NT from the human body fluid samples was performed using immunoaffinity two-dimensional (2D) liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an HP 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) and a switching valve (Valco Instruments, Houston, TX, USA) plumbed in-line with a pump and interfaced to an API 4000 mass spectrometer (Applied Biosystems/MDS-Sciex, Toronto, Canada) operated in the negative ion electrospray and multiple reaction monitoring modes.

NOx was measured using a fluorescent assay as previously described<sup>22</sup>.

*Immunoassays for MMPs, cytokines, chemokines, aggrecan fragments, aggrecan epitope 846 and cartilage oligomeric matrix protein*

MMP-1, MMP-3 and MMP-9 were measured in plasma from patients with RA using a multiplex sandwich-based enzyme-linked immunosorbent assay (ELISA) format (Meso Scale Discovery, Gaithersburg, MD, USA). Typically, a 5- to 10-fold dilution of biological fluid was analyzed. For measurement of cytokines and chemokines, approximately 25 µl of each sample was analyzed for 42 different human antigens as defined in the manufacturer's protocol (HCYTO-80K-42PMCX; Millipore, Billerica, MA, USA): epidermal growth factor (EGF), eotaxin, fibroblast growth factor (FGF-2), flt3 ligand (Ftl3L), fractalkine, granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), growth-regulated oncogene (GRO), interferon alpha-2 (IFNα2),

interferon gamma (IFN $\gamma$ ), interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-1 receptor antagonist (ra), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-15, IL-17, interferon-gamma inducible protein-10 (IP-10), monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage-derived chemokine (MDC), macrophage-inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$ , soluble CD40 ligand (sCD40L), soluble IL-2 receptor  $\alpha$  (sIL-2R $\alpha$ ), transforming growth factor  $\alpha$  (TGF $\alpha$ ), TNF $\alpha$ , TNF $\beta$ , vascular endothelial growth factor (VEGF), platelet-derived growth factor  $\alpha$  a (PDGF-AA), PDGF-AB/BB, and regulated upon activation, normal T-cell expressed and secreted (RANTES). Only those antigens showing a change with treatment are reported here.

Biomarker immunoassay results for a part of the synovial fluid samples of Cohort 3 were from published reports<sup>16-19</sup>. Assay methodologies for aggrecan ARGS-neoepitope, aggrecan epitope 846, cartilage oligomeric matrix protein (COMP), MMP-1, MMP-3 and cross-linked type II collagen C-telopeptides (CTX-II) were as described<sup>16-19</sup>. CRP was measured using a nephelometric immunoassay.

### *Statistical analysis*

Data were analyzed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA) and Sigmaplot 12.3 to assess normal distribution of values and to calculate mean values, standard deviations, confidence intervals and statistical significance. Either an unpaired Student's *t*-test with *P* value < 0.05 or

a one-way analysis of variance, followed by the Holm-Sidak test for multiple comparisons of datasets, was used to determine level of significance between mean values. If values were not normally distributed, log-transformed values were analyzed. The Spearman rank correlation coefficient was used to examine the relationship between two potential markers for disease activity using two-tailed  $P$  values of  $< 0.05$  as criteria for statistical significance.

A regression analysis was performed using the data derived from the RA study. Besides plasma NT values, the demographic variables of age, gender, alcohol consumption, smoking history and hormonal status (pre- or post-menopausal) were included in the analysis.

## **Results**

Comparison of plasma and synovial fluid NT levels in all healthy volunteers and all patients with OA showed that plasma NT was significantly elevated in the OA group ( $P < 0.001$ ; Fig. 1A). In the subset of patients with OA evaluated in Cohort 2, urinary NT did not show a statistically significant difference versus controls for this cohort (Fig. 1A): 104.6 ng/mg creatinine (95% confidence interval [CI] 69.6, 139.7) versus 104.1 ng/mg creatinine (95% CI 78.5, 129.6), respectively, despite higher plasma levels of NT (704.1 pg/ml [95% CI 626.8, 781.4], versus 536.4 pg/ml [95% CI 454.7, 618.2]) for controls.

We found a statistically significant elevation of synovial fluid NT in the OA, joint injury and pseudogout groups when compared with the control group ( $P < 0.001$ ; Fig. 1B). In these joint fluid samples, concentrations of NT were significantly correlated with levels of ARGS-aggrecan neopeptide generated by aggrecanase cleavage of aggrecan ( $P \leq 0.001$ ), cross-linked C-telopeptides of type II collagen ( $P < 0.001$ ), and with protein levels of MMP-1 ( $P = 0.008$ ) and MMP-3 ( $P \leq 0.001$ ; Table I). In contrast, no statistically significant correlations were found between NT and COMP ( $P = 0.655$ ) or aggrecan epitope 846 ( $P = 0.43$ ), or between NOx and any of the other biomarkers ( $P \geq 0.16$ ).

In a smaller group of patients with OA prior to knee replacement surgery and controls, the synovial fluid to plasma NT ratio was higher for the OA group when compared with the control group ( $P = 0.0047$ ; Fig. 2) despite no statistically significant increase in synovial fluid NOx in patients with OA ( $57.1 \mu\text{M}$  [95% CI 45.5, 68.7] versus  $56.2 \mu\text{M}$  [95% CI 45.2, 67.2] for controls,  $P = 0.9112$ ). Although OA plasma NOx showed an apparent elevation ( $30.4 \mu\text{M}$  [95% CI 24.5, 36.3]) versus controls ( $23.0 \mu\text{M}$  [95% CI 19.5, 26.6];  $P = 0.0346$ ), the synovial fluid to plasma NOx ratio was not statistically different between the OA and control groups (Fig. 2;  $P = 0.8837$ ).

Patients with RA showed increased levels of plasma NT when compared with healthy controls ( $P < 0.001$ ; Fig. 3, Table II). Interestingly, a decrease in plasma NT ( $P < 0.05$ ) was observed after 6 months of anti-TNF therapy but not after 3 months of treatment (Fig. 3, Table II). Compared with NT, the temporal

modulation of MMP-3 and MMP-1 levels was more rapid and corresponded more closely to the pattern observed for both CRP and IL-6, both of which were reduced by 3 months of treatment. As expected, DAS28-3 CRP was also decreased by the anti-TNF treatment (Table II).

Before the initiation of anti-TNF therapy, we observed a correlation between plasma NT and plasma levels of MMP-1 and MMP-3 (Table III). In addition to a strong correlation between plasma NT and CRP (Table III), we found a strong correlation between percentage change in  $\log_{10}$  NT and the percentage change in the  $\log_{10}$  DAS28-3 CRP score when adjusted for age, alcohol consumption, and gender after 6 months of anti-TNF therapy (Fig. 4, Table IV). At 26 weeks of treatment, changes in NT level were predictive of changes in DAS28-3 CRP scores when age, gender, alcohol consumption and all second- and third-level interactions were included (Fig. 4, Table IV).

## **Discussion**

A genuine need exists for clinical biomarkers for joint damage and disease progression in arthritis. Using a novel assay for the quantification of NT, we demonstrated increased levels of NT in synovial fluid from patients with OA, joint injury, and pseudogout versus controls. NT was also elevated in plasma from patients with OA and RA compared with healthy controls. In RA, our results demonstrate for the first time that anti-TNF therapy decreased plasma NT. This decrease in plasma NT correlated with markers of RA disease activity, including

MMP-1, MMP-3, CRP and DAS28-3 CRP. This is the first time that a biomarker of oxidative/nitrative damage like NT has been shown to correlate strongly with a clinical disease activity tool like DAS28-3 CRP.

Previous studies have shown the presence of iNOS in activated monocytes and a reduction in iNOS levels following anti-TNF therapy<sup>23, 24</sup>. However, NO reacts in a diffusion-limited manner with superoxide anion to form peroxynitrite, a powerful oxidant that has been linked to tissue injury. Because NT is a stable end product of peroxynitrite formation, it should better reflect disease-associated tissue injury than iNOS levels. The apparent delay in plasma NT reduction, relative to other markers and DAS28-3 CRP, may be explained by alternative pathways of iNOS regulation or by the possibility that plasma NT is a marker for chronic tissue damage rather than an acute response marker for inflammation like CRP or MMPs. A further possible explanation for the delayed plasma NT reduction is provided by our previous observation in a rodent study that the clearance of nitrated proteins was slower than that of NO<sub>x</sub> or free NT<sup>25</sup>. Disease-associated markers like rheumatoid factor may well be among these nitrated proteins.

In order to evaluate the utility of NT as a biomarker for arthritis and joint injury, we measured NT levels in urine, plasma and synovial fluid. We found that of the biological fluids we examined for NT, plasma and synovial fluid displayed the most consistent elevation in levels of this potential biomarker for disease activity. Moreover, NT in these fluids correlated well with previously established

markers for joint damage. Surprisingly, we did not observe a similar pattern for urinary NT. Thus, we analyzed NT in synovial fluid, which should directly reflect the underlying molecular processes affecting joint integrity, and also NT in plasma, which should contain NT generated from the joint as well as from other sources.

We chose to compare our results with NOx (nitrite and nitrate) because we had found NOx to be a more robust signal in biological fluids than nitrite alone and because peroxynitrite preferentially breaks down into nitrate (greater than 90% of the NOx signal) if it fails to oxidize surrounding molecular targets like protein, lipids and nucleic acids<sup>1, 26</sup>. With that said, in this study we did not observe a correlation between NOx (usually predominantly nitrate in plasma) and NT when measured in the same sample (data not shown).

Our present study demonstrated elevated synovial fluid and plasma levels of NT in OA, RA, joint injury and pseudogout. The lack of any appreciable difference in synovial fluid NOx in all samples, including controls, reveals NT to be a better biomarker for joint damage than NOx in these same fluids. Importantly, synovial fluid NT (but not NOx) was correlated with synovial fluid markers of joint tissue degradation (ARGS-aggrecan, CTX-II, MMP-1, MMP-3), but not with synovial fluid COMP and aggrecan epitope 846, which have been suggested to reflect joint tissue synthesis and repair<sup>27</sup>. Similar to our results in OA, NT in plasma from patients with RA correlated well with plasma markers of disease and joint destruction including CRP, interferon  $\gamma$ -induced protein 10 and MMP-3, all of which are increased in RA and may contribute to joint pathophysiology<sup>28</sup>.



In summary, we have shown NT, but not NO<sub>x</sub>, to be increased in synovial fluid and plasma from patients with arthritis and joint injury. The synovial fluid to plasma ratio of NT was greater than one in OA patients, suggesting local joint production of NT, a marker for peroxynitrite, the powerful oxidant generated by the reaction of NO with superoxide. Furthermore, plasma NT was increased in RA patients, responded to anti-TNF treatment, and was predictive of changes in DAS28-3 CRP following treatment. Although further studies are warranted in larger, prospective patient cohorts, our results suggest that NT in synovial fluid and plasma may serve as a biomarker for joint destruction and that it may be used to monitor therapeutic efficacy of disease-modifying treatment. Thus, the identification of NT as a potential biomarker may enable better selection and stratification of individuals with active joint disease and may help provide a more effective assessment of their response to disease-modifying therapeutics.

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## **Contributions**

1. **Thomas P Misko (corresponding author)** was the principal investigator in the study, provided data analyses and interpretation, and reviewed the manuscript drafts and provided comments.

2. **Melissa R Radabaugh, Maureen Highkin, Mark Abrams, and Olga Friese** analyzed samples, collected and interpreted data, reviewed and provided comments to manuscript drafts.
3. **Candace Bramson, Marie Pierre Hellio Le Graverand, L Stefan Lohmander, and Doina Roman** managed the clinical evaluation of patients and the collection of their biological fluids, preparation of the manuscript, were involved in data analyses and interpretation, and provided substantial reviews and comments to the manuscript drafts.
4. **Robert Gallavan** provided statistical analyses, as well as reviewed and commented on manuscript drafts.
5. **All authors** reviewed and approved the final manuscript prior to journal submission.
6. **Joseph Oleynek** provided medical writing support during the preparation of this manuscript, but does not meet the ICMJE guidelines for authorship.

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## **Conflict of interests**

L.S. Lohmander declares no conflict of interest.

## **Ethics approval**

Samples were obtained from studies performed with local ethics committee approval and in accordance with the guidelines of the Declaration of Helsinki and Good Clinical Practice.

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## Figure Legends

**Fig. 1.** (A) Plasma levels of nitrotyrosine (NT) in patients with OA ( $n = 143$ ) patients compared with controls ( $n = 174$ ), urinary NT levels in OA ( $n = 20$ ) and controls ( $n = 20$ ). (B) Synovial fluid (SF) NT values (one-way analysis of variance [ANOVA] of  $\log_{10}$ -transformed data, followed by the Bonferroni multiple comparisons test) in patients with OA ( $n = 115$ ), joint injury (anterior cruciate ligament [ACL] and/or meniscus injury) ( $n = 206$ ) and pseudogout ( $n = 31$ ) compared with non-arthritic controls ( $n = 46$ ). Values for SF nitrate/nitrite (NOx) from controls ( $n = 50$ ), patients with OA ( $n = 125$ ), joint injury ( $n = 254$ ) and pseudogout ( $n = 34$ ). Bars show mean values with 95% confidence intervals.

**Fig. 2.** NT ratios for matched SF and plasma samples (late-stage OA patients at the time of joint replacement, Clinomics BioSciences Inc., Pittsfield, MA, USA) for the OA group ( $n = 40$ ) compared with non-OA controls ( $n = 37$ ). NOx ratios for OA patients ( $n = 40$ ) and controls ( $n = 40$ ). Bars show mean values with 95% confidence intervals.

**Fig. 3.** Plasma levels of nitrotyrosine (NT) from rheumatoid arthritis (RA;  $n = 18$ ) were compared with healthy volunteer (HV) controls ( $n = 21$ ) and to plasma levels following 3 and 6 months of anti-tumor necrosis factor (TNF) therapy ( $n = 17$ ). Values were analyzed using one-way ANOVA, followed by Bonferroni multiple comparisons test. Bars show mean values with 95% confidence intervals.



**Fig. 4.** Relationship between the predicted percentage change in  $\log_{10}$  disease activity scores measured by 28 tender and swollen joint counts and C-reactive protein levels (DAS28-3 CRP) and the observed percentage change in  $\log_{10}$  DAS28-3 CRP clinical score after 6 months of anti-TNF therapy.

**Table I**

Relationship of SF NT and NOx to biomarkers of joint destruction and repair

	SF ARGS	SF COMP	SF MMP-3	SF MMP-1	SF 846	SF CTx2B4
SF NT correlation coefficient	0.286	0.052	0.241	0.252	0.081	0.437
<i>P</i> value	< 0.001	0.655	< 0.001	0.008	0.43	< 0.001
<i>n</i>	87	75	190	109	98	174
SF NOx correlation coefficient	-0.012	0.149	-0.022	-0.015	-0.005	-0.061
<i>P</i> value	0.904	0.16	0.752	0.869	0.957	0.39
<i>n</i>	100	90	216	127	113	199

846, 846 epitope of aggrecan; ARGS, ARGS-neoepitope generated by aggrecanase cleavage of aggrecan interglobular domain; COMP, cartilage oligomeric matrix protein; CTx2B4, Type II cross-linked C-telopeptide 2B4 epitope; MMP, matrix metalloproteinase; NOx, nitrate and nitrite; NT, nitrotyrosine; SF, synovial fluid.

Variable number of assay results available due to limitations in SF sample volume.

**Table II**

RA plasma biomarkers that responded to anti-TNF therapy

Clinical score/biomarker	Baseline	3 months	6 months	Healthy volunteers
DAS28-3 CRP	6.12 (5.79, 6.46)	4.05 (3.58, 4.53) <0.001	3.74(3.28, 4.19) <0.001	ND
IL-6 (pg/ml)	151.3 (101.6, 200.9)	74.8 (32.4, 117.3) <0.005	73.7 (30.2, 117.2) <0.001	0.75 (0.75, 0.75) <0.001
MMP-3 (ng/ml)	71.9 (20.1, 123.7)	22.1 (12.0, 32.2) 0.008	15.5 (9.82, 21.1) 0.002	10.1 (7.50, 12.7) <0.001
MMP-1 (ng/ml)	38.2 (23.5, 52.8)	15.6 (12.2, 19.0) <0.001	14.2 (10.6, 17.7) <0.001	6.6 (4.23, 8.88) <0.001
NT (pg/ml)	3774 (2711, 4838)	2955 (2354, 3556) 0.111	2374 (2014, 2734) 0.006	1257 (1105, 1410)<0.001
IP-10 (pg/ml)	352.8 (192.4, 513.2)	316.5 (147.3, 485.7) 0.503	131.9 (90.7, 173.2) 0.014	217.4 (166, 269) 0.672
CRP (µg/ml)	33.16 (18.0, 48.3)	9.41 (6.68, 12.1) <0.001	7.79 (5.70, 9.90) <0.001	ND

Plasma was analyzed from RA patients ( $n = 18$ ) before and after anti-TNF treatment, and from healthy volunteers ( $n = 21$ ).

Mean values (95% confidence interval) P for each group were compared to the baseline (pre-treatment group) using one-way

ANOVA, followed by the Holm-Sidak multiple comparison test. Significance was assessed for log<sub>10</sub>-transformed data for IL-6, MMP-3, MMP-1, NT, IP-10 and CRP.

CRP, C-reactive protein; DAS, disease activity score; IL-6, interleukin-6; IP-10, interferon  $\gamma$ -induced protein 10; MMP, matrix metalloproteinase; ND, not determined; NT, nitrotyrosine.

**Table III**

Correlation between key plasma markers of disease activity prior to initiation of anti-TNF therapy in patients with RA ( $n = 18$ )

Biomarker	Correlation with	Spearman coefficient	<i>P</i> value
NT	CRP	0.6549	0.0032
	MMP-3	0.5934	0.0094
	IP-10	0.4915	0.0383
	MMP-1	0.4138	0.0878
DAS28-3 CRP	sCD40L	0.4716	0.0482
	MMP-9	0.5501	0.0180
	EGF	0.4964	0.0361
sCD40L	IP-10	-0.7434	0.0004
	EGF	0.5769	0.0122
MMP-3	MMP-1	0.4923	0.0380
	CRP	0.5304	0.0236
MMP-1	MCP-1	-0.5170	0.0280
MMP-9	IL-1 $\beta$	0.5173	0.0279
	TNF- $\alpha$	0.4770	0.0453
	VEGF	0.4801	0.0437
	GM-CSF	0.5480	0.0186
	MIP-1 $\beta$	0.5516	0.0176
	IL-2	0.6969	0.0013

IL-10                      0.5608                      0.0155

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CRP, C-reactive protein; DAS28-3 CRP, disease activity score measured by 28 tender and swollen joint counts and C-reactive protein levels; EGF, epidermal growth factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; IP-10, interferon  $\gamma$ -induced protein 10; MIP-1 $\beta$ , macrophage inflammatory protein 1 $\beta$ ; MMP, matrix metalloproteinase; NT, nitrotyrosine; RA, rheumatoid arthritis; sCD40L, soluble CD40 ligand; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; VEGF, vascular endothelial growth factor.

**Table IV**

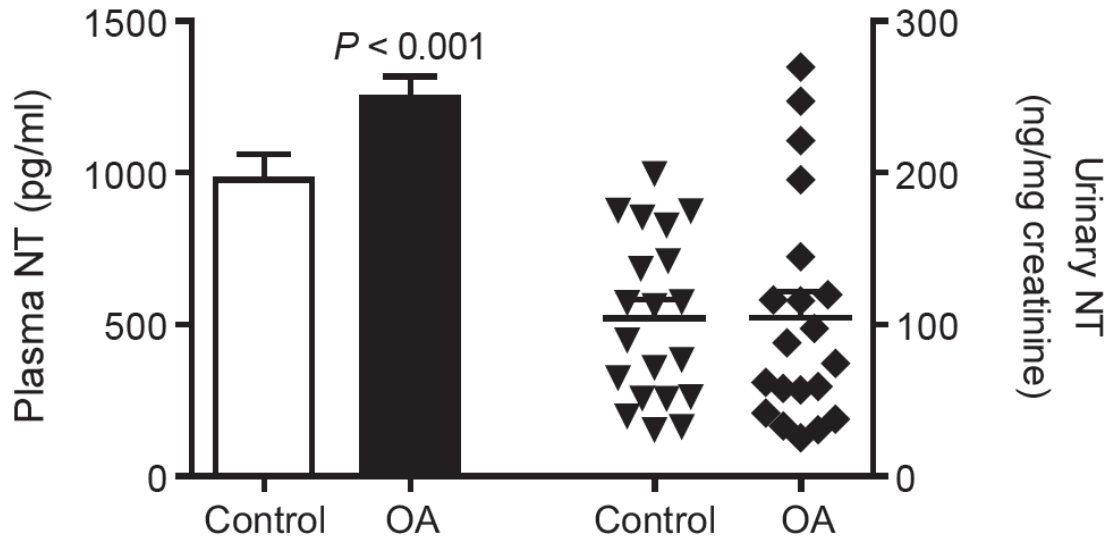
Parameters for model predicting percentage change in  $\log_{10}$  DAS28-3 CRP (6-month time point)

Parameter	Parameter estimate	<i>P</i> value	Adjusted $R^2$ value
Intercept	-29.612	0.653	0.871
Hormone status	-16.015	0.034	
Age	0.078	0.948	
Gender	-24.487	0.717	
Age $\times$ gender	0.814	0.514	
Alcohol	-8.864	0.019	
Age $\times$ alcohol	0.136	0.034	
Gender $\times$ alcohol	12.962	0.014	
Age $\times$ gender $\times$ alcohol	-0.246	0.011	
Percentage change log NT	1.131	0.014	

DAS28-3 CRP, disease activity score measured by 28 tender and swollen joint counts and C-reactive protein levels; CRP, C-reactive protein; NT, nitrotyrosine.

Figure 1

A)



B)

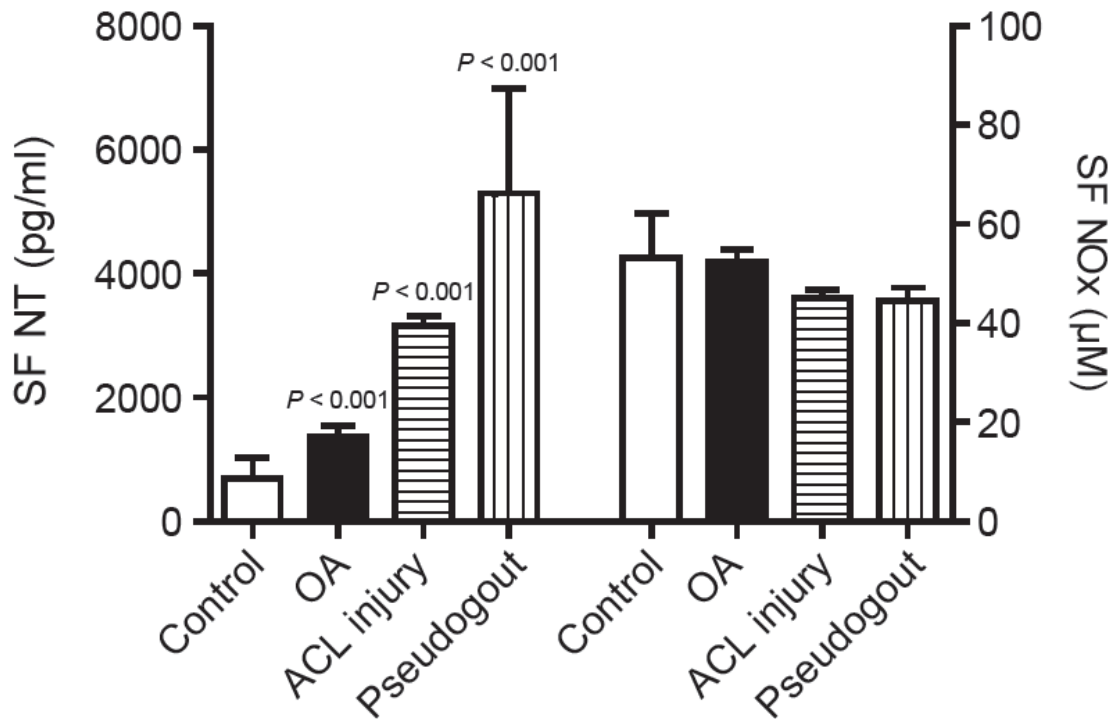




Figure 2

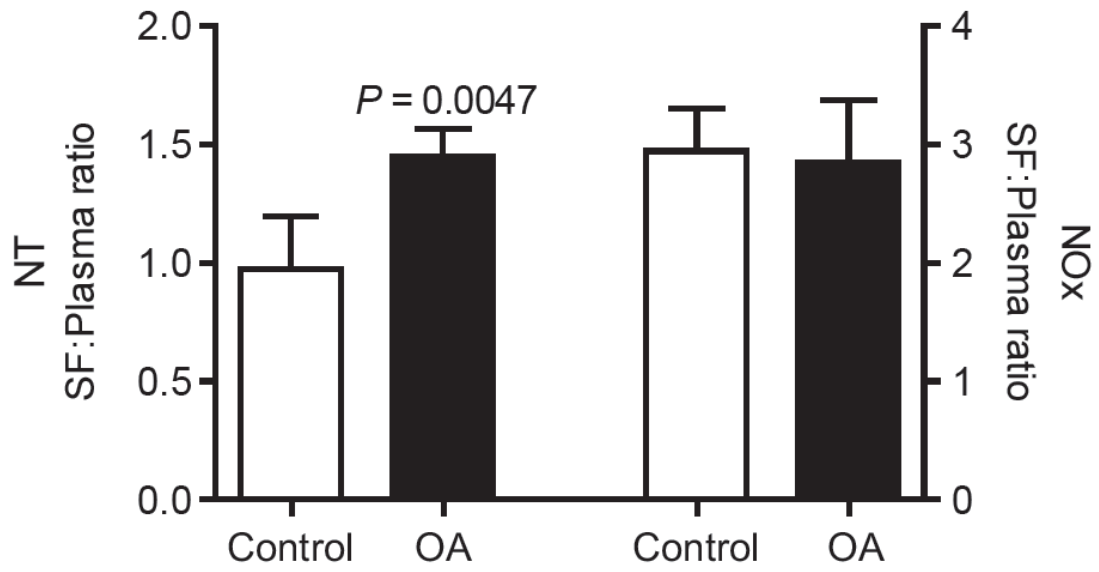


Figure 3

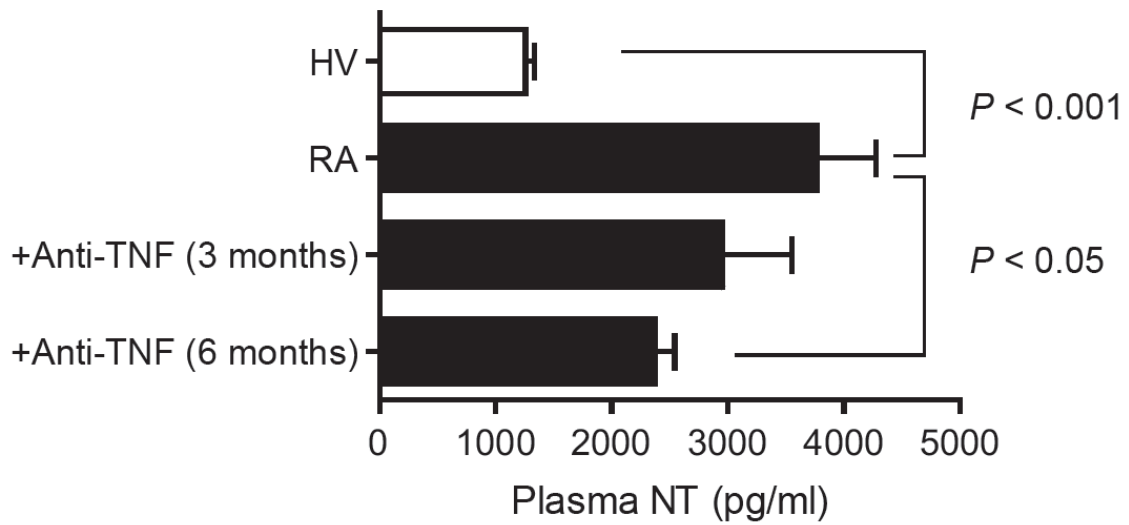


Figure 4

