

# Salivary Antioxidants and Metalloproteinases in Juvenile Idiopathic Arthritis

Riva Brik,<sup>1,5</sup> Irit Rosen,<sup>1</sup> Dana Savulescu,<sup>2,5</sup> Iris Borovoi,<sup>2,5</sup> Moshe Gavish,<sup>2,5</sup> and Rafael Nagler<sup>2,3,4,5</sup>

<sup>1</sup>Meyer Children's Hospital, Haifa, Israel; <sup>2</sup>Department of Molecular Pharmacology, Technion, Israel Institute of Technology, Haifa, Israel; <sup>3</sup>Oral Biochemistry Laboratory, Technion, Israel Institute of Technology, Haifa, Israel; <sup>4</sup>Rambam Medical Center, Technion, Israel Institute of Technology, Haifa, Israel; and <sup>5</sup>Department of Pediatrics and Pediatric Rheumatology Service, Bruce Rappaport Faculty of Medicine, Technion, Israel Institute of Technology, Haifa, Israel

Juvenile idiopathic arthritis (JIA) is the most common autoimmune inflammatory disease in children; joint inflammation is the hallmark of the disease. Thirty-five children with JIA were studied, of whom 26 had active disease and 14 were receiving anti-TNF therapy (5 with Infliximab, 9 with Etanercept). Sixteen healthy controls also were studied. Saliva samples were obtained for analysis of anti-oxidant status, metalloproteinases (MMPs) and sialochemistry. The total antioxidant status was significantly higher in the saliva of all JIA patients, whether treated ( $P = 0.014$ ) or not treated ( $P = 0.038$ ) with anti-TNF agents. The increase in antioxidant status (TAS) in the saliva of the active patients was nearly two times higher than that of non-active patients ( $P = 0.01$ ). MMP levels were significantly lower in JIA patients than in controls. MMP-9, MMP-3 and MMP-2 were lower in JIA patients *without* anti-TNF treatment by 36.7% ( $P = 0.01$ ), 30.0% ( $P = 0.0001$ ) and 10.7% ( $P = 0.0001$ ), respectively. A greater reduction in MMP levels was observed in the group of patients treated *with* anti-TNF drugs: MMP-9, MMP-3 and MMP-2 were lower than in controls by 51.1% ( $P = 0.0001$ ), 61.5% ( $P = 0.0001$ ) and 55.4% ( $P = 0.0001$ ), respectively. Children with JIA exhibited a significantly higher salivary antioxidant activity and significantly lower MMP levels. Anti-TNF treatment was associated with a further decrease in MMP levels in the saliva of JIA patients while an active state of JIA was associated with a further increase in the salivary antioxidant activity. Anti-TNF treatment may modulate the degradation process during the course of arthritis by inhibition of the activity of MMP.

© 2010 The Feinstein Institute for Medical Research, [www.feinsteininstitute.org](http://www.feinsteininstitute.org)

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2009.00096

## INTRODUCTION

Juvenile idiopathic arthritis (JIA) is the most common rheumatic disease in children, with at least three primary modes of onset: systemic, polyarticular and oligoarticular types (1). The severity of the disease varies between its active and non-active states and follow up requires multiple blood tests, which can be a major drawback in children. The pathogenesis of JIA is characterized by prolonged chronic inflammation of the synovial membranes, accompanied by recruitment of mononuclear cells and phagocytes into the synovial fluid (2). The accompanying T-cell abnormalities and pathological

characteristics of this chronic synovitis suggest an immune cell-mediated pathogenesis (3). Indeed, proinflammatory cytokines such as TNF- $\alpha$  have been found to play a pivotal role in the pathogenesis of JIA, and anti-TNF drugs are used routinely in the clinical setup (4–6). Two of these anti-TNF drugs are Infliximab (Schering-Plough Ltd, Shire Park, Welwyn Garden City, Hertfordshire, UK) and Etanercept (Wyeth Pharmaceuticals, Maidenhead, Berkshire, UK), which differ in both mechanism of action as well as level of their related side effects (6). Both drugs have their drawbacks: aside from their high cost, they become less effective

as therapy continues and their dosage often needs to be altered or even replaced with the other drug. This situation requires intensive follow-up and multiple blood tests carried out by professional medical personnel. Therefore, salivary testing would be a major advantage due to its noninvasive nature, which would be much less traumatic for the child and could even be done at home (7,8).

Oxidative stress is increasingly recognized as one of the major factors contributing to the chronic inflammatory process within the inflamed joint. We have previously reported increased activity of antioxidant enzymes in the serum and saliva of JIA patients and other salivary alterations, indicating a specific disruption of the salivary glands as part of the JIA pathogenesis (9). Also, Walton *et al.* (10) previously reported salivary gland involvement in JIA, expressed by a decrease in both salivary flow rates. In yet another study, our group found that the antioxi-

---

**Address correspondence and reprint requests to Rafael Nagler, Department of Oral and Maxillofacial Surgery and Oral Biochemistry Laboratory, Rambam Medical Center, P.O. Box 9602, Haifa 31096 Israel. Phone: 972-4-8520989; Fax: 972-4-8542557; E-mail: [nagler@tx.technion.ac.il](mailto:nagler@tx.technion.ac.il).**

Submitted July 26, 2009; Accepted for publication December 26, 2009; Epub ([www.molmed.org](http://www.molmed.org)) ahead of print December 28, 2009.

dant profile of saliva secreted by rheumatoid arthritis (RA) patients showed remarkably increased levels of antioxidant enzymes both in saliva and in serum, especially in severely affected patients, and also a decrease in salivary rate (11). Furthermore, involvement of the salivary glands in adults with RA has been recognized for quite some time. Minor salivary glands of RA patients have been shown to be infiltrated markedly with lymphocytes, with B cells predominating over T cells (12). Other studies showed that the typical minor salivary gland alterations in RA include fibrosis, acinar atrophy and lympho-plasma cell sialadenitis (13). In other studies, it has been reported that matrix metalloproteinases (MMPs) 2, 3 and 9 were found to be increased in the serum and in the synovial fluid of JIA patients (14–17). The MMPs are a family of zinc-dependent endopeptidases capable of degrading all components of the extracellular matrix, and MMPs contribute significantly to tissue destruction. The pathognomonic destruction of cartilage and bone in JIA is mediated largely by proteolytic enzymes, among which the MMPs play a critical role (15,18,19).

MMPs have not been studied in the saliva of JIA patients nor as part of a salivary antioxidant profile and/or composition in JIA patients. Neither have MMPs been studied with respect to the activity status of JIA or the effects of the anti-TNF therapy. The purpose of the current study was to conduct a comprehensive salivary antioxidant and compositional analysis and examination of MMPs in JIA patients, and to correlate these to both the activity status of the disease and the effects of anti-TNF therapy.

## MATERIALS AND METHODS

### Patients and Methods

This study was approved by the Human Studies Ethics Committee of Rambam Medical Center, Haifa, and each participant's parent signed an informed consent form. Thirty-five patients with JIA according to the International League of Associations for Rheumatology (ILAR)

criteria (20) participated in the study, 28 females and 7 males, with a mean age of  $12.1 \pm 3.9$  years old. None of the patients suffered from any kind of local oral inflammatory disease or from any other systemic disease other than JIA. Of the 35 patients, 7 had systemic, 14 had polyarticular and 14 had pauciarticular course of disease, while 26/35 had active disease and 9/35 had non-active JIA, as defined by established criteria, at the time of the study (21). All the patients were followed up at the Pediatric Rheumatology Clinic, Meyer Children's Hospital, by the same pediatric rheumatology expert (RB). Of the 35 patients, 17 had long-standing disease (> 5-year duration) while 2 were treatment free, 17 were on methotrexate (MTX; Hospira UK Ltd, Queensway, Royal Leamington Spa, Warwickshire, UK) and 14 received anti-TNF therapy (5 with Infliximab and 9 with Etanercept). Sixteen age-matched children (mean age  $12.19 \pm 3.09$  years old) constituted the control group. Saliva samples were obtained from each participant and stored for analysis, as previously described by Brik *et al.* (9).

### Immunoreactivity Assay (ELISA) for MMP Levels

Saliva samples were centrifuged (800g, 10 min, 4°C), and the pellets were suspended in 150 µL of lysis buffer (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 10% glycerol, pH 7.8). Following 30 min incubation at room temperature, the samples were centrifuged (11,000g, 10 min, 4°C). Protein concentrations in the supernatants were determined. A volume containing 50 ng of protein was transferred to a 1.5-mL vial and all samples were brought to the same volume of 500 µL with the addition of PBS. The solutions were mixed well and 100 µL of each sample was added to enzyme-linked immunosorbent assay (ELISA)-plate wells (nunc-immunoplate; Thermo Fisher Scientific). The plate was covered and stored overnight at 4°C. The next day, each well was washed three times with 100 µL PBS-Tween solution (PBS-T, PBS containing 0.05% Tween 20) and a volume of 100 µL

of 1% BSA PBS-T blocking solution (PBS containing 0.05% Tween 20 and 1% BSA) was added to each well. After 1 h incubation at room temperature, 100 µL of primary antibody was added to each well. For MMP9 we used a monoclonal rabbit anti-human antibody (1:1000; Sigma-Aldrich, Saint Louis, MO, USA). For MMP3 we used a monoclonal rabbit anti-human antibody (1:500; Sigma-Aldrich, Saint Louis, MO, USA). For MMP2 we used a monoclonal rabbit anti-human antibody (1:1000; Sigma-Aldrich, Saint Louis, MO, USA). Following 2 h incubation at room temperature, the plate was washed as described above and a volume of 100 µL of peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000; Jackson ImmunoResearch, West Grove, PA, USA) was added to each well. Following 2 h incubation at room temperature, the plate was washed as described above. To achieve color development, we added 100 µL of 3,3',5,5'-tetramethylbenzidine solution (TMB) (Southern Biotech) to each well. After 1–2 min, we added 100 µL of stopping reagent to each well (10% sulphuric acid). Absorbencies of the samples were measured at 450 nm directly after the addition of the stopping reagent, using a Zenith 200 ELISA reader (Anthos, Eugendorf, Austria).

### General Sialochemistry and Oxidative Analysis

The salivary analysis was performed as previously described (9,22) and included calcium (Ca), phosphate (P) and total protein (TP) as well as a comprehensive salivary oxidative analysis. The analysis included (a) two general measurements of salivary antioxidant potential: total antioxidant status (TAS) and ImAnOx; (b) one antioxidative molecule: uric acid (UA) and (c) two antioxidant enzymes: superoxide dismutase (SOD) and salivary peroxidase (SPO). Analysis also included one oxidative parameter—carbonyls—which represents the oxidative damage to proteins.

### TAS

The assay used was based on a commercial kit supplied by Randox (USA) in

which metmyoglobin in the presence of iron is turned into ferrylmyoglobin. Incubation of the latter with the Randox reagent ABTS results in the formation of a blue-green colored radical which can be detected at 600 nm (23).

### Imanox

An ELISA colorimetric test kit (Immundiagnostik AG, Bensheim, Germany) was used to measure total salivary antioxidative capacity quantitatively, as described previously (24). Briefly, saliva samples were added to a defined amount of exogenous hydrogen peroxide ( $H_2O_2$ ), partial elimination of the latter. The residual  $H_2O_2$  was determined by an enzymatic reaction involving the conversion of tetramethylbenzidine (TMB) to a colorimetric product. Following addition of a stop solution to the samples, absorbance was measured at 450nm, using a Zenith 200 ELISA reader (Anthos, Eugendorf, Austria). To quantify the absorbance values, we used a calibrator provided by the manufacturer.

### UA Concentration

Uric acid concentration was measured with a kit supplied by Sentinel CH (Milano, Italy) as previously described (23). In the assay, uric acid is transformed by uricase into allantoin and hydrogen peroxide, which, under the catalytic influence of peroxidase, oxidizes the chromogen (4-aminophenazone/N-ethyl-methylanilin propan-sulphonate sodic). This reaction forms a red compound whose intensity of color is proportional to the amount of uric acid present in the sample and is read at a wavelength of 546 nm.

### SPO

Peroxidase activity was measured in both the patients' serum and saliva according to the NBS assay as described previously (23). Briefly, the colorimetric change induced by the reaction between the enzyme and the substrate, Dithiobis 2-Nitrobenzoic Acid (DTNB) in the presence of mercapto-ethanol, was read at a wavelength of 412 nm for 20 s.

### SOD

Total activity of SOD isoenzymes (Cu/Zn-SOD and Mn-SOD) was measured using the xanthine oxidase/2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) method. This method is a spectrophotometric assay for SOD based on tetrazolium salt 3'-[1-(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate reduction by xanthine-xanthine oxidase. The method is a modification of the NBT assay. XTT is reduced by the superoxide anion ( $O_2^{\bullet-}$ ) generated by xanthine oxidase. Formazan is read at 470 nm. SOD inhibits this reaction by scavenging the  $O_2^{\bullet-}$ . One unit of the enzyme is defined as the amount of enzyme needed for 50% inhibition of absorption in the absence of the enzyme (23).

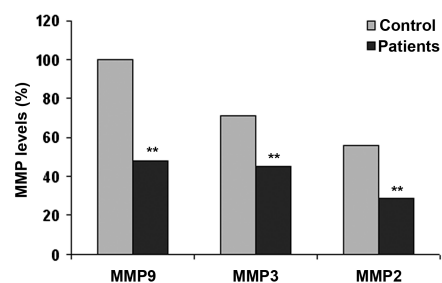
### Detection of Protein Oxidation (Protein Carbonyl Assay)

An ELISA colorimetric test kit (Cayman Chemical, New Zealand) was used to quantitatively measure the products of protein oxidation (carbonyls) in saliva samples. Saliva samples were centrifuged (800g, 10 min, 4°C), and the pellets were suspended in 150  $\mu$ L of lysis buffer (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 10% glycerol, pH 7.8). Following 30 min incubation at room temperature, the samples were centrifuged (11,000g, 10 min, 4°C) and the supernatants were stored at -20°C. On the day of the carbonyl analysis, the supernatants were thawed and protein concentrations were determined. A total of 20  $\mu$ g was transferred to a 1.5-mL vial and all samples were brought to the same volume of 100  $\mu$ L with the addition of water of high pressure liquid chromatography grade (HPLC). We added 0.8 volumes of ice cold 28% trichloroacetic acid (TCA), mixed well, and after 10 min of incubation on ice, the tubes were centrifuged (10,000g, 3 min, 4°C). Supernatants were aspirated carefully without disturbing the pellet; 5  $\mu$ L of EIA buffer (1 M phosphate solution containing 1% BSA, 4 M NaCl, 10 mM EDTA and 0.1% sodium azide) and 15  $\mu$ L diluted 2,4-dinitrophenol (DNP)

solution were added to samples according to the manufacturer's instructions. Following 45 min incubation at room temperature, 5  $\mu$ L of each sample were taken to a parallel set of 1.5-mL vials containing 1 mL EIA buffer. The solutions were mixed thoroughly and 200  $\mu$ L of each sample was added to ELISA-plate wells. The plate was covered and stored overnight at 4°C. The next d, the plate was washed three times with EIA buffer (250  $\mu$ L per well) and 250  $\mu$ L of diluted blocking solution (provided by the manufacturer) was added to each well. After 30 min incubation at room temperature, the wells were washed as described above and 200  $\mu$ L of diluted anti-DNP-biotin-antibody was added to each well. The plate was incubated for 1 h at 37°C. Following incubation, the plate was washed and 200  $\mu$ L of diluted streptavidin-HRP were added to each well. After 1 h incubation at room temperature the plate was washed as described above. To achieve color development, we added 200  $\mu$ L of chromatin reagent (provided by the manufacturer) to each well. After 5 min, we added 100  $\mu$ L of stopping reagent to each well. Absorbencies of the samples were measured at 450 nm directly after the addition of the stopping reagent, using a Zenith 200 ELISA reader (Anthos). To quantify the absorbance values, we performed the same procedure for standard and control samples provided by the manufacturer, and created a standard curve.

### Statistical Analysis

Data concerning the levels of various parameters evaluated in saliva were observed and calculated. Because of the large inborn variability of parameters in saliva, median values were calculated. The results of saliva parameters between subgroups of patients (two or more) were compared by the Kruskal-Wallis test (a multiple-comparison nonparametric test). The correlation coefficients between the pairs of salivary parameters levels were analyzed using the Spearman correlation analysis. Sensitivity and specificity values were calculated as the fraction of obser-



**Figure 1.** Median levels of salivary MMPs in healthy controls (Control salivary samples;  $n = 16$ ) and JIA patients (Patient salivary samples;  $n = 35$ ). The data is presented as percentage out of MMP9 levels in the control group. The significance values (\*\* $P < 0.01$ ) were calculated for each group in comparison to its respective control.

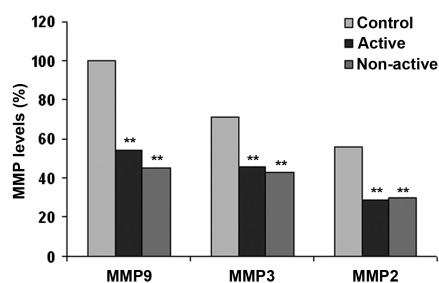
vations, which were correctly classified. A  $P$  value smaller than 0.05 was regarded as statistically significant.

## RESULTS

### MMP Levels

A significant decrease of MMPs evaluated was found in JIA patients compared with control healthy children. The median values of MMP-9, MMP-3 and MMP-2 in the controls were 0.994, 0.668 and 0.524, respectively. In the JIA children these values were lower by 52.2% ( $P = 0.0001$ ), 36.2% ( $P = 0.0001$ ) and 47.2% ( $P = 0.0001$ ), respectively. The reduction in the MMP levels was even greater in JIA children being treated with anti-TNF therapy as compared with those untreated (without anti-TNF). The median MMP-9, MMP-3 and MMP-2 levels were lower in JIA patients without anti-TNF treatment by 36.7% ( $P = 0.01$ ), 30.0% ( $P = 0.0001$ ) and 10.7% ( $P = 0.0001$ ), respectively. A greater reduction in MMP levels was observed in the group of patients treated with anti-TNF drugs: median MMP-9, MMP-3 and MMP-2 levels were lower than in controls by 51.1% ( $P = 0.0001$ ), 61.5% ( $P = 0.0001$ ) and 55.4% ( $P = 0.0001$ ), respectively.

The salivary median concentrations of MMP-2, MMP-3 and MMP-9 in the anti-TNF treated children were 0.234, 0.411



**Figure 2.** Median levels of salivary MMPs in healthy controls (Control salivary samples;  $n = 16$ ), patients with an active state of JIA (Active patient salivary samples;  $n = 26$ ) and patients with a non-active state of JIA (Non-active patient salivary samples;  $n = 9$ ). The data is presented as percentage out of MMP9 levels in the control group. The significance values (\*\* $P < 0.01$ ) were calculated for each group in comparison to its respective control.

and 0.424, respectively, lower by 50% ( $P = 0.0006$ ), 12.2% ( $P = 0.048$ ) and 29.1% ( $P = 0.036$ ) than in the untreated children (which were lower than in controls).

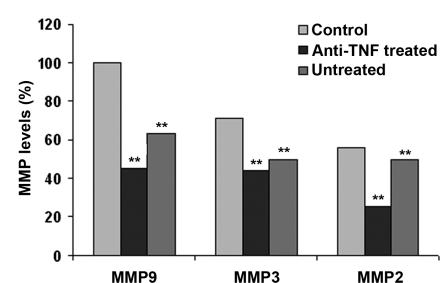
In contrast to the anti-TNF therapy, the status of disease activity did not influence the level of reduction. MMP levels were reduced in a similar manner in the JIA children regardless of disease activity status and the reduced MMP levels were not statistically different; in fact, they were almost identical in active JIA and non-active JIA children (Figure 1–3).

### General Sialochemistry

The salivary calcium concentrations were altered in a similar manner as those of the MMPs in the control and JIA children. Phosphate concentrations demonstrated opposite trends though these did not reach statistical significance.

Thus a significant decrease in calcium concentration was found in JIA patients compared with healthy control children. The median value of calcium in the control group was 2.35 mg/dL, whereas in the JIA children this value was lower by 23.5% ( $P = 0.045$ ).

The median salivary phosphate concentrations of the control and JIA children were not significantly different (NS): 13.3 mg/dL and 10.9 mg/dL, respectively.



**Figure 3.** Median levels of salivary MMPs in healthy controls (Control salivary samples;  $n = 16$ ), patients treated with anti-TNF (Anti-TNF treated patient salivary samples;  $n = 14$ ) and untreated patients (Untreated patient salivary samples;  $n = 21$ ). The data is presented as percentage out of MMP9 levels in the control group. The significance values (\*\* $P < 0.01$ ) were calculated for each group in comparison to its respective control.

The phosphate concentrations of the anti-TNF treated and untreated children were similar, 13.6mg/dL and 12.9 mg/dL, respectively. In the JIA active versus JIA non-active children these values were 13.1 mg/dL (NS) and 14.0 mg/dL (NS), respectively. The salivary total protein concentrations in the controls and JIA patients were 50.5 mg/dL (NS) and 49.3 mg/dL (NS), respectively. Neither were any significant alterations found in the total protein concentrations of the JIA subgroups: active versus non-active or anti-TNF treated versus untreated subgroups.

### Oxidative Analysis

In contrast to the MMPs, most of the analyzed antioxidative parameters demonstrated the opposite trend, that is, a substantial increase in salivary antioxidants in the JIA children (Table 1). The median salivary antioxidant values of the TAS, ImAnOx, UA, SOD and SPO in the controls were 0.27 mmol/L, 193 umol/L, 0.70 mg/dL, 1.2 U/mL and 0.51, respectively, while that of the carbonyls (the only oxidative parameter analyzed) was 0.087 nmol/mg. The TAS, ImAnOx, UA and carbonyls increased substantially in the JIA children, more so when the disease was active, while the SOD and the

**Table 1.** Summary of oxidative stress related data for measurements of total salivary antioxidant status levels (TAS), total antioxidative capacity (ImAnOx), activity of three salivary antioxidants (Uric acid, SOD, SPO) and the salivary protein oxidation levels (carbonyls).<sup>a</sup>

Group	TAS, mmol/L	ImAnOx, (umol/L)	Uric acid, mg/dL	SOD, U/mL	SPO, OD <sup>b</sup>	Carbonyls, nmol/mg
Control						
n	16	13	16	16	16	16
Range	(0.14–0.48)	(6–333)	(0.0–2.1)	(0.69–4.61)	(0.215–0.55)	(–0.215 to 1.3)
Median	0.27	193	0.70	1.205	0.515	0.087
All patients						
n	35	26	35	33	35	32
Range	(0.19–1.28)	(158–389)	(0.0–5.2)	(0.64–3.28)	(0.41–0.553)	(–0.128 to 1.55)
Median	0.39	241	0.80	1.31	0.485	0.161
<i>p</i> <sup>2c</sup>	0.01 <sup>d</sup>	0.049	0.57	0.709	0.009 <sup>c</sup>	0.444
Anti-TNF treated patients						
n	14	12	14	14	14	13
Range	(0.26–0.58)	(158–389)	(0.0–5.2)	(0.64–3.26)	(0.428–0.523)	(–0.13 to 1.55)
Median	0.39	239	0.75	1.07	0.476	0.25
<i>p</i> <sup>2c</sup>	0.0144 <sup>e</sup>	0.07	0.643	0.826	0.014 <sup>e</sup>	0.188
Untreated patients						
n	21	14	21	20	21	19
Range	(0.19–1.28)	(159–315)	(0.0–2.3)	(0.8–3.28)	(0.41–0.553)	(–0.1 to 0.63)
Median	0.37	241	1.0	1.395	0.496	0.122
<i>p</i> <sup>2c</sup>	0.0384 <sup>e</sup>	0.254	0.599	0.474	0.032 <sup>e</sup>	0.869
Active-JIA patients						
n	26	18	26	24	26	23
Range	(0.23–1.28)	(158–370)	(0.0–5.2)	(0.8–3.28)	(0.41–0.553)	(–0.13 to 1.55)
Median	0.437	248	1.7	1.35	0.48	0.166
<i>p</i> <sup>2c</sup>	0.001 <sup>d</sup>	0.062 <sup>e</sup>	0.60	0.553	0.006 <sup>d</sup>	0.607
Non-active-JIA patients						
n	9	8	9	9	9	9
Range	(0.19–0.50)	(159–389)	(0.1–1.7)	(0.64–3.26)	(0.45–0.52)	(–0.03 to 0.88)
Median	0.26	241	0.7	1.26	0.51	0.132
<i>p</i> <sup>2c</sup>	0.976	0.469	0.648	0.82	0.213	0.336

<sup>a</sup>For calculation of *P* values, all groups were compared with the control group.

<sup>b</sup>OD, optical density (absorbance).

<sup>c</sup>*P*<sup>2</sup>, Kruskal–Wallis test.

<sup>d</sup>*P* ≤ 0.01.

<sup>e</sup>*P* ≤ 0.01.

SPO values remained virtually unchanged. The change in the TAS, ImAnOx and SPO median levels reached statistical significance and more so when the disease was active. Hence, in the JIA children (all patients group) the TAS was higher by 44% (*P* = 0.01), the ImAnOx by 24.8% (*P* = 0.04), the SPO by 6.1% (*P* = 0.009), the carbonyls by 85% (NS), the UA by 14.2% (NS) and the SOD by 8.7% (NS). In the JIA-active subgroup the median levels of UA, TAS and carbonyls were higher by 142% (*P* = 0.01), 68% (NS) and 25% (NS), respectively, as compared with the JIA non-active children (Table 1). The further increase in the antioxidant

potential of the JIA-active group was also accompanied by total prevention of an increase in carbonyls, and hence the carbonyl concentration of the controls and the JIA-active children was identical.

The anti-TNF treatment made no difference with respect to the JIA-induced increase in antioxidants, and there were no statistical differences in the antioxidant parameters among the anti-TNF treated versus untreated subgroups (Table 1).

#### Spearman Correlation Analysis

The Spearman analysis revealed high correlation rates for MMP-3 and MMP-2;

MMP-3 and MMP-9; and MMP-2 and MMP-9 which were *r* = 0.89 (*P* = 0.0001); *r* = 0.80 (*P* = 0.0001) and *r* = 0.79 (*P* = 0.0001), respectively. The analysis revealed moderate correlation rates for MMP-3 and Ca; MMP-9 and Ca and MMP-2 and Ca which were *r* = 0.46 (*P* = 0.001); *r* = 0.40 (*P* = 0.007) and *r* = 0.41 (*P* = 0.004), respectively.

#### Sensitivity, Specificity

The sensitivity values for salivary MMP-2, MMP-3, MMP-9 and TAS concentrations (based on cutoff value = mean ± 1 STD), were 80%, 74%, 66% and 51%, respectively, while for the speci-

ficity values they were 81%, 81%, 88% and 75%, respectively.

## DISCUSSION

The currently reported data demonstrate a significant enhancement of the salivary antioxidant system in JIA patients which is even greater when the disease is in its active state. This was demonstrated by various analyzed parameters, including the uric acid molecule (which is considered responsible for no less than 70% of the salivary antioxidant potential (25–27) and the two general salivary antioxidant analyses performed—TAS and ImAnOx. Concurrently we report, for the first time, that salivary MMPs are reduced in JIA and more so following anti-TNF therapy. This is accompanied by a similar reduction in salivary calcium. These results are important at the diagnostic level and also for their value concerning the pathogenesis of the disease, considering the highly significant reductions concomitantly demonstrated for all three MMPs examined and the high correlation rates among these reductions. Simple kits for salivary MMP detection may be used by children or their parents for diagnostic purposes. The relatively high sensitivity and specificity scores obtained may be further improved if various MMPs were examined simultaneously. However, the results of JIA-related reductions of MMPs 2, 3 and 9 in the saliva are puzzling, as these specific MMPs were found to be increased in the serum and in the synovial fluid of JIA and rheumatoid arthritis (RA) patients (14–17). It is well established that most enzymes found in the saliva originate from the salivary or oral mucosal cells and not from the serum (28). This could explain an absence of increase in the salivary MMP in JIA, but not a decrease. Therefore, we should look for a mechanism which is related to JIA and its local effects on saliva. In contrast to what is expected due to data reporting the increase of MMP-2, MMP-3 and MMP-9 in the serum, the fact that all three MMPs were reduced in the saliva seems to indicate the presence

of a local mechanism. The currently reported increase in various salivary antioxidant parameters is of no surprise; in recent years, oxidative stress has become increasingly recognized as one of the major factors contributing to the chronic inflammatory process both in RA and in JIA patients (9,11,29–33). Furthermore, increased MMP expression and activity was reportedly induced by the greater oxidative stress in general and also in rheumatoid synovial cells, as was shown in numerous studies; and vice versa, MMP expression and activity was decreased by antioxidants such as vitamin E or SOD (18,34–40). It is also worth noting recent reports demonstrating that the bioflavonoid Hesperidin protects against the effects of nicotine by reversing the nicotine-induced parallel increase of both oxidative stress and enhanced MMP expression (19,41,42). In another study, bioflavonoids, which are antioxidants and antiinflammatory drugs, inhibited the elevation of intracellular calcium and the production of the proinflammatory cytokines TNF- $\alpha$  and IL-6 in a model of mast cell-mediated inflammation (43). Oxidative stress also was shown to induce various inflammatory mediators of rheumatoid arthritis, such as prostaglandins formed by Cox-2 in the synovium (44). Such proinflammatory cytokines have been shown to induce MMPs and, therefore, it is not surprising that anti-TNF therapies have been found to reduce serum MMP levels in patients with RA (11–14). Indeed, Mentzel *et al.*, (45) who studied a rat model for antigen-induced arthritis, reported that TNF- $\alpha$  increased total MMP activity, levels of IL-6 and oxidant nitric oxide (NO) in the supernatants of synoviocytes, and induced the expression of MMP9 (as did IL-1 $\beta$ ). There is a demonstrated association between calcium release from various cells following their exposure to oxidative stress which is reported to be accompanied by increased MMP activity (34–37,46). There also are reports demonstrating that calcium-phosphate crystals normally associated with rheumatic syndromes stimulate a variety of inflamma-

tory mediators such as prostaglandin E (2), NO, IL-1 $\beta$  and MMPs (47,48).

In summary, we suggest that the increase in salivary antioxidant capacity in JIA leads to a local abrogation of its pathological network, resulting in substantial hindrance of produced carbonyls as well as a significant reduction in salivary MMPs and calcium. This was enhanced further by the anti-TNF therapy, which intervened with another pivotal component of this network, the major proinflammatory mediator. The significance of these results at the diagnostic level has been discussed earlier. However, we think that this may have possible implications on future therapy as well, and we suggest adding antioxidant therapy to the JIA patients, both locally and systemically.

## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

## REFERENCES

- Cassidy JT, Petty RE. Juvenile Rheumatoid Arthritis. In: Cassidy JT, Petty RE, eds. *Textbook of Pediatric Rheumatology*. Philadelphia: Saunders 2001:218–38.
- Bosco MC *et al.* (2009) The hypoxic synovial environment regulates expression of vascular endothelial growth factor and osteopontin in juvenile idiopathic arthritis. *J. Rheumatol.* 36:1318–29.
- Cush JJ, Lipsky PE. (1991) Cellular basis of rheumatoid inflammation. *Clin. Orthop. Related Res.* 265:9–22.
- Hashkes PJ, Laxer RM. (2005) Medical treatment of juvenile idiopathic arthritis. *JAMA.* 294:1671–84.
- Borchers AT, *et al.* (2006) Juvenile idiopathic arthritis. *Autoimmun. Rev.* 5:279–98.
- Chikanza IC. (2002) Juvenile Rheumatoid Arthritis—therapeutic perspectives. *Pediatr. Drugs* 4:335–48.
- Zurawa-Janicka D, *et al.* (2006) Preferential immunoglobulin oxidation in children with juvenile idiopathic arthritis. *Scand. J. Rheumatol.* 35:193–200.
- Lotito AP, *et al.* (2004) Nitric oxide-derived species in synovial fluid from patients with juvenile idiopathic arthritis. *J. Rheumatol.* 31:992–7.
- Brik R, Livnat G, Pollack S, Catz R, Nagler R. (2006) Salivary gland involvement and oxidative stress in juvenile idiopathic arthritis: novel observation in oligoarticular-type patients. *J. Rheumatol.* 33:2532–7.

10. Walton AG, Welbury RR, Foster HE, Wright WG, Thomason JM. (2002) Sialochemistry in juvenile idiopathic arthritis. *Oral Dis.* 8:287–90.
11. Nagler RM, Salameh F, Reznick AZ, Livshits V, Nahir AM. (2003) Salivary gland involvement in rheumatoid arthritis and its relationship to induced oxidative stress. *Rheumatology.* 42:1231–41.
12. Sullivan S, et al. (1978) Impairment of lacrimal and salivary secretion and cellular immune responses to salivary antigens in rheumatoid arthritis. *Ann. Rheum. Dis.* 37:164–7.
13. Markkanen SO, Syrjanen SM, Lappalainen R, Markkanen H. (1989) Assessment of labial salivary gland changes in patients with rheumatoid arthritis by subjective and quantitative methods. *Appl. Pathol.* 7:233–40.
14. Gattorno M, et al. (2002) Synovial membrane expression of matrix metalloproteinases and tissue inhibitor 1 in Juvenile Idiopathic Arthritis. *J. Rheumatol.* 29:1774–9.
15. Peake NJ, et al. (2005) Levels of matrix metalloproteinase (MMP)-1 in paired sera and synovial fluids of juvenile idiopathic arthritis patients: relationship to inflammatory activity, MMP-3 and tissue inhibitor of metalloproteinases-1 in a longitudinal study. *Rheumatology.* 44:1383–9.
16. Gattorno M, et al. (2002) Serum and synovial fluid concentrations of matrix metalloproteinases 3 and its tissue inhibitor 1 in Juvenile Idiopathic Arthritis. *J. Rheumatol.* 29:826–31.
17. Peake NJ, Foster HE, Khawaja K, Cawston TE, Rowan AD. (2006) Assessment of the clinical significance of gelatinase activity in patients with juvenile idiopathic arthritis using quantitative protein substrate zymography. *Ann. Rheum. Dis.* 65:501–7.
18. Hirai Y, et al. (2001) Effects of nitric oxide on matrix metalloproteinase-2 production by rheumatoid synovial cells. *Life Sci.* 68:913–20.
19. Balakrishnan A, Menon VP. (2007) Antioxidant properties of hesperidin in nicotine-induced lung toxicity. *Fundam. Clin. Pharmacol.* 21:535–46.
20. Petty RE, et al. (1998) Revision of the proposed classification criteria for juvenile idiopathic arthritis: Durban 1977. *J. Rheumatol.* 25:1991–4.
21. Brunner HI, Lovell DJ, Finch BK, Gianni EH. (2002) Preliminary definition of disease flare in juvenile rheumatoid arthritis. *J. Rheumatol.* 29:1058–64.
22. Nagler RM, Lischinsky S, Diamond E, Klein I, Reznick AZ. (2001) New insights into salivary lactate dehydrogenase of human subjects. *J. Lab. Clin. Med.* 137:363–9.
23. Nagler RM, Klein I, Zarzhevsky N, Drigues N, Reznick AZ. (2002) Characterization of the differentiated antioxidant profile of human saliva. *Free Radic. Biol. Med.* 32:268–77.
24. Hershkovich O, Shafat I, Nagler RM. (2007) Age-related changes in salivary antioxidant profile: possible implications for oral cancer. *J. Gerontol. A Biol. Sci. Med. Sci.* 62:361–6
25. Moore S, Calder KAC, Miller NJ, Rice-Evans CA. (1994) Antioxidant activity of saliva and periodontal disease. *Free Radic. Res.* 21:417–25.
26. Kondakova I, Lissi EA, Pizarro M. (1999) Total reactive antioxidant potential in human saliva of smokers and non-smokers. *Biochem. Mol. Biol. Int.* 6:911–20.
27. Terao J, Nagao A. (1991) Antioxidative effect of human saliva on lipid peroxidation. *Agric. Biol. Chem.* 55:869–72.
28. Nagler RM, Hershkovich O, Lischinsky S, Diamond E, Reznick AZ. (2002) Saliva analysis in the clinical setting: revisiting an underused diagnostic tool. *J. Investig. Med.* 50:214–25.
29. Darlington LG, Stone TW. (2001) Antioxidant and fatty acids in the amelioration of rheumatoid arthritis and related disorders. *Br. J. Nutr.* 85:251–69.
30. Mantle D, Falkous G, Walker D. (1999) Quantification of protease activities in synovial fluid from rheumatoid and osteoarthritis cases: comparison with antioxidant and free radical damage markers. *Clin. Chem. Acta.* 284:45–58.
31. Blake DR, et al. (1989) Hypoxic-reperfusion injury in the inflamed human joint. *Lancet.* 8633:289–93.
32. Babior BM. (2000) Phagocytes and oxidative stress. *Am. J. Med.* 109:33–44.
33. Harris ED Jr. (1990) Rheumatoid arthritis. Pathophysiology and implications for therapy. *N. Engl. J. Med.* 18:1277–89.
34. Mandal M, Das S, Chakraborti T, Mandal A, Chakraborti S. (2003) Role of matrix metalloproteinase-2 in oxidant activation of Ca<sup>2+</sup> ATPase by hydrogen peroxide in pulmonary vascular smooth muscle plasma membrane. *J. Biosci.* 28:205–13.
35. Mandal A, et al. (2004) Matrix metalloproteinase-2-mediated inhibition of Na<sup>+</sup>-dependent Ca<sup>+</sup> uptake by superoxide radicals (O<sub>2</sub><sup>-</sup>) in microsomes of pulmonary smooth muscle. *IUBMB Life.* 56:267–76.
36. Mandal A, et al. (2005) Role of MMP-2 in inhibiting Na<sup>+</sup> dependent Ca<sup>2+</sup> uptake by H<sub>2</sub>O<sub>2</sub> in microsomes isolated from pulmonary smooth muscle. *Mol. Cell. Biochem.* 270:79–87.
37. Chakraborti S, Mandal A, Das S, Chakraborti T. (2005) Role of MMP-2 in PKC $\delta$ -mediated inhibition of Na<sup>+</sup> dependent Ca<sup>2+</sup> uptake in microsomes of pulmonary smooth muscle: involvement of a pertussis toxin sensitive protein. *Mol. Cell. Biochem.* 280:107–17.
38. Cox MJ, Hawkins UA, Hoit BD, Tyagi SC. (2004) Attenuation of oxidative stress and remodeling by cardiac inhibitor of metalloproteinase protein transfer. *Circulation.* 109:2123–8.
39. Tiku ML, Shah R, Allison GT. (2000) Evidence linking chondrocyte lipid peroxidation to cartilage matrix protein degradation. Possible role in cartilage aging and the pathogenesis of osteoarthritis. *J. Biol. Chem.* 275:20069–76.
40. Ishii Y, et al. (2003) Induction of matrix metalloproteinase gene transcription by nitric oxide and mechanisms of MMP-1 gene induction in human melanoma cell lines. *Int. J. Cancer.* 103:161–8.
41. Balakrishnan A, Menon VP. (2007) Effect of hesperidin on matrix metalloproteinases and antioxidant status during nicotine-induced toxicity. *Toxicology.* 238:90–8.
42. Balakrishnan A, Menon VP. (2007) Protective effect of hesperidin on nicotine induced toxicity in rats. *Indian J. Exp. Biol.* 45:194–202.
43. Park HH, et al. (2008) Flavonoids inhibit histamine release and expression of proinflammatory cytokines in mast cells. *Arch. Pharm. Res.* 31:1303–11.
44. Honda S, et al. (2000) Induction of COX-2 expression by nitric oxide in rheumatoid synovial cells. *Biochem. Biophys. Res. Commun.* 268:928–31.
45. Mentzel K, Brauer R. (1998) Matrix metalloproteinases, IL-6, and nitric oxide in rat antigen-induced arthritis. *Clin. Exp. Rheumatol.* 16:269–76.
46. Pariente JA, Camello C, Camello PJ, Salido GM. (2001) Release of calcium from mitochondrial and nonmitochondrial intracellular stores in mouse pancreatic acinar cells by hydrogen peroxide. *J. Membr. Biol.* 179:27–35.
47. Molloy ES, Morgan MP, McDonnell B, O'Byrne J, McCarthy GM. (2007) BCP crystals increase prostacyclin production and upregulate the prostacyclin receptor in OA synovial fibroblasts: potential effects on mPGES1 and MMP-13. *Osteoarthritis Cartilage.* 15:414–20.
48. Molloy ES, McCarthy GM. (2006) Basic calcium phosphate crystals: pathways to joint degeneration. *Curr. Opin. Rheumatol.* 18:187–92.