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New serum biochemical markers (Coll 2-1 and Coll 2-1 NO$_2$) for studying oxidative-related type II collagen network degradation in patients with osteoarthritis and rheumatoid arthritis

Michelle Deberg Ph.D.†, Alain Labasse Bc.S.†, Stephan Christgau Ph.D.‡, Paul Cloos Ph.D.‡,
Dennis Bang Henriksen Ph.D.‡, Jean-Pierre Chapelle M.D., Ph.D.§, Brigitte Zegels Bc.S.†,
Jean-Yves Reginster M.D., Ph.D.† and Yves Henrotin Ph.D.†‖¶*

† *Bone and Cartilage Research Unit, University of Liège, Sart Tilman, 4000 Liège, Belgium*
‡ *Nordic Bioscience, Herlev Hovedgade 207, Herlev, Denmark*
§ *Clinical Biology, CHU, Sart Tilman, 4000 Liège, Belgium*
‖ *Centre for Immunology, CHU, Sart Tilman, 4000 Liège, Belgium*
¶ *Centre for Oxygen Research and Development, CHU, Sart Tilman, 4000 Liège, Belgium*

Summary

Objective: Protein nitration is a prominent feature of inflammatory processes in the joint. We have developed immunoassays specific for a peptide of the α-helical region of type II collagen ^{108}HRGYPGLDG^{116} (Coll 2-1) and its nitrated form ^{108}HRGY(NO$_2$)PGLDG^{116} (Coll 2-1 NO$_2$) in biological fluids.

Design: Coll 2-1 and Coll 2-1 NO$_2$ peptides were injected into rabbits. Two antisera (D3 and D37) were selected for their specificity and affinity and used to develop specific immunoassays. Coll 2-1 and Coll 2-1 NO$_2$ were measured in sera of 242 healthy subjects (N), 67 patients with primary knee osteoarthritis (OA) and 19 patients with rheumatoid arthritis (RA).

Results: In healthy subjects, Coll 2-1 and Coll 2-1 NO$_2$ concentrations were 125.13 ± 3.71 nM and 0.16 ± 0.08 nM, respectively. In OA and RA, Coll 2-1 and Coll 2-1 NO$_2$ serum levels were found to be significantly increased compared to controls of the same range of age (Coll 2-1: OA: 200.80 ± 8.98 nM, RA: 172.30 ± 19.05 nM, normal: 126.60 ± 6.70 nM and Coll 2-1 NO$_2$: OA: 0.26 ± 0.02, RA: 0.38 ± 0.05, normal: 0.12 ± 0.01 nM). Coll 2-1 NO$_2$ levels were significantly more elevated in RA than in OA patients ($P < 0.05$). As a consequence, the ratio Coll 2-1 NO$_2$/Coll 2-1 was 1.6 times higher in RA than in OA subjects. No relationship was found between the radiological OA severity and the levels of Coll 2-1 and Coll 2-1 NO$_2$ in serum. Coll 2-1 NO$_2$, but not Coll 2-1, was correlated with C-reactive protein in the sera of OA and RA patients.

Conclusions: The determination of both Coll 2-1 and Coll 2-1 NO$_2$ in serum of arthritic patients seems to be a promising useful tool for the detection of oxidative-related cartilage degradation episode. Further, these markers could be helpful for monitoring the effects of anti-inflammatory or antioxidant drugs on cartilage degradation.
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Key words: Biochemical markers, Collagen, Cartilage, Free radicals, Oxidative nitrosylation, Inflammation.

Introduction

Type II collagen is the major structural protein in cartilage and making up approximately 50% of the extracellular cartilage matrix. In arthritis, type II collagen is enzymatically and mechanically degraded and fragments are released into synovial fluid. Type II collagen-derived fragments have been extensively investigated as potential markers of cartilage remodelling in osteoarthritis (OA) and rheumatoid arthritis (RA). Different strategies have been developed including the measurement in biological fluids and tissue explants of type II collagen propeptides[1−4] or degradation products[5−9]. Type II collagen C-propeptide and N-propeptide, released extra-cellularly from the newly synthesized molecule, have been investigated as markers of type II collagen synthesis. Type II

collagen N- and C-propeptide levels increase in OA cartilage[3,4]. Type II collagen fragments released during collagen processing are also present in circulation and can be measured as markers of collagen degradation. To date, different antibodies against degradation products of type II collagen have been described in the literature[5−9]. Hollander et al.[5] have developed an immunoassay specific for the peptide CB11B generated by cyanogen bromide cleavage of the α1(II) chain. Another approach applied by Poole and co-workers consisted in producing polyclonal antibodies directed against neo-epitopes generated through cleavage of type II collagen by interstitial collagenases[6]. These immunoassays were used for quantifying denatured type II collagen in cartilage after the enzymatic digestions of biopsies. These studies concluded that type II collagen denaturation is increased in OA cartilage and that collagenase activity plays a key role in this process[5,6]. In contrast, the C2C neo-epitope did not vary in synovial fluid from patients with OA or with inflammatory joint diseases[10]. Downs *et al.*[8] have developed an enzyme-linked

immunosorbent assay (ELISA) specific for the C-terminal neo-epitope of the 3/4 fragment of type II collagen generated by collagenase cleavage. Urinary levels of this marker have been reported to be 2.5 times higher in OA patients than in healthy controls. Recently, Christgau et al.[9] have described an immunoassay specific for CartiLaps or CTX-II. Urinary levels of this peptide are significantly elevated in patients with OA or RA compared with age-matched controls[9]. In OA, urinary CTX-II correlated with minimal joint space width at baseline and with increased rate of progression of joint damage over 1 year[4,11,12].

Peptide nitration is mainly caused by interaction of aromatic amino acids with peroxynitrite anion (ONOO$^-$), a strong oxidant formed by the reaction of nitric oxide ($^{\bullet}$NO) and superoxide anion (O$_2^{\cdot-}$). Tyrosine, phenylalanine and tryptophan residues are particularly sensitive to nitration[13]. As demonstrated for type I collagen, type II collagen is susceptible of nitration by peroxynitrite[14]. High levels of nitrite/nitrate have been found in the serum of patients with OA and RA indicating that production of ONOO$^-$ is increased in these diseases[15]. Furthermore, chondrocytes can produce both O$_2^{\cdot-}$ and $^{\bullet}$NO[16–18] and nitrotyrosine has been found in cartilage of arthritic patients[19]. It has also been reported that N-iminoethyl-L-lysine, a selective inhibitor of the inducible nitric oxide synthase, reduces the progression of experimental OA induced in dog[20]. Altogether, these findings indicate that $^{\bullet}$NO or derived reactive oxygen species play a major role in the structural changes in arthritis and suggest that cartilage matrix components can be nitrated in situ and thereafter released in the synovial fluid.

In this paper, we describe the development, technical validation and clinical evaluation of two new immunoassays for the measurements of type II collagen-derived fragments. One assay was specific for the sequence [108]HRGYPGLDG[116] derived from the triple helical region of type II collagen (Coll 2-1) and the other for its nitrated form (Coll 2-1 NO$_2$). The development of specific immunoassay for the measurement of nitrated type II collagen fragments in biological fluids seems relevant to study the impact of the oxidative damage in cartilage pathophysiology. These assays have been validated in serum of healthy subjects and of patients with OA and RA.

Materials and methods

REAGENTS AND BUFFERS FOR IMMUNOASSAYS

All reagents were purchased from VWR and Sigma-Aldrich unless otherwise stated. The coating buffer was 0.08 M NaHCO$_3$, pH 9.6. The blocking buffer was composed of 1.5 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, 2 mM KCl, 138 mM NaCl, 0.5% (w/v) bovine serum albumin (BSA), 5.3 g/100 ml lactose monohydrate, pH 7.2. The washing buffer was a solution of 25 mM Tris, 50 mM NaCl, pH 7.3. The standard curve and the dilution of samples, when it was necessary, were done in 10 mM phosphate buffer saline (PBS), 138 mM NaCl, 0.7% (w/v) BSA, 0.1% (v/v) Tween 20, pH 7.0, for the Coll 2-1 immunoassay and in 50 mM Tris, 138 mM NaCl, 0.7% (w/v) BSA, 0.1% (v/v) Tween 20, pH 8.0, for the Coll 2-1 NO$_2$ immunoassay. The dilutions of the antisera and of the secondary antibody were done in 10 mM PBS, 138 mM NaCl, 0.2% (w/v) BSA, 0.1% (v/v) Tween 20, pH 7.0, for the Coll 2-1 immunoassay and in 50 mM Tris, 138 mM NaCl, 0.2% (v/v) BSA, 0.1% (v/v) Tween 20, pH 8.0, for the Coll 2-1 NO$_2$ immunoassay.

COLL 2-1 AND COLL 2-1 NO$_2$ SYNTHESIS

The peptide [108]HRGYPGLDG[116] (Coll 2-1) derived from the α-helix of type II collagen (accession number in GenBank: P02468) and its nitrated form [108]HRGY(NO$_2$)PGLDG[116], Coll 2-1 NO$_2$, were synthesized by standard 9-fluorenylmethyloxycarbonyle solid-phase peptide synthesis (HBTU/HOBt protocol)[21]. Peptide purity was confirmed by reversed phase high performance liquid chromatography (RP-HPLC), mass spectrometry and amino acid analysis. The purity of both peptides exceeded 95% as assessed by RP-HPLC analysis. These peptides were conjugated to thyroglobulin by using glutaraldehyde as linker[22]. The Coll 2-1 sequence is found only in type II and XI collagens with 100% of homology. The sequence is not found in other extracellular matrix proteins such as human type I, III, VI and IX collagens, fibronectin and aggrecan (Swiss Prot and Blast databases).

IMMUNIZATION

Rabbits were injected intra-peritoneally with 1 ml of the conjugated peptides (0.5 mg/ml) emulsified in equal volumes with complete Freund's adjuvant. Injections were repeated four times every month using the same peptide concentration as that of the first injection in incomplete Freund's adjuvant. Ten days after the last injection, the rabbits were sacrificed. Blood was drawn, allowed to clot for 30 min and centrifuged for 10 min at 2500 rpm at 4°C to prepare serum. Serum was stored at −20°C until biomarker measurement. Each month, a blood sample was taken and the antiserum was screened by titration experiment for the presence of anti-Coll 2-1 and anti-Coll 2-1 NO$_2$ antibodies. The antisera with the highest titres were selected for the following experiments.

NITRATION OF NATIVE HUMAN TYPE I COLLAGEN, HUMAN TYPE II COLLAGEN AND BSA MOLECULES

ONOO$^-$ in alkaline solution was prepared from NaNO$_2$ and H$_2$O$_2$ as previously described[23]. To eliminate the excess of H$_2$O$_2$, ONOO$^-$ was treated with MnO$_2$ and ONOO$^-$ concentration was determined spectrophotochemically at 302 nm ($\varepsilon = 1700$ M^{-1} cm^{-1}). Stock solutions were stored at −80°C. The nitration of human type I collagen (Sigma-Aldrich), human type II collagen (purified according to the method described in Henrotin et al.[24]) and BSA was performed as follows: 1.6 mg of protein was dissolved in 1 ml of 10 mM PBS, pH 7.4, containing 1 mM Fe^{3+} ethylenediaminetetraacetic acid (EDTA) and mixed rapidly with 2 mM ONOO$^-$ (final concentration). This procedure allowed the optimal nitration (nitration verified by an ELISA specific for nitrotyrosine, TCS CellWorks Ltd, UK) of the molecules without damaging their structure as demonstrated by sodium dodecyl sulphate−polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown).

ANTISERUM SPECIFICITY

The specificity/cross-reactivity of the two selected antisera (D3 and D37) was investigated by competitive inhibitions procedure. Coll 2-1, Coll 2-1 NO$_2$, native human type I and II collagens, human nitrated type I and II collagens, human heat denatured type I and II collagens (obtained by heating a solution of native human type I and II collagens at 100°C for 30 min), BSA, nitrated BSA and 3-nitro-L-tyrosine residue were used as competitors. Briefly,

immunoplates were coated overnight at 4°C with 100 µl of the antigen (Coll 2-1 or Coll 2-1 NO$_2$) both conjugated to BSA by Bis[sulfosuccinimidyl] suberate (BS^3) (40 ng/ 100 µl). After washing, the plates were blocked with 400 µl of saturation buffer at room temperature. Fifty microlitres of buffer with or without the different competitors at increasing concentrations (from 10^{-5} to 10^{-11} M) and 100 µl of antiserum diluted to obtain 1.5 of optical density were incubated for 1 h at room temperature. Microplates were then washed, 100 µl of a goat antibody conjugated to horseradish peroxidase (Biosource Europe), diluted at 1/ 5000, was added and incubated for 1 h at room temperature. After washing, 100 µl of freshly prepared enzyme substrate [3,3′,5,5′-tetramethylbenzidine (TMB), Biosource Europe] was added into each well. The reaction was stopped with 100 µl of H$_3$PO$_4$ 4 M. The colouration was read at 450 nm, corrected for absorbance at 650 nm.

IMMUNOASSAY FOR COLL 2-1 PEPTIDE

Coll 2-1 conjugated to BSA by BS^3 was coated by adding 100 µl of a solution at 50 ng/ml for 48 h at 4°C. Microtitre plates were subsequently blocked with 400 µl/well of blocking buffer for 90 min at room temperature. Fifty microlitres of calibrators (synthetic peptide) or unknown samples were applied to the wells, followed by 100 µl of D3 antibody, diluted 1/40,000, and incubated for 1 h at room temperature. During the procedure, a competition for binding the antibody takes place between the immobilized peptide and the peptide contained in the samples. After washing, 100 µl of peroxidase-conjugated goat antibodies to rabbit IgG (Biosource Europe), diluted 1/5000, were incubated for 1 h at room temperature. After washing, 100 µl of freshly prepared enzyme substrate (TMB, Biosource Europe) was added into each well. After 15 min, the reaction was stopped with 100 µl of 4 M H$_3$PO$_4$. The colouration was read with a microplate reader (Labsystem) at 450 nm, corrected for absorbance at 650 nm.

IMMUNOASSAY FOR COLL 2-1 NO$_2$

Coll 2-1 NO$_2$ was conjugated to biotin according to the method described by Rosenquist *et al.*[25]. One hundred microlitres of this biotinylated peptide at 1.25 ng/ml was added to each well of streptavidine-coated plates (Exiqon, Denmark) and incubated for 1 h at room temperature. Fifty microlitres of calibrators or unknown samples and 100 µl of D37, diluted 1/500,000, were distributed in 96-well plate and incubated for 1 h at room temperature. The detection of the antibody bound and the revelation were realized using the same procedure that described for Coll 2-1 immunoassay.

EXPERIMENTAL POPULATIONS

To establish reference values for Coll 2-1 and Coll 2-1 NO$_2$, sera were collected from 242 healthy ambulatory subjects attending a blood donor centre, none of whom was subjected to bone radiological and/or scintigraphy and none of whom had any evidence of arthritis or other inflammatory disease. None was currently taking any medication known to modify arthritic disease or influence joint metabolism. This group was composed by 170 men and 72 women, aged from 20 to 65 years (mean $\pm$ SD: 41.8 $\pm$ 13.0 years). Women were aged 42.7 $\pm$ 10.4 years and men 42.8 $\pm$ 14.5 years. Sera of 67 successive patients (47 women and 20 men aged over 45 years, mean $\pm$ SD: 65.0 $\pm$ 20.1 years)

with knee OA diagnosed according to the clinical and radiological criteria of the American College of Rheumatology (ACR) were collected. Disease severity was graded on the basis of Kellgren and Lawrence radiographic system. The OA population was mainly composed of patients with grade 2 (4/67 had a grade 1, 45/67 had a grade 2, 15/67 had a grade 3 and 3/67 had a grade 4). All subjects had a normal leukocytosis. None of these patients were taking any potential structure modifying drugs. Coll 2-1 and Coll 2-1 NO$_2$ concentrations were also measured in serum samples of patients with RA ($n = 19$). The RA patients (three men, 16 women) had an average age of 60 years (mean $\pm$ SD: 59.6 $\pm$ 12.6 years) and an average disease duration of 8 years and all had active disease at the sampling time [according to the ACR criteria of RA (revised 1987 criteria)]. The RA patients did not receive glucocorticoids but were treated with Non Steroidal Anti-Inflammatory Drugs or Disease Modifying Anti-Rheumatic Drugs i.e., methotrexate.

For all the participants, serum levels of C-reactive protein (CRP) were measured by a highly sensitive CRP assay (Berhing, Marburg, Germany).

All study subjects gave their written informed consent to participate, and the studies were approved by the ethical committee of the University of Liège.

STATISTICAL METHODS

Statistical studies were performed using SPSS software version 11.0 software (SPSS, sigma Stat) and S-PLUS 6.2 for windows. The Coll 2-1 and Coll 2-1 NO$_2$ values in the different groups of patients were expressed as mean $\pm$ s.e.m. The Coll 2-1 NO$_2$/Coll 2-1 ratio was also calculated. The non-parametric Mann−Whitney U test was used to compare the OA or RA patients and healthy subjects of the same range of age. To determine the influence of gender and ageing on Coll 2-1, Coll 2-1 NO$_2$ concentrations and Coll 2-1 NO$_2$/Coll 2-1 ratio, we performed a regression analysis. To find which subclasses of age of healthy subjects were different to the others, we realized an analysis of one-way variance. The correlations between the CRP and the two markers and between the two markers were estimated by the non-parametric Spearman's rank correlation coefficient. Data were considered statistically significant when P value was below 0.05 (two-tailed test).

Results

ANTISERUM SPECIFICITY

Two antisera, D3 and D37, with a high specificity for Coll 2-1 and Coll 2-1 NO$_2$, respectively, were identified. D3 did not recognize human native type I and II collagens, human heat denatured type I and II collagens, and BSA suggesting that D3 was specific for the linear form of Coll 2-1 [Fig. 1(A)]. D3 also recognized, with the same affinity, the nitrated form of Coll 2-1.

D37 showed a high affinity for Coll 2-1 NO$_2$. Thus, the cross-reactivity of non-nitrated peptide (Coll 2-1) and human nitrated type II collagen with D37 was calculated to 0.02% and less than 0.08%, respectively. Furthermore, D37 did not recognize human nitrated type I collagen, native human type I and II collagens, nitrated BSA, BSA and 3-nitro-L-tyrosine residue [Fig. 1(B)]. The very high concentrations of Coll 2-1 and nitrated collagen type II needed to displace the Coll 2-1 NO$_2$/D37 binding suggested that D37 was specific for Coll 2-1 NO$_2$.
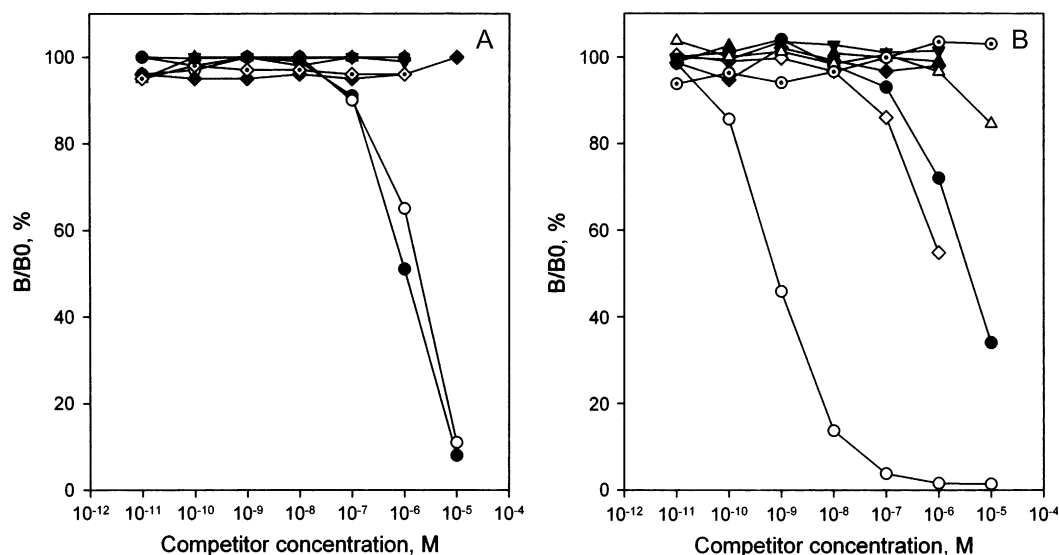
Fig. 1. Competitive inhibitions of D3 and D37. Competitive inhibitions of (A) D3 with Coll 2-1 (●), Coll 2-1 NO$_2$ (○), native type II collagen (◆), type I collagen (▲), denatured type II collagen (◈), denatured type I collagen (⬤) and BSA (▼) and (B) D37 with Coll 2-1 (●), Coll 2-1 NO$_2$ (○), type II collagen (◆), nitrated type II collagen (◇), type I collagen (▲), nitrated type I collagen (△), BSA (▼), nitrated BSA (▽) and 3-nitro-L-tyrosine (⊙). B/B0 represents the ratio of coated antigen bound in the presence of free antigen to the coated antigen bound in the absence of free antigen.

ASSAYS CHARACTERISTICS

ELISA for Coll 2-1 peptide

The lower detection limit of the assay, defined as the concentration corresponding to three SD above the mean of 10 determinations of the zero calibrator, was 17 nM. The working range of this assay was established by calculating the coefficients of variation (CVs) of each calibrator in 10 independent calibration curves. CVs were below 10% between 2000 and 10 nM. The within-run (intra-assay) precision, assessed by measuring three serum samples 10 times in the same run, and the between-run (inter-assay) precision, determined from the same samples ($n = 3$) assayed on 10 plates, were below 10% (Table I). Linearity was shown by diluting the samples serially and comparing the observed values with those expected. Typical recovery rate of 92.0−109.3% was noted in a range of dilution between 2 and 16 fold (Table II). Spiking recovery, determined by addition of known quantities of Coll 2-1

peptide (from 25 to 500 nM) into sera ranged from 101.8% to 106.5% (Table III).

ELISA for Coll 2-1 NO$_2$ peptide

The limit of detection of Coll 2-1 NO$_2$ assay was 25 pM and the working range was between 0.01 and 10 nM. In serum ($n = 3$), the intra- and inter-assays CVs were less than 10% (Table I). The dilution curves of sera were parallel to the standard curve (Table II). The spike recovery of Coll 2-1 NO$_2$ in sera was between 106.2% and 132.2% (Table III).

COLL 2-1 AND COLL 2-1 NO$_2$ LEVELS IN SERA OF HEALTHY, OA AND RA SUBJECTS

The mean concentrations of Coll 2-1 and Coll 2-1 NO$_2$ in the serum of normal subjects ($n = 242$) were 125.13 ± 3.71 nM and 0.16 ± 0.08 nM, respectively. By a regression

Table I
Assay imprecision of Coll 2-1 and Coll 2-1 NO$_2$ immunoassays in serum

Sample	Intra-assay		Sample	Inter-assay	
	Coll 2-1 concentration (mean ± SD) (nM)	CV (%)		Coll 2-1 concentration (mean ± SD) (nM)	CV (%)
Coll 2-1 assay					
1	95.07 ± 7.2	7.6	1	112.84 ± 10.49	9.3
2	109.86 ± 9.11	8.3	2	123.08 ± 11.69	9.5
3	173.48 ± 15.09	8.7	3	184.16 ± 16.57	9.0
Average		8.2	Average		9.3
Coll 2-1 NO$_2$ assay					
	Coll 2-1 NO$_2$ concentration (mean ± SD) (nM)			Coll 2-1 NO$_2$ concentration (mean ± SD) (nM)	
1	0.14 ± 0.01	8.6	1	0.14 ± 0.01	8.9
2	0.17 ± 0.01	6.6	2	0.16 ± 0.01	10.1
3	0.39 ± 0.02	5.4	3	1.61 ± 0.17	10.7
Average		6.9	Average		9.9

Table II

Dilution test of three serum samples in Coll 2-1 and Coll 2-1 NO$_2$ immunoassays

Dilution	A	B	C
Coll 2-1 in serum, nM (%)*			
1:2	597.30 (99.5)	684.91 (93.8)	785.29 (98.0)
1:4	282.80 (94.3)	340.53 (93.3)	373.01 (93.3)
1:8	139.95 (93.3)	176.59 (96.7)	196.60 (98.3)
1:16	81.98 (109.3)	84.01 (92.0)	93.10 (93.2)
Coll 2-1 NO$_2$ in serum, nM (%)*			
1:2	0.52 (100)	1.06 (91.4)	0.12 (92.3)
1:4	0.27 (103.8)	0.62 (106.9)	0.06 (92.3)
1:8	0.10 (76.9)	0.35 (120.7)	−

*Undiluted sample = 100%.

analysis, we observed that neither the age and nor the gender modified the Coll 2-1 serum level. In contrast, Coll 2-1 NO$_2$ concentration and the Coll 2-1 NO$_2$/Coll 2-1 ratio decreased with the age ($P < 0.001$). When the population was stratified by age in 5-year brackets, Coll 2-1 serum level did not vary significantly between the age groups (20−65 years) [Fig. 2(A)]. In contrast, the level of Coll 2-1 NO$_2$ and the ratio Coll 2-1 NO$_2$/Coll 2-1 of the group 26−30 years were significantly higher than in the older groups (Coll 2-1 NO$_2$: $0.0001 < P < 0.05$ and Coll 2-1 NO$_2$/Coll 2-1: $0.0001 < P < 0.05$) [Fig. 2(B and C)]. When subjects aged from 46 to 55 years corresponding to the early post-menopausal women were removed, Coll 2-1 NO$_2$ level was higher ($P = 0.003$) in premenopausal women (aged under 46 years and with regular menstruation) (0.22 ± 0.02 nM) than in late postmenopausal women (aged above 55 years) (0.12 ± 0.01 nM). Furthermore, in individuals younger than 45 years, Coll 2-1 NO$_2$ serum levels were significantly higher in women than in men (0.22 ± 0.02 nM vs 0.16 ± 0.01 nM, $P = 0.003$), whereas levels are identical in both sexes after 55 years old (0.12 ± 0.01 nM vs 0.12 ± 0.01 nM). No significant correlation was observed between Coll 2-1 and Coll 2-1 NO$_2$ concentrations in healthy subjects aged from 20 to 65 years ($r = -0.10$).

In comparison with healthy subjects of the same range of age, Coll 2-1 concentration was 1.6 and 1.4 times higher in OA patients ($P < 0.001$) and in RA patients ($P < 0.05$), respectively (normal: 126.60 ± 6.70 nM, OA: 200.80 ± 8.98 nM and RA: 172.30 ± 19.05 nM). Coll 2-1 NO$_2$ concentration doubled in OA patients ($P < 0.001$) and was increased by 3.2 times ($P < 0.001$) in RA patients compared to the healthy subjects (OA: 0.26 ± 0.02, RA: 0.38 ± 0.05 and normal: 0.12 ± 0.01 nM) [Fig. 3(A and B)]. The ratio Coll 2-1 NO$_2$/Coll 2-1 was increased in both OA and RA subjects, but to a lesser extent in OA. In healthy population, the mean ratio Coll 2-1 NO$_2$/Coll 2-1 value was
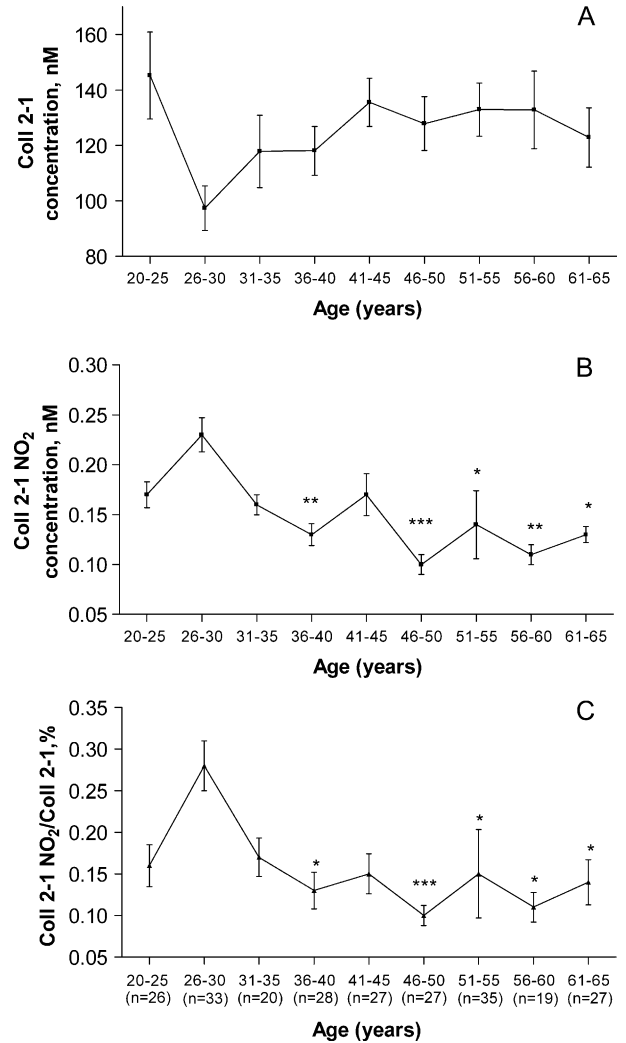

Fig. 2. Concentrations of Coll 2-1 (A) and Coll 2-1 NO$_2$ (B) and ratio Coll 2-1 NO$_2$/Coll 2-1 (C) in 242 healthy subjects aged from 20 to 65 years and stratified by 5 years. Results were expressed as mean $\pm$ s.e.m. The concentration of Coll 2-1 NO$_2$ and the ratio Coll 2-1 NO$_2$/Coll 2-1 of the subclass 26−30 are different to those of the subclasses 36−40, 46−50, 51−55, 56−60, 61−65 years. Statistical analysis was performed by an analysis of one-way variance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

0.13 ± 0.02 but it increased to 0.15 ± 0.01 in OA and 0.25 ± 0.03 in RA (normal vs OA, $P = 0.03$; normal vs RA, $P < 0.001$; OA vs RA, $P < 0.001$) [Fig. 3(C)]. We have also studied the relationship between the levels of Coll 2-1 and

Table III

Analytical recovery of Coll 2-1 and Coll 2-1 NO$_2$ added to serum samples. The concentration range in the unspiked samples was 81−500 nM and 0.05−0.15 nM for Coll 2-1 and Coll 2-1 NO$_2$, respectively

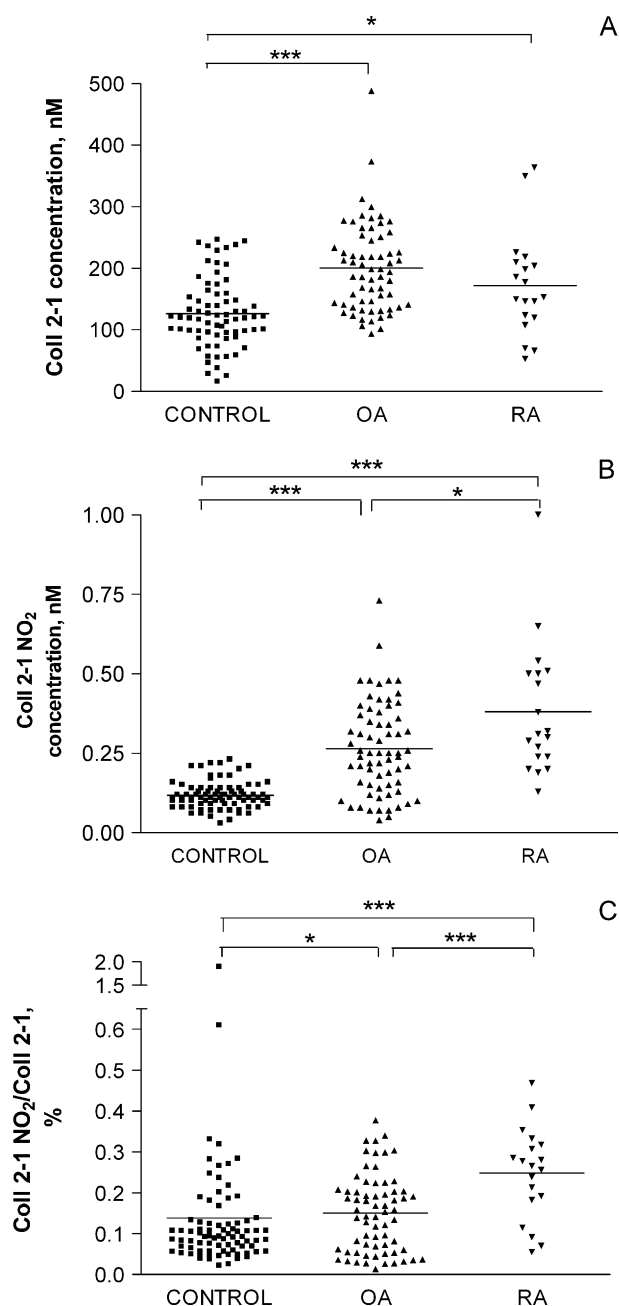| Coll 2-1 assay		Coll 2-1 NO$_2$ assay	
Added peptide (nM)	Recovery (%) (mean $\pm$ SD)	Added peptide (nM)	Recovery (%) (mean $\pm$ SD)
+0	−	+0.00	−
+25	104.9 ± 0.0	+0.10	106.2 ± 6.0
+50	105.6 ± 9.2	+0.25	119.4 ± 18.7
+100	106.5 ± 26.1	+0.50	129.3 ± 27.6
+500	101.8 ± 5.0	+1.00	132.2 ± 21.8
Average	104.7	Average	121.9

Fig. 3. Individual values of serum Coll 2-1 (A), Coll 2-1 NO_2 (B) and of the ratio Coll 2-1 NO_2/Coll 2-1 (C), in 67 OA, in 19 RA patients and in 80 age-matched healthy subjects. Statistical analysis was performed by the non-parametric Mann−Whitney U test: $*P < 0.05$; $***P < 0.001$.

Coll 2-1 NO_2 in serum and radiological severity of knee OA graded according the Kellgren and Lawrence scale (noted 1−4). Whatever the severity of OA radiological lesions, Coll 2-1 and Coll 2-1 NO_2 were elevated in OA patients compared to healthy subjects ($P < 0.05$), but there was no significant difference between Kellgren and Lawrence subgroups (data not shown). In OA and RA populations, Coll 2-1 NO_2, but not Coll 2-1, was significantly correlated (OA: $r = 0.27$; RA: $r = 0.23$, $P < 0.05$) with the CRP values, indicating that Coll 2-1 nitration is related to the intensity of inflammatory reaction.

Discussion

Here, we described two novel immunoassays, one specific for a peptide derived from the triple helical part of the type II collagen molecule (Coll 2-1) and the other for its nitrated form (Coll 2-1 NO_2). We selected this peptide-sequence because it contains a tyrosine residue susceptible for nitration. Interestingly, phenylalanine and tryptophan, two other aromatic residues sensitive to nitration, were absent of our selected peptide. Chondrocytes express both nicotin-amide adenine dinucleotide phosphate oxidase and $\overset{\cdot}{\text{N}}\text{O}$ synthases[16,26] and can produce high levels of $\overset{\cdot}{\text{N}}\text{O}$ and $O_2^{\div}$ in pathological conditions[19]. As it is now well accepted that oxidative damage is involved in cartilage matrix degrada-tion[27], we have anticipated that nitrated Coll 2-1 could reflect the oxidative-related cartilage degradation.

A systematic research of the Coll 2-1 amino acids sequence in Swiss Prot and Blast databases revealed that the Coll 2-1 peptide is only found in the $\alpha 1$ chain of type II collagen and in the $\alpha 3$ chain of type XI collagen. Both collagen types are very specific to cartilage and are only found in trace amounts in other tissues. Moreover, type XI collagen makes up only about 1% wt/wt relative to $\alpha 1(II)$ chain[28] suggesting that the contribution of type XI collagen in our immunoassays is probably minor. Type XI collagen is embedded in the type II collagen fibrils and its processing occurs with type II collagen. Therefore, we can hypothesize that Coll 2-1 fragments are simultaneously released from type II and XI collagens and that they reflect the degradation of cartilage matrix.

Based on these considerations, we have developed two immunoassays for measuring Coll 2-1 and Coll 2-1 NO_2 in serum. These immunoassays have a good performance with respect to precision, recovery, linearity and specificity. The antisera D3 is specific for the sequence Coll 2-1 and does not cross-react with any other collagen or protein tested in this study in spite of a 66% sequence homology with the $\alpha(2)$ chain of type I collagen. In parallel, we have developed an immunoassay for the nitrated form of Coll 2-1. Despite a very low cross-reactivity (<0.1%) with Coll 2-1 and nitrated type II collagen, the antiserum (D37) can be considered as specific for Coll 2-1 NO_2. It does not cross-react with the other nitrated proteins and with free tyrosine residues. With regards to the long-term handling and assaying of the serum samples, we have shown that type II collagen α-helix fragments (Coll 2-1 and Coll 2-1 NO_2) resisted at least at five freeze−thaw cycles. Moreover, lipids, haemoglobin and bilirubin (concentrations up to 5 g/L, 200 μM and 250 μM, respectively) do not interfere with Coll 2-1 and Coll 2-1 NO_2 measurements in serum (data not shown). These findings are important for potential applica-tions of the marker in a routine clinical laboratory.

The current assays were further characterized by analysing sera from different groups of subjects. In normal subjects, whatever the gender, Coll 2-1 concentrations remain relatively constant from age 20−65 suggesting that Coll 2-1 increase in arthritic patients is not the conse-quence of ageing but reflects disease process. In contrast, Coll 2-1 NO_2 level was more elevated in the younger subjects. This elevation could be explained by the more intensive professional and sport activities performed by subjects of this group. Coll 2-1 NO_2 levels are higher in premenopausal women than in men of the same range of age whereas Coll 2-1 levels are similar in both sexes. The increased level of Coll 2-1 NO_2 in premenopausal women suggests that Coll 2-1 nitration is associated with oestrogen levels. This hypothesis is supported by previous studies

demonstrating that nitrite/nitrate plasma levels were positively correlated with 17β-oestradiol levels in pre- and postmenopausal women and that hormone replacement therapy increased NO_2^-/NO_3^- levels[29]. Nevertheless, further *in vitro* and *in vivo* investigations are needed before concluding on the role played by oestrogens on cartilage matrix nitration.

We have also observed that the Coll 2-1 concentration is significantly increased in serum of OA patients compared to healthy controls, indicating that the rate of type II collagen degradation is increased in OA. This finding corroborates previous studies demonstrating an increase of type II collagen breakdown products in urine[9], synovial fluid[30] and cartilage explants[6] of OA patients. Interestingly, in OA and RA populations, Coll 2-1 NO_2, but not Coll 2-1, was significantly correlated with CRP levels. Although we cannot exclude an extra-articular nitration of Coll 2-1, these findings suggest that Coll 2-1 peptide nitration is directly related to synovium inflammation and that Coll 2-1 NO_2 could be a promising marker of arthritic disease activity. However, we are aware that our study has some limitations. The clinical data represent quite small OA and RA cohorts and the clinical performance of these markers will require additional validation in larger clinical studies. However, the determination of Coll 2-1, a specific peptide of cartilage, and its nitrated form in biological fluids or chondrocytes-conditioned culture medium has the potential to be a useful tool in understanding the role of reactive nitrogen species in cartilage homeostasis and degradation.

In conclusion, we have designed two immunoassays for quantifying in serum a fragment derived from the triple helix of type II collagen in its native and nitrated form. These assays could be useful to elucidate type II collagen degradation and oxidative damages in inflammatory joint diseases.

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