CONCISE REPORT

Evaluation of free and peptide bound collagen crosslink excretion in different skeletal diseases

A Müller, K Jakob, G E Hein

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Objective: To investigate urinary fractions of free and peptide forms of collagen crosslinks in patients with rheumatoid arthritis (n=50), osteoarthrosis (n=38), psoriatic arthritis (n=38) and in healthy volunteers (33 adults, 17 children).
Methods: Pyridinoline (PYD) and deoxypyridinoline (DPD) were measured by high performance liquid chromatography.
Results: In rheumatoid arthritis (RA) all fractions of PYD and DPD were significantly raised compared with osteoarthritis, psoriatic arthritis, and healthy controls. PYD and DPD correlated with disease activity in RA. In RA the collagen degradation resulted in primarily peptide bound forms.
Conclusion: The correlation between total peptide bound or free collagen crosslinks in different chronic joint diseases varies; however, this variation does not allow for a reliable differentiation between inflammatory and degenerative joint diseases.

Pyridinoline (PYD), a trifunctional 3-hydroxypyridinium crosslink[1] and its analogue, deoxypyridinoline (DPD),[2] are two non-reducible collagen crosslinks. PYD occurs in type I and II collagen[3] and, with the exception of skin, sclerae, and cornea, in most connective tissues—that is, bone and cartilage. DPD originates mainly from bone.[3] When collagen fibrils are degraded into molecular fragments as a result of osteoclastic resorption, PYD and DPD are released into the circulation. They are then cleared by the kidney and excreted through urine, where they can be detected in peptide bound and free forms.[4] It has been reported that 40% of these crosslinks are excreted into urine as free and 60% in the peptide bound form.[4]

Yet it remains unclear whether the determination of free or peptide bound crosslinks can provide more information on a possibly different collagen catabolism in patients with different diseases, compared with healthy adults and children.

The aim was to investigate the fractions of free and peptide bound crosslinks as well as the total excretion in patients with rheumatoid arthritis (RA), psoriatic arthritis (PsA), and osteoarthrosis (OA) compared with healthy adults and children. Inflammation markers and steroid dose were included in further correlation studies. Healthy children were investigated to discover possible differences due to growth.

SUBJECTS AND METHODS

Healthy controls

Fifty healthy volunteers were included (33 healthy adults (HA), 12 female, 21 male, mean (SD) age 41.0 (7.8) years and 17 healthy children (HC), nine female, eight male, mean (SD) age 7.7 (3.3) years). None had a previous history of metabolic bone disease, and none were receiving drugs affecting calcium absorption, excretion, or bone metabolism.

Rheumatoid arthritis

This group included 44 female and six male patients, mean age 54.9 (15.9) years with definite RA. To determine the disease activity, serum C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were measured. The daily steroid dose was also recorded.

Osteoarthritis

This group included 38 patients with proven OA of the knee joints (30 female, eight male, mean age 59.0 (11.7) years).

Psoriatic arthritis

This group included 21 female and 25 male patients, mean age 47.2 (11.2) years. PsA was diagnosed using the criteria of Moll and Wright modified by Bennett.[5]

Sample collection and storage

Fasting second void urine samples were taken in the morning without any predetermined diet and centrifuged (3000 *g*) for 10 minutes, and aliquots for the different assays were stored at –20°C. All the assays were performed within 12 months of sample collection.

Urinary assays of free and peptide bound crosslinks by HPLC

High performance liquid chromatography (HPLC) assays of total PYD and DPD (tPYD, tDPD) were performed using hydrolysed urinary samples as described previously.[6 7] The free form of PYD and DPD (fPYD, fDPD) was measured without prior hydrolysis, and acidified with 6 N HCl by the same HPLC assay. Peptide bound crosslinks (pPYD, pDPD) were obtained by calculating the difference between the level of total and free crosslinks.

Statistical analysis

The Mann-Whitney U test for two independent samples, the Kruskal-Wallis H test for several independent samples, and the bivariate correlation analysis (Spearman's r_s) were used for statistical evaluation performed by the SPSS (version 10.0) software. A normal distribution was excluded using the Kolmogorov-Smirnov test.

RESULTS

Table 1 shows the values (mean (SD)) and the confidence interval of the free and peptide bound forms, and total PYD and DPD by HPLC in HA, HC, RA, OA, and PsA.

In RA, all fractions of PYD and DPD were significantly raised compared with those in OA and PsA. All patient groups

Abbreviations: CRP, C reactive protein; DPD, deoxypyridinoline; ESR, erythrocyte sedimentation rate; f, free; HA, healthy adults; HC, healthy children; HPLC, high performance liquid chromatography; OA, osteoarthritis; p, peptide bound; PsA, psoriatic arthritis; PYD, pyridinoline; RA, rheumatoid arthritis; t, total

Table 1 Mean (SD) and confidence interval of free, peptide bound, and total PYD, DPD, and PYD/DPD by HPLC and Ntx by immunoassay in RA, PsA, OA, HA, and HC groups

	RA	PsA	OA	HA	HC
Number	50	46	38	33	17
fPYD (nmol/mmol Cr)	34.9 (23.6) (6.8 to 100)	16.6 (7.6) (8.9 to 41)	15.8 (7.0) (5.0 to 36.3)	16.0 (6.6) (7.9 to 35.2)	116 (30) (51 to 156)
pPYD (nmol/mmol Cr)	44.9 (29.7) (7.0 to 144)	24.5 (15.9) (8.2 to 99)	19.3 (7.4) (9.1 to 39.0)	6.3 (4.1) (0.12 to 36.9)	72.8 (49.5) (2.2 to 155)
tPYD (nmol/mmol Cr)	79.8 (45.7) (19.2 to 200)	41.1 (19.4) (19.9 to 123)	35.1 (12.9) (17.1 to 70)	22.3 (12.4) (8.9 to 72)	189 (72) (57 to 295)
fDPD (nmol/mmol Cr)	8.5 (5.7) (0.46 to 24.6)	5.5 (4.0) (2.1 to 24.0)	6.1 (2.9) (1.2 to 12.2)	5.9 (5.1) (0.57 to 24.0)	28.2 (10.0) (11.4 to 58)
pDPD (nmol/mmol Cr)	12.9 (12.4) (0.3 to 57.5)	6.8 (8.3) (0.4 to 49)	6.2 (5.1) (0.43 to 22.9)	2.5 (2.1) (0.5 to 15.4)	35.1 (24.1) (1.6 to 89)
tDPD (nmol/mmol Cr)	21.4 (17.0) (3.3 to 63)	12.0 (8.6) (3.4 to 52)	12.3 (6.3) (3.9 to 29.7)	8.2 (6.3) (1.2 to 31.3)	63.3 (26.4) (16.7 to 125)
fPYD/fDPD	4.4 (1.9) (1.2 to 9.7)	3.6 (1.4) (0.81 to 7.1)	3.0 (1.4) (0.76 to 6.7)	4.3 (3.9) (0.72 to 21.7)	4.3 (0.8) (2.4 to 5.9)
pPYD/pDPD	8.5 (17.2) (0.8 to 91)	7.9 (12.2) (1.7 to 64)	6.6 (9.8) (0.84 to 55)	4.1 (8.6) (0.6 to 52)	4.8 (11.9) (0.2 to 51)
tPYD/tDPD	4.4 (1.9) (1.2 to 10.0)	3.9 (1.7) (1.6 to 9.9)	3.3 (1.4) (0.98 to 7.7)	3.3 (1.6) (1.5 to 20.3)	3.0 (0.4) (2.4 to 3.8)

f, free; p, peptide bound; t, total, measured by HPLC
The crosslinks were expressed as nmol/mmol creatinine (Cr), except for the qotients of the crosslinks. The values in brackets are the 95% confidence intervals.
All measurements showed a significant difference among the groups as calculated by the Kruskal-Wallis test.
A significance between two groups was determined by the Mann-Whitney U test; significances are reported in the text.

showed significantly higher levels of tPYD than the adult control group. Also for pPYD, there were significant differences between all patient groups compared with HA. The group of healthy children showed the highest levels of PYD and DPD in all fractions.

The levels of the fractions of tDPD and pDPD in all patient groups were significantly raised compared with those in HA. Yet for fDPD there were no significant differences between OA, PsA, and HA.

For the tPYD/tDPD ratio, there were significant differences between patient groups and HA and HC, except for OA. For the pPYD/pDPD ratio we found significant differences between the patient groups and HC and HA, and for the ratio fPYD/fDPD differences were only found between RA and HA and between HC and OA and PsA. For all fractions we found the highest ratio in patients with RA. The ratio of pPYD/pDPD was more pronounced in RA than in HA and HC, resulting in the most clear differences between patient groups and the healthy controls.

Figure 1 presents the ratio of free to total (f/t) PYD and DPD. There was no significant difference in f/tPYD and f/tDPD. But, it was shown that the share of fDPD was higher in the patient groups, whereas the reverse was true for HA and HC.

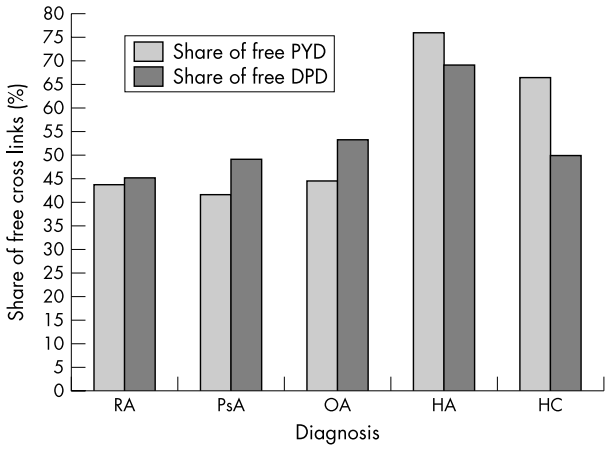

Figure 1 Free PYD and DPD shown as a share of the total amount of PYD and DPD.

Correlation studies between the inflammation markers ESR and CRP in serum and free, total, and peptide bound crosslinks in patients with RA showed that tPYD (r_s=0.45, p=0.01), fPYD (r_s=0.47, p<0.001), pPYD (r_s=0.39, p=0.02), tDPD (r_s=0.37, p=0.02), and fDPD (r_s=0.46, p<0.001) correlated significantly with ESR, and that CRP correlated significantly with pPYD (r_s=0.34, p=0.03), tDPD (r_s=0.35, p=0.02), and pDPD (r_s=0.34, p=0.03). There was no significant effect of the steroid dose on the excretion of crosslinks or N-terminal crosslinking telopeptide of type I collagen.

DISCUSSION

Little is known about the release and catabolism of pyridinium crosslinks in tissue, their uptake into the blood circulation, further cleavage, and urinary excretion. In particular, no data exist which show possible different catabolic pathways of collagen within various skeletal diseases. We have assessed the urinary fractions of PYD and DPD by HPLC in healthy adults and children, and also in RA, OA, and PsA. We found different patterns of metabolism/excretion of crosslinks containing collagen fragments and portions of free crosslinks in healthy adults, children, and different patient groups. The high levels of crosslink excretion in healthy children is obviously based on a quantitatively increased, but qualitatively similar, bone and cartilage degradation during the growth period compared with healthy adults.

It has been reported that the fractions of PYD and DPD differed between postmenopausal healthy women and osteoporotic women.[8] Randall *et al* stated that the free fraction of DPD showed a downward trend with lower disease severity in Paget's disease.[9] They proposed a model in which collagen fragments containing pyridinoline are degraded to free pyridinolines with a rate limiting step. In contrast, Ebeling *et al* reported no change in fDPD in menopausal women or in women receiving hormone replacement therapy, and found no changes in tDPD.[10] Colwell and Eastell investigated the renal clearance and fractional clearance of pyridinium crosslinks in serum, and suggested that some of the free fractions of crosslinks excreted into urine are produced in the kidney.[11]

A comparison of free and peptide bound forms of these crosslinks in RA and OA has, thus far, only been investigated by Takahashi *et al*.[12] As of yet, no publications report these data for healthy children or for patients with PsA. Our study shows that in adults, patients with RA have the highest levels of free and peptide bound forms of PYD and DPD. In OA and PsA, no significant differences were found between fPYD and tPYD, and for fPYD, as compared with HA; the same was true for fDPD. In contrast, we were able to show significant differences between these groups for pPYD in OA and PsA and also for tPYD, tDPD, and pDPD as compared with HA. The highest levels of all fractions of PYD and DPD were found in HC.

Only RA showed significantly raised levels of fPYD and fDPD. Probably, this indicates that there are different degradation pathways in different pathophysiological circumstances—for example, different collagenase activities in different disease groups lead to different amounts of free crosslinks.

Also in the ratio tPYD/tDPD, we found significant differences in RA compared with the other patient groups and compared with HC and HA, which might be explained by a higher degradation of collagen fragments containing PYD, as also reported by Takahashi *et al*.[12] The ratios of PYD/DPD in all fractions did not differ between HC and HA, a further indication of qualitative analogue processes in collagen turnover/degradation of healthy volunteers of different ages. We found no published reports on the ratios fPYD/fDPD or pPYD/pDPD. For the ratio fPYD/fDPD there are similar levels for RA and HC/HA, whereas these ratios in PsA and OA are significantly decreased. For the ratio pPYD/pDPD we showed increased levels in all disease groups compared with HA/HC. This also supports the hypothesis that, in inflammatory skeletal diseases the degradation pathway mainly results in protein bound crosslinks, possibly because the cleavage pathway to free crosslinks is restricted.

For the correlations between free and total crosslinks, we found a stronger correlation for PYD in all groups compared with DPD; further evidence showing that PYD containing collagen-fragments are degraded distinctively in different skeletal diseases.

Our results showed a higher share of free crosslinks in the normal volunteers (fig 1) than those of Abbiati *et al*,[13] who found 50% for both, and Knott and Bailey,[4] who found 40%. However, our chromatographic method is validated and the determination procedure includes an internal standard, allowing for improved intra- and interassay variation coefficients.

Overall, the results of our investigations into free, protein bound, and total collagen crosslinks indicate the probability that differences do exist between healthy subjects and those with skeletal disease. Differences in collagen breakdown pathways in different joint diseases are likely, yet it is still not possible, by the determination of free crosslinks, to distinguish clearly the type of skeletal disease—that is, between inflammatory and degenerative skeletal disease.

Further details on the relation between the excretion rate of crosslinks and disease activity/severity are given in our previous publications.[7 14]

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Authors' affiliations

A Müller, G E Hein, Department of Internal Medicine IV, Rheumatology and Osteology, University of Jena, Jena, Germany
K Jakob, Helios Hospital Erfurt, Department of Anaesthesiology and Intensive Care, Erfurt, Germany

Correspondence to: Dr A Müller, Department of Internal Medicine IV, Rheumalabor, 07740 Jena, Germany; i5admu@med.uni-jena.de

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