

## ASSESSMENT OF URINARY HYDROXYPYRIDINIUM CROSS-LINKS MEASUREMENT IN OSTEOARTHRITIS

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### SUMMARY

The aim of this study is to re-evaluate urinary collagen cross-links, previously proposed as markers of osteoarthritis (OA). The urinary excretion of collagen cross-links, pyridinoline (PYD) and deoxypyridinoline (DPD), was measured using high-performance liquid chromatography (HPLC) in 114 patients with OA, 19 patients with rheumatoid arthritis (RA) and 40 healthy subjects. An increase in PYD and DPD, expressed per millimole of creatinine, was confirmed in RA. However, PYD and DPD in patients with hip OA, knee OA and polyOA were similar, and did not differ from controls. In patients with radiographic end-stage OA, PYD and DPD were significantly higher than in patients with an early OA, but not significantly higher than in controls. The PYD/DPD ratio did not vary with the OA stage. Thus, urinary collagen cross-links are not elevated in OA, but could reflect bone sclerosis and/or erosion in late OA.

**KEY WORDS:** Pyridinium cross-links, Osteoarthritis, Rheumatoid arthritis.

THE covalent bridges or cross-links between the adjacent molecules of collagen stabilize the extracellular matrix of connective tissues. Owing to their natural fluorescent properties, the pyridinium cross-links, pyridinoline (PYD) and deoxypyridinoline (DPD), have been identified in many connective tissues, essentially in bone and cartilage [1, 2]. DPD is mostly found in bone, while PYD is found both in skeletal and vascular connective tissues [1, 3]. Unlike hydroxyproline, pyridinium cross-links are released following mature collagen degradation in peptide-free forms and are excreted unchanged in urine. Pyridinium cross-links are measured by high-performance liquid chromatography (HPLC), paper chromatography and enzyme-linked immunoassay (ELISA) [4]. Both paper chromatography and ELISA do not allow PYD and DPD to be distinguished. Quantitative analysis using ion-paired reversed-phase liquid chromatography followed by fluorescent detection has been improved [5].

Pyridinium cross-links have already been proposed as an index of bone resorption in Paget's disease [6], primary hyperparathyroidism [7], bone metastasis [8] and osteoporosis [9]. PYD and DPD were also investigated in osteoarthritis (OA) and rheumatoid arthritis (RA). They were suggested as markers of joint degradation [10-15]. These studies led us to correlate urinary pyridinium cross-links with the measurement of joint space narrowing in patients with hip OA [16]. However, initial results obtained in such patients clearly conflicted with reported studies. The present

work is a re-evaluation of urinary concentrations of pyridinium cross-links in OA patients.

### PATIENTS AND METHODS

#### *Patients*

The OA group was made up of 114 consecutive patients, referred to the rheumatology clinic for symptomatic OA. There were 47 hip OA (coxOA), 31 knee OA (gonOA) and 36 patients with generalized OA (polyOA). All these patients fulfilled the ACR criteria for OA [17]. A normal control group was composed of 40 healthy volunteers. A joint disease control group was composed of 19 patients with classic RA, according to the ARA criteria. Age, sex, menopausal status, weight and body mass index are given in Table I.

Anteroposterior roentgenograms of lumbar rachis, pelvis and knees taken in the weight-bearing position, a sunrise view of the patellofemoral joints and dorsal X-ray of the hands were performed in OA patients and normal controls. Films were read by a single experienced observer who was blinded to the patient's status. X-ray OA was scored as follows: stage 1, minimal joint space loss and/or osteophytosis; stage 2, substantial but incomplete joint space loss; stage 3, total and focal joint space narrowing; stage 4, nearly complete joint space narrowing and subchondral bone erosion (intraobserver reproducibility:  $k = 0.76$ ) [18]. In patients with bilateral OA, the most severely affected side was analysed.

Westergren erythrocyte sedimentation rate (ESR) (mm/h), routine blood haematology and biochemistry tests (red and white cell counts, alkaline phosphatase, alanine aminotransferase, calcium, phosphorus, glycaemia, albumin and creatinine) were performed in OA and RA patients. None of the patients suffered from renal impairment, diabetes or a metabolic bone disease.

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TABLE I  
Characteristics of patients with osteoarthritis (OA), rheumatoid arthritis (RA) and controls (median and s.d.)

	N	Age	Weight	BMI	Sex ratio M/F	Post-menopausal/ non-post-menopausal
Controls	40	42.9 (12)	70.56 (13.4)	24.37 (4.2)	21/19	9/10
OA	114	62.44 (11.5)	73.72 (12.5)	27.74 (4.2)	49/65	50/15
RA	19	58.3 (18)	66.67 (15)	24.75 (4)	8/11	7/4

#### Measurement of urinary pyridinoline and deoxypyridinoline

Morning urine samples were collected from all patients and stored at  $-20^{\circ}\text{C}$  prior to analysis. Urine samples were centrifuged at 1000 g during 10 min and an aliquot of urine was hydrolysed by heating with an equal volume of 12 M HCl at  $116^{\circ}\text{C}$  during 16 h, to convert all cross-links to the free form. The hydrolysates were fractionated by partition chromatography on CF1 cellulose and the pyridinium compounds were quantified by reverse-phase, isocratic, ion-paired HPLC as described by James *et al.* [19]. The ion-pairing agent used was the octane sulphonic acid. Concentrations of urinary pyridinium cross-links were expressed relative to values of urinary creatinine (nmol/mmol Cr). The coefficient of variation of the PYD and DPD assay was 14 and 15%, respectively.

#### Statistical analysis

A comparison between two groups was performed using Student's *t*-test. Analysis of variance was used when there were more than two groups. Correlations were studied using simple linear regression.

### RESULTS

Figure 1 shows the individual values of PYD and DPD for each group. Means and standard deviations according to age and sex are given in Table II. PYD and DPD excretion, as well as the PYD/DPD ratio, were unrelated to sex, age, menopausal status, weight or body mass index (BMI) in each of the control, OA and RA groups. In healthy control individuals, mean values and standard deviations of PYD and DPD/mmol of creatinine were  $34.5 \pm 4.9$  and  $6.9 \pm 1.1$  nmol/mmol, respectively. The PYD/DPD ratio was  $8.1 \pm 1.9$  nmol/mmol. The strong correlation between PYD and DPD ( $r = 0.76$ ;  $P < 0.001$ ) is illustrated in Fig. 2.

Urinary concentrations of PYD and DPD in OA patients were slightly but not significantly lower than those in the control group ( $30.2 \pm 1.7$  and  $6.2 \pm 0.4$  nmol/mmol, respectively). The PYD/DPD ratio was unmodified in OA ( $5.5 \pm 0.2$  nmol/mmol). PYD was sometimes increased in polyOA, but no significant difference was found between patients with hip OA, knee OA and polyOA (Fig. 3). Urinary concentrations of hydroxypyridinium cross-links according to radiographic scores were studied in patients with hip or knee OA. They clearly increased with radiographic scores, but the difference between the four groups was not significant. When pooling patients with early OA (X-ray score 1 and 2) and patients with late

OA (X-ray score 3 and 4), PYD and DPD were, respectively, 1.47- and 1.4-fold higher in late OA than in early OA ( $P = 0.003$  and  $0.04$ , respectively) (Fig. 4). The PYD/DPD ratios were  $4.4 \pm 1.3$  and  $6.1 \pm 2.7$ , respectively. The urinary excretion of PYD and DPD was unrelated to NSAID uptake.

PYD and DPD were increased by 42.5 and 29.7%, respectively, in RA patients, when compared with normal controls. The difference was significant

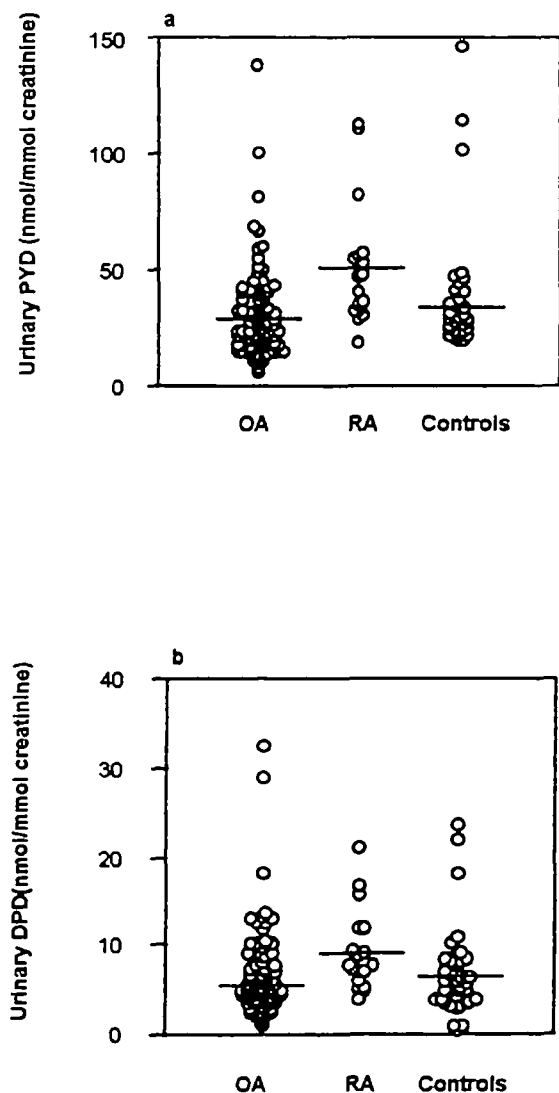


FIG. 1.—Urinary concentrations of pyridinoline (PYD) (a) and deoxypyridinoline (DPD) (b) in patients with osteoarthritis (OA) and rheumatoid arthritis (RA) and controls. All concentrations are expressed relative to urinary creatinine.

TABLE II

Pyridinium cross-link values (median and s.d.) in patients with osteoarthritis (OA), rheumatoid arthritis (RA) and controls relative to sex and menopausal status. Values are expressed relative to urinary creatinine

	Controls			OA			RA		
	N	PYD (nmol/mmol)	DPD (nmol/mmol)	N	PYD (nmol/mmol)	DPD (nmol/mmol)	N	PYD (nmol/mmol)	DPD (nmol/mmol)
Males	21	33.77 ± 3.9	6.71 ± 1.9	49	27.45 ± 2.2	4.9 ± 0.4	8	47.61 ± 3.9	8.58 ± 1.6
Non-post-menopausal females	10	32.86 ± 6.6	7.89 ± 2.4	15	22.9 ± 2.1	4.9 ± 0.7	4	52.3 ± 10.9	7.72 ± 1.49
Post-menopausal females	9	37.78 ± 8.6	6.34 ± 1.9	50	33.17 ± 2.8	7.42 ± 0.7	7	53.2 ± 10.5	10.4 ± 2.2
Total	40	34.5 ± 4.9	6.92 ± 1.1	114	30.17 ± 1.7	6.21 ± 0.4	19	50.66 ± 5.9	8.98 ± 1.04

( $P < 0.02$ ) for PYD only. The difference with the OA group was significant for both parameters ( $P = 0.018$  and  $P = 0.02$ , respectively). The PYD/DPD ratio was  $6 \pm 0.5$ . PYD and DPD were significantly ( $P < 0.05$ ) higher, by 104.1 and 69.7%, respectively, in RA patients treated with steroids than in RA patients without steroids (Table III). The latter were not significantly different from the normal controls. The PYD/DPD ratio did not vary with the steroid intake. There was no correlation between cross-links and ESR.

#### DISCUSSION

The present work does not confirm the previously reported increased urinary concentrations of PYD and DPD in OA [13, 15].

The difference between our results and those of previous studies does not seem to be in relation to the controls. Our number of normal controls is rather higher than that of previous studies, in some of which the same control group was used repeatedly [11, 12, 15]. Means and standard deviations of the present normal control group were also close to those of previous studies in which the same HPLC method was used [10, 19]. There were more women and old patients in our OA group than in the control group. The urinary excretion of pyridinium cross-links is similar in men and pre-menopausal women. It was also found to be unrelated to age. An increased excretion of PYD and DPD in peri- and post-menopausal women was demonstrated [20, 21]. However, increasing the number of menopausal women in our control group would have increased the normal values accordingly.

The selection of OA patients could be an explanation

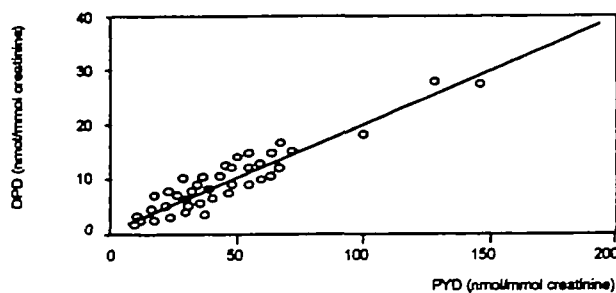


FIG. 2.—Correlation between urinary pyridinoline (PYD) and urinary deoxypyridinoline (DPD) values in controls. Spearman test:  $P < 0.001$  and  $r = 0.76$ .

for our results. MacDonald *et al.* [15] found a higher urinary excretion of PYD in gonOA than in coxOA and polyOA. Astbury *et al.* [14] demonstrated that the urinary excretion of pyridinium cross-links could be related to joint damage. The present work does not support the findings of MacDonald *et al.* and rather

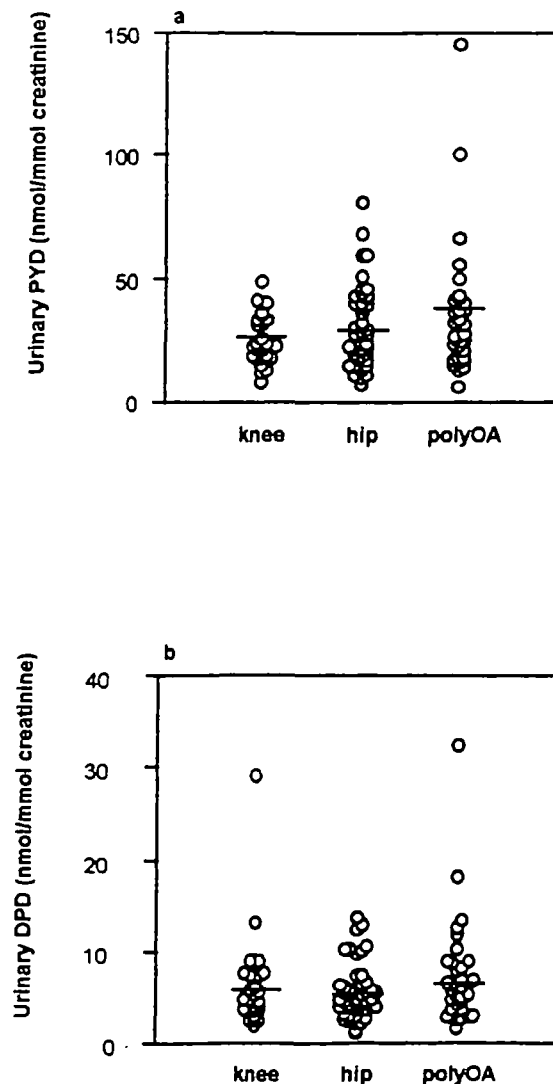


FIG. 3.—Urinary pyridinoline (PYD) (a) and deoxypyridinoline (DPD) (b) values in osteoarthritis (OA) subgroups. All concentrations are expressed relative to urinary creatinine.

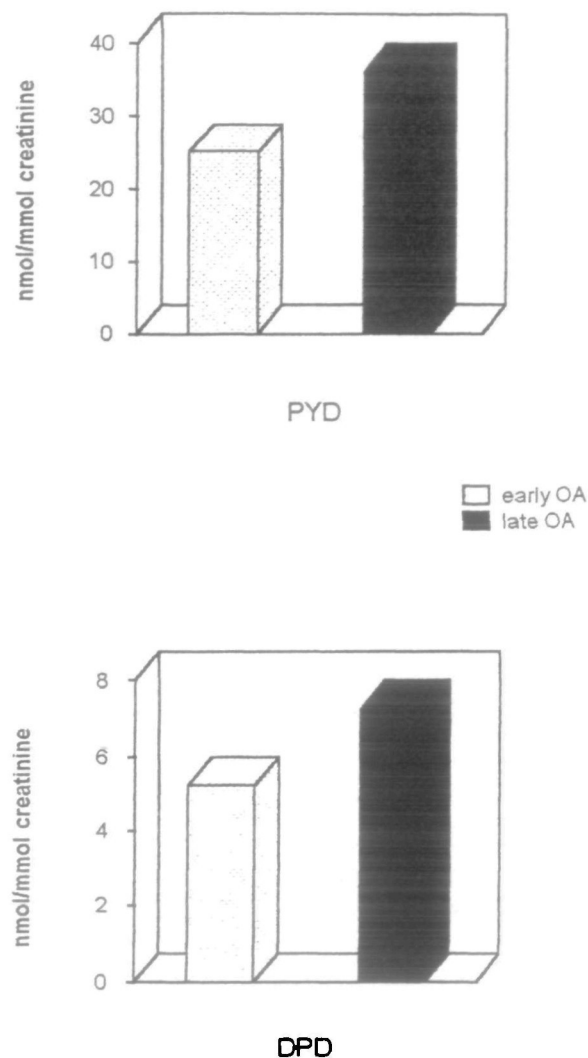


FIG. 4.—Urinary pyridinoline (PYD) and deoxypyridinoline (DPD) values relatively to radiographic scores. Patients were divided into early OA (X-ray scores 1 or 2) and late OA (X-ray scores 3 or 4). PYD and DPD increased significantly in late OA:  $P = 0.003$  and  $P = 0.04$ , respectively.

suggests an increase in some cases of polyOA. It demonstrates that PYD and DPD excretion increased significantly with the radiological stage of OA. Although there was no significant difference between the late OA subgroup and the normal group, the selection of a larger number of patients with late OA might have made the difference between OA and the control significant.

The tissular topography of hydroxyypyridinium

TABLE III  
Pyridinium cross-link values (median and s.d.) relative to steroid intake

	N	PYD	DPD	DPD/PYD
RA + steroids	9	78.7 ± 7.2	12.9 ± 1.8	7.1 ± 0.9
RA - steroids	10	38.5 ± 2.3	7.6 ± 0.4	5.3 ± 0.3
Total	19	50.6 ± 5.9	8.8 ± 1.04	6.1 ± 0.4

cross-links has been studied in detail [1, 2]. DPD could be considered as a sensitive marker of bone degradation since it is mostly found in bone and dentine, and the turnover of the latter tissue is negligible [1]. PYD is a major collagen cross-link in bone, cartilage and other connective tissues such as muscle and intervertebral discs [1, 22]. Although bone resorption is probably the major source of urine hydroxyypyridinium cross-links [13, 14], the PYD/DPD ratio may help to define collagen degradation in cartilage relative to other tissues. In agreement with previous studies in OA patients [10, 13], PYD and DPD were highly correlated ( $r = 0.75$ ,  $P < 0.001$ ), and the PYD/DPD ratio was unchanged. In patients with advanced OA, both PYD and DPD were significantly increased and the PYD/DPD ratio was also unchanged. Thus, most probably, the observed increased excretion of pyridinium cross-links in patients with late OA reflects the bone erosion and/or the increased sclerotic bone remodelling of joint epiphysis that occurs in the advanced stages of OA. Unfortunately, bone sclerosis was not graded in our score of OA [18]. The unchanged excretion of PYD and DPD in earlier stage OA suggests that the markers certainly do not reflect collagen degradation of the articular cartilage.

An increased urinary excretion of pyridinium cross-links has been reported in RA [12, 13, 14, 23, 24]. A small control group of RA patients was included in this study to test the validity of our measurements. We were able to confirm a dramatic increase in the urinary excretion of PYD and DPD in RA patients. Interestingly, this increase was only found in patients treated with steroids. Since joint destruction in RA patients was not investigated, the results suggest that an increase in urinary PYD and DPD was related to severe forms of RA justifying steroids, and/or with the increased bone resorption induced by steroids.

#### ACKNOWLEDGEMENT

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