

# The Effects of Sulphasalazine on Urinary Excretion of the Hydroxypyridinium Crosslinks of Collagen in Patients with Rheumatoid Arthritis

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Secondary osteoporosis is a feature of rheumatoid arthritis (RA). In recent years, several attempts have been made to develop specific markers for monitoring connective tissue metabolism in arthritic diseases. Our purpose, in this study was to assess pyridinium crosslinks (PYD and DPYD) excretion in relation to the activity of RA (changes related to sulphasalazine treatment).

Fourty premenopausal female patients with active RA (mean age;  $36.0 \pm 7.2$  years), 20 postmenopausal women with active RA (mean age;  $60.0 \pm 6.8$  years), 23 postmenopausal women with OA (mean age;  $56.1 \pm 6.6$  years) and 17 premenopausal healthy subjects (mean age;  $28.3 \pm 4.28$  years) were enrolled in our study. All of the 40 premenopausal female patients with active RA were given sulphasalazine. The mean follow up period for these patients was  $10.3 \pm 1.1$  months. In all of these patients, urine samples were collected both in the active and in the inactive periods. Urine PYD and DPYD levels were measured by ELISA.

Urine PYD levels were significantly higher in the active period ( $14.01 \pm 3.16$  nmol/mmol cr) than in the inactive ( $8.25 \pm 4.23$  nmol/mmol cr) period in patients with premenopausal RA ( $p < 0.05$ ). Urine PYD levels were significantly high in postmenopausal active RA patients ( $19.06 \pm 3.26$  nmol/mmol cr) compared to premenopausal active and ind inactive, postmenopausal inactive RA patients, osteoarthritis and healthy controls. Urine DPYD excretion was similar in patients with premenopausal RA in the active ( $7.46 \pm 2.13$  nmol/mmol cr) and inactive periods ( $5.08 \pm 0.87$  nmol/mmol cr) ( $p > 0.05$ ).

In active premenopausal RA patients, a correlation was found between PYD excretion and RAI, ESR, CRP and functional capacity ( $r=0.5729$   $p < 0.01$ ,  $r=0.5953$   $p < 0.01$ ,

$r=0.6125$   $p < 0.01$  and  $r=0.6232$ ,  $p < 0.01$  respectively). But in the inactive period, no such correlation was was evident. In disease activity parameters did not correlate with DPYD excretion in either the active or the inactive period.

As a result, urine PYD excretion was significantly high in patients with active RA. During sulphasalazine treatment, urine PYD levels decreased. This is attributed to improvement in bone destruction.

**Key Words:** Pyridinium crosslinks, Sulphasalazine, Rheumatoid arthritis

## INTRODUCTION

Chronic joint disease is characterized by alterations in the extracellular matrix metabolism of articular cartilage, adjacent bone and the surrounding connective tissues, and by inflammatory changes in the synovial membrane. In recent years, several attempts have been made to develop specific markers for monitoring connective tissue metabolism in arthritic diseases. In an attempt to gain a more sensitive and specific index of cartilage and bone breakdown, assays for the hydroxypyridinium crosslinks of collagen have been developed.<sup>1-3</sup>

Secondary osteoporosis is a feature of rheumatoid arthritis (RA). Generalised osteoporosis is also a well documented feature of late disease, although its primary causes are poorly understood. Evidence exist which suggests that bone loss in RA occurs rapidly and early during the disease cause,<sup>4,5</sup> and it is therefore, desirable to study patients as soon as possible. Additionally, by doing

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so, associations between disease activity and bone loss can be studied.

The pyridinium crosslinks, pyridinoline (PYD) and deoxypyridinoline (DPYD) are constituents of mature cartilage and measurements of their concentrations in urine reflect the rate of collagen degradation. Both crosslinks are present in bone collagen. Deoxypyridinoline is bone specific, and is found in type I collagen only,<sup>6</sup> but PYD is particularly prevalent as the major crosslink in cartilage, and is found in both skeletal and vascular connective tissue. The pyridinium compounds are products of intermediate aldehyde mediated crosslinks of collagen. Pyridinoline involves hydroxylysine and is predominant crosslink of cartilage, whereas DPYD involves lysine form and DPYD is primarily located in the collagen matrix of bone collagen.<sup>7</sup> Following mature collagen degradation, these crosslinks are released in free peptide forms and excreted unchanged in urine.

Our purpose was to assess pyridinium crosslinks excretion in relation to the activity of RA (changes related to sulphasalazine treatment).

## MATERIALS AND METHODS

Forty premenopausal patients with RA (mean age;  $36.0 \pm 7.2$  years, mean disease duration;  $2.2 \pm 1.3$  years), attending the Department of Immunology at the Medical School of Ankara University, were enrolled in this study. All patients with RA met the criteria of the American Rheumatism Association (ARA).<sup>8</sup> The subjects: twenty postmenopausal women with active RA (mean age;  $60.0 \pm 6.8$  years, mean disease duration;  $8.41 \pm 4.12$  years), and 23 postmenopausal women with OA (all of them had moderate OA characterized by multi-joint involvement, cartilage loss, osteosclerosis and osteophyte formation, mean age;  $56.1 \pm 6.6$  years); 17 premenopausal healthy subjects (mean age;  $28.3 \pm 4.28$  years) served as controls.

The forty premenopausal active RA patients had received no corticosteroids or disease modifying drugs. All of these patients had taken nonsteroid anti-inflammatory drugs. All 40 patients were followed prospectively. Twenty-three of the patients had positive rheumatoid factor

(RF). Three patients had typical rheumatoid nodules. No other extraarticular manifestations were noted and no patient had renal disease. All of these patients were given sulphasalazine (2 gr/day), NSAIDs and some patients ( $n=11$ , 27%) were given low dosage prednisolone (range 2-4 mg/day, for less than a 2 month period). The mean following period was  $10.3 \pm 1.1$  months. Urine samples were studied in both active and inactive period. The patients without remission were excluded. Disease activity and functional capacity were prospectively assessed in all patients by the same clinician. Disease activity and functional capacity were assessed monthly. Measures of disease activity included the number of tender joints, the number of swollen joints, the Ritchie articular index,<sup>9</sup> duration of morning stiffness (minute), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Functional capacity was assessed using a Dutch equivalent of the Stanford Health Assessment Questionnaire.<sup>10</sup> Active disease was defined by the presence of at least 3 of 5 criteria;  $\geq 3$  swollen joints,  $\geq 6$  tender joints, early morning stiffness  $\geq 45$  minutes, ESR  $\geq 28$  mm/h and CRP  $\geq 15$  ng/L. Complete blood cell count and routine chemistry were determined before initiating of sulphasalazine treatment.

Twenty postmenopausal active RA patients used multiple DMARDs (5 had sulphasalazine, NSAIDs and chloroquin, 9 had chloroquin and NSAIDs, 3 had auranofin and NSAIDs, and 3 had methotrexate, sulphasalazine and NSAIDs) during their disease but did not receive any treatment for osteoporosis. Ten of the 20 patients had positive RF. All of the postmenopausal active RA patients had been taking a low dosage of prednisolone (maximum 4 mg/day) for a short time (maximum 3 month). Postmenopausal women with OA were not taking any medication known to influence calcium metabolism.

Urine samples were collected for 24 hours during clinical and laboratory evaluation. Samples were stored at  $-70^{\circ}\text{C}$  until assayed. Pyridinoline and DPYD were measured by ELISA (Pyrilinks and Pyrilinks-D, Metra Biosystems, USA). Concentrations of urinary PYD and DPYD are expressed as fractions urinary creatinine (nmol/mmol cr).

Statistical Analysis: Differences between groups

were determined using the Paired *t* test, the Duncan test and the Mann-Whitney U-test. Correlations were tested by linear regression analysis.

## RESULTS

The demographic and descriptive details of the RA patients are shown in Table 1. Urine PYD levels were significantly higher in the active period ( $158.5 \pm 35.8$  mg/dl,  $14.01 \pm 3.16$  nmol/mmol cr) compared to the inactive ( $93.3 \pm 47.9$  mg/dl,  $8.25 \pm 4.23$  nmol/mmol cr) period in patients with premenopausal RA ( $p < 0.05$ ). Urine PYD levels were lowest in healthy controls ( $54.4 \pm 16.5$  mg/dl,  $4.81 \pm 1.46$  nmol/mmol cr). Urine PYD levels were significantly higher in postmenopausal active RA patients ( $215.6 \pm 36.9$  mg/dl,  $19.06 \pm 3.26$  nmol/mmol cr) than in the other groups (Fig. 1).

Deoxypyridinoline excretion was similar in patients with premenopausal RA during the active ( $84.3 \pm 24.1$  mg/dl,  $7.46 \pm 2.13$  nmol/mmol cr) and inactive periods ( $57.4 \pm 9.8$  mg/dl,  $5.08 \pm 0.87$  nmol/mmol cr) ( $p > 0.05$ ). Urine DPYD levels were lowest in the healthy controls ( $31.7 \pm 13.8$  mg/dl,  $2.80 \pm 1.21$  nmol/mmol cr). Urine DPYD excretion was similar for OA patients ( $57.8 \pm 16.6$  mg/dl,  $5.11 \pm 1.46$  nmol/mmol cr) and postmenopausal ( $69.3 \pm 24.2$  mg/dl,  $6.12 \pm 2.13$  nmol/mmol cr) active patients ( $p > 0.05$ ) (Fig. 2).

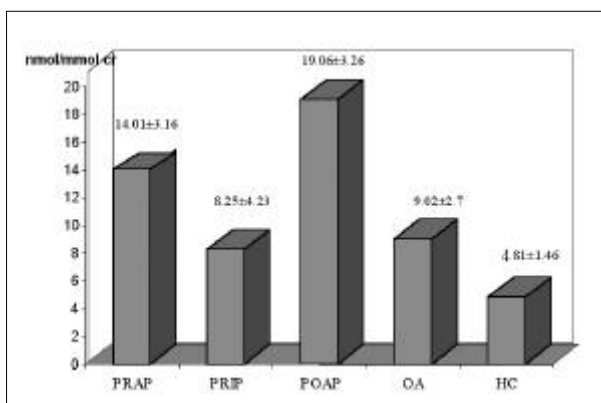
Pyridinoline excretion was significantly higher

in RF(+) patients than in RF(-) patients in both the active and the inactive premenopausal groups ( $p < 0.01$  and  $p < 0.05$  respectively). In postmenopausal patients with RA; no significant differences were found between the RF(+) and the RF(-) groups. On the other hand, DPYD excretion was high in active RF (+) premenopausal patients with RA than in RF (-) patients ( $p < 0.05$ ). In postmenopausal patients with RA, DPYD excretion was higher in RF (+) patients than in RF (-) patients ( $p < 0.05$ ). These results are shown in Table 2.

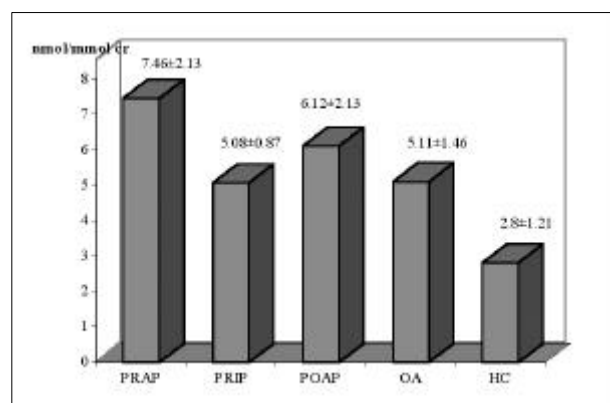
In active premenopausal RA patients, no correlation was found between DPYD excretion and disease duration, functional capacity, ESR, CRP and RAI, although PYD excretion was found to be correlated with RAI, ESR, CRP and functional capacity ( $r=0.5729$   $p < 0.01$ ,  $r=0.5953$   $p < 0.01$ ,  $r=0.6125$   $p < 0.01$  and  $r=0.6232$ ,  $p < 0.01$  respectively).

**Table 1.** The Demographic and Descriptive Details of All RA Patients

	Premenopausal active patients	Postmenopausal active patients
Age (years)	$36.0 \pm 7.6$	$60.0 \pm 6.8$
Disease duration (years)	$2.2 \pm 1.3$	$8.41 \pm 4.12$
ESR (mm/hour)	$38.5 \pm 12.3$	$42.3 \pm 7.6$
CRP (0-5gr/Lt)	$12.4 \pm 4.6$	$16.2 \pm 5.9$
HQA (0-3 scale)	$1.4 \pm 0.9$	$1.7 \pm 1.2$
RAI (0-72 scale)	$15.6 \pm 11.3$	$18.4 \pm 10.4$



**Fig. 1.** The mean values of urine PYD between the groups. PRAP: Premenopausal active patients with RA. PRIP: Premenopausal inactive patients with RA. POAP: Postmenopausal active patients with RA. OA: Patients with osteoarthritis. HC: Healthy controls.



**Fig. 2.** The mean values of urine DPYD between the groups. PRAP: Premenopausal active patients with RA. PRIP: Premenopausal inactive patients with RA. POAP: Postmenopausal active patients with RA. OA: Patients with osteoarthritis. HC: Healthy controls.

**Table 2.** Urine PYD and DPYD Excretions in RF(+) and RF (-) Patients with RA

Patients	n	PYD	DPYD
		nmol/mmol cr	nmol/mmol cr
RF (+) Premenopausal active RA	23	18.4 ± 2.51	9.33 ± 1.55
RF (-) Premenopausal active RA	17	11.2 ± 4.33	5.44 ± 3.78
RF (+) Premenopausal inactive RA	23	10.2 ± 1.55	6.22 ± 1.2
RF (-) Premenopausal inactive RA	17	6.32 ± 6.82	4.02 ± 0.48
RF (+) Postmenopausal active RA	10	22.47 ± 5.22	7.64 ± 1.47
RF (-) Postmenopausal active RA	10	17.06 ± 2.42	4.11 ± 1.88

In postmenopausal active RA patients, correlations were found between PYD excretion and disease duration, functional capacity, CRP and ESR ( $r=0.5697$   $p<0.01$ ,  $r=0.4917$   $p<0.05$ ,  $r=0.6745$   $p<0.01$  and  $r=0.6281$   $p<0.05$  respectively). No correlation were found for urine DPYD excretion and disease duration, functional capacity, ESR, CRP and RAI ( $p>0.05$  for all).

## DISCUSSION

In RA, although the exact cause of the osteopenic process has not yet been identified, many studies have suggested that bone loss results from a multifactorial processes (involving, for example, cytokine secretion, disease duration and severity, corticosteroid, physical activity, and the menopause). The cellular basis of bone loss has not been established; though the majority of kinetic and biochemical studies have indicated the bone formation is decreased,<sup>5,11,12</sup> another reported evidence for increased bone turnover.<sup>13</sup>

Biochemical markers of bone resorption and formation may be helpful in identifying bone disease by allowing the estimation of the rate of bone loss and the response to treatment. In untreated RA patients, bone loss is predominantly juxta articular in the early stages of the disease.<sup>14</sup> Moreover, total bone loss is known to correlates significantly with disease duration and parameters of disease activity.<sup>15</sup> Both pyridinium crosslinks are present in bone collagen, and these components have been studied widely in the assesment of bone turnover in osteoporosis.<sup>16</sup> Results suggest

that the urinary pyridinium crosslinks, PYD and DPYD, appear to provide specific markers of bone resorption,<sup>17,18</sup> and information on stage activity and the efficacy of drug therapy in arthritic disease.<sup>1,19</sup> Therefore urine pyridinium crosslinks excretion has been studied in patients with RA and OA,<sup>1,17,20,21</sup> and preliminary studies show that urinary PYD is elevated in patients with RA and osteoporosis.<sup>1,17,21,22</sup> A number of studies have shown that urine PYD and DPYD excretion are high<sup>1,19-24</sup> in RA. A further study also showed elevated crosslink production in RA and OA, but failed to demonstrate any effect of NSAIDs on the production of crosslinks.<sup>25</sup> In RA patients, indices of disease activity were reported to be correlated with a fall in PYD levels following treatment with second line drugs.<sup>1,26</sup> We also found urine PYD levels significantly high in premenopausal patients with active RA than during the inactive period. Low urine PYD levels reflects reduced of destruction of bone and vascular connective tissues. On the other hand, positive correlations were found between activation criterias (i.e., CRP, ESR, functional capacity and RAI) and urine PYD levels. Furumitsu et al reported similar results,<sup>27</sup> and suggested that increased PYD in RA serum may originate primarily from affected joints. Garner et al<sup>28,29</sup> found that high levels of PYD is associated with increased risk of progression of joint destruction over 1 year in early RA and OA.

In an another study, it was reported that urinary PYD and DPYD were not elevated in OA.<sup>30</sup> Takahashi et al<sup>31</sup> reported that bone and joint disorders did not affect the PYD content in articular cartilage. In an another study, no signifi-

cant difference between the excretion of PYD and DPYD was found in patients with ankylosing spondylitis and healthy controls.<sup>32</sup> In our study, urine PYD level was significantly higher in patients with OA than in the healthy controls, although no difference was found between OA patients and premenopausal patients with inactive RA. This data shows that RA patients have significantly higher urine PYD levels than healthy controls even during the inactive period. The earliest manifestation in RA is juxta articular osteoporosis which can occur within weeks of disease onset and is a characteristic feature of the disease,<sup>14,15</sup> and this situation may continue in inactive period.

In our study, no difference was found between active and inactive premenopausal RA patients in terms of urine DPYD levels. Although a significantly difference was found between the patient groups and the healthy controls, we obtained similar results in the patient groups. Clair et al<sup>33</sup> compared levels of PYD, DPYD and Type I collagen peptides in patients with RA and in controls and analyzed their day to day variabilities. They found increased collagen degradation in RA patients, and that PYD was high compared with the controls, although no significant increase in DPYD levels was evident. They also found no significant correlation between DPYD and PYD levels and disease activation criterias. Also, in our study, urine DPYD levels did not correlate with any of the disease activation criterias. Choy et al reported that PYD and DPYD were decreased by roughly 25% after treatment with DMARD's.<sup>34</sup>

In our study, PYD excretion was significantly higher in RF(+) patients than in RF(-) patients. This is due to serious bone destruction and aggressive disease progression, in patients with RF(+). It was also reported that DPYD is elevated in patients taking steroids demonstrating the deteriorous effects of steroids on bone resorption, even as low doses.<sup>23</sup> The effect of long-term steroid use upon increasing pyridinium crosslink excretion was apparent, but it appears that short-term intraarticular steroid injection may improve collagen degradation in OA.<sup>33,35</sup> The role of low corticosteroid doses (<10 mg/day) upon bone loss in RA patients is controversial.<sup>36</sup> Laan et al reported

that long-term low dosage prednisone therapy caused osteoporosis in postmenopausal patients with RA.<sup>37</sup> In our study, we chosed patients who had received less than 4 mg/day prednisolone, so that the possible effects of prednisolone on PYD and DPYD excretion were minimized.

Urine PYD and DPYD excretion values are generally determined by HPLC, but we chosed ELISA. The ELISA, determination of free pyridinolines is less sensitive than pyridinium crosslinks measurement by high performance liquid chromatography,<sup>3</sup> and our results could have been effected by our method choice.

We found that due to the induction of bone resorption, urine PYD excretion was significantly elevated in patients with active RA. During sulphasalazine treatment, urine PYD levels were found to decrease, but urine DPYD levels did not change. This is attributed to improvement in bone destruction.

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