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## EXTENDED REPORTS

## Purine enzyme activities in recent onset rheumatoid arthritis: are there differences between patients and healthy controls?

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### Abstract

**Objective**—Purine enzyme activities may predict the effectiveness of azathioprine treatment and be associated with increased deaths from infectious diseases. In rheumatoid arthritis, patients show variable responses to azathioprine and a higher percentage of death is caused by infections. The aim of the study was to investigate possible rheumatoid arthritis associated abnormalities of purine enzyme activities by measuring several of these enzymes in patients with recent onset rheumatoid arthritis before treatment with disease modifying antirheumatic drugs or prednisone.

**Methods**—23 patients with recent onset rheumatoid arthritis and 28 healthy controls were studied. Activities of the enzymes 5'-nucleotidase, purine nucleoside phosphorylase (PNP), hypoxanthine guanine phosphoribosyltransferase (HGPRT), and thiopurine methyltransferase (TPMT) were measured. Assessment of disease activity and blood sampling for routine measurements and HLA typing were done simultaneously.

**Results**—Purine enzyme activities did not differ between patients and healthy controls. Enzyme activities had no significant relations with indices of disease activity or rheumatoid factor titre or with the rheumatoid arthritis associated HLA types. Activity of 5'-nucleotidase decreased with age ( $P \leq 0.05$ ) and was lower by about 27% ( $P = 0.007$ ) in males than in females.

**Conclusions**—In rheumatoid arthritis patients, neither the variability in azathioprine effectiveness nor the increased death rate from infections can be explained by pre-existing abnormalities in the activities of the purine enzymes 5'-nucleotidase, PNP, HGPRT, or TPMT at an early stage of the disease, before disease modifying antirheumatic drugs or prednisone treatment. Besides adjustment for age, results of studies involving purine 5'-nucleotidase activity should also be adjusted for sex.

Azathioprine is used in the treatment of immunological diseases and to prevent graft rejection in transplant recipients. After ingestion the drug is rapidly converted to 6-mercaptopurine (6-MP), a drug which is used in the treatment of leukaemia. 6-MP is a substrate in three metabolic pathways<sup>1</sup>: catabolism to 6-thiouric acid by xanthine oxidase, methylation to methylmercaptopurine metabolites by thiopurine methyltransferase (TPMT), and conversion by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) to 6-thioinosine-monophosphate which consequently results in formation of cytotoxic 6-thioguanine nucleotides<sup>2</sup> (figure)

Rheumatoid arthritis patients treated with azathioprine show variable responses ranging from successful reduction of disease activity to occurrence of side effects like leucopenia, pancytopenia, gastrointestinal complaints, hepatotoxicity, or hypersensitivity.<sup>1-9</sup>

Nowadays four different enzyme disturbances are known to be related to the effect of azathioprine treatment. Patients with the Lesch-Nyhan syndrome (inborn HGPRT deficiency) are resistant to azathioprine<sup>10</sup> and persons with TPMT deficiency<sup>11-13</sup> or low activity of purine 5'-nucleotidase<sup>11-13</sup> are prone to develop bone marrow toxicity. Finally the simultaneous use of azathioprine and allopurinol, a competitive inhibitor of xanthine oxidase, increases toxicity of azathioprine so that the dosage of azathioprine has to be reduced to approximately 25%.<sup>1</sup>

Rheumatoid arthritis may be connected to purine metabolism not only by the use of azathioprine but also by the disease itself since it involves at very least a dysregulation of the immune system, expressed by autoimmunity and changes in lymphocyte subsets.<sup>14</sup> In rheumatoid arthritis, there is probably also a deficiency of the immune system, since it has been established that death due to infectious diseases occurs more often in rheumatoid arthritis patients than in the general population.<sup>15,17</sup> Early death due to infection is a well known consequence of a number of immune deficiency syndromes, which in turn are related to purine enzyme deficiencies, for example T lymphocyte malfunction due to

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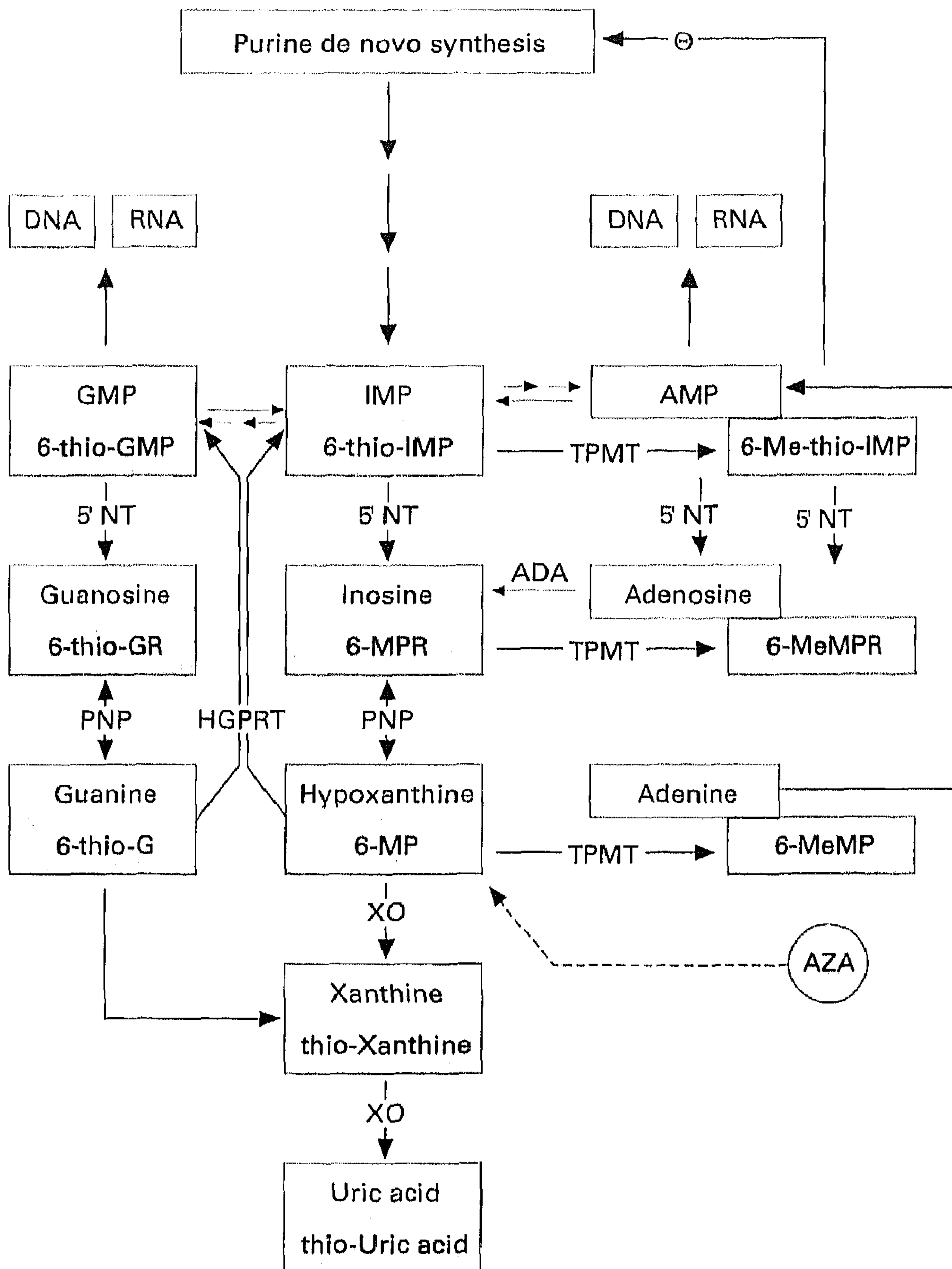
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Simplified scheme of purine and azathioprine (AZA) (6-mercaptapurine) metabolism. (Thio)purines: GMP, guanosine monophosphate; IMP, inosine monophosphate; AMP, adenosine monophosphate; AZA, azathioprine; MP, mercaptopurine; MPR, mercaptopurineribonucleoside; GR, guanosine ribonucleoside; G, guanine; Me stands for methyl. (Thio)purine enzymes: 5'NT, 5'-nucleotidase; PNP, purine nucleoside phosphorylase; ADA, adenosine deaminase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; XO, xanthine oxidase; TPMT, thiopurine methyltransferase.

deficiency of purine nucleoside phosphorylase (PNP)<sup>18, 19</sup> and (adult onset) primary hypogammaglobulinaemia and combined immunodeficiency due to deficiency of 5'-nucleotidase.<sup>20, 21</sup>

Despite the fact that there are no previous data showing that subtle changes in enzyme levels have an impact on azathioprine effectiveness or susceptibility to infections, the above mentioned observations still raise the question of whether the purine enzymatic make up in rheumatoid arthritis patients is also different. In addition, it would be very useful if purine enzyme activities could give some prediction about the outcome of azathioprine treatment.

As far as we know there are only two reports addressing differences between rheumatoid arthritis patients and healthy controls, one of which is a recent pilot study from our own group, showing differences in purine enzyme activities of 5'-nucleotidase and PNP,<sup>22</sup> the other showing differences in 5'-nucleotidase and adenosine deaminase activity.<sup>23</sup> The difference between these studies and the one presented here is that the first studies were

done in rather heterogeneous groups of patients. The influence of different disease modifying antirheumatic drugs, prednisone, other medications, duration of the disease, age, and sex could not be excluded.

To detect possible differences present at the beginning of the disease (rheumatoid arthritis associated) and to obviate several confounding variables from the two previous reports, we have studied a homogeneous group of rheumatoid arthritis patients within the first year of diagnosis and before treatment with a disease modifying antirheumatic drug or prednisone.

The study protocol was approved by the ethics committee of the hospital.

## Methods

### PATIENTS AND CONTROLS

In the period from February 1993 to April 1994, 23 rheumatoid arthritis patients and 28 healthy controls entered the study. They were all Caucasian. Patients had to meet the following criteria: the 1987 ARA criteria,<sup>24</sup> disease duration less than one year and no previous treatment with disease modifying antirheumatic drugs or prednisone. The use of non-steroidal antiinflammatory drugs (NSAID) or paracetamol was allowed. Patients with other coexisting diseases were excluded, as were persons using allopurinol.

To avoid influences of possible circadian rhythms, clinical assessments and blood sampling were done between 9 and 11 am for all participants.

### CLINICAL ASSESSMENTS

Indices of disease activity included patients assessments of pain and general health (GH) on visual analogue scales (VAS) of 10 cm (0 = best possible, 10 = worst possible), the Ritchie articular index (RAI), the number of swollen joints, and the degree of morning stiffness. The disease activity score (DAS<sub>d</sub>)<sup>25</sup> was calculated by using the RAI, the number of swollen joints, the VAS-GH, and the erythrocyte sedimentation rate (ESR).

### LABORATORY ASSESSMENTS

Venous blood samples for determination of purine enzyme activities, IgM-RF (ELISA, positive if > 10 IU ml<sup>-1</sup>), HLA types, and routine blood tests (patients only) were collected immediately after the clinical assessments. Routine blood analyses included haematological and biochemical profiles, serum uric acid, the ESR (Westergren, mm in 1 h), and C-reactive protein (mg ml<sup>-1</sup>). Tissue typing of all patients for HLA class I and II (DR/DQ) was performed by standard techniques.

To determine purine enzyme activities of 5'-nucleotidase, PNP, and HGPRT, blood was collected in 10 ml Vacutainer tubes containing polystyrene granules (Becton and Dickinson). Mononuclear cells were obtained by Ficoll-Isopaque (density 1.077 g ml<sup>-1</sup>, Nycomed) gradient centrifugation of defibrinated blood and in these cells the enzyme activities were measured by a new high performance liquid chromatography method.<sup>26</sup> Enzyme activities

are expressed in nmol  $10^{-6}$  mononuclear cells  $h^{-1}$  of incubation. To measure the TPMT activity in red cell lysate as described by Weinshilboum *et al.*,<sup>27</sup> peripheral blood was collected in 10 ml Monoject tubes containing 150 USP units of lithium heparin (Sherwood Medical). TPMT activity is expressed in pmol  $10^{-6}$  erythrocytes  $h^{-1}$  of incubation.

All enzyme assays were carried out in quadruplicate.

#### STATISTICAL ANALYSIS

Pearson's correlation analyses were used to study relations between purine enzyme activities and indices of disease activity, between indices of disease activity and age, and between enzyme activities and rheumatoid factor. Since the distributions of 5'-nucleotidase (patients and controls) and HGPRT (patients) were not quite normal, Spearman's rank correlation analyses were also performed. Two sample Student *t* tests were used to compare enzyme activities in HLA positive and HLA negative patients (and additionally Mann-Whitney tests with respect to 5'-nucleotidase and HGPRT). Influence of age on enzyme activities was analysed by linear regression analysis. Differences between patients and healthy controls and between the two sexes with respect to enzyme activities (and additionally with respect to the square root of 5'-nucleotidase) were examined by a two way analysis of covariance adjusting for age.

The statistics were processed by SAS computer software (SAS Institute, Cary, NC, USA) and by NCSS computer software (version 5.1, 1988, Dr J L Hintze, North Kayville, Utah, USA). Test results were considered to be significant at  $P \leq 0.05$ .

## Results

### STUDY POPULATION

Characteristics of patients and healthy controls are given in table 1. All patients had normal laboratory values for kidney and liver function and a normal serum urate. Only minor abnormalities were found in haematological variables (low haemoglobin concentration, raised platelet count) and were considered to be attributable to activity of the disease (data not shown).

RELATIONS BETWEEN ENZYME ACTIVITY, DISEASE ACTIVITY, RHEUMATOID FACTOR, AND HLA TYPE  
Pearson's coefficients of correlation for the relations between enzyme activities and the indices of disease activity (including the DAS<sub>4</sub>) are presented in table 2. They varied from

Table 3 Linear regression analysis results for examining the influence of age on purine enzyme activity. Estimates show the yearly change in enzyme activity

|          | Early RA patients |      |       |      | Healthy controls |      |       |      |
|----------|-------------------|------|-------|------|------------------|------|-------|------|
|          | 5'NT              | PNP  | HGPRT | TPMT | 5'NT             | PNP  | HGPRT | TPMT |
| Estimate | -0.21             | 1.1  | 0.06  | 0.02 | -0.37            | 0.7  | 0.08  | 0.08 |
| SE       | 0.10              | 0.8  | 0.05  | 0.06 | 0.13             | 1.0  | 0.13  | 0.09 |
| P value  | 0.05              | 0.22 | 0.26  | 0.74 | 0.009            | 0.51 | 0.55  | 0.36 |

RA, rheumatoid arthritis. Other abbreviations, see legend to table 2.

SE, standard error of the estimate; enzyme activities of 5'NT, PNP and HGPRT are expressed in nmol  $10^{-6}$  cells  $h^{-1}$  of incubation; TPMT activity in pmol  $10^{-6}$  cells  $h^{-1}$  of incubation.

Table 1 Characteristics of patients and controls. Values are mean (SD)

|                                    | RA patients (n=23)        | Controls (n=28)           |
|------------------------------------|---------------------------|---------------------------|
| Age in years [range]               | 54.0 (14.0)<br>[23 to 73] | 43.6 (10.4)<br>[26 to 63] |
| Male/female (n)                    | 13/10                     | 11/17                     |
| Disease duration in months [range] | 4.3 (3.5)<br>[1 to 12]    |                           |
| RF positive patients (n)           | 20                        |                           |
| Disease activity indices           |                           |                           |
| ESR (Westergren)                   | 31.2 (22.4)               |                           |
| Ritchie articular index            | 11.8 (6.7)                |                           |
| No of swollen joints               | 16.3 (9.2)                |                           |
| VAS general health                 | 42.0 (23.5)               |                           |
| DAS <sub>4</sub>                   | 4.1 (1.3)                 |                           |
| HLA type:                          |                           |                           |
| positive/negative (n)              |                           |                           |
| DR1                                | 5/15                      |                           |
| DR2                                | 2/18                      |                           |
| DR3                                | 6/14                      |                           |
| DR4                                | 10/9                      |                           |

RA, rheumatoid arthritis; RF, rheumatoid factor; ESR, erythrocyte sedimentation rate; VAS, visual analogue scale; DAS<sub>4</sub>, disease activity score; HLA, human lymphocyte antigen.

-0.34 to 0.46. None of these correlation coefficients reached significance except the one between 5'-nucleotidase activity and ESR. However, checking the latter correlation by a Spearman analysis, which in this case is probably better in view of the not quite normal distribution, no significance was found. Furthermore, no relevant correlations were found between indices of disease activity and age. A relation between the rheumatoid factor titre and the activity of the tested enzymes could not be demonstrated (data not shown).

Enzyme activities in HLA positive and HLA negative patients were examined for HLA-DR1, HLA-DR2, HLA-DR3, and HLA-DR4. There were no differences (data not shown).

### ENZYME ACTIVITY—INFLUENCE OF AGE

In rheumatoid arthritis patients as well as in healthy controls only 5'-nucleotidase activity showed a significant decrease with increase of age ( $P = 0.05$  and  $P = 0.009$  respectively) (table 3). The estimates of the yearly decrease ranges from 0.21 nmol  $10^{-6}$  cells  $h^{-1}$  in rheumatoid arthritis patients to 0.37 nmol  $10^{-6}$  cells  $h^{-1}$  in the healthy controls.

Table 2 Coefficients of correlation according to Pearson (and Spearman) between indices of disease activity and purine enzyme activity and between indices of disease activity and age in rheumatoid arthritis patients

|        | RAI              | Swollen jts No   | ESR             | VAS-GH           | DAS <sub>4</sub> |
|--------|------------------|------------------|-----------------|------------------|------------------|
| 5'NT   | -0.02<br>(-0.12) | 0.25<br>(0.15)   | 0.46<br>(0.37)* | 0.26<br>(0.19)   | 0.22<br>(0.10)   |
| PNP    | 0.25             | -0.05            | -0.29           | -0.13            | 0.05             |
| HGPRT* | -0.19<br>(-0.10) | -0.22<br>(-0.21) | -0.02<br>(0.07) | -0.001<br>(0.09) | -0.16<br>(-0.01) |
| TPMT   | -0.33            | -0.34            | 0.20            | -0.10            | -0.27            |
| Age    | 0.02             | -0.05            | -0.04           | 0.23             | 0.01             |

RAI, Ritchie articular index; ESR, erythrocyte sedimentation rate; DAS<sub>4</sub>, disease activity score; Swollen jts No, number of swollen joints; VAS-GH, visual analogue scale general health; 5'NT, 5'-nucleotidase; PNP, purine nucleoside phosphorylase; HGPRT, hypoxanthine guanine phosphoribosyltransferase; TPMT, thiopurine methyltransferase.

\* Critical value (n=23) for Pearson (and Spearman): 0.41 (0.42); \* n=22.

ENZYME ACTIVITY—INFLUENCES OF GROUP, SEX, AND AGE

Results of the two way analysis of covariance examining the influences of group, sex, and age on enzyme activities are shown in the tables 4 and 5. No significant differences between rheumatoid arthritis patients and controls, averaged over both sexes, could be established. Interaction tests showed that mean patient-control differences were not clearly different in the two sexes.

Males showed a significant lower ( $P < 0.007$ ) mean 5'-nucleotidase activity than females (averaged over both groups:  $\pm 4$  activity units or 27% lower activity). Mean male 5'-nucleotidase activity was 80% and 68% of the female activity respectively for the rheumatoid arthritis group and the control group.

A significant ( $P = 0.005$ ) decrease of 5'-nucleotidase activity with age was again found. This decrease amounted about 0.23 activity units per year. Activities of PNP and HGPRT showed a tendency to increase with age ( $P = 0.10$  and  $P = 0.07$ , respectively) about 1.1 and 0.09 activity units per year, respectively. (For 5'-nucleotidase activity, similar results were found after a square root transformation.)

### Discussion

Our previous pilot study indicates that activities of some purine enzymes in rheumatoid arthritis differ from those in healthy controls and are associated with the outcome of azathioprine treatment, whereas there is no clear relation between enzyme activity and disease activity.<sup>22</sup> However, the group of patients in that study is heterogeneous and the influence of disease modifying antirheumatic drugs, prednisone, other medications, disease duration, age, and sex cannot be excluded. It was to determine if the differences in enzyme activities between rheumatoid arthritis and controls are related to the disease itself, the drugs used, or other confounding variables that we started the present study. In contrast to the other studies,<sup>22-23</sup> the enzyme activities are measured solely in recent onset rheumatoid arthritis patients not previously treated with a disease modifying antirheumatic drug or prednisone.

One of the pitfalls may be that enzyme activity reflects disease activity.<sup>28</sup> Our tests are reas-

Table 4 Adjusted least squares means (with standard errors) for enzyme activities, classified by patient-control status and sex on the basis of a two way analysis of covariance model at a mean age of 48.3 years

|       |          | Male       | Female     |
|-------|----------|------------|------------|
| 5'NT  | RA       | 15.6 (1.9) | 19.6 (2.0) |
|       | Controls | 13.7 (1.9) | 20.4 (1.6) |
| PNP   | RA       | 199 (16)   | 219 (17)   |
|       | Controls | 201 (16)   | 223 (14)   |
| HGPRT | RA       | 9.0 (1.1)  | 12.0 (1.1) |
|       | Controls | 12.3 (1.6) | 12.2 (1.1) |
| TPMT  | RA       | 22.3 (1.3) | 24.9 (1.3) |
|       | Controls | 23.0 (1.3) | 21.5 (1.1) |

RA, rheumatoid arthritis. Other abbreviations, see legend to table 2.

The male/female numbers for patients and controls are 13/10 and 11/17, respectively, except for the HGPRT activity with ratios of 12/10 and 5/11.

Table 5 Testing results ( $P$  values) from two way analyses of covariance examining the influence of group and sex on purine enzyme activities with age as covariable

|                       | 5'NT  | PNP  | HGPRT | TPMT |
|-----------------------|-------|------|-------|------|
| Group                 | 0.78  | 0.86 | 0.34  | 0.31 |
| Sex                   | 0.007 | 0.18 | 0.13  | 0.67 |
| Age                   | 0.005 | 0.10 | 0.07  | 0.31 |
| Group/sex interaction | 0.48  | 0.94 | 0.11  | 0.10 |

Abbreviations, see legend to table 2.

suring on this issue. Most correlation coefficients show little or no relation between enzyme activities and indices of disease activity.

In accordance with a previous study,<sup>29</sup> 5'-nucleotidase activity is significantly age dependent, showing decreasing values with increasing age. The degree of change is comparable for patients and controls. A combined analysis with adjustment for sex does not change this finding. It may be that age is also a factor influencing PNP and HGPRT activities. Although the change was not significant, both enzyme activities tended to increase with age.

Activity of 5'-nucleotidase is also subject to a substantial influence of sex and as far as we know this has not been reported before. A combined analysis of patients and controls with adjustment for age shows a 27% lower 5'-nucleotidase activity in males than in females. The underlying mechanism is not known but the difference in hormonal status may possibly play a role.

Susceptibility, prognosis, and clinical course of rheumatoid arthritis are related to the rheumatoid factor status and some HLA types.<sup>30-32</sup> We could not show a clear correlation between enzyme activities and rheumatoid factor titre, nor did we find differences in enzyme activities between positive and negative groups of the HLA types DR1, DR2, DR3, and DR4.

In contrast to previous reports,<sup>22-23</sup> we did not find any difference in purine enzyme activities between patients with early rheumatoid arthritis and controls. Confounding variables like age, sex, duration of the disease, and medication (disease modifying antirheumatic drugs, prednisone, others) are avoided by means of the study design and statistical analysis. All of our patients used NSAID and a few used paracetamol. Only two patients used co-medication by inhalation for chronic obstructive pulmonary disease. Their enzyme activities were well within the ranges of the other patients. This means that the only important differences between patients and controls are the use of NSAID and the presence of rheumatoid arthritis. To our knowledge, nothing is known about the influence of NSAID on 5'-nucleotidase, PNP, or HGPRT activities. More is known about NSAID and red blood cell TPMT activity. One report mentions a gender difference<sup>33</sup> while others do not.<sup>34-35</sup> The same report<sup>33</sup> shows that chronic disease in general is associated with somewhat higher TPMT levels and that NSAID are associated with lower levels of TPMT. Although a gender difference was not

found in our study, the possibility that opposite influences of NSAID and chronic disease mask a difference with healthy controls cannot be entirely excluded. However, based on our results we conclude, with some reservation about TPMT activity, that the variability in response to azathioprine treatment and the increased death rate from infectious diseases cannot be explained by pre-existing (rheumatoid arthritis associated) differences in the enzyme activities tested.

The previous reports about rheumatoid arthritis patients, as we mentioned above, were based on patient groups characterised by heterogeneity in disease duration and medication. In addition, the enzyme activities were determined by different enzyme assays, the type of 5'-nucleotidase investigated differed (membrane bound 5'-nucleotidase<sup>21</sup> versus total 5'-nucleotidase (ectoplasmic and cytoplasmic 5'-nucleotidase),<sup>22</sup> and in neither report were enzyme activities adjusted for age and sex. For these reasons a comparison of previous data and the data presented in this study is hardly possible. Furthermore, it is known that activities of some purine enzymes are not evenly distributed among the several mononuclear cells or cell types<sup>36-37</sup> or different stages of maturation,<sup>38</sup> and that with age percentile changes occur in lymphocyte subsets and maturity.<sup>39</sup> It is conceivable that age, disease duration, or disease modifying antirheumatic drug use may cause a shift in the subtype or maturity of peripheral blood mononuclear cells, and that different disease modifying antirheumatic drugs may have different induction effects on enzyme activities. Further analyses of these assumptions are needed and some are under investigation now.

In summary, no differences were found in purine enzyme activities of 5'-nucleotidase, PNP, HGPRT, or TPMT between patients with early rheumatoid arthritis before disease modifying antirheumatic drug or prednisone treatment and healthy controls. The tested enzyme activities have no associations with disease activity, nor with the rheumatoid factor titre, nor with the rheumatoid arthritis associated HLA types. Our study confirms the significant decrease of 5'-nucleotidase activity with age. It also shows a tendency, although not significant, towards increasing activities of PNP and HGPRT with age. A new finding is that 5'-nucleotidase activity in males is about 27% lower than in females.

In future studies, enzyme activity of 5'-nucleotidase should be adjusted both for age and sex, and studies on activities of PNP and HGPRT should be evaluated for possible influences of age.

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