Inter-Individual Variability of Serum Xanthine Oxidase Activity in Patients with Inflammatory Bowel Disease

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Submitted
Abstract

Background Thiopurines play an essential role in the management of inflammatory bowel diseases (IBD, i.e. Crohn’s disease and ulcerative colitis). Over the past decade, several strategies to optimize treatment with thiopurines have been evaluated. One of these therapy optimization strategies is the co-administration of allopurinol, a xanthine-oxidase (XO) inhibitor, to low-dose thiopurine therapy. In this study, we aimed to assess the inter-individual variability of XO-activity between IBD-patients.

Methods We assessed xanthine oxidase activity in serum of IBD-patients of two medical centers in The Netherlands using the Amplex® Red Xanthine/Xanthine Oxidase Assay Kit.

Results We observed a high inter-individual variability of XO-activity in 119 patients, with a median activity of 16 µU/ml/hour (range 1 - 85 µU/ml/hour). The XO-activity was influenced by gender (male 19.5 vs. female 14.0 µU/ml/hour, P < 0.01), patient’s age (Pearson’s correlation r = 0.21, P = 0.02) and duration of IBD (r = 0.23, P = 0.01). The activity of XO was not affected by the type of IBD, smoking status, body mass index or (type of) thiopurine use (P > 0.05).

Conclusions With this study, we describe the inter-individual variability of XO-activity in IBD-patients and show that XO-activity is positively associated with male gender and patient’s age.
**Introduction**

Inflammatory bowel disease (IBD) is the collective term of ulcerative colitis (UC) and Crohn’s disease (CD). Thiopurines, especially the conventional derivatives azathioprine (AZA) and mercaptopurine (MP) have been widely accepted as first-line immunosuppressive therapy and have proven to be effective in maintaining steroid-free remission. [1, 2] Azathioprine is a pro-drug, which needs to be converted into MP and is subsequently activated by an enzymatic pathway, the so-called purine salvage pathway, to the pharmacologically active metabolites 6-thioguaninenucleotides (6-TGN). Xanthine oxidase (XO) degrades MP into 6-thiouric acid (6-TUA) while thiopurine S-methyltransferase (TPMT) can convert MP to 6-methylmercaptopurine (6-MMP). [3] (Fig. 1)

Approximately 40% of the IBD patients are able to maintain remission during therapy with conventional thiopurine derivatives. [4, 5] One mechanism possibly contributing to ineffectiveness of thiopurine therapy might be increased XO activity, thus decreasing the amount of MP available for biotransformation into effective 6-TGN metabolites. [6, 7]

Over recent years, several strategies to optimize individual thiopurine therapy have been suggested. Measurement of thiopurine metabolites and subsequent co-administration of allopurinol to patients with a skewed metabolism has proven its clinical value. [6, 8-10] Allopurinol acts as an XO inhibitor, therefore a possible significant role for XO activity in effectiveness of thiopurine therapy is assumed. [11] Little is known concerning the range of XO variability in IBD patients. [10, 12, 13]

The primary objective of this study was to determine the inter-individual variability of XO activity in IBD patients. The secondary aim was to determine which variables influenced XO activity.
Methods

Patient selection
In this retrospective cross-sectional study, we identified IBD patients from a database consisting of all IBD-patients from one academic (VU University Medical Center, Amsterdam, The Netherlands) and one district referral hospital (St. Anna Hospital, Geldrop, The Netherlands). Diagnosis of IBD was ascertained by standard clinical, radiological, histological and endoscopic criteria. [14] The patient characteristics extracted from the clinical patient charts were gender, age, type, localization, classification (according to the Montreal guidelines) and duration of IBD, current and historical medication status, surgical history and smoking status. [14] Duration of IBD was calculated from the date of diagnosis to the date of blood withdrawal.

Analysis of samples
Xanthine oxidase activity was determined in serum using the Amplex® Red Xanthine/Xanthine Oxidase Assay Kit (A22182, Invitrogen, Carlsbad, CA, USA). [15] Briefly, xanthine oxidase catalyzes the oxidation of hypoxanthine to uric acid and superoxide. Superoxide subsequently degrades spontaneously to hydrogen peroxide (H₂O₂) and in the presence of horseradish peroxide this reacts with the Amplex® Red reagent to produce a red-fluorescent oxidation product, resofurine. [16] The lower limit of detection of this kit was 1 µU/ml/hour, the lower limit of quantification was 2 µU/ml/hour.

Venous blood samples were collected in 4mL tubes from all patients. After collecting serum, this was stored at -80°C. Prior to analysis, the samples were thawed to 4°C overnight. In each cycle, 36 serum samples were analyzed. Patients were matched according to gender, age at blood withdrawal and diagnosis, and homogeneity between the tests was ensured. The XO activity was expressed in µU/ml/hour, using the (T45-blank)-(T15-blank) as reference points on the standard curve obtained from the kit. [15]
**Statistical analysis**
Continuous variables were expressed as median with range or mean with standard deviation, according to distribution. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, IBM, Armonk, New York, USA). A \( P \)-value less than 0.05 was considered significant. To determine differences between 2 groups the Mann-Whitney-U test or the Chi square method was used, according to distribution. Correlations were assessed using the Spearman correlation coefficient. To determine possible confounding variables linear regression was applied.

**Results**

**Patient characteristics**
In total, 144 patients were included, of which 91 (63%) patients were treated in an academic hospital and 53 (37%) in a referral hospital. The male to female ratio was 9:10, with a median age of 39 years (range 19 - 86) at the time of blood withdrawal. Crohn’s disease and UC were diagnosed in 77 (54%) and 67 (46%) patients, respectively. Fifty-eight patients (49%) used thiopurine therapy at the time of blood withdrawal, of which the majority used AZA (94%). The median duration of IBD was 7 years (range 0 - 35). Results were obtained in the serum of 119 patients. The remaining 25 patients were excluded due to errors in assay execution. Patient characteristics of the included patients were summarized in Table 1.

**Xanthine oxidase activity**
Xanthine oxidase activity ranged from 1 to 85 µU/ml/hour, with a median activity of 16 µU/ml/hour. Median xanthine oxidase activity was higher in men than in women (19.5 vs. 14.0 µU/ml/hour, \( P < 0.01 \), Fig. 2) and there was a positive correlation with patient’s age \( (r = 0.21, P = 0.02) \) and the duration of the disease \( (r = 0.23, P = 0.01) \). The activity of XO was not affected by the type of IBD, Montreal classification, type of hospital (academic/non-academic), smoking status, body mass index, prior IBD-related surgery or (type of) thiopurine use \( (P > 0.05) \).
**Table 1** Demographic characteristics of included patients.

<table>
<thead>
<tr>
<th></th>
<th>Total (n=119)</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>60</td>
<td>(50)</td>
</tr>
<tr>
<td>Female</td>
<td>59</td>
<td>(50)</td>
</tr>
<tr>
<td><strong>Diagnosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn’s disease (CD)</td>
<td>62</td>
<td>(52)</td>
</tr>
<tr>
<td>Ulcerative colitis (UC)</td>
<td>57</td>
<td>(48)</td>
</tr>
<tr>
<td><strong>Montreal classification of UC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1-E2-E3</td>
<td>11-18-28</td>
<td>(19-32-49)</td>
</tr>
<tr>
<td><strong>Montreal classification of CD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1-B2-B3</td>
<td>30-20-12</td>
<td>(48-32-20)</td>
</tr>
<tr>
<td>L1-L2-L3</td>
<td>13-23-26</td>
<td>(21-37-42)</td>
</tr>
<tr>
<td><strong>Prior IBD-related surgery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>36</td>
<td>(30)</td>
</tr>
<tr>
<td>No</td>
<td>83</td>
<td>(70)</td>
</tr>
<tr>
<td><strong>Thiopurine use at time of blood withdrawal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>58</td>
<td>(49)</td>
</tr>
<tr>
<td>No</td>
<td>61</td>
<td>(51)</td>
</tr>
<tr>
<td><strong>Median age at blood withdrawal (range)</strong></td>
<td>40 (19-86) years</td>
<td></td>
</tr>
<tr>
<td><strong>Median duration of IBD at blood withdrawal (range)</strong></td>
<td>7 (0-35) years</td>
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</table>

**Discussion**

In this retrospective cross-sectional study amongst 119 IBD patients, we observed a wide inter-individual variety of XO activity. Our results are consistent with previous studies who found a 4 - 10 fold inter-individual variation of XO activity in healthy, unrelated subjects. [6, 17, 18] Xanthine oxidase activity in our cohort ranged from 1 - 85 µU/ml/hour with a median of 16 µU/ml/hour. This wide spread in activity might be due to allelic variants in the gene coding for the XO enzyme. [19, 20] In this study, we aimed to identify variables which might affect XO activity.
Inter-individual variability of serum xanthine oxidase activity in patients with inflammatory bowel disease

First, we observed a direct significant relationship to gender. Previously, conflicting results have been published regarding the relationship between gender and XO activity. In some studies, no difference in XO activity between men and women were observed, whereas in other studies a higher XO activity was observed in either women or men. [12, 13, 17, 18, 21] These discrepancies might be explained by the applied method to determine XO activity. In these studies, the urinary caffeine 1U/(1X+1U) metabolic ratio was measured and considered to represent XO activity, which was calculated from this ratio, without considering other enzymes that might metabolize caffeine. [22] In our study, XO activity was measured directly in serum, using hypoxanthine as a substrate.
Second, we found a correlation between XO activity and age \((r = 0.21)\), which was in line with an earlier observation. [23] This outcome was supported by previous data correlating age with the level of uric acid, which is the end-product of the reaction catalyzed by XO. [24] When evaluating the thiopurine metabolism, a higher XO activity causes shunting towards the XO pathway (hyperoxidators), thereby decreasing the bioavailability of MP for transformation into 6-TGN. [6] Consequently, thiopurines might be less effective at an older age, which was also concluded in a recent systematic review. [25] To our knowledge, no data are currently available monitoring XO activity prospectively with age and correlating this with thiopurine metabolite levels.

Third, duration of IBD correlated positively with XO activity. However, this relationship was not confirmed when stratification for age was applied \((P = 0.13)\). In case IBD turns out to be an independent factor to influence XO activity, the underlying mechanism could be upregulation of xanthine oxidase activity in (active) inflammatory bowel disease, a phenomenon earlier described in patients with celiac disease. [26] Further prospective trials are necessary to support this theory. In this study, unfortunately, no data were available regarding the activity of disease at the moment of blood withdrawal.

Whereas this study is the first to determine XO activity in a large cohort of IBD patients, there are some limitations which need to be taken into account. First, data regarding concomitant therapy were missing in this study. Second, the activity index of IBD was not known at the time of blood withdrawal, making it difficult to draw any conclusions about the relation of XO activity and IBD activity status. Third, we did not measure hepatic XO activity in our study. Since the liver is the major store of this enzyme, we cannot draw firm conclusions of total XO activity in our patients. However, since no data are available describing the correlation between serum and hepatic XO activity and previous literature showed similar correlations for XO activity and gender, we believe this does not impact the quality of these data. [18]
Conclusion
We demonstrated a wide inter-individual variability in XO activity amongst 119 IBD patients. Further, we identified that male gender and higher age contributed to higher XO activity.
References


