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OBSERVATIONAL RESEARCH

Serum uric acid increases in patients with systemic autoimmune rheumatic diseases after 3 months of treatment with TNF inhibitors

Lenka Hasikova[1,2] · Marketa Pavlikova[3] · Hana Hulejova[1] · Petr Kozlik[4] · Kveta Kalikova[5] · Aparna Mahajan[6] · Martin Herrmann[6] · Blanka Stiburkova[1,7] · Jakub Zavada[1,2]

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Abstract

In patients with gout, the serum uric acid (SUA) is usually lower during acute gouty attacks than during intercritical periods. It has been suggested that systemic inflammatory response can cause this phenomenon. The objective is to determine whether therapy with TNF inhibitors (TNFis) affects SUA levels in patients with systemic autoimmune rheumatic diseases (SARDs) and whether SUA changes correlate with pro-inflammatory cytokines or with the oxidative stress marker allantoin. In this study, SUA, CRP, creatinine, MCP-1, IFN-α2, IFN-γ, Il-1β, IL-6, IL-8, IL-10, IL-12, IL-17a, IL-18, IL-23, IL-33, TNF-α, and allantoin levels were measured prior to and after 3 months of TNFis treatment in patients with SARDs. The values obtained in the biochemical assays were then tested for associations with the patients' demographic and disease-related data. A total of 128 patients (rheumatoid arthritis, $n=44$; ankylosing spondylitis, $n=45$; psoriatic arthritis, $n=23$; and adults with juvenile idiopathic arthritis, $n=16$) participated in this study. Among the entire patient population, SUA levels significantly increased 3 months after starting treatment with TNFis (279.5 [84.0] vs. 299.0 [102.0] μmol/l, $p<0.0001$), while the levels of CRP, IL-6, IL-8, and MCP-1 significantly decreased. Male sex was the most powerful baseline predictor of ΔSUA in univariate and multivariate models. None of the measured laboratory-based parameters had statistically significant effects on the magnitude of ΔSUA. 3 months of anti-TNF therapy increased the levels of SUA in patients with SARDs, but neither the measured pro-inflammatory cytokines nor the oxidation to allantoin appeared responsible for this effect.

Keywords Uric acid · Inflammation · Rheumatic diseases · Cytokines · Oxidative stress

Introduction

Patients with gout tend to have lower SUA levels during an acute gout attack than during the intercritical period (i.e. the time between acute gout attacks) [1–3], but the mechanisms responsible for this phenomenon remain unclear. A drop in SUA levels was also observed during systemic inflammatory response (SIR) provoked by orthopedic surgery, suggesting that lowering in SUA concentrations is associated with SIR unrelated to gout [4]. Decreased SUA levels were also reported upon administration of human recombinant IL-6 to patients with refractory thrombocytopenia [5] implicating its causative effect. One study suggested that the decrease in SUA observed during an acute gout attack may be mediated by SIR-induced increase in urinary uric acid (UUA) excretion [6], while other study has failed to confirm this [4]. Circulating SUA might also be consumed in reactions with the free radicals generated during the SIR. Uric acid is the predominant antioxidant in the extracellular environment

✉ Jakub Zavada
zavada@revma.cz

[1] Institute of Rheumatology, Na Slupi 4, 128 50 Prague 2, Czech Republic

[2] Department of Rheumatology, First Faculty of Medicine, Charles University, Prague, Czech Republic

[3] Department of Probability and Mathematical Statistics, Faculty of Mathematics and Physics, Charles University, Prague, Czech Republic

[4] Department of Analytical Chemistry, Faculty of Science, Charles University, Prague, Czech Republic

[5] Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Prague, Czech Republic

[6] Department of Internal Medicine 3, Rheumatology and Immunology, Friedrich-Alexander University of Erlangen-Nürnberg, Erlangen, Germany

[7] Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic

[7–9] scavenging free radicals and thus preventing oxidative damage [10]. Despite the lack of uricase activity in humans [11], uric acid can be non-enzymatically oxidized into allantoin and other products by reactive oxygen species (ROS) [12]. During episodes of systemic inflammation, where oxidative stress is involved, levels of allantoin may rise as a consequence of more rapid oxidation of urate to allantoin. Allantoin has been used as a biomarker for monitoring oxidative status both in vitro and in vivo [13–16]. In this context, significantly increased levels of allantoin have been found in patients with active rheumatoid arthritis (RA) [16] and with gout [17] compared to healthy controls.

While in the setting of acute SIR, the levels of SUA seem to be inversely correlated with markers of inflammation, in a population-based study, SUA concentrations were shown to be positively correlated with IL-6, C-reactive protein (CRP), and TNF-α [18, 19]. Uric acid released by injured cells has been shown to be a damage-associated molecular pattern (DAMP) able to activate the inflammasome [20, 21] and to stimulate the production of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, in human mononuclear cells [22, 23]. Interestingly, SUA levels were also found to be positively correlated with multiple pro-inflammatory cytokines released during severe exacerbations of malaria, and have been implicated in its pathogenesis [24]. This evidence suggests that uric acid could exert bidirectional effects in systemic inflammatory conditions such as RA. Of clinical importance, both chronic inflammation and hyperuricemia have been independently associated with increased cardiovascular risk not only in the general population or gout, but also in patients with systemic autoimmune rheumatic diseases [25, 26].

The aim of the present study was to investigate the effect of TNF inhibition on SUA levels in patients with systemic autoimmune rheumatic diseases (SARDs) including RA, ankylosing spondylitis (AS), psoriatic arthritis (PsA), and juvenile idiopathic arthritis (JIA). In addition, we assessed whether the ΔSUA concentration was associated with the levels of the most important pro-inflammatory cytokines concentrations as well as the oxidative stress marker allantoin.

Methods

Subjects

In this study, we used clinical data and frozen serum and plasma samples collected at baseline and first follow-up (3 months) from a prospective cohort of 128 patients with clinically and serologically highly active SARDs (44 with RA, 45 with AS, 23 with PsA, and 16 with JIA) starting therapy with TNFis (cohort 1). The patients were treated between 2008 and 2017 at the Institute of Rheumatology, Prague and their clinical data were captured in the database of the Czech biologic's registry ATTRA. The ATTRA registry is a national prospective cohort study with mandatory registration for all patients with RA, AS, PsA, and JIA who start treatment with biologic agents. The ATTRA study was approved by the Czech Multicentre Research Ethics Committee (no. 201611 S300) and all of the subjects provided their written consent for the collection and storage of biological samples and clinical data prior to participation. All procedures were performed in accordance with the Declaration of Helsinki.

In the Czech Republic, treatment with TNFis is covered in cases of RA (and adult patients with JIA and polyarticular involvement) when the DAS28 exceeds 5.1 despite the availability of conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs), for PsA, if the disease is not adequately controlled with csDMARDs and, for AS, if the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) exceeds 4 and the CRP/ESR are elevated above typical healthy levels. Selected patients met the classification criteria. For AS, we have used New York criteria, for PsA either Moll and Wright criteria, or CASPAR criteria (either set was applicable). The diagnosis of RA and JIA was made by their treating physician, who was expected to apply the current ACR/ACR-EULAR or ILAR criteria, respectively. At baseline, these patients had highly active diseases (i.e. fulfilled the eligibility criteria for TNFis therapy) and also had CRP levels above 10 mg/l. Patient information including demographic parameters, disease activity, concomitant medications, and comorbidities was collected from the clinical questionnaire responses in addition to specific disease activity at 3 months post-clinical intervention.

In addition, to analyze the relationships between ΔSUA, UUA excretion, and the other parameters, urine spot samples were obtained from ten patients with SARDs (eight with RA, two with PsA) initiating TNFis between 2016 through 2017 (cohort 2). These patients were also selected using the criteria described above. This study was approved by the Institutional Ethics Committee of Institute of Rheumatology, Prague, Czech Republic (no. 10113/2016).

Laboratory analyses

In every case, sera and plasma were separated within 60 min of being drawn from the patients and then immediately aliquoted and frozen at − 80 °C until further biochemical analysis. All of the analytes were measured in serum collected before and after 3 months of TNFis treatment except for allantoin, which was analyzed in plasma.

The concentrations of serum CRP, creatinine, and SUA and UUA and urine creatinine were determined using the Beckman Coulter AU system with Beckman Coulter CRP

Latex, Creatinine (Enzymatic), and Uric Acid kits (Beckman Coulter, Brea, CA, USA). The CRP Latex Kit was calibrated for high sensitivity, according to the manufacturer's instructions.

We employed flow cytometry bead-based immunoassays (LEGENDplex™ Human Inflammation Panel, BioLegend, San Diego, CA, USA) to quantify the concentrations of pro-inflammatory cytokines (MCP-1, IFN-α2, IFN-γ, Il-1β, IL-6, IL-8, IL-10, IL-12p70, IL-17a, IL-18, IL-23, IL-33, and TNFα). Data were analyzed using Kaluza Analysis Software (Beckman Coulter).

To estimate UUA excretion (only in cohort 2), fractional excretion of uric acid (FeUA) was determined using the equation: (UUA/SUA) × (serum creatinine/urinary creatinine) × 100. The results are given in percentages.

Plasma allantoin levels were assessed using the Agilent Infinity 1290 system coupled with the Triple Quad 6460 tandem mass spectrometer (Agilent Technologies, Waldbronn, Germany). An ACQUITY UPLC BEH Amide Column (100 mm × 2.1 mm, 1.7 µm particle size) (Waters, Milford, MA, USA), thermostated at 30 °C, was used for the analysis. The mobile phases consisted of 0.1% formic acid in deionized water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The flow rate of the mobile phase was maintained at 0.4 ml/min. The optimized gradient program [(time (min))/volume% B] was 0/95, 1/95, 3/50, 4/20, 5/95, and 10/95. The injection volume was 2 µl, and the samples were kept at 5 °C. The tandem mass spectrometry measurement was performed in multiple reaction-monitoring mode using positive electrospray ionization. The precursor and product ions were *m/z* 159 and *m/z* 116, respectively (collision energy 5 V and fragmentor voltage 60%). The ion source was set as follows: gas temperature: 350 °C, gas flow: 10 l/min, nebulizer pressure: 310 kPa, and capillary voltage: 4000 V. For sample preparation, 300 µl of 100% acetonitrile was added to 100 µl of plasma, shaken (vortex), and centrifuged (10 min/14,000 rpm). 100 µl of supernatant was transferred into chromatographic vials. For the statistical analyses, the ratio between the values at baseline and those after 3 months of therapy with TNFis were used.

Statistical analyses

Data are summarized as means with standard deviation (SD) or as medians with interquartile range [IQR]. Medians are mostly used for laboratory measurements, whose distribution was skewed to the left. Baseline characteristics between diagnostic groups were compared using ANOVA and the Kruskal–Wallis test for continuous variables and Fisher's exact test for categorical ones. Changes in measurements from baseline and at 3 months after initiating TNFis treatment were evaluated using the Wilcoxon signed-rank test. For the exploratory analysis of changes in various interleukin concentrations, the *p* values from their respective Wilcoxon tests were corrected for multiple comparisons using the Benjamini–Hochberg method.

To explore the relationship between ΔSUA and various demographic and laboratory-based characteristics, first univariate and then stepwise multiple linear regression models were constructed to look for association with the patients' baseline characteristics (sex, diagnosis, age, BMI, and weight) and the significantly changing laboratory-based parameters. Laboratory-based predictors required log transformation for the sake of the model fit. The level of statistical significance was set to 0.05. All analyses were performed in the statistical language and environment R, version 3.5.0.

Results

Study population

We studied 128 patients (57 females, 45%) with a mean age of 43.9 (SD 14.5, range 18–81) years, who had started TNFis therapy. Their baseline characteristics are presented in Table 1. Diagnostic groups significantly differed in their proportion of females (patients with AS and PsA—lower percentage of females, patients with JIA and RA—lower percentage of males), in their age (patients with JIA and AS are younger than those with PsA and RA), slightly in their weight (biggest difference—patients with PsA weighed more than those with JIA), in their glucocorticoids usage (highest among patients with RA), and in their NSAIDs usage (highest among patients with AS, JIA, and PsA).

ΔSUA

As shown in Table 2, the levels of SUA in our patients were significantly lower before treatment with TNFis (279.5 [84.0] µmol/l) than after 3 months of treatment (299.0 [102.0] µmol/l, $p < 0.0001$) with a median difference of 19.0 [56.6] µmol/l.

Other laboratory-based parameters

Several laboratory-based parameters, namely, CRP (31.2 [26.7] vs. 2.3 [4.2] mg/l, $p < 0.0001$), IL-6 (62.0 [143.5] vs. 13.3 [24.7] pg/ml, $p < 0.0001$), IL-8 (82.9 [101.0] vs. 57.2 [62.5] pg/ml, $p = 0.0117$), and MCP-1 (962.6 [528.5] vs. 878.7 [457.2] pg/ml, $p = 0.0016$) were significantly higher at baseline than 3 months later. The concentrations of the remaining cytokines (IFN-α2, IFN-γ, Il-1β, IL-10, IL-12p70, IL-17a, IL-18, IL-23, IL-33, and TNFα) and of allantoin did not significantly differ as a result of TNFis treatment. Relative to baseline, serum creatinine levels

Table 1 Baseline characteristics for the cohort 1 ($n = 128$) and for the four diagnostic groups

	n	Total 128	RA 44	AS 45	PsA 23	JIA 16	*p* value
Female	*n* (%)	57 (44%)	33 (75%)	7 (16%)	6 (26%)	11 (69%)	< 0.001*
Age (years)	Mean (SD)	43.9 (14.5)	56.4 (10.4)	35.4 (9.8)	46.7 (12.1)	30.0 (8.5)	< 0.001
Weight (kg)	Mean (SD)	74.2 (15.1)	73.6 (15.4)	73.5 (16.2)	81.3 (11.4)	67.7 (12.8)	0.0436
BMI (kg/m^2) ($N = 80$)[a]	Mean (SD)	26.4 (4.8)	26.6 (5.4)	26.5 (4.7)	26.4 (4.5)	25.3 (4.0)	0.9936
CRP (mg/l)	Median [IQR]	31.2 [26.7]	22.5 [22.4]	40.6 [23.9]	23.4 [21.6]	44.8 [22.5]	< 0.001**
DAS28-CRP ($n = 82$)[b]	Mean (SD)	5.79 (1.02)	5.99 (0.86)		5.17 (1.17)	6.14 (0.82)	0.003
BASDAI[b] ($n = 40$)	Mean (SD)	n.a.		6.22 (2.13)			n.a.
Concomitant glucocorticoids	*n* (%)		33 (75.0%)	6 (13.3%)	6 (26.1%)	8 (50.0%)	< 0.001*
Concomitant NSAIDs	*n* (%)		11 (25.0%)	30 (66.7%)	12 (52.2%)	10 (62.5%)	< 0.001*
Concomitant COX-2 inhibitors	*n* (%)		2 (4.5%)	2 (4.4%)	0 (0%)	1 (6.3%)	0.738*
TNFis	Etanercept, *n* (%)	23 (18.0%)	15 (34.1%)	5 (11.1%)	1 (4.3%)	2 (12.5%)	0.038*
	Adalimumab, *n* (%)	42 (32.8%)	13 (29.5%)	17 (37.8%)	9 (39.1%)	3 (18.8%)	
	Infliximab, *n* (%)	43 (33.6%)	10 (22.7%)	19 (42.2%)	6 (26.1%)	8 (50%)	
	Golimumab, *n* (%)	18 (14.0%)	6 (13.6%)	4 (8.9%)	5 (21.7%)	3 (18.8%)	
	Certolizumab pegol, *n* (%)	2 (1.6%)	0 (0%)	0 (0%)	2 (8.7%)	0 (0%)	

Unless stated otherwise, Fisher's exact test in a one-factor ANOVA was used to determine group differences

n.a. not applicable

**p* value from Fisher's exact test. For the TNFis, the *p* value was simulated without certolizumab pegol

***p* value from Kruskal–Wallis ANOVA

[a]For 48 (38%) patients the data on weight and hence BMI were missing, most for AS (21.47%), and JIA (9.56%) patients

[b]DAS28-CRP is not measured for AS patients, BASDAI is measured for AS patients only

increased significantly after 3 months of TNFis treatment (68.0 µmol/l [21.0] vs. 73.0 [19.0] mg/l, $p < 0.0001$) (Table 2).

Relationship between ΔSUA and patient parameters

The regression analysis showed that the ΔSUA was more pronounced in males (model estimate 32.5 µmol/l [55.5]) than in females (model estimate 6 µmol/l [56.0]) (Fig. 1). No other patients' baseline characteristics (diagnosis, age, BMI, weight) did have an influence on ΔSUA, neither univariately nor in the model together with sex.

Relationship between ΔSUA and laboratory-based parameters

In the univariate and multivariate regression models for predicting ΔSUA, no change over time in the (log-transformed) levels of CRP, pro-inflammatory cytokines, oxidative stress marker—allantoin, nor any change in the reported use of NSAIDs, conferred a statistically significant effect on the magnitude of ΔSUA (Fig. 2). The use of glucocorticoids (in the model together with sex) did not have an influence on ΔSUA.

Relationship between ΔSUA and change of FeUA in cohort 2

In cohort 2 (ten patients total: four males, six females; eight patients with RA, two with PsA), the levels of SUA were significantly lower at baseline than after 3 months of TNFis treatment (267.5 [133.3] vs. (342.5 [147.3] µmol/l, $p = 0.0059$), but on a group level, there was no significant difference in FeUA before and after TNFis treatment (Table 2).

Discussion

To the best of our knowledge, this is the first study comprehensively evaluating the effect of treatment with TNFi on SUA level in patients with SARDs. We have observed an increase in SUA levels in the whole cohort with SARDs after abrogation of systemic inflammation using TNFis. These results correspond with an earlier report from Waldron et al. demonstrating that SUA levels decrease during SIR provoked by orthopedic surgery in patients without gout [4] or after administration of human recombinant IL-6 to patients with refractory thrombocytopenia [5]. Taken together, systemic inflammation and/or certain pro-inflammatory

Table 2 Laboratory parameters at baseline and after 3 months of therapy with TNFis

Analyte	Pre-TNFis treatment, median [IQR] or mean (SD)	Post-3-month TNFis treatment, median [IQR] or mean (SD)	Median difference between pre- and post-treatment values, median [IQR] or mean (SD)	p value*
SUA, μmol/l[a]	279.5 [84.0]	299.0 [102.0]	−19.0 [56.8]	< 0.0001
Serum CRP, mg/l	31.2 [26.7]	2.3 [4.2]	27.0 [26.4]	< 0.0001
Serum creatinine, μmol/l	68.0 [21.0]	73.0 [19.0]	−3.0 [9.0]	< 0.0001
IFN-α2, pg/ml, n = 93	101.4 [222.3]	103.3 [184.8]	5.4 [78.9]	0.3469
IFN-γ, pg/ml, n = 72	83.6 [354.5]	67.7 [208.8]	9.9 [123.7]	0.0830
IL-10, pg/ml, n = 95	5.2 [8.9]	5.1 [6.8]	0.3 [6.5]	0.3469
IL-12p70, pg/ml, n = 98	2.2 [3.9]	2.6 [3.4]	−0.1 [2.6]	0.7129
IL-17a, pg/ml, n = 82	40.1 [55.3]	32.7 [54.7]	−0.2 [31.2]	0.5032
IL-18, pg/ml, n = 95	112.6 [177.8]	112.8 [219.7]	5.5 [108.5]	0.4627
IL-1β, pg/ml, n = 100	31.0 [60.1]	30.6 [47.0]	2.6 [30.5]	0.3469
IL-23, pg/ml, n = 100	53.4 [108.4]	36.4 [79.7]	−0.5 [48.5]	0.4083
IL-33, pg/ml, n = 99	32.5 [101.3]	29.4 [87.1]	0.8 [40.8]	0.3782
IL-6, pg/ml, n = 99	62.0 [143.5]	13.3 [24.7]	45.3 [140.1]	< 0.0001
IL-8, pg/ml, n = 99	82.9 [101.0]	57.2 [62.5]	10.8 [76.6]	0.0117
MCP-1, n = 101	962.6 [528.5]	878.7 [457.2]	119.8 [344.9]	0.0016
TNF-α, pg/ml, n = 95	16.8 [35.8]	18.4 [27.7]	0.7 [18.9]	0.3469
FeUA, n = 10	6.9 [5.4]	6.2 [3.8]	0.03 [4.5]	0.9219
Allantoin, n = 99			−6.0 [255]**	
DAS28-CRP[b], n = 82	5.79 (1.02)	2.74 (1.05)	3.01 (1.15)	< 0.0001
BASDAI[b], n = 40	6.27 (2.07)	2.06 (1.45)	4.05 (2.49)	< 0.0001

*Wilcoxon signed-rank test was used to compare the difference between paired samples. In the exploratory analysis of interleukins changes, p values from their respective Wilcoxon tests were corrected for multiple comparisons using the Benjamini–Hochberg method

**For statistical analyses, the ratio between the values at baseline and values after 3 months of treatment with TNFi were used (1.030 [0.8455])

[a]The reference range of SUA is 208–428 μmol/l for men and 155–357 μmol/l for women. (1 mg/dl SUA = 59.48 μmol/l)

[b]DAS28-CRP is not measured for AS patients, BASDAI is measured for AS patients only

cytokines appear to have an impact on SUA levels in patients with gout as well as with other inflammatory conditions.

The mechanism underlying the decrease in SUA associated with systemic inflammation remains elusive. Urano et al. [6] reported that the SUA percentage upon onset of an acute gout attack correlated with CRP and IL-6 levels, as well as with increased UUA excretion, estimated by the percentage change in FeUA during the attack. In the present study, we analyzed the levels of CRP and 13 pro-inflammatory cytokines in patients with SARDs and did not observe any significant associations between the magnitude of the ΔSUA and the investigated cytokines or CRP levels.

We also measured FeUA in a small cohort of patients, which suggested that FeUA was not largely affected by TNFis treatment in this group. However, the small number of patients (n = 10) precluded us from drawing any firm conclusions on the relationship between ΔFeUA, ΔSUA, and other parameters of interest.

An alternative hypothesis to explain mechanism of SUA decrease during SIR involves the increased production of ROS during inflammation and the resulting degradation of SUA to allantoin [12]. In patients with Wilson's disease, an inherited disorder of copper metabolism characterized by the impairment of copper incorporation into ceruloplasmin and enhancement of oxidative stress, SUA levels were found to be markedly reduced, whereas levels of allantoin were elevated. The degradation of SUA by free radicals was proposed to be responsible for this phenomenon [27]. In the field of rheumatic diseases, both patients with active rheumatoid arthritis [16] and with gout [17] were reported to have significantly increased plasma levels of allantoin compared to healthy controls. We have analyzed allantoin as a stable biomarker of oxidative stress, but we have not found any correlation between the amount of the change of allantoin and the change of SUA or other analyzed parameters.

Interestingly, we observed more pronounced changes in SUA in males than in females. This phenomenon appeared not to be confounded by diagnosis (more males were represented in the AS and PsA groups), BMI or generally higher SUA levels in males. We can speculate that sex hormones may play a role in increased responsibility of SUA levels to

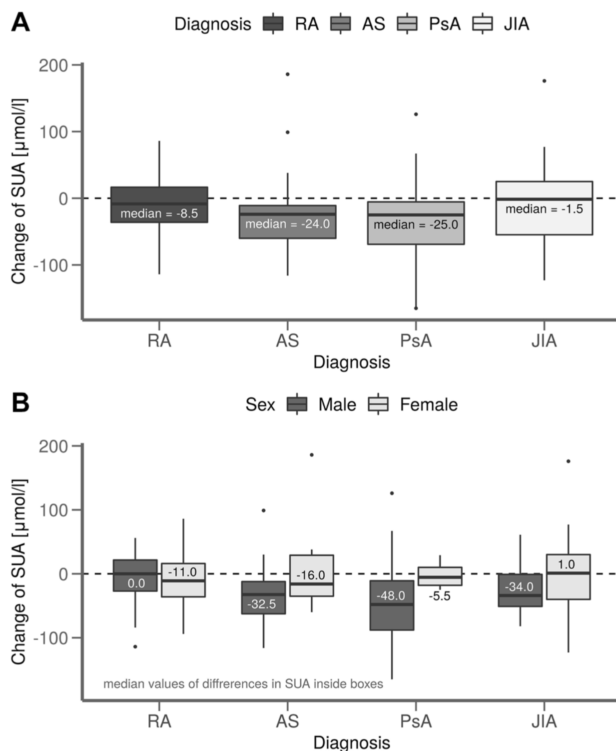

Fig. 1 Change of SUA (ΔSUA) according to sex and diagnosis before (SUA_{M0}) and after 3 months (SUA_{M3}) of therapy with TNFis. **a** ΔSUA by diagnosis. **b** ΔSUA by diagnosis and sex. Note: $\Delta SUA = SUA_{M0} - SUA_{M3}$; hence, a negative ΔSUA represents an increase in SUA level in response to TNFis therapy

SIR in male patients. We have not seen differences in ΔSUA according to age.

We have observed significant decrease of the levels of CRP, IL-6, IL-8, and MCP-1 after treatment with TNFi, which is consistent with the previous studies [28, 29]. TNFα is a crucial cytokine in the pathogenesis of immune-mediated inflammatory diseases including RA, PsA, JIA, AS, or inflammatory bowel diseases. TNFα can induce expression of other pro-inflammatory cytokines such as IL-1β, IL-6, or IL-8 [30]. Promotion of the inflammatory response by TNFα can occur directly by stimulating IL-1 secretion or by inducing other pro-inflammatory cytokines, such as IL-6, which contributes to the production of acute phase reactants [31]. It has been assumed that suppressing TNFα can block the entire inflammatory response [32]. However, in several studies, the cytokine profile after treatment with TNFi was heterogeneous (as in our study) and TNFα levels did not decrease in subjects treated with TNFi, despite clinical improvement [29, 33].

Our study has several limitations. First, we studied patients with different types of rheumatic inflammatory diseases as proxies for patients with gout. The chronic nature and autoimmune pathophysiology shared by these SARDs may differ from the acute flares characteristic of gout, as may the mechanisms underlying the coincident changes in SUA levels. Second, the difference in the use of NSAIDs before and after initiation of TNFis therapy may have confounded our results, particularly because NSAIDs are known to confer uricosuric effects [34]. However, when we analyzed the patient-reported use of NSAIDs, based on their clinical questionnaire responses, we could not detect a relationship between the use of NSAIDs and the ΔSUA, although underreporting still may have played a role. The use of glucocorticoids could also potentially affect SUA level [35, 36]. However, most of our patients had stable dose of glucocorticoids at baseline and after 3 months of anti-TNF treatment. After adjustment for sex, the use of glucocorticoids did not confer a statistically significant effect on the ΔSUA. Third, we did not evaluate the patients' dietary patterns in the present study. We can speculate that food intake decreases, or its composition changes during periods of active disease. Fasting has been shown to induce decreased SUA levels, despite the lack of significant body weight changes [37]. Finally, we did not analyze the intestinal excretion of uric acid, known to account for about one-third of urate excretion [38, 39], due to the invasive nature of the procedures required to collect this sample.

Our study has also several strengths. We studied a large cohort of patients with highly active SARDs starting first-line therapy with a single class of potent anti-inflammatory drugs with a targeted mechanism of action. We maintained a consistent time frame for sample collection, and measured large number of analytes, all of which have been implicated in inflammation-induced SUA fluctuations. In conclusion, we have shown that TNFi-induced abrogation of systemic inflammation in patients with several SARDs was associated with a significant increase in SUA. None of the measured pro-inflammatory cytokines appeared to be the driver of this observed change in SUA, nor the oxidization of SUA to allantoin to be the responsible mechanism. Further studies are needed to clarify the pathophysiology of lowering of SUA levels during an SIR.

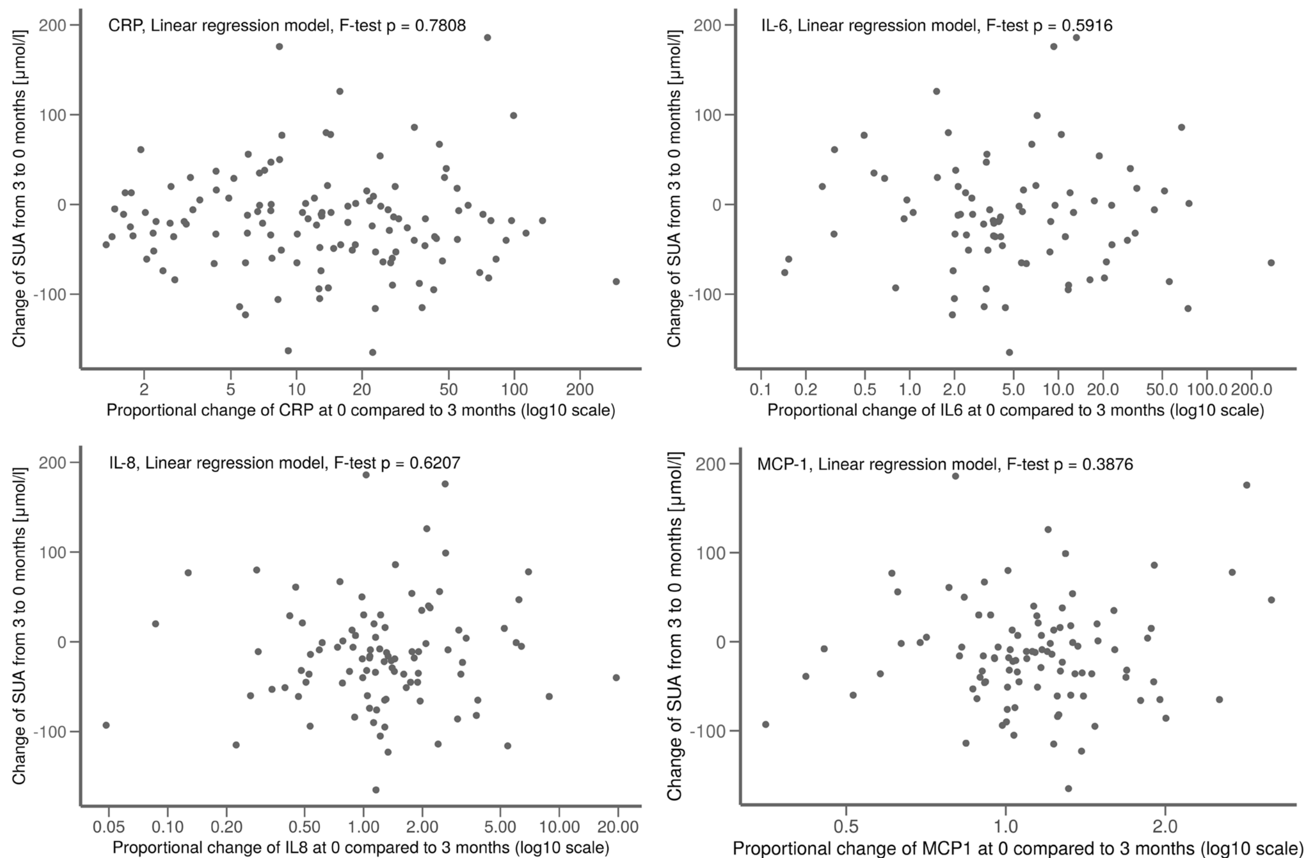

Fig. 2 Scatterplots of ΔSUA on (log-transformed) change in CRP and other selected pro-inflammatory cytokines with fitted regression lines

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Author contributions All authors were involved in drafting the manuscript or revising it critically for content. LH planned and performed most of the measurements, conducted data analysis, and wrote the manuscript. MP conducted statistical data analysis and prepared the figures. HH and AM helped with measurements of cytokines and with analyzing data. PK and KK carried out mass spectrometric measurements. MH and BS provided scientific input and interpretation of data. JZ designed the project, supervised its conduct, and helped to write the manuscript. The final manuscript has been seen and approved by all authors.

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Compliance with ethical standards

Conflict of interest All authors declare that there is no conflict of interest regarding the publication of this article.

Ethical approval All procedures in this study were in accordance with the ethical standards of the institutional and national research committee (Czech Multicentre Research Ethics Committee, no. 201611 S300 and Institutional Ethics Committee of Institute of Rheumatology, Prague, Czech Republic, no. 10113/2016) and with the 1964 Helsinki declaration and its later amendments.

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Functional non-synonymous variants of *ABCG2* and gout risk

Blanka Stiburkova[1,2], Katerina Pavelcova[1,3], Jakub Zavada[1], Lenka Petru[1,3], Pavel Simek[1], Pavel Cepek[1], Marketa Pavlikova[1] Hirotaka Matsuo[4], Tony R. Merriman[5] and Karel Pavelka[1]

Abstract

Objectives. Common dysfunctional variants of ATP binding cassette subfamily G member 2 (Junior blood group) (*ABCG2*), a high-capacity urate transporter gene, that result in decreased urate excretion are major causes of hyperuricemia and gout. In the present study, our objective was to determine the frequency and effect on gout of common and rare non-synonymous and other functional allelic variants in the *ABCG2* gene.

Methods. The main cohort recruited from the Czech Republic consisted of 145 gout patients; 115 normouricaemic controls were used for comparison. We amplified, directly sequenced and analysed 15 *ABCG2* exons. The associations between genetic variants and clinical phenotype were analysed using the *t*-test, Fisher's exact test and a logistic and linear regression approach. Data from a New Zealand Polynesian sample set and the UK Biobank were included for the p.V12M analysis.

Results. In the *ABCG2* gene, 18 intronic (one dysfunctional splicing) and 11 exonic variants were detected: 9 were non-synonymous (2 common, 7 rare including 1 novel), namely p.V12M, p.Q141K, p.R147W, p.T153M, p.F373C, p.T434M, p.S476P, p.D620N and p.K360del. The p.Q141K (rs2231142) variant had a significantly higher minor allele frequency (0.23) in the gout patients compared with the European-origin population (0.09) and was significantly more common among gout patients than among normouricaemic controls (odds ratio = 3.26, $P < 0.0001$). Patients with non-synonymous allelic variants had an earlier onset of gout (42 *vs* 48 years, $P = 0.0143$) and a greater likelihood of a familial history of gout (41% *vs* 27%, odds ratio = 1.96, $P = 0.053$). In a meta-analysis p.V12M exerted a protective effect from gout ($P < 0.0001$).

Conclusion. Genetic variants of *ABCG2*, common and rare, increased the risk of gout. Non-synonymous allelic variants of *ABCG2* had a significant effect on earlier onset of gout and the presence of a familial gout history. *ABCG2* should thus be considered a common and significant risk factor for gout.

Key words: gout, urate transport, ABCG2

CLINICAL SCIENCE

Rheumatology key messages

- Dysfunctional genetic variants of *ABCG2*, common and rare, markedly increased the risk of gout.
- Dysfunctional genetic variants of *ABCG2* associate with an earlier onset of gout.
- *ABCG2* should be considered a common and significant risk factor for gout.

[1]Institute of Rheumatology, [2]Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University, General University Hospital in Prague, [3]Department of Rheumatology, First Faculty of Medicine, Charles University, Prague, Czech Republic, [4]National Defense Medical College, Saitama, Japan and [5]Department of Biochemistry, University of Otago, Dunedin, New Zealand

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Correspondence to: Blanka Stiburkova, Institute of Rheumatology, Na Slupi 4, 128 50 Prague 2, Czech Republic.
E-mail: stiburkova@revma.cz

Introduction

Over the past decade, genome-wide association studies and meta-analyses have revealed over 30 common sequence variants influencing hyperuricaemia or gout, mostly in urate transporters [1]. Recently, novel gout risk loci *HIST1H2BF-HIST1H4E*, *NIPAL1* and *FAM35A* were

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identified [2]. However, detailed knowledge of the degree to which genetic variants predict serum uric acid (SUA) concentrations remains limited.

Uric acid (UA) is the end product of purine metabolism in humans. Transport mechanisms for UA are localized mainly in the proximal tubules of the kidneys, where UA is extensively filtered and reabsorbed with some (~10%) excreted [3]. The intestine can also excrete UA; it is estimated that up to one-third of UA may be excreted into the gut and this fraction may increase in patients with chronic renal failure [4]. SUA concentrations are highly heritable (proportion 0.38–0.63 [5–8]), consistent with a significant genetic component.

Genes that influence the level of SUA via renal UA excretion primarily encode urate transporters such as URAT1 (*SLC22A12*) and GLUT9 (*SLC2A9*). The heritable secretion component of urate homeostasis is principally mediated by the product of the ATP-binding cassette, subfamily G, member 2 (*ABCG2/BCRP*) gene. It is expressed on the plasma membranes of a variety of tissues, including the placenta, pharynx, bladder, brain and kidney, where it mediates the efflux of xenobiotics [9, 10]. Recent studies suggest that ABCG2 also plays an important role in intestinal excretion [11, 12]. Decreased UA excretion caused by ABCG2 dysfunction is a common mechanism of hyperuricaemia. The polymorphism rs2231142, allelic variant p.Q141K, results in a 53% reduction in UA transport with at least 10% of all gout cases in people of European ancestry attributable to this variant [13–16]. Moreover, a significant association between rs2231142 and an increased risk of a poor response to allopurinol has been described [17–19]. The aim of the present study was to determine the effect of common and rare non-synonymous and other functional allelic variants in the *ABCG2* gene in patients with gout, and investigate the relationship between rs2231142 and the response to allopurinol.

Methods

Subjects

The main cohort of 145 subjects with gout was selected from patients of the Institute of Rheumatology, Prague, the Czech Republic. The control group of 115 normouricaemic subjects was selected from the personnel of the Institute of Rheumatology. Gouty arthritis was diagnosed according to the 1977 American Rheumatism Association preliminary criteria [20]. Patients suffering from secondary gout and other purine metabolic disorders associated with pathological concentrations of SUA were excluded. For each patient, a family history of gout, age of disease onset, and details of their gout treatment were recorded.

Excess production of UA associated with purine metabolic disorders was excluded through investigation of purine metabolites. For this reason, two separate measurements were performed: one set of samples was taken while patients were receiving allopurinol/febuxostat treatment, and the second set of samples was taken 72 h after temporary suspension or before initiation of allopurinol/febuxostat treatment.

To specifically study the p.V12M (rs2231137) variant, a New Zealand sample set of Māori and Pacific (Polynesian) ancestry (929 cases and 861 controls [21]) was typed for the surrogate rs4148153 using the Illumina CoreExome platform (Illumina, Inc., San Diego, CA, USA). It was necessary to use the surrogate because rs2231137 was not included on the CoreExome platform and imputation was not possible owing to the lack of a reference haplotype sample set from the Polynesian population. Data for rs2231137 were extracted from the publicly available UK Biobank (2432 cases and 102 989 controls).

The study was conducted in accordance with the Declaration of Helsinki. Before entering the study, each patient signed an informed consent regarding biological sample collection, storage, and genetic testing. All tests were performed in accordance with standards set by the institutional ethics committees, which approved the project in Prague (no.6181/2015) and New Zealand (no. MEC 05/130/10). UK Biobank data were accessed under approval no. 12611.

Clinical and biochemical investigations

Biochemical analytes were measured using a Beckman Coulter AU system (Beckman Coulter, Brea, CA, USA). High performance liquid chromatography determination of hypoxanthine, xanthine and oxypurinol in urine were performed on an Alliance 2695 and a 2998 photodiode array detector (Waters, Milford, MA, USA) as described previously [22].

PCR amplification of *ABCG2* and sequence analysis

Genomic DNA of the Czech data set was extracted from EDTA whole blood using a QIAmp DNA Mini Kit (Qiagen, GmbH., Hilden, Germany). All protein-coding exons (2–16) were amplified using PCR and purified using a PCR DNA Fragments Extraction Kit (Geneaid, New Taipei City, Taiwan). DNA sequencing was performed with a DNA sequencer (Applied Biosystems 3130 Genetic Analyzer; Thermo Fisher Scientific, Waltham, MA, USA). The genotypes of allelic variants in the Czech control cohort were determined using PCR with allele-specific primers. Primer sequences and PCR conditions are available upon request. The reference sequence was defined as version ENST00000237612.7; chromosome 4: 88 090 269–88 158 912 reverse strand (www.ensembl.org). The reference protein sequence was defined as Q9UNQ0 (http://www.uniprot.org/uniprot).

Prediction of the possible impact of finding non-synonymous allelic variants on protein function was determined using PolyPhen, Provean, Mutation Taster, SIFT, Human Splicing Finder and MutPred predictive software.

Statistical analysis

The data were summarized as absolute and relative frequencies, means (S.D.) and/or medians (with interquartile range; IQR), where appropriate. Linear and logistic regression models were used to examine association of allelic

variant with SUA and with gout patient/normouricaemic status, respectively. Comparisons of patient characteristics between different groups of patients, according to presence/absence of allelic variants, were performed using Student's two-sample *t* test, Wilcoxon's test, chi-square test or Fisher's exact test, as appropriate.

To replicate the study of Roberts *et al.* [17] in the Czech cohort, we divided the gout patients based on their response to allopurinol treatment according to Roberts *et al.*'s definition (i.e. good responders were defined as having SUA on treatment $\leqslant$357 μmol/l = 6 mg/dl with an allopurinol dose $\leqslant$300 mg, poor responders as having SUA on treatment >357 μmol/l = 6 mg/dl with allopurinol dose >300 mg). We were, however, unable to verify compliance by measuring the allopurinol metabolite oxypurinol as was done by Roberts *et al.* [17]. Differences in the rs2231142 allele frequency between good and poor responders were tested using Fisher's exact test and a logistic regression model.

The New Zealand Polynesian and UK Biobank p.V121M association analyses were adjusted by age and sex with the Polynesian sample set additionally adjusted by the number of self-reported Polynesian grandparents with gout. Gout was ascertained in the New Zealand sample set by clinical examination and in the UK Biobank by a combination of urate-lowering therapy and self-reporting of doctor-diagnosed gout [20].

All analyses were performed in the statistical language and environment R, version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria) with Rmeta used for the p.V12M meta-analysis. The level of statistical significance was set at 0.05.

Results

Subjects

The main demographic and biochemical characteristics of the subjects are summarized in Table 1. Our cohort consisted of 145 individuals with primary gout. In total 48 patients (33%) had a positive family history of gout; of those, 30 patients had first-degree relatives affected, 8 patients had second-degree relatives affected and 10 patients did not provide information about affected relatives.

Sequencing analysis of *ABCG2*

In the *ABCG2* gene, 18 intronic variants and 11 exonic sequence variants (one not annotated) were detected (Table 2 and supplementary Table S1, available at *Rheumatology* Online). In the case of c.689 + 1 G > A, related to an individual with severe gouty phenotype, two abnormal ABCG2 splicing variants were identified: r.[532_689del] deletion of exon 6, and r.[532_689del; 944_949del] deletion of exon 6 and deletion of the first six base pairs of exon 9. These deletions, as we published previously, lead to a frameshift, a premature stop codon, a mis-localized ABCG2 signal on the plasma membrane and no urate transport activity in HEK293 cells [24].

Of the exonic variants, nine were non-synonymous: p.V12M (rs2231137), p.Q141K (rs2231142), p.R147W (rs372192400), p.T153M (rs753759474), p.F373C (rs752626614), p.T434M (rs769734146), p.S476P (not annotated), p.D620N (rs34783571) and a three base deletion p.K360del (rs750972998). Heterozygous p.V12M was detected in seven individuals. Heterozygous variants p.R147W, p.T153M, p.F373C, p.T434M, p.K360del and p.S476P were detected once, and variant p.D620N was detected twice. *In silico*, all seven allelic variants with unknown and/or having a rare minor allele frequency (i.e. <0.01) were predicted as probably damaging. The p.Q141K variant was present with a significantly higher minor allele frequency (MAF; 0.23, 55 heterozygotes/6 homozygotes) in the Czech cohort of gout patients compared with the European-origin population (MAF = 0.09) and the worldwide population (MAF = 0.12). In total, 71 patients (49.0%) harboured at least one of these nine non-synonymous variants. Of those, 11 harboured two non-synonymous variants: six had two copies of p.Q141K, five had one copy of p.Q141K and one of the other identified non-synonymous variants. There was no patient with three or more copies of identified non-synonymous variants. The identified variants, their genotype distribution, alternative allele frequency in the Czech gout cohort and database source are shown in Table 2 and supplementary Table S1, available at *Rheumatology* Online. Positions of identified non-synonymous allelic variants in the membrane topology model of ABCG2 are showed in Fig. 1.

Association between allelic variants in *ABCG2*, presence of gout and levels of hyperuricaemia

The results from our association analyses are shown in supplementary Table S1, available at *Rheumatology* Online. A univariate association of SUA, measured on allopurinol/febuxostat treatment, with allelic variants showed the minor alleles of rs2231138 and rs2231165 to be potentially positively associated with increased SUA concentration (P = 0.038 and P = 0.022, respectively). Similarly, the minor alleles of rs2231156 and rs2231165 showed a positive association with increased SUA, off-treatment (P = 0.041 and P = 0.043, respectively). These variants showed associations significant at the 0.05 level; however, they were not statistically significant with the Bonferroni correction for multiple comparisons applied. The comparison of the gout group *vs* normouricaemic group showed a strong association between rs2231142 and gout/normouricaemic status: the variant frequency in gout patients was almost triple the frequency in normouricaemic controls (23% *vs* 8%, odds ratio (OR) = 3.26, 95% CI: 1.96, 5.36, P < 0.0001).

Association between allelic variants in *ABCG2* and response to allopurinol

There were 42 (29%) good responders and 9 (6%) poor responders. The rest of the cohort (94 patients, 65%) had either higher SUA with lower doses of allopurinol (50 patients) or lower SUA when treated with higher doses of allopurinol (9 patients); 35 patients had missing data for genotype, dose or UA

Table 1 Main demographic, biochemical and genetic characteristics of the subjects (n = 145)[a]

Characteristic	N (%)	
Sex		
Male	131 (90.3)	
Female	14 (9.7)	
Familial occurrence	48 (33.1)	
First degree	30 (20.7)	
Second degree	8 (5.5)	
No information provided	10 (6.9)	
Allopurinol treatment	116 (80.0)	
Febuxostat treatment	14 (9.7)	
At least one non-synonymous variant[b]	71 (49.0)	
	Mean (s.d.)	Range
Age, years	55.5 (13.5)	14–90
Age of onset, years	44.7 (14.9)	13–84
SUA on treatment, μmol/l(n = 134, M/F: 123/11)	377.0 (98.1)	163–725
SUA off treatment, μmol/l(n = 90, M/F: 79/11)	441.7 (94.3)	245–683
FEUA on treatment(n = 134, M/F: 123/11)	3.75 (1.86)	0.90–11.76
FEUA off treatment(n = 87, M/F: 76/11)	3.92 (1.50)	0.75–11.27
BMI, kg/m^2 (n = 114)	29.5 (4.8)	19.5–43.4
eGFR, ml/min (n = 134)	86.5 (20.9)	22.9–127.9
Plasma oxypurinol, μmol/l(n = 96)	70.5 (48.3)	4.3–270.4
	Median (IQR)	Range
Treatment dose, mg[c](n = 130, M/F: 118/12)	300 (100)	0–900

The differences in characteristic between male and female gout patients were mostly non-significant. [a]For some parameters, there were missing data; in cases where the missing data amounted to 5% or more, the real n is mentioned in parentheses. [b]Exon non-synonymous allelic variant as described in Table 2. [c]Febuxostat dose recomputed so that 40 mg febuxostat = 300 mg allopurinol. eGFR: estimated glomerular filtration rate; F: female; FEUA: excretion fraction of uric acid; M: male; SUA: serum uric acid.

concentration, or received treatment other than allopurinol. Characteristics of good and poor responders are presented in Table 3.

The minor allele frequency of rs2231142 was numerically higher in patients who responded poorly to allopurinol therapy (OR = 1.78, 95% CI: 0.41, 7.75; P = 0.440; see Table 3), but the result was not statistically significant. Adjustment for gender, BMI, estimated glomerular filtration rate and SUA concentration without the use of urate-lowering therapy did not change the results, although large confidence intervals suggest that the sample was too small.

The presence of identified non-synonymous allelic variants was two times higher in patients who responded poorly to allopurinol therapy (6 of 9, 67%) compared with good responders (14 of 42, 33%; OR = 4.00, 95% CI: 0.87, 18.42; P = 0.075). The result was not statistically significant; however, this might change with a larger data set.

Association between allelic variants in *ABCG2* and age of onset of gout

In the Czech cohort, the mean age of gout onset was 44.9 years. Remarkably, we detected a non-synonymous allelic variant in the *ABCG2* gene in seven of eight patients (88%) with very early gout onset (between ages 10 and 20 years). Of those, the p.Q141K variant was present in six patients. In the group with early onset between 21 and 30 years, the non-synonymous allelic variants were detected in 12 of 21 patients (57%). On the other hand, these variants were under-represented when the age of onset was over 61 years (6 of 20, 30%). This shows an apparent shift in proportions of patients with non-synonymous alleles who are over-represented in earlier age of onset categories and under-represented in older age of onset categories $\chi 2$-test for trend in proportions, P = 0.010). The median age of onset among patients with any non-synonymous allelic variant was 42 years, while among patients without non-synonymous allelic variants it was 48 years (Wilcoxon's test P = 0.014). Under a dose–response model, the median age of onset among those with two non-synonymous alleles was 31 years (in a linear regression model: $\beta = -4.9$ meaning a shift of 4.9 to earlier age of onset with each extra copy of a non-synonynous allele; P = 0.013).

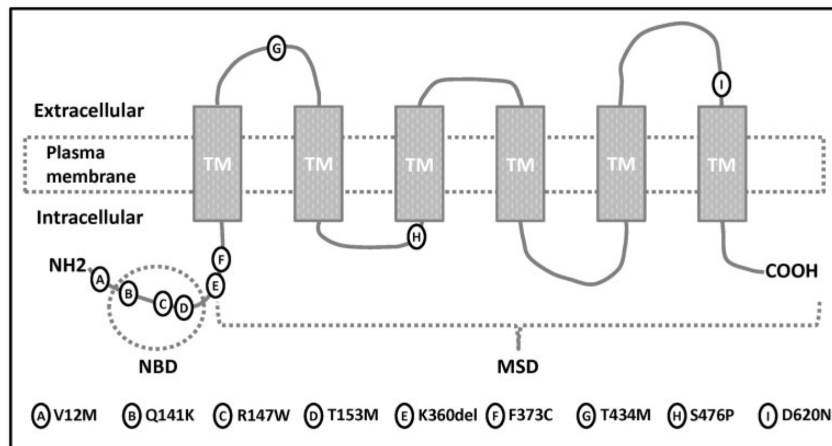
As for family history, patients with non-synonymous variants had familial gout in 29 of 71 cases (40.8%),

Table 2 Identified non-synonymous *ABCG2* allelic variants, genotype distribution and alternative allele frequency in the gout cohort and database in 145 subjects with primary gout

Reference SNP number	Amino acid substitution or deletion	Heterozygotes/ homozygotes	Allele frequency in study subjects	Allele frequency in Caucasian population	*In silico* prediction software					
					PolyPhen	SIFT	PROVEAN	Mutation taster	Human splicing finder	MutPred
rs2231137	p.V12M	7/0	0.02	0.06	Benign (0.003)	Tolerated (1)	Neutral (0.656)	Polymorphism (21)	Potential alteration	0.114
rs2231142	p.Q141K	55/6	0.23	0.09	Benign (0.035)	Tolerated (0.19)	Neutral (−1.588)	Polymorphism (53)	No impact	0.214
rs372192400	p.R147W	1/0	0.003	0.0001	Probably damaging (0.999)	Damaging (0)	Deleterious (−7.146)	Disease causing (101)	Potential alteration	0.804 (deleterious)
rs753759474	p.T153M	1/0	0.003	N/A	Benign (0.268)	Damaging (0.04)	Neutral (−2.415)	Polymorphism (81)	Potential alteration	0.387
rs750972998	p.K360del	1/0	0.003	0.007	N/A	N/A	Neutral (0.9)	Polymorphism (N/A)	Potential alteration	NS
rs752626614	p.F373C	1/0	0.003	N/A	Probably damaging (0.988)	Damaging (0)	Deleterious (−7,828)	Disease causing (205)	No impact	0.627
rs769734146	p.T434M	1/0	0.003	N/A	Probably damaging (0.223)	Tolerated (0.02)	Deleterious (−3.369)	Disease causing (81)	Potential alteration	0.482
N/A	p.S476P	1/0	0.003	N/A	Probably damaging (0.979)	Tolerated (0.06)	Deleterious (−3.16)	Disease causing (N/A)	No impact	0.702
rs34783571	p.D620N	2/0	0.007	0.004	Probably damaging (0.028)	Tolerated (0.07)	Deleterious (−3.331)	Disease causing (23)	No impact	0.158
N/A	r.[532_689del], r.[532_689del; 944_949del]	1/0	0.003	N/A	N/A	N/A	N/A	N/A	N/A	N/A

N/A: not available; SNP: single nucleotide polymorphism.

Fig. 1 Position of identified allelic variants in membrane topology model of ABCG2



while patients without non-synonymous variants had familial gout in 19 of 74 cases (25.7%). This association showed borderline significance (OR = 1.96, 95% CI: 0.97, 3.96; Fisher's test, P = 0.053). The relationship between a family history of gout, onset of gout and allelic variants in *ABCG2* is shown in Fig. 2.

Association of p.V12M with gout: meta-analysis

Our data showed that the p.V12M alternative allele was under-represented in the Czech gout cases (frequency = 0.02) compared with the general European population (frequency = 0.06, P = 0.036). While we did not test for an association with gout, these observations are consistent with previous studies showing the p.12M allele to be protective against gout in Han Chinese and Taiwanese Aboriginal sample sets [24]. Therefore we tested p.V12M for association with gout in a NZ Polynesian sample set (using surrogate rs4148153) and in the UK Biobank cohort, and meta-analysed with the Tu *et al.* [25] and Zhou *et al.* [26] studies (Fig. 3). There was evidence of a p.V12M association with both the Polynesian (OR = 0.76, P = 0.005) and UK Biobank sample sets (OR = 0.81, P = 0.02), with the p.12 M allele conferring a protective effect. A meta-analysis using published data indicates strong evidence that p.12 M has a gout protective effect (OR = 0.73, P < 0.0001).

Discussion

In this study, we identified a total of 29 sequence variants [one intronic splicing variant, one hitherto unpublished allelic non-synonymous variant, six non-synonymous variants with unknown and/or a very rare MAF (i.e. <0.01) and two non-synonymous common variants] in the *ABCG2* gene in a Czech gout cohort. The new findings of this study are the following: identification of novel non-synonymous allelic variant p.S476P; the presence of non-synonymous allelic variants in *ABCG2* was significantly higher in the gout cohort compared with the common population; earlier onset of gout was associated with the presence of non-synonymous allelic variants in the ABCG2 transporter.

ABCG2 is highly variable in the human population. Most of the variants are rare, and only two common non-synonymous allelic variants (MAF ⩾ 0.01), p.V12M and p.Q141K, have been identified. A previous study showed that ABCG2 common dysfunction causes hyperuricaemia by two complementary mechanisms: increased urate levels in the blood caused by reduced urate excretion by the kidneys, and renal urate overload caused by reduced urate excretion by the intestines [27]. The functional characterization and impact of most variants, except for p.Q141K, which accounts for approximately one-half the reduction of UA transport, are currently unknown.

The structural model for ABCG2 focuses on the organization and alignment of residues within six transmembrane spanning domains. No identified variant in our cohort was present in transmembrane segments. The variant p.V12M, localized in the short and flexible N-terminal region, had a lower minor allele frequency (MAF = 0.02) in the Czech cohort of gout patients than in the European-origin population (MAF = 0.06). A significant association between p.V12M and gout has been reported in separate samples from different ethnic groups, for example, Taiwanese Han, Taiwan Aborigines [25] and Chinese Han [26]. These findings were extended in this study by a meta-analysis of diverse population groups. The meta-analysis confirmed the protective effect of the p.12 M allele. This variant is genetically independent of p.Q141K (r^2 = 0.002 in Europeans) and thus it represents an ABCG2 effect additional to that of p.Q141K. Data from the Japanese population also showed a different haplotype for p.V12M and dysfunctional variants p.Q126X and p.Q141K, which supports the independent protective effect of p.V12M [14]. However, tagging experiments have shown that p.V12M has no measurable effect on the processing or function of the ABCG2 protein [28]. In the Czech cohort, the seven carriers of p.V12M allele had a disease onset at a relatively younger age (range 23–69,

TABLE 3 Baseline demographics, frequency of rs2231142 and its association with allopurinol response among patients with gout

Variable	Good responder (n = 42)	Poor responder (n = 9)	P-value[a]
Familial occurrence, n (%)	9 (21.4)	3 (33.3)	1.0000[b]
Age, mean (range), years	61.0 (25-90)	64.3 (50-74)	0.4333
Male, n (%)	36 (85.7)	7 (100)	0.5749[b]
BMI, mean (S.E.), kg/m^2	28.4 (4.5)	33.0 (4.0)	0.0245
eGFR, mean (S.E.), ml/min	87.1 (18.2)	72.0 (12.0)	0.0214
Serum urate, mean (S.E.), μmol/l	300.3 (47.0)	421.8 (76.6)	<0.0001
Allopurinol dose, mean (range), mg/day	233.3 (100–300)	588.9 (400–800)	<0.0001
Plasma oxypurinol, mean (S.E.), μmol/l	76.7 (45.2)	90.4 (29.6)	0.4178
Off serum urate, mean (S.E.), μmol/l	405.3 (64.8)	439.1 (86.4)	0.2654
rs2231142			
GG, N (relative frequency)	29 (0.69)	5 (0.56)	0.4589[b]
GT, N (relative frequency)	13 (0.34)	4 (0.44)	
TT, N (relative frequency)	0 (0.00)	0 (0.00)	
MAF, N (relative frequency)	13 (0.15)	4 (0.22)	0.4589[b]
Non-synonymous allele variants			
None, N (relative frequency)	28 (0.67)	3 (0.33)	0.1289[b]
At least one, N (relative frequency)	14 (0.33)	6 (0.67)	
Association of *ABCG2* SNP rs2231142 with allopurinol response in patients with gout/hyperuricaemia			P-value[c]
Unadjusted OR (95% CI)	1.78 (0.41, 7.75)		0.4395
Adjusted on age, BMI (95% CI)	2.97 (0.35, 25.06)		0.3168
Adjusted on GFR (95% CI)	1.54 (0.32, 7.29)		0.5894
Adjusted on off serum urate (95% CI)	2.86 (0.49, 16.64)		0.2415
Association of any identified non-synonymous variant with allopurinol response in patients with gout/hyperuricaemia			P-value[c]
Unadjusted OR (95% CI)	4.00 (0.87, 18.42)		0.0752

[a]If not stated otherwise, good *vs* poor responders are compared using two-sample *t*-test. [b]Good *vs* poor responders are compared using Fisher's exact test. [c]OR estimates, 95% CIs and P-values come from logistic regression models with poor/good response as dependent variable, presence of *ABCG2* SNP rs2231142 as predictor and different covariates as specified in the table. eGFR: estimated glomerular filtration rate; G: guanine; GFR: glomerular filtration rate; MAF: minor allele frequency; T: thymine.

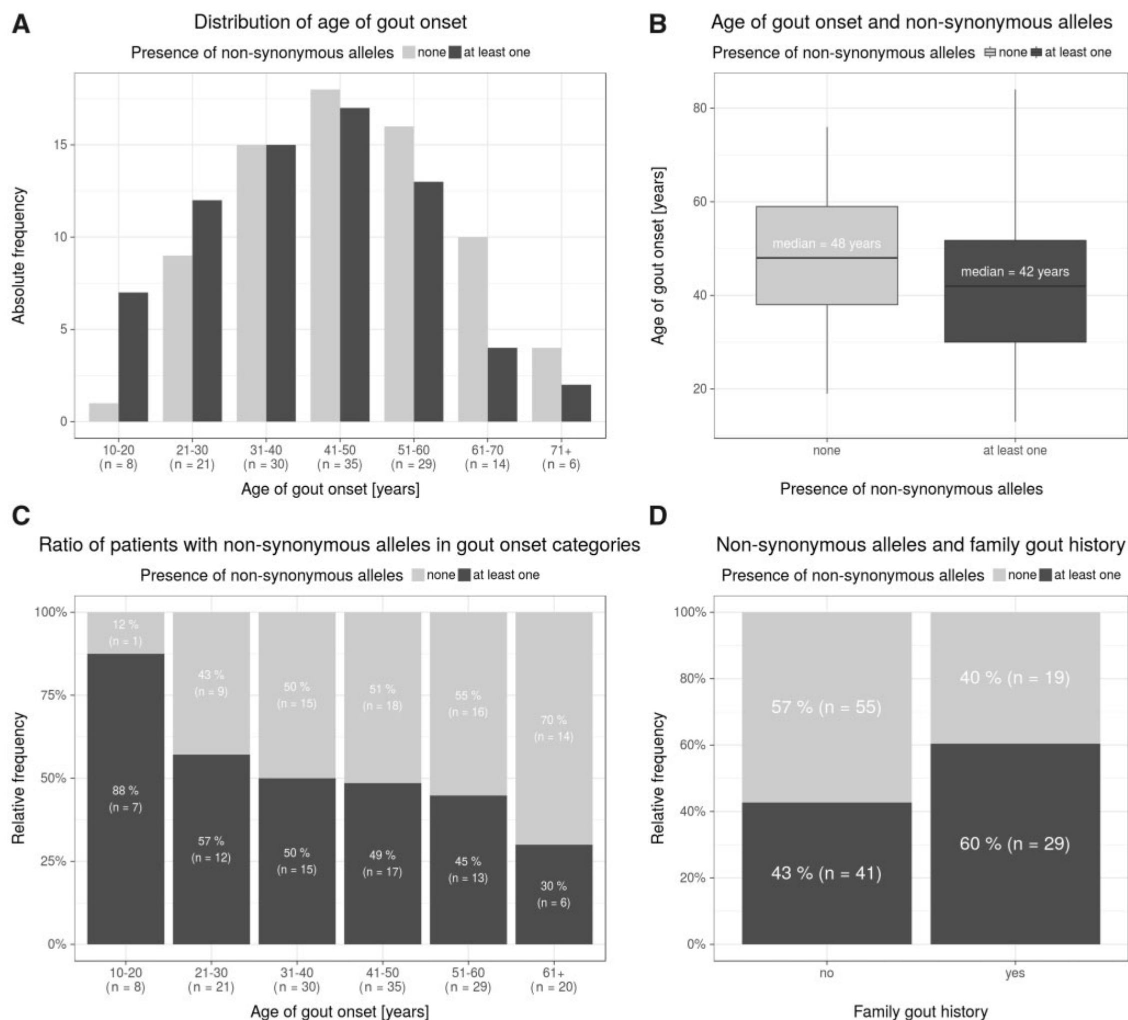
median 41), without notable differences in family gout history relative to the whole cohort. Based on the Czech data alone, we cannot support or contradict the possible protective role of p.V12M.

In the Czech cohort, the p.Q141K variant was present with a significantly higher allele frequency, almost triple the frequency in normouricaemic controls (23% *vs* 8%, P < 0.0001) and higher than in the European-origin population (9%, P < 0.0001). p.Q141K is located in the nucleotide binding domain, the most conserved region in all ABC proteins (where the domain binds and cleaves ATP). The prevalence of this variant is 1–5% in the African, 9% in the Caucasian, and 30% in the Asian population [15]. Our results confirm that this particular alteration in the *ABCG2* gene is a common cause of hyperuricaemia. This finding is consistent with previous studies in which rs2231142 has been associated with hyperuricaemia and gout in individuals of European, Han Chinese, Japanese and African American ancestry [14, 15, 29–32]; however, this association was not found in Māori subjects from New Zealand [32].

The rare variants p.R147W (Caucasian MAF = 0.0001) and p.T153M (Caucasian MAF unknown) were identified in our gout cohort in one heterozygous individual each (MAF = 0.003). These variants were localized close to p.Q141K, in the nucleotide-binding domain, and are probably damaging through disruption of ATP binding. The in-frame three nucleotide deletion p.K360del (Caucasian MAF = 0.007) and p.F373C (Caucasian MAF unknown), also identified in one heterozygous individual each, were located in the intracellular membrane-spanning domain. Variant p.T434M (Caucasian MAF unknown), identified in one heterozygous individual, was located in the first extracellular loop between transmembrane domains 1 and 2. The novel variant p.S476P was located in the first intracellular loop between transmembrane domains 2 and 3. Variant p.D620N (Caucasian MAF = 0.0004), identified in two heterozygous individuals, was located in the last extracellular loop between transmembrane domains 5 and 6. This is a specific region with an unusual conformation (consecutive V-shaped helices partially inserted into the membrane) with an experimentally verified N-glycosylation site [33]. These variants have not been characterized yet in ABCG2 functional studies, but the nature of these variants suggests that they may have a functional impact: analysis using PolyPhen software, Provean and Mutation Taster predicted that substitutions p.R147W, p.F373C, p.T434M, p.S476P and p.D620N could possibly be damaging. Analyses using other predictive software such as SIFT, Human Splicing Finder and MutPred were not consistent and emphasize the need for experimental functional characterization.

An association analysis in the Czech cohort showed a relationship between SUA and three single nucleotide

Fig. 2 Family history, age distribution and allelic variants in the *ABCG2* gene

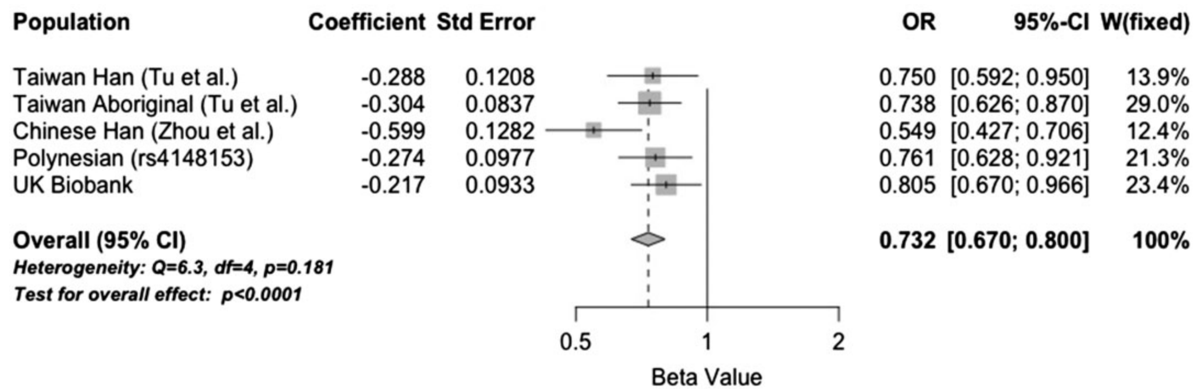


(**A**) Histogram of age of gout onset among patients with and without any of the nine exon non-synonymous alleles. (**B**) Box-and-whiskers plot of age of gout onset among patients with and without any of the nine exon non-synonymous alleles. (**C**) Proportion of patients with and without any of the nine exon non-synonymous alleles in age of gout onset (by decades). (**D**) Proportion of patients with and without any of the nine exon non-synonymous alleles among patients with and without gout family history.

polymorphisms within the *ABCG2* locus, marked by intronic variants rs2231138, rs2231165 and rs2231156. These variants showed a significant association with the concentration of SUA ($P < 0.05$) during hyperuricaemic treatment (rs2231138, rs2231165) and after 72 h without treatment (rs2231156, rs2231165). However, the associations were not statistically significant after the Bonferroni correction. A comparison of genotype distribution showed a lower MAF in the gout group *vs* the Caucasian population for rs2231138 (0.021 *vs* 0.044) and rs2231165 (0.010 *vs* 0.021), and a higher MAF in rs2231156 (0.159 *vs* 0.073). However, the size of the studied groups was not sufficiently large for a detailed analysis of such rare variants with very low MAFs. Furthermore, this finding could have been caused by a linkage disequilibrium with previously reported and more strongly associated common variants that include rs2231142 and intronic rs2622629 [34].

Although we did not find a statistically significant association between rs2231142 and an increased risk of a poor response to allopurinol, the directionality of the ORs of this analysis support the hypothesis that the dysfunctional p.Q141K variant may be associated with a poor response to allopurinol in patients with gout. It is further supported by the increased occurrence of all identified non-synonymous allele variants among poor responders compared with good responders. We can theorize that genotyping rs2231142 may someday be a useful tool for more effective allocation of gout patients relative to therapeutic interventions (e.g. uricosurics and/or febuxostat) as part of the ‘personalized medicine’ concept.

Fig. 3 Meta-analysis of p.V12M for association with gout



ABCG2 dysfunctional variants have a strong impact on the progression of hyperuricaemia. A study in a cohort of 5005 Japanese participants reported that the ABCG2 population-attributable risk percentage (PAR%) for hyperuricaemia was 29.2%, which is much higher than those of the other typical environmental risks, that is, overweight/obesity (BMI $\geqslant$ 25.0; PAR% = 18.7%), heavy drinking [>196 g/week (male) or >98 g/week (female) of pure alcohol; PAR% = 15.4%] and ageing ($\geqslant$60 years old; PAR% = 5.74%) [35].

Gout usually occurs between the fourth and sixth decade of life. In a large cohort study of 23 857 incident gout patients from the UK, the mean age of onset of gout was 61.9 (s.d. 14.5) years [36]. However, the number of patients experiencing onset at a younger age is now increasing [37]. A study of 705 Japanese male gout cases described that 88.2% of early-onset patients (twenties or younger) were positive for mild to severe ABCG2 dysfunctional variants. Severe ABCG2 dysfunction particularly increased the risk of early-onset gout (OR = 22.2, $P = 4.66 \times 10^{-6}$) [38]. Our results confirm that common dysfunction of ABCG2 is a significant cause of familial and/or early-onset gout.

Our study has several strengths. First, our analysis of all *ABCG2* exon regions was complete and thorough and we believe that it has not been done before to such an extent in a gout cohort. Second, in the selection of our gout cohort, we controlled for and excluded several potential confounders of secondary hyperuricaemia/gout and other purine metabolic disorders associated with pathological concentrations of SUA. Some limitations of this study should be acknowledged. First, the size of the studied groups was not large enough to rule out that some functional variants may have gone undetected. Second, we studied genetic variants in transcribed regions and exon–intron boundaries only and therefore genetic variants outside these regions would have gone undetected.

Conclusions

In conclusions, our finding of one intronic splicing and one novel, six very rare and two common non-synonymous *ABCG2* allelic variants in a sample of 145 gout patients suggests that the *ABCG2* gene should be considered a strong and common risk for gout. This finding is supported by the significant effect of these variants, especially the dysfunctional variant p.Q141K, on those with early-onset gout and the presence of a familial gout history. Genotyping the rare variants of ABCG2 in conjunction with its common variants will eventually contribute to evaluation of an individual's risk for gout, a wider selection of effective treatments and better understanding of why some patients respond poorly to treatment, especially in patients with a family history and/or early gout onset.

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Study conception and design: B.S.; clinical observation: J.Z. and K.P.; acquisition of data: B.S., Kat.P., P.S., P.C., M.P. and T.R.M.; analysis and interpretation of data: B.S., Kat.P., M.P., L.P., J.Z., M.H. and T.R.M.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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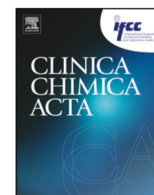

Case Report

Genetic background of uric acid metabolism in a patient with severe chronic tophaceous gout

Lenka Petru [a,b], Katerina Pavelcova [a,b], Ivan Sebesta [c,d], Blanka Stiburkova [a,d,*]

[a] Institute of Rheumatology, Prague, Czech Republic
[b] Department of Rheumatology, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic
[c] Institute of Medical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic
[d] Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic

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ABSTRACT

Hyperuricemia depends on the balance of endogenous production and renal excretion of uric acid. Transporters for urate are located in the proximal tubule where uric acid is secreted and extensively reabsorbed: secretion is principally ensured by the highly variable *ABCG2* gene. Enzyme hypoxanthine–guanine phosphoribosyltransferase (HPRT) plays a central role in purine metabolism and its deficiency is an X-linked inherited metabolic disorder associated with clinical manifestations of purine overproduction. Here we report the case of a middle-aged man with severe chronic tophaceous gout with a poor response to allopurinol and requiring repeated surgical intervention. We identified the causal mutations in the *HPRT1* gene, variant c.481G>T (p.A161S), and in the crucial urate transporter *ABCG2*, a heterozygous variant c.421C>A (p.Q141K). This case shows the value of an analysis of the genetic background of serum uric acid.

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1. Introduction

Uric acid (UA) is the end product of purine metabolism in humans. Urate homeostasis depends on a balance between production and the complex process of secretion and reabsorption in the renal proximal tubule and excretion in the intestine. The production is derived from *de novo* biosynthesis, dietary purine intake, and turnover of tissue nucleic acids. Hypoxanthine-guanine phosphoribosyltransferase deficiency (HPRTD) is one of the most common inborn errors of purine metabolism. This X-linked disorder (OMIM 308000) is classified into distinct forms. Partial HPRTD (#300323) is associated with a clinical manifestation of purine overproduction that results in increased UA synthesis – patients are at risk of gout and urate nephrolithiasis. However, some patients develop a variable spectrum of neurological manifestations, such as motor disability and intellectual impairment, (Lesch–Nyhan variants). Classical features of severe deficiency, Lesch–Nyhan syndrome (#300322), are characterized by neurological and behavioral abnormalities and the overproduction of uric acid. Neurologic and behavioral disabilities may include dystonia, choreoathetosis, ballismus, spasticity, hyperreflexia, mental retardation, and aggressive and impulsive behaviors. Patients often develop persistent and severe self-injurious behaviors [1].

HPRTD shows an X-linked inheritance pattern: female carriers have somatic cell mosaicism of HPRT activity and are usually asymptomatic, with enzyme activity in erythrocytes within normal limits. However, females with complete HPRTD have been described [2]. Female carriers with normal excretion of hypoxanthine and xanthine, and hyperuricemia and/or gout have also been reported [3]. It is noteworthy that heterozygotes for partial HPRT deficiency had significantly diminished HPRT activity in hemolysates compared with heterozygotes for Lesch-Nyhan syndrome. This suggests that selection against HPRT-deficient erythrocyte precursors is more intensive in Lesch-Nyhan syndrome carriers [4].

A diagnosis of HPRTD is determined according to the following scheme: (1) hyperuricemia and hyperuricosuria (a consequence of uric acid overproduction) with urinary hypoxanthine and xanthine elevation is present; (2) HPRTD is confirmed by low HPRT activity in erythrocytes; (3) results are confirmed by molecular genetics. Hyperuricemia

Abbreviations: HPRT, hypoxanthine-guanine phosphoribosyltransferase; UA, uric acid; HPRTD, hypoxanthine-guanine phosphoribosyltransferase deficiency; TG, tophaceous gout; PCR, polymerase chain reaction.

* Corresponding author at: Institute of Rheumatology, Na Slupi 4, 128 50 Prague 2, Czech Republic.
E-mail address: stiburkova@revma.cz (B. Stiburkova).

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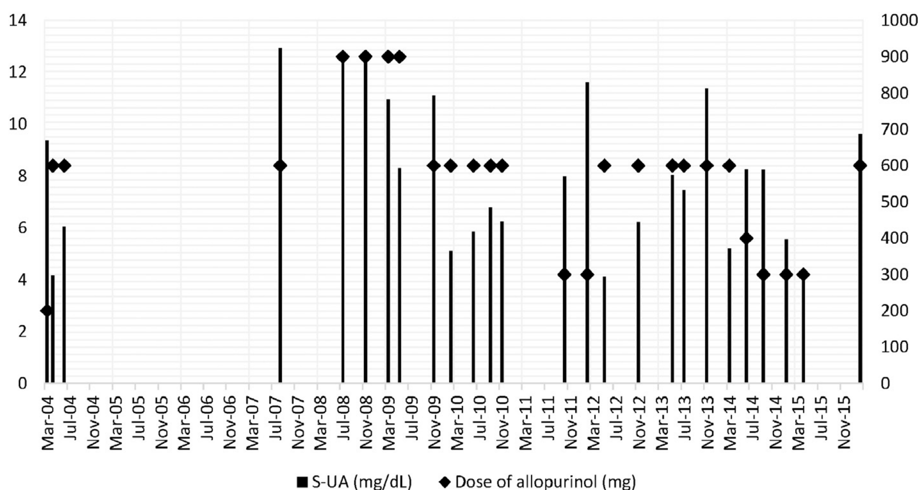

Fig. 1. Serum uric acid level (mg/dl, ref. ranges 3–7 mg/dl) and dosage of allopurinol (mg) in a patient with partial HPRT deficiency, years 2004–2016.

can be treated with febuxostat or allopurinol. No sustained drug therapy has proven uniformly effective for treatment of the neurological problems associated with Lesch-Nyhan syndrome. A reduction in self-injurious and aggressive behavior in children was reported after treatment with *S*-adenosylmethionine on five LND (Lesch–Nyhan disease) patients in an open-label clinical trial [4,5]. However, in an open-label, dose-escalation trial of the drug on 14 LND patients, the authors found that only four patients were able to tolerate the drug; however, when tolerated some beneficial effects were reported [6].

As we mentioned before, the balance between UA production and excretion influences serum UA concentrations. The renal excretion of UA is determined by glomerular filtration followed by secretion, and reabsorption in the proximal tubules. The secretion part of UA transport is principally ensured by the highly variable *ABCG2* gene. Common dysfunction of ABCG2 exporter has proved to be a significant genetic cause of hyperuricemia and gout [7,8].

This is the report on HPRT deficiency complicated by ABCG2 dysfunction allelic variant in patient with severe tophaceous gout.

2. Case report

Our patient was a 41-year-old Caucasian man, who suffered from severe chronic tophaceous gout (TG). He had the first episode of acute podagra at the age of 13. Since then he has had recurrent gout attacks with

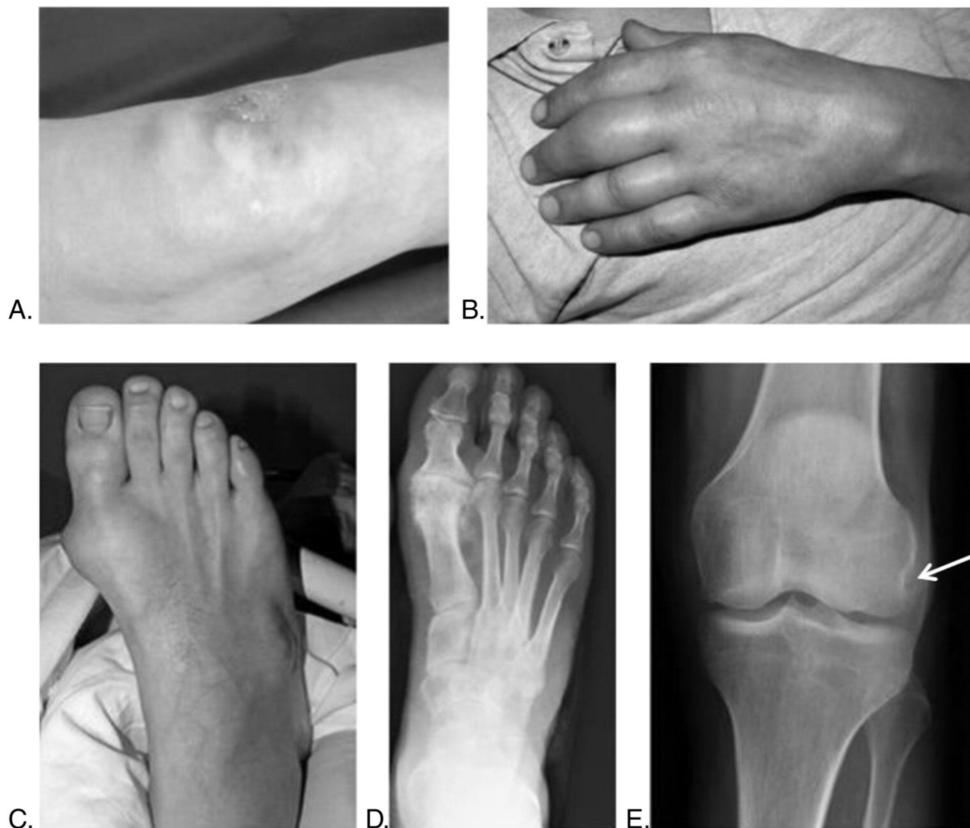

Fig. 2. Pictures of a 41-year-old patient with partial HPRT deficiency with chronic tophaceous gout. (A) Tophi on the left knee after spontaneous drainage (February 2016), (B) left hand during acute gouty attack (September 2009), (C) tophi on the right metatarsus (September 2009), (D) radiograph of the foot with the radiographic hallmark findings of chronic gout (February 2016), (E) radiograph of the left knee with a small round radiolucency on the lateral femoral condyle (suspected gouty tophus) (February 2016).

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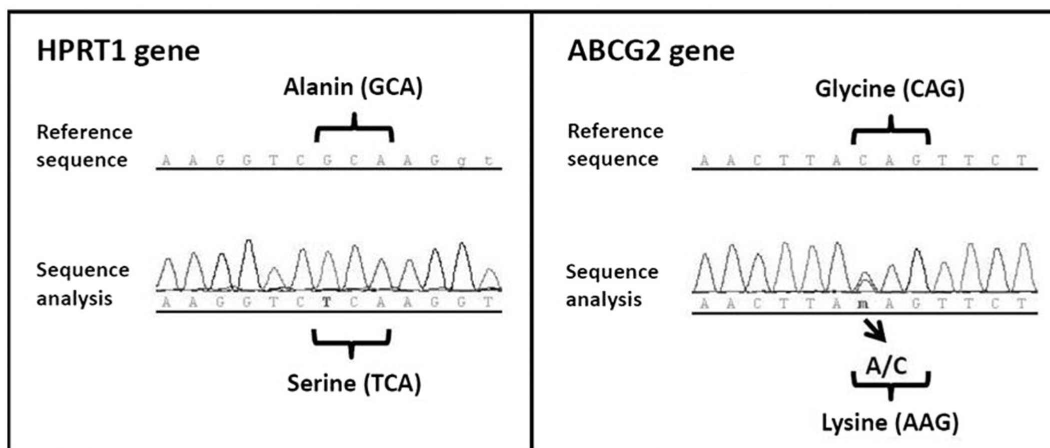

Fig. 3. Chromatogram showing part of the *HPRT1* and *ABCG2* gene sequence. The figures show allelic variant p.A167S (c.481G>T) in the *HPRT*1 gene and allelic variant p.Q141K (c.421C>A) in the *ABCG2* gene of a patient with partial HPRT deficiency.

multiple joint affections. Nephrolithiasis developed at age 22. The patient also had a substantial family history of renal disease and/or gout. His mother had gout and nephrolithiasis, she died at age 55 from pyelonephritis complications. The patient has 13 siblings; all his brothers suffer from renal disease.

At the age of 29, the patient was examined at the Institute of Inherited Metabolic Disorders. Detailed metabolic investigations were performed, as described [9]. Significant hyperuricemia (10.1 mg/dl) and elevated urinary hypoxanthine (116.3 mmol/mol Creatinine, ref. ranges: <30.0 mmol/mol Cr) was found. An enzyme assay of HPRT in lysed erythrocytes showed 0.0 nmol/mgHb/h (ref. ranges: 80–140 nmol/l mgHb/h) and the investigation of intact erythrocytes revealed 45% residual HPRT activity (performed in Purine Research Laboratory, UMDS, London University, Dr. H.A. Simmonds) [10]. Patients with residual enzyme activity of HPRT usually present with at least some minor neurological abnormalities, however, in this case there were no complaints of any neurological symptoms. However, very mild neurological manifestations cannot be excluded since the full neurological exam was not performed (due to patient's poor cooperation). The patient has been treated with allopurinol, in different doses, since the age of 13 years (Fig. 1). Other drugs included corticosteroids (regularly), nonsteroidal anti-inflammatory drugs, and colchicine (intermittently). However, despite treatment with full doses of allopurinol, long-term serum UA levels were poorly controlled and TG eventually developed (Fig. 2). Adherence to allopurinol in the patient was confirmed using the following tests: in 2004 we found plasma levels of xanthine: 19.6 μmol/l (ref. ranges < 9 μmol/l); oxypurinol: 40.0 (ref. ranges < 100 μmol/l) and urinary xanthine: 384.4 mmol/mol Cr (ref. ranges < 25 mmol/mol Cr). In 2009 we found plasma levels of xanthine: 34.6 μmol/l and oxypurinol: 70.2. Tophi were repeatedly surgically removed. Recently he was admitted to our hospital because of his worsening condition. Serum UA level was 9.6 mg/dl, tophi on his right metatarsus and left knee drained spontaneously. Chronic gout was seen on X-rays. A renal ultrasound showed progression of urate nephropathy and his serum creatinine level was 1.32 mg/dl (ref. ranges 0.5–1.2 mg/dl). The diagnosis of partial HPRTD was confirmed through molecular genetic analysis, which revealed the nonsynonymous allelic variant c.481G>T (p.A161S, *rs137852484*). This pathogenic variant was previously described in a patient with partial HPRTD and gout (#300323) [11–13]. Because of the inefficacy of allopurinol and signs of mild renal insufficiency, the urate-lowering therapy was changed to febuxostat with an improvement in serum UA levels.

To explore the cause of TG in our patient with long term treatment with high doses of allopurinol, we performed PCR amplification and sequencing analysis of all exon regions and exon/intron boundaries of *ABCG2*. We identified five intronic variants and heterozygous allelic variant c.421C>A (p.Q141K, *rs2231142*), Fig. 3.

3. Discussion and conclusions

Serum UA concentration is a complex phenotype influenced by both genetic and environmental factors as well as their interactions. HPRTD is considered a rare but well-defined disorder. Our case shows that genetic background can influence both production and excretion of UA in one patient. We identified in the *HPRT1* gene the variant c.481G>T, and in the crucial urate transporter *ABCG2*, a heterozygous variant c.421C>A: this allelic variant resulted in a 53% reduction in transport of UA and has been hypothesized to cause at least 10% of all gout cases in Caucasians [14]. Moreover, recently a significant association of this variant c.421C>A with an increased risk of a poor response to allopurinol was described [15,16].

Clinical manifestations of purine overproduction, which results in increased UA synthesis, can coexist with common and/or rare allelic variants of proteins that have been shown to transport urate in the kidney proximal tubules. Recently, unpublished *HPRT1* gene variant c.206A>T in Japanese adolescent boy with hyperuricemia, gout and decreased erythrocyte HPRT activity was reported [17]. In addition, two introns (homozygous *rs505802* in *SLC22A12*, heterozygous *rs1014290* in *SLC2A9*) and two exons nonsynonymous located variants (heterozygous *rs2231142* in *ABCG2*, homozygous *rs1165196* in *SLC17A1*) associated with hyperuricemia were found. Combination treatment with benzbromarone and febuxostat had a significant effect in reducing the urate level in this patient.

The novelty of our case is that it reveals the significance of genetic background in UA. Genetic analysis will eventually lead to a wider selection of effective treatments and better comprehension of treatments failures.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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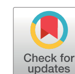
Rapid and reliable HILIC-MS/MS method for monitoring allantoin as a biomarker of oxidative stress



Petr Kozlik[a,*], Lenka Hasikova[b,c], Blanka Stiburkova[b,d], Jakub Zavada[b,c], Kveta Kalikova[e]

[a] Department of Analytical Chemistry, Faculty of Science, Charles University, Prague, Czech Republic
[b] Institute of Rheumatology, Prague, Czech Republic
[c] Department of Rheumatology, First Faculty of Medicine, Charles University, Prague, Czech Republic
[d] Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic
[e] Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Prague, Czech Republic

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ABSTRACT

Allantoin is an excellent biomarker of oxidative stress in humans as the main product of uric acid oxidation by reactive oxygen species. Yet, allantoin determination is still not routinely performed in clinical laboratories. Therefore, we developed a fast, simple, selective, and sensitive UHPLC-MS/MS method for allantoin determination in human serum using an isotopically labeled internal standard. Our analytical protocol provided high sensitivity by mass spectrometry detection and high throughput by HILIC-MS/MS analysis within 4 min, with one-step serum sample preparation approximately within 7 min. Lastly, our protocol was fully validated to demonstrate its reliability in allantoin determination in human serum. The method showed an excellent linear range from 0.05 to 100 μM, with precision ranging from 1.8 to 11.3% (RSD), and with accuracy (relative error %) within ± 6.0%. The method was then applied to analyze the concentration of allantoin in serum samples from 71 patients with chronic gout without treatment with xanthine oxidase inhibitors. The median serum allantoin concentration in the cohort was 2.8 μM (n = 71). Overall, our simple analytical protocol has the potential to be easily implemented in clinical routine practice for monitoring allantoin as a key oxidative stress biomarker.

1. Introduction

Uric acid is the predominant antioxidant in the extracellular environment [1] and thus can scavenge reactive oxygen species (ROS) [2]. Despite the lack of uricase activity in humans, uric acid can be nonenzymatically oxidized into allantoin and other products by ROS [3]. Over the years, allantoin has emerged as a reliable biomarker for monitoring oxidative status both in vitro and in vivo [4–6], and many studies have demonstrated elevated levels of allantoin in different diseases, including diabetes [7], gout [8], and cystic fibrosis [9], among others.

Allantoin analysis remains a challenge mainly due to its low concentrations in biological fluids and high polarity and to the lack of a chromophore for UV detection. Until recently, allantoin was analyzed by LC with UV detection after its conversion to glyoxylic acid and derivatization with dinitrophenylhydrazine [10], but this method was not sensitive enough for allantoin measurement in clinical samples. For such purpose, several GC-MS-based methods were developed for allantoin determination [11,12]. However, the time-consuming allantoin derivatization step is a major limitation of GC-MS methods. Hence, allantoin has been recently measured using fluorimetric [13] and chemoluminescence [14] assays. These techniques nevertheless involve a relatively expert sample workup, and the robustness of these methods is persistently questioned. Alternatively, some LC methods have also been used for allantoin analysis in recent years [10,15–21], but reversed-phase chromatography is not fully suitable for this type of analyte because allantoin is a very polar molecule. For this reason, it is more appropriate to use HILIC. Accordingly, Tolun et al. pioneered the use of HILIC-MS/MS for allantoin determination in urine samples [22]. In addition, Turner et al. developed HILIC-MS/MS under gradient elution using a 5-μm particle size LC column for allantoin determination in plasma, synovial fluid, and urine [23], and Chung et al. developed HILIC-MS/MS under isocratic conditions using 3-μm particle size LC column for allantoin determination in plasma. Yet, despite the methods described above, allantoin determination is still challenging and not routinely performed in clinical laboratories.

* Corresponding author. Charles University, Faculty of Science, Department of Analytical Chemistry, Hlavova 8, 128 43 Prague 2, Czech Republic.
E-mail address: kozlik@natur.cuni.cz (P. Kozlik).

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Based on the above, the aim of the present study was to design simple analytical protocol for measuring allantoin in serum as the biomarker of oxidative stress in patients with chronic gout. These patients show significantly higher plasma levels of allantoin than healthy controls [8]. For fast determination of this important biomarker in serum samples we established and validated a simple and reliable UHPLC method in HILIC with MS/MS. The development of this new method aimed to create a simple and robust protocol for routine clinical laboratories with a high sample throughput. Accordingly, we applied this new method to measure allantoin concentration in serum samples from a large cohort of patients with chronic gout without treatment with xanthine oxidase inhibitors. Further, this developed analytical protocol will be used in medical research for testing the hypothesis that the treatment with xanthine oxidase inhibitors (allopurinol or febuxostat) may affect, inter alia, serum allantoin levels.

2. Experimental

2.1. Chemicals

Acetonitrile (LC-MS grade), water (LC-MS grade), formic acid (LC-MS grade), acetic acid (LC-MS grade), ammonium acetate (LC-MS grade), ammonium formate (LC-MS grade), and allantoin standard (purity ≥ 98%) were purchased from Sigma-Aldrich (St Louis, MO, USA). The internal standard (IS), allantoin-$^{13}C_2$, $^{15}N_4$ (purity ≥ 99.6%), was supplied by Clearsynth Labs (Mumbai, India).

2.2. Preparation of solutions

Stock solutions of allantoin and IS at a concentration of 5 mM were prepared by dissolving the appropriate amount of compounds in 50% acetonitrile. Calibration solutions were prepared in the concentration range from 100 to 0.05 μM. IS was added to each calibration solution at 1 μM. Quality control (QC) samples at concentrations of 1, 10, and 50 μM were prepared by spiking allantoin into patient serum samples with the lowest allantoin concentration available, and QC sample at a concentration of 0.05 μM by diluting a stock solution of allantoin with neat matrix (80% acetonitrile). All QC samples were fortified with IS at a final concentration of 1 μM.

2.3. Samples and samples preparation

In this study, we used frozen serum (−80 °C) from 71 patients with chronic gout. Gout was diagnosed according to the 1977 American Rheumatism Association preliminary criteria. The study was conducted in accordance with the Declaration of Helsinki. All patients provided their written consent regarding biological sample collection and storage prior to participation. All tests were performed in accordance with standards set by the institutional ethics committee (no. 6181/2015). Before the analysis, the serum samples were deproteinized by acetonitrile, adding 60 μL of 100% acetonitrile (containing IS at the concentration of 1.33 μM) to 20 μL of serum and performing deproteinization in an Eppendorf tube by vortexing for 15 s. Then, the serum samples were centrifuged at 16500 × *g* for 6 min, and 50 μL of supernatant was transferred into LC vials.

2.4. Instrumentation and experimental conditions

Measurements were performed using a UHPLC system Agilent 1290 with a Triple Quad 6460 mass spectrometer (Agilent Technologies, Waldbronn, Germany). When developing the method, two different HILIC columns were tested: Acquity UPLC BEH Amide (100 mm × 2.1 mm, 1.7 μm particle size) and Acquity UPLC BEH HILIC (100 mm × 2.1 mm, 1.7 μm particle size) from Waters (Milford, MA, USA). Different mobile phases were tested, including ammonium acetate and ammonium formate at different pH and ionic strengths, and formic acid and acetic acid at different concentrations. The final HILIC conditions consisted of the Acquity BEH Amide column; isocratic elution by a mobile phase composed of acetonitrile and 0.1% formic acid 90/10 (*v/v*). The flow rate of the mobile phase was maintained at 0.3 mL/min, and the injection volume was 2 μL. The temperature of the column was kept at 30 °C and samples were thermostated at 5 °C. QqQ mass spectrometer was equipped with electrospray ionization with Agilent Jet Stream technology using thermal gradient technology of super-heated nitrogen as a sheath gas to improve ion production and desolvation. The ion source was set as follows: gas temperature: 300 °C, gas flow: 7 L/min, sheath gas temperature: 300 °C, sheath gas flow: 8 L/min, nebulizer pressure: 310 kPa and capillary voltage: 6000 V. The MS/MS measurements (operated in positive mode) were performed in the SRM mode. Two SRM transitions were monitored for allantoin: Quantifier transition was 159 > 116 (collision energy 2 V and fragmentor voltage 50 V) and a qualifier transition was 159 > 99 (collision energy 8 V and fragmentor voltage 50 V). The transition 165 > 120 (collision energy 2 V and fragmentor voltage 50 V) was monitored for IS. MS spectra (scan mode and fragmentation of precursor ion) of allantoin and its internal standard are displayed in the Supplementary Material Figs. S1 and S2.

3. Results and discussion

3.1. Method development and optimization

Several analytical parameters were optimized concerning the high throughput, robustness, and sensitivity of the UHPLC-HILIC-MS/MS method. Two HILIC columns were tested. We observed the typical HILIC retention behavior of allantoin in both columns: Increased allantoin retention with the increase in acetonitrile content in the mobile phase. The Acquity BEH Amide column provided a better peak shape and narrower peak than the Acquity BEH HILIC column under tested conditions. The effects of the ionic strength (1, 5, 10 mM ammonium formate, pH 3.0) and pH (pH values 3.0 and 4.0 of 5 mM ammonium formate and 5.0 of 5 mM ammonium acetate) of the aqueous part of the mobile phase on retention, peak shape, and MS intensity were tested. No significant effects on retention time and peak shape were observed when changing the ionic strength and pH of the mobile phase. However, a marked effect on the MS signal was observed. Buffers (ammonium acetate and formate) significantly decreased the MS signal of allantoin by approximately 5 fold in comparison with 0.1% formic acid. A higher concentration (1%) of formic acid or acetic acid in the mobile phase reduced allantoin ionization as well. For this reason, 0.1% formic acid was selected as the aqueous part of the mobile phase. Due to the high throughput of our HILIC method, we operated under isocratic elution conditions using a UHPLC column with sub-2μm particle size. The final composition of the mobile phase consisted of acetonitrile and 0.1% formic acid 90/10 (*v/v*). Allantoin eluted in approximately 2.5 min, and the total time of the analysis was only 4 min, which is a considerably faster analysis than previously published HILIC-MS/MS methods for allantoin determination in plasma [7,23]. Moreover, thanks to the use of isocratic elution, the method is easily transferable to different UHPLC instruments from different vendors, which is crucial for simple method implementation in different clinical laboratories. Although previously published methods [7,23] have used electrospray in negative mode, we observed improved allantoin intensity and signal-to-noise ratio in positive ionization mode, which was likely caused by differences in HILIC chromatography. We used transition for allantoin quantification (m/z 159 > 116), which was the most intensive [22]. To reduce the cleaning time of the ion source, we switched the MS six-port valve to waste for the first 2 min and for the last 1 min of analysis. Moreover, we injected just 2 μL, which helped us to maintain the ion source as clean as possible. Routine protocols in clinical laboratories require keeping sample treatment as simple as possible for high reliability. Accordingly, protein precipitation was chosen for sample

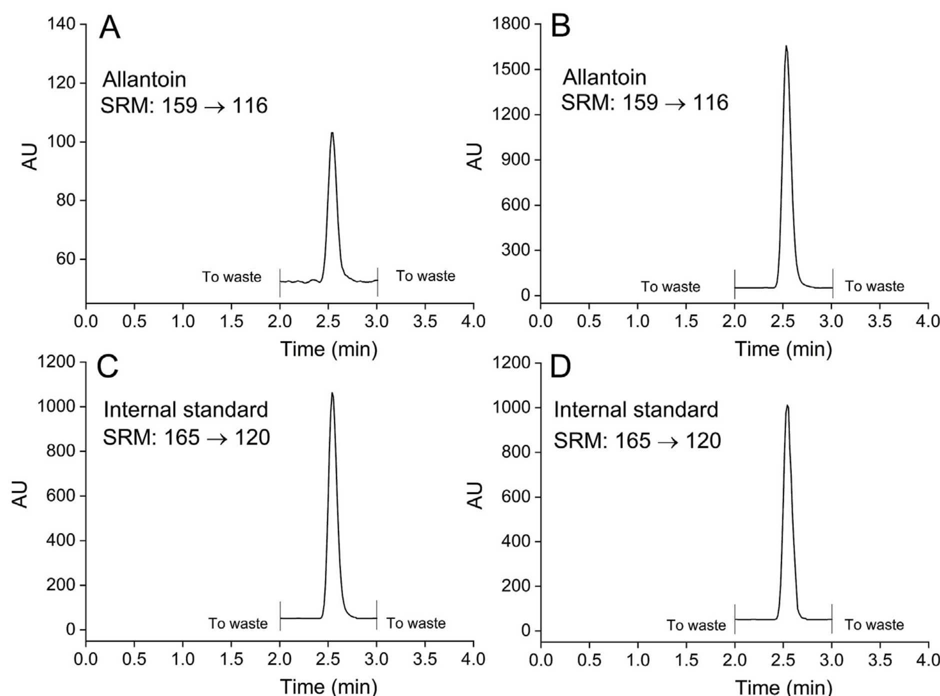

Fig. 1. SRM chromatograms of allantoin and its internal standard obtained under optimized HILIC-MS/MS conditions for A, LLOQ level (c = 0.05 μM) and its internal standard (C) and for B, patient sample (c = 1.83 μM) and its internal standard (D).

preparation considering its simplicity and small sample volume requirements. Our protocol operated only with 20 μL of serum, which was precipitated with 60 μL of 100% acetonitrile containing IS. The total time of sample preparation was approximately 7 min. SRM chromatograms of allantoin at LLOQ level and of one patient sample, obtained under optimized HILIC-MS/MS conditions (see "Instrumentation and the experimental conditions" section), are shown in Fig. 1.

3.2. Method validation

The method was validated in terms of linearity, LOD, lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), accuracy, precision, selectivity, recovery, carry-over effect, matrix effects, robustness and stability of QC samples and clinical patient serum samples.

3.2.1. Calibration, linearity, LLOQ, ULOQ, and LOD

Because allantoin naturally occurs in human serum, the calibration curve was constructed in the neat matrix with nine concentrations by plotting the ratio of the peak area of the analyte to that of deuterium-labeled IS against analyte concentration. Calibration was performed every day before measuring patient samples. The calibration curve was statistically analyzed by $1/x^2$ weighted linear regression analysis using the least-squares regression method, which improved the accuracy in low concentrations. The average calibration equation with its standard deviations is shown in Table 1. LLOQ, which was the lowest calibration standard, was 0.05 μM with precision and accuracy up to 10% (back-calculated). ULOQ, which was the highest calibration standard, was 50 μM with precision and accuracy up to 5% (back-calculated). It is not expected that the concentration of allantoin in humans could be higher than that value. The accuracy and precision of back-calculated concentrations of other calibration points were within 3% of the nominal concentration.

To confirm the reliability of our results derived from the calibration curve, we also compared the results with the standard addition method at six different patient serum samples. The advantage of the standard addition method lies in using the exact same matrix for every individual sample and directly quantifying the endogenous analyte without using deuterium-labeled IS. Conversely, the standard addition method is time-consuming and requires a large amount of sample [24]. Thus, it is not suitable for the analysis of large clinical sample sets. We used the Lord's range test to determine the conformity of the two sets of results [25]. This test is useful in the comparison of small samples and its advantage is the simplicity of the calculation. We tested differences in the experimentally obtained concentration means (three repeated determination) at the probability $P = 0.05$. We calculated u value as a difference of obtained means divided by the sum of the range of a set of data and compared it with the critical value. If the calculated u value exceeds the critical value (the critical value for the tested set is $u_{crit} = 0.636$) [25], the concentration means obtained by two tested methods are statistically different. Results of the Lord's range test are listed in the Supplementary Material Table S1. No significant difference was observed between results derived from the calibration curve method in the neat matrix and standard addition methods. Therefore, the calibration curve method was used to measure clinical patient

Table 1
Validation parameters of the HILIC-MS/MS method for targeted allantoin determination in clinical serum samples.

Parameter	Allantoin
Linear range (μM)	0.05–100
Regression equation	$y = 0.9988(\pm 0.0076)x + 0.0066(\pm 0.0029)$
Coefficient of determination	≥0.9997
LOD (μM)	0.0001
LLOQ (μM)	0.05
ULOQ (μM)	100
Intra-day accuracy (RE,%), $n = 5$	± 4.0
Inter-day accuracy (RE,%), $n = 5$	± 6.0
Intra-day precision (RSD,%), $n = 5$	≤9.6
Inter-day precision (RSD,%), $n = 5$	≤11.3
Recovery (%), $n = 3$	99.1–100.4
Matrix effect (%), $n = 6$	70–105

serum samples.

The linearity was evaluated through the calibrations providing coefficients of determination (R^2) higher than 0.9997 and back-calculated concentrations of calibration points (mentioned above). General accepted literature limits based on back-calculated concentrations for verifying linear range should lie between ± 15% for all the calibration range, and between ± 20% when LLOQ is reached [26]. Obtained coefficients of determination and back-calculated concentrations confirmed acceptable method linearity.

LOD value was determined as 3.3 × σ/S ratio, where σ is the baseline noise obtained from the neat matrix, and S is a slope of the regression line (based on peak heights) obtained from the linearity data. The highest LOD was 0.1 nM (calibration on different days showed a little bit different LOD values), which showed satisfactory sensitivity for allantoin determination in clinical patient serum samples.

3.2.2. Accuracy, precision, and recovery

Method accuracy and precision were evaluated by measuring 5 replicates at four different concentrations of 0.05, 1, 10, and 50 μM, representing the LLOQ, low, mid and high concentration samples in two different days. The accuracy was expressed as the relative error (RE, %; calculated as ((measured concentration – expected concentration)/expected concentration)*100), and the precision expressed by repeatability as the relative standard deviation (RSD). Because of no free allantoin sample matrix, we used patient serum samples with the lowest allantoin concentration available and spiked allantoin into them to cover concentrations of 1, 10, and 50 μM. For the LLOQ QC sample, we used a neat matrix spiked with standard solutions. The accuracy and precision results are listed in Table 2. The inter-day and intra-day precisions (RSD %) ranged from 1.8 to 11.3%, and the accuracy (RE %) was within ± 6.0%. Recovery was evaluated by comparing the area of the isotopic-labeled allantoin standard peak of the pre-protein-precipitation spiked serum sample with that of the corresponding post-protein-precipitation spiked sample at three concentrations (1, 10, and 50 μM). The recovery ranged from 99.1 to 100.4%, as shown in Table 1.

3.2.3. Selectivity, matrix effect

Method selectivity was monitored by injecting six patient serum samples (mass spectrometer was set in scan mode). These chromatograms showed no interfering compound within the retention time window of allantoin. Moreover, the developed method uses a tandem mass spectrometer in specific SRM mode, which ensures high selectivity.

Matrix effect was evaluated at two concentration levels (1 and 50 μM) of six patient serum samples. It was determined by comparing the area of the isotopic-labeled allantoin standard peak of the post-protein-precipitation spiked serum sample with that of the neat matrix (without matrix effect). Matrix effect was calculated according to following equation [27]: (peak area of the post-protein-precipitation spiked serum/peak area obtained in the neat matrix)*100. The matrix effect ranged from 70 to 105%. Higher matrix effect (decreasing absolute signal up to 70% signal in a neat matrix) was observed at lower concentrations and varied randomly between patient serum samples. Matrix effect is common in the analysis of biological samples. Therefore, the use of an isotopic-labeled standard can solve these problems since the analyte/IS response ratio is expected to remain unaffected, even when the absolute responses of the analyte and IS are affected significantly [24].

3.2.4. Carry-over, robustness, and stability of samples

The carry-over was evaluated by injecting a neat matrix blank after the ULOQ sample. Sample carry-over was verified by visual inspection of chromatograms of the neat matrix blank. The carry-over observed after injecting ULOQ samples was not higher than 1% of LLOQ. The robustness of the method was examined by varying the chromatographic conditions, such as column temperature (± 5 °C), flow rate (± 0.05 mL/min), acetonitrile volume, and formic acid concentration in the mobile phase (± 10%). The ratio of allantoin to IS peak areas was monitored. The HILIC-MS/MS method for allantoin determination showed high robustness because changes in chromatographic conditions had no significant effect on the ratio between allantoin and its IS peak areas. QC and patient serum samples were stable for up to 72 h when stored in the autosampler at 5 °C.

3.3. Application to clinical samples

The newly developed and validated UHPLC-HILIC-MS/MS method was applied to the measurement of allantoin concentration in serum samples from 71 patients with chronic gout without treatment with xanthine oxidase inhibitors. The samples were prepared and analyzed according to the optimized and validated protocol explained in the experimental section. Data were expressed as median with interquartile range (IQR). The median allantoin concentration in serum for the entire cohort was 2.8 μM (IQR 2.0–3.6, $n = 71$), which is slightly lower than previously published concentrations in plasma samples of patients with acute gout [8]. We divided patients into groups according to age and body mass index (BMI). Differences among medians of the age and BMI groups were identified by analysis of variance (ANOVA), setting the significance level to 0.05. In Fig. 2, results expressed as box plots show allantoin concentration in serum over different age and BMI ranges. Allantoin concentrations measured in serum showed no significant differences among various age and BMI groups of patients with chronic gout. The developed and validated method will be further used to measure the same set of patients after treatment with xanthine oxidase inhibitors, and the data will be compared and correlated with other clinical parameters, e.g., creatinine and uric acid levels in serum, for medical purposes.

4. Conclusion

Although allantoin is a reliable oxidative stress biomarker in humans, its determination is still not routinely performed in clinical laboratories. In this study, we developed and validated a fast, selective and robust UHPLC-MS/MS method based on HILIC for allantoin determination in serum samples from gout patients within only 4 min. In our protocol, we use an easy, one-step, protein precipitation method with acetonitrile for sample preparation, which is directly compatible with HILIC and MS. Sample consumption is only 20 μL, which is

Table 2
Accuracy and precision of the HILIC-MS/MS method ($n = 5$ replicates; for 2 days).

Allantoin concentration (μM)	Intra-day ($n = 5$)	RSD (%)	RE (%)	Inter-day ($n = 5$)	RSD (%)	RE (%)
	Measured concentrations (μM) (mean ± SD)			Measured concentrations (μM) (mean ± SD)		
0.05	0.052 ± 0.005	9.6	4.0	0.053 ± 0.006	11.3	6.0
1	0.980 ± 0.031	3.2	−2.0	1.023 ± 0.034	3.4	2.3
10	10.158 ± 0.183	1.8	1.6	10.260 ± 0.246	2.4	2.6
50	48.950 ± 1.126	2.3	−2.1	48.550 ± 1.748	3.6	−2.9

SD, standard deviation; RSD, relative standard deviation; RE, relative error.

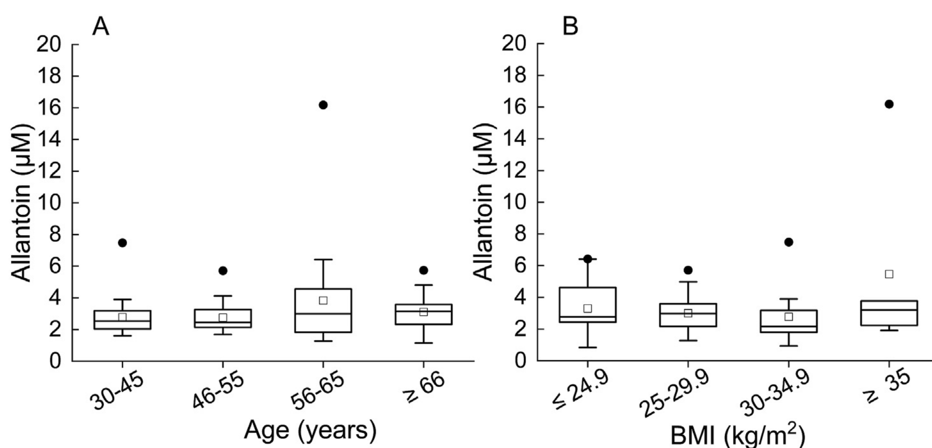

Fig. 2. Box plots of allantoin concentration in patient serum over A: different age groups (years): 30–45 (n = 17), 46–55 (n = 16), 56–58 (n = 18), ≥66 (n = 20), and B: BMI ranges (kg/m^2): ≤24.9 (n = 11), 25–29.9 (n = 34), 30–34.9 (n = 21), ≥35 (n = 5). Box plots show medians with 25th and 75th percentiles as boundaries. Whiskers are for maximum and minimum values, respectively. Solid circles indicate outliers, and squares stand for means.

particularly relevant when considering the value of patient serum samples. Here, we proved the reliability of the method for monitoring allantoin concentration in human sera by full validation. We applied our protocol to large set of serum samples from patient with chronic gout without treatment with xanthine oxidase inhibitors. This newly developed UHPLC-HILIC-MS/MS method with one-step sample preparation provides high sample throughput, which is suitable for large cohorts. In addition, UHPLC-MS/MS instruments are commonly used in clinical laboratories. Therefore, our method has the potential to be implemented in clinical laboratories as a routine test to monitor allantoin as an oxidative stress biomarker.

Declaration of competing interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2019.113509.

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