Journal of
Clinical Medicine

MDPI

Article

Evaluation of the Influence of Genetic Variants of *SLC2A9* (GLUT9) and *SLC22A12* (URAT1) on the Development of Hyperuricemia and Gout

Katerina Pavelcova [1,2], Jana Bohata [1,2], Marketa Pavlikova [3], Eliska Bubenikova [1,2], Karel Pavelka [1] and Blanka Stiburkova [1,4,*]

[1] Department of Molecular Biology and Immunogenetics, Institute of Rheumatology, 128 50 Prague, Czech Republic; pavelcova@revma.cz (K.P.); bohata@revma.cz (J.B.); bubenikova@revma.cz (E.B.); pavelka@revma.cz (K.P.)
[2] Department of Rheumatology, First Faculty of Medicine, Charles University, 128 50 Prague, Czech Republic
[3] Department of Probability and Mathematical Statistics, Faculty of Mathematics and Physics, Charles University, 186 75 Prague, Czech Republic; marketa@ucw.cz
[4] Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague, 120 00 Prague, Czech Republic
* Correspondence: stiburkova@revma.cz; Tel.: +420-234-075-319

Received: 1 July 2020; Accepted: 1 August 2020; Published: 4 August 2020

Abstract: Urate transporters, which are located in the kidneys, significantly affect the level of uric acid in the body. We looked at genetic variants of genes encoding the major reabsorption proteins GLUT9 (*SLC2A9*) and URAT1 (*SLC22A12*) and their association with hyperuricemia and gout. In a cohort of 250 individuals with primary hyperuricemia and gout, we used direct sequencing to examine the *SLC22A12* and *SLC2A9* genes. Identified variants were evaluated in relation to clinical data, biochemical parameters, metabolic syndrome criteria, and our previous analysis of the major secretory urate transporter ABCG2. We detected seven nonsynonymous variants of *SLC2A9*. There were no nonsynonymous variants of *SLC22A12*. Eleven variants of *SLC2A9* and two variants of *SLC22A12* were significantly more common in our cohort than in the European population ($p = 0$), while variants p.V282I and c.1002+78A>G had a low frequency in our cohort ($p = 0$). Since the association between variants and the level of uric acid was not demonstrated, the influence of variants on the development of hyperuricemia and gout should be evaluated with caution. However, consistent with the findings of other studies, our data suggest that p.V282I and c.1002+78A>G (*SLC2A9*) reduce the risk of gout, while p.N82N (*SLC22A12*) increases the risk.

Keywords: gout; hyperuricemia; urate transporters; sequencing; *SLC2A9*; *SLC22A12*

---

1. Introduction

Uric acid is the final product of purine metabolism in humans. If the balance between uric acid production and excretion is impaired, hyperuricemia can occur [1]. Since uric acid is poorly soluble, at higher concentrations, in the blood, monosodium urate crystals can form [2]. In the early stages, hyperuricemia is asymptomatic; however, over time, monosodium urate crystals can lead to gout, a form of inflammatory arthritis. In addition to gout, hyperuricemia is also associated with kidney disease, hypertension, cardiovascular disease, and type 2 diabetes mellitus [3–5].

Uric acid levels are influenced by various factors, such as the intake of dietary purines, the formation of endogenous purines, the excretion of uric acid via the kidneys and intestines, genetic predisposition, medications, and health conditions [1,6]. Different studies indicate that genetic factors are involved in 25–73% of cases [7]. GWAS studies have shown an association between hyperuricemia and gout

and dysfunction of urate transporters [8,9]. These urate transport proteins are located primarily in the proximal tubules of the kidneys, and they are responsible for the excretion and reuptake of uric acid [1]. Variants of the genes that encode urate transporters are associated with both hyperuricemia and, in very rare cases, hypouricemia.

The major excretion urate transporter is ABCG2, while the GLUT9 and URAT1 proteins are important for reabsorption [6].

The *SLC2A9* gene (ENSG00000109667, solute carrier family 2 member 9, located on chromosome 4p16) encodes glucose transporter 9 (GLUT9). It occurs in two isoforms, GLUT9a, which is located on the basolateral membrane and GLUT9b, which is located on the apical membrane of the proximal tubules in the kidneys [2]. GLUT9 provides urate reuptake, and single-nucleotide polymorphisms (SNPs) of *SLC2A9* are associated not only with hyperuricemia and gout, but also with renal hypouricemia type 2 (OMIM(Online Mendelian Inheritance in Man) # 612076) [10].

The *SLC22A12* gene (ENSG00000197891, solute carrier family 22 member 12, located on chromosome 11q13) encodes urate transporter 1 (URAT1). Genetic variants of this gene lead, as in the case of *SLC2A9*, to hyperuricemia and gout and rare cases to hypouricemia type 1 (OMIM # 220150) [11].

The *ABCG2* gene (ENSG00000118777, located on chromosome 4q22) encodes the ATP-binding cassette sub-family G member 2 protein (ABCG2), which is the major secretor of uric acid. In addition to the kidneys, the ABCG2 protein is also located in the intestines, where it facilitates up to one-third of the excretion of uric acid [12]. In our previous work, we reported that genetic variants of the *ABCG2* gene (ENSG00000118777) increases the risk of developing gout, especially the common nonsynonymous variant p.Q141K (*rs2231142*) [13]. These variants are also associated with early disease onset, as confirmed by the findings of our study using a cohort of patients with pediatric-onset primary hyperuricemia and gout [14].

There are other urate transporters in the proximal tubules that are also responsible for uric acid transport, i.e., NPT1 (solute carrier family 17 member 1, *SLC17A1*), NPT4 (solute carrier family 17 member 3, *SLC17A3*), OAT4 (solute carrier family 22 member 11, *SLC22A11*), OAT10 (solute carrier family 22 member 13, *SLC22A13*), and MRP4 (ATP binding cassette subfamily C member 4, *ABCC4*) [2,15]. However, recent evidence suggests that these proteins have less impact on uric acid levels in the blood than GLUT9, URAT1, and ABCG2 [2,16].

The aims of our study were to identify which variants of the *SLC2A9* and *SLC22A12* genes existed in a cohort of 250 individuals with primary hyperuricemia and gout, and at what frequency they existed. We also intended to determine whether the variants were associated with uric acid levels and/or other important factors related to the development of hyperuricemia and gout. Polymorphisms of the *ABCG2* gene, biochemical parameters, and metabolic syndrome markers in this cohort were previously investigated in one of our other studies [13].

2. Experimental Section

The cohort consisted of 177 patients with primary gout and 73 patients with primary hyperuricemia under care from The Institute of Rheumatology. The gout diagnosis was determined using criteria developed by the American College of Rheumatology (ACR) Board of Directors and the European League Against Rheumatism (EULAR) Executive Committee [17]. The hyperuricemia group included individuals with elevated levels of uric acid (women > 360 μmol/L and men > 420 μmol/L). Increased levels of uric acid had to be repeatedly detected over a period of at least four weeks.

In our previous study, we examined 234 individuals from our cohort in search of pathogenic variants of the *ABCG2* gene [13]. The advantage of using this cohort was that we had already excluded individuals suspected of secondary hyperuricemia and secondary gout from our study. Using questionnaires filled out by physicians, we noted the presence of chronic kidney disease, active malignancy, diabetes, hypertension, or severe psoriasis. Furthermore, the age of onset of the first signs of gout and the patient's family history of this disease were noted. In addition, an extensive

biochemical examination was performed from peripheral blood samples. These same data were also recorded for an additional 16 individuals who were added to the cohort used in this, our current study.

Prior to data collection, all 250 participants signed informed consent. Ethics approval for this study was obtained from the Ethics Committee of the Institute of Rheumatology (reference number 6181/2015).

In order to identify SNPs of the *SLC2A9* and *SLC22A12* genes, PCR amplification and sequencing were performed. Peripheral blood was collected into EDTA tubes, and total DNA was isolated by using QIAamp DNA Mini Kits (Qiagen, Hilden, Germany) and stored immediately at −20 °C until analysis.

Specific PCR primers for coding regions of the *SLC2A9* and *SLC22A12* genes were designed, and PCR reaction conditions were optimized. For analysis of *SLC22A12*, the longest transcript, ENST00000377574, coding 553 amino acids and containing 10 exons was chosen. Other transcripts of *SLC22A12* were shorter but did not differ in the amino acid sequence. As for the *SLC2A9* gene, it occurs in two transcripts that differ in exon 3. The longer transcript, ENST00000264784, contains 540 amino acids and PCR primers were designed for all twelve exons. In the shorter transcript, ENST00000506583, coding 511 amino acids, exons 1 and 2 were missing. In addition to the twelve exons in *SLC2A9*, PCR primers were also designed for exon 3 in which the amino acid sequence differs, in exon 3, from the longer transcript, ENST00000264784. The remaining exons of the two transcripts have the same sequence.

PCR products were first verified using electrophoretic analysis with 2% agarose gels.

Following electrophoresis, Presto 96 Well PCR Cleanup Kits (Geneaid, New Taipei City, Taiwan) were used to purify PCR products.

To determine nucleic acid sequences, purified PCR products were analyzed using an Applied Biosystems 3130 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA), i.e., a 4-capillary electrophoretic instrument based on the Sanger sequencing method.

For evaluation of the data, reference sequences of the *SLC2A9* and *SLC22A12* transcripts listed in the Ensembl database were needed. We used Lasergene (DNASTAR) software (version 10.1.2, www.dnastar.com) to search for SNPs having the sequences of the individuals in our cohort.

Data were summarized as medians with interquartile ranges (IQR) or as absolute and relative frequencies where appropriate. Continuous characteristics between patients with hyperuricemia and patients with gout were compared using the Wilcoxon two-sample test; categorical characteristics were compared using the Fisher exact test. The binomial test was used for comparisons of sample minor allele frequencies (MAF) with population MAFs; results with p-values < 0.0001 were considered statistically significant. Differences in MAF between patients with hyperuricemia and with gout were explored using the Fisher exact test. Associations of the allelic variants with biochemical measurements (serum uric acid, creatinine, FEUA) and anamnestic data (age of onset of hyperuricemia or gout) were explored using the Kruskal-Wallis nonparametric ANOVA.

Associations between the allelic variants and hypertension were examined using the Fisher exact test. The level of statistical significance was set at 0.05; the Benjamini-Hochberg adjustment for multiple comparisons was used wherever appropriate. All analyses were performed using statistical language and environment R, version 3.6.3 (www.r-project.org).

3. Results

The characteristics of the cohort are summarized in Tables 1 and 2. Basic clinical data and biochemical data relevant for hyperuricemia are also included. The overview also indicates how many individuals have the p.Q141K variant of the *ABCG2* gene, which significantly increases the risk of gout since it reduces urate transport capacity.

Table 1. Main demographic and genetic characteristics of the hyperuricemic (n = 68) and gout patients (n = 182).

	All (Number)	All (%)	Hyperuricemic (Number)	Hyperuricemic (%)	Gout (Number)	Gout (%)	Fisher Test *p*-Value
sex (men/women)	214/36	85.6/14.4	48/20	70.6/29.4	166/16	91.2/8.8	0.0002
familial occurrence of gout	97	59.8	31	48.3	66	63.5	0.0480
no treatment	58	23.2	30	44.1	28	15.4	
treatment with allopurinol	175	70.0	38	55.9	137	75.3	<0.0001
treatment with febuxostat	17	6.8	0	0.0	17	9.3	
p.Q141K-wild type	147	58.8	44	64.7	103	56.6	
p.Q141K-heterozygous variant	87	34.8	19	27.9	68	37.4	0.3682
p.Q141K-homozygous variant	16	6.4	5	7.4	11	6.0	
hypertension	100	52.8	24	58.6	76	50.6	0.3551

Fisher exact test for comparisons between categorical variables in hyperuricemia and gout cohorts. p.Q141K, variant of the *ABCG2* gene.

Table 2. Main clinical and biochemical characteristics of the hyperuricemic (n = 68) and gout patients (n = 182).

	All Median (IQR)	All Range	Hyperuricemic Median (IQR)	Hyperuricemic Range	Gout Median (IQR)	Gout Range	Wilcoxon Test *p*-Value
age of onset [years]	40.0 (28.0)	1.2–84	27.0 (40.5)	1.2–76	42.0 (24.0)	11–84	0.0026
age [years]	51.5 (25.0)	3–90	36.0 (42.0)	3–78	54.0 (21.0)	11–90	<0.0001
BMI	28.4 (5.8)	16–50	28.1 (6.4)	16–41	28.4 (5.4)	19.5–50	0.0822
WHR	1.0 (0.1)	0.6–1.7	1.0 (0.1)	0.7–1.3	1.0 (0.1)	0.6–1.7	0.0038
SUA off treatment [μmol/L]	460.0 (123.8)	181–683	446.0 (111.0)	253–608	462.0 (124.5)	181–683	0.6298
SUA on treatment [μmol/L]	375.0 (134.0)	163–808	424.0 (140.0)	240–628	372.0 (128.0)	163–808	0.0515
FEUA [fraction]	3.6 (1.7)	0.8–20	3.8 (2.0)	1.6–20	3.6 (1.6)	0.8–14.3	0.6066
GFR.MDRD	86.0 (27.6)	24–426	88.0 (36.0)	28–426	86.0 (26.0)	24–154	0.2312
serum creatinine [μmol/L]	80.5 (19.8)	26–226	79.0 (19.2)	26–132	81.5 (20.5)	47–226	0.0240
CRP	3.5 (6.4)	0.2–224.4	1.9 (4.6)	0.2–153.1	4.0 (6.4)	0.2–224.4	0.0025

Wilcoxon two-sample test for comparisons between continuous variables in hyperuricemic and gout cohorts. IQR, interquartile ranges; WHR, waist-hip ratio; SUA, serum uric acid; FEUA, excretion fraction of uric acid; GFR.MDRD, estimation of glomerular filtration rate; CRP, C-reactive protein. Note: These are data from the initial examination at the Institute of Rheumatology. At this time, uric acid levels were in the reference range in five individuals diagnosed with hyperuricemia.

An overview of the variations found in our cohort of 250 individuals of the *SLC2A9* and *SLC22A12* genes is presented in Table 3. No nonsynonymous variants were found of the *SLC22A12* gene; however, five synonymous variants were detected: p.N82N, p.H86H, p.H142H, p.A416H, and p.L437L. We also identified three intronic variants.

In the *SLC2A9* gene, we detected seven nonsynonymous variants. Six of them were found in transcript ENST00000264784 (p.G25R, p.T275M, p.D281H, p.V282I, p.R294H, p.P350L) and the p.A17T variant was detected in exon 3 of transcript ENST00000506583. We also identified five synonymous variants in transcript ENST00000264784: p.L108L, p.T125T, p.I168I, p.L189L, and p.S515S. In transcript ENST00000264784 of the *SLC2A9* gene, we detected 16 intron variants and a novel variant, c.1002 + 68C > T, which is not yet listed in the Ensembl (Ensembl Genome Browser, www.ensembl.org) and NCBI (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) databases. We analyzed this variant using the Human Splicing Finder; the result was that this mutation probably has no impact on splicing. By examining the intron-exon boundaries of exon 3 of transcript ENST00000506583, we discovered three additional intronic variants.

Statistical analysis using the binomial test revealed genetic variants that were significantly more common in our cohort of 250 individuals with hyperuricemia and gout compared to their frequency in the European population (data from the Ensembl database). The variants of the *SLC2A9* gene were p.L108L, p.T125T, p.L18L, c.151-60T>C, c.249+35C>T, c.249+119G>A, c.250-40A>G, c.410+49A>G, c.1002+72G>A, c.63+18delT, and c.-40-45G>A ($p = 0$). A higher allelic frequency was found in *SLC22A12* for variants c.662-7C>T and c.955-38G>A ($p = 0$). On the other hand, some variants of the *SLC2A9* gene had higher MAFs in the European population, namely p.V282I, p.A17T, c.535+67A>G, c.1002+78A>G, c.1113+9A>C, c.1114-89G>C, and c.-40-13T>C ($p = 0$).

Table 4 shows the results of the Fisher test comparing differences in the occurrence of genetic variants in individuals with hyperuricemia vs. patients with gout. Interestingly, variants p.A17T (OR (odds ratio) = 3.44, $p = 0.0023$, p-value adjusted = 0.0432) and c.-40-13T>C (OR = 3.18, $p = 0.0306$, p-value adjusted = 0.2510) of *SLC2A9* were observed to be more frequent in patients with gout. In contrast, variants c.249 + 119G > A (OR = 0.42, $p = 0.0012$, p-value adjusted = 0.0432), c.151-60T>C (OR = 0.49, $p = 0.0035$, p-value adjusted = 0.0432) and c.249+35C>T (OR = 0.49, $p = 0.0042$, p-value adjusted = 0.0432) were more frequently found in the hyperuricemia subgroup. All associations except for c.-40-13T>C were statistically significant after adjustment for multiple comparisons.

Table 3. SNPs in *SLC2A9* and *SLC22A12* that were identified in a cohort of 250 patients with primary hyperuricemia and gout.

Variant	Gene	Region of the Gene	Reference SNP Number	Wild Type Homozygotes (Number)	Wild Type/Variant Heterozygotes (Number)	Variant Allele Homozygotes (Number)	Allelic Variant MAF	European Population MAF	Binomial Test *p*-Value
p.G25R, c.73G>A	*SLC2A9*	exon 1	*rs2276961*	44	109	97	0.606	0.528	0.0005
p.R294H, c.881G>A	*SLC2A9*	exon 7	*rs3733591*	161	78	11	0.200	0.191	0.6087
p.V282I, c.844G>A	*SLC2A9*	exon 7	*rs16890979*	195	51	4	0.118	0.214	0.0000
p.T275M, c.824C>T	*SLC2A9*	exon 7	*rs112404957*	244	6	0	0.012	0.009	0.4690
p.D281H, c.841G>C	*SLC2A9*	exon 7	*rs73225891*	238	12	0	0.024	0.029	0.5945
p.P350L, c.1049C>T	*SLC2A9*	exon 8	*rs2280205*	54	123	73	0.538	0.484	0.0176
p.A17T, c.49G>A	*SLC2A9*	exon 3	* *rs6820230*	222	0	28	0.112	0.297	0.0000
p.L108L, c.322T>C	*SLC2A9*	exon 3	*rs13113918*	7	48	195	0.876	0.800	0.0000
p.T125T, c.375G>A	*SLC2A9*	exon 3	*rs10939650*	10	58	182	0.844	0.752	0.0000
p.I168I, c.504C>T	*SLC2A9*	exon 4	*rs3733589*	237	13	0	0.026	0.045	0.0397
p.L189L, c.567T>C	*SLC2A9*	exon 6	*rs13125646*	7	47	196	0.878	0.801	0.0000
p.S515S, c.1545C>T	*SLC2A9*	exon 12	*rs144428359*	243	7	0	0.014	0.007	0.0944
c.150+24A>G	*SLC2A9*	intron 1–2	*rs2276962*	241	9	0	0.018	0.042	0.0050
c.150+65C>T	*SLC2A9*	intron 1–2	*rs2276963*	239	11	0	0.022	0.054	0.0007
c.151-60T>C	*SLC2A9*	intron 1–2	*rs2240722*	44	52	154	0.720	0.528	0.0000
c.249+35C>T	*SLC2A9*	intron 2–3	*rs2240721*	42	46	162	0.740	0.528	0.0000
c.249+119G>A	*SLC2A9*	intron 2–3	*rs2240720*	45	25	180	0.770	0.601	0.0000
c.250-40A>G	*SLC2A9*	intron 2–3	*rs28592748*	8	48	194	0.872	0.800	0.0000
c.410+29G>T	*SLC2A9*	intron 3–4	*rs16891971*	246	4	0	0.008	0.026	0.0069
c.410+49A>G	*SLC2A9*	intron 3–4	*rs772544951*	249	1	0	0.002	0.000	0.0000
c.535+67A>G	*SLC2A9*	intron 4–5	*rs3733590*	236	14	0	0.028	0.071	0.0000
c.681+25G>A	*SLC2A9*	intron 5–6	*rs13115193*	50	109	91	0.582	0.505	0.0006
c.681+13C>T	*SLC2A9*	intron 5–6	*rs202000076*	248	2	0	0.004	0.001	0.0901
c.682-31C>T	*SLC2A9*	intron 5–6	*rs4292327*	142	97	11	0.238	0.224	0.4528
c.1002+68C>T	*SLC2A9*	intron 7–8	*NA*	249	1	0	0.002	NA	NA
c.1002+72G>A	*SLC2A9*	intron 7–8	*rs1050991059*	249	1	0	0.002	0.000	0.0000
c.1002+78A>G	*SLC2A9*	intron 7–8	*rs6823877*	128	71	51	0.346	0.651	0.0000
c.1113+9A>C	*SLC2A9*	intron 8–9	*rs2280204*	196	48	6	0.120	0.200	0.0000
c.1114-89G>C	*SLC2A9*	intron 8–9	*rs114361719*	249	1	0	0.002	0.028	0.0000
c.63+18delT	*SLC2A9*	intron 3–4	* *rs61256984*	1	236	13	0.524	0.299	0.0000
c.-40-13T>C	*SLC2A9*	5′ UTR	* *rs6449237*	232	0	18	0.072	0.293	0.0000
c.-40-45G>A	*SLC2A9*	5′ UTR	* *rs752032126*	249	0	1	0.004	0.000	0.0000
p.N82N, c.246C>T	*SLC22A12*	exon 1	*rs3825017*	248	2	0	0.004	0.004	1.0000

Table 3. *Cont.*

Variant	Gene	Region of the Gene	Reference SNP Number	Wild Type Homozygotes (Number)	Wild Type/Variant Heterozygotes (Number)	Variant Allele Homozygotes (Number)	Allelic Variant MAF	European Population MAF	Binomial Test *p*-Value
p.H86H, c.258C>T	*SLC22A12*	exon 1	*rs3825016*	37	106	107	0.640	0.706	0.0014
p.H142H, c.426T>C	*SLC22A12*	exon 2	*rs11231825*	36	106	108	0.644	0.706	0.0027
p.A416A, c.1248A>G	*SLC22A12*	exon 7	*rs1630320*	0	0	250	1.000	1.000	1.0000
p.L437L, c.1309T>C	*SLC22A12*	exon 8	*rs7932775*	154	77	19	0.230	0.202	0.1191
c.662-7C>T	*SLC22A12*	intron 3–4	*rs373881060*	245	5	0	0.010	0.000	0.0000
c.1598+18C>T	*SLC22A12*	intron 9–10	*rs11231837*	152	79	19	0.234	0.199	0.0566
c.955-38G>A	*SLC22A12*	intron 5–6	*rs368284669*	248	2	0	0.004	0.000	0.0000

SNPs found in the *SCL2A9* gene in transcript ENST00000506583 are marked with an asterisk (*) sign, others come from longer transcript ENST00000264784. Genetic variants of the *SLC22A12* gene originate from transcript ENST00000377574. The minor allele frequency (MAF) in our cohort was compared to the European MAF using the binomial test.

Table 4. Comparison of genetic variants in individuals with primary hyperuricemia and patients with primary gout.

Variant	Individuals with Hyperuricemia				Patients with Gout				OR	Fisher Test *p*-Value	Benjamini -Hochberg Method: *p*-Value Adjusted
	Wild Type Homozygotes (Number)	Wild Type/Variant Heterozygotes (Number)	Variant Allele Homozygotes (Number)	Variant Allele MAF	Wild Type Homozygotes (Number)	Wild Type/Variant Heterozygotes (Number)	Variant Allele Homozygotes (Number)	Variant Allele MAF			
p.G25R	7	33	28	0.654	37	76	69	0.588	0.75	0.1829	0.7374
p.R294H	42	22	4	0.221	119	56	7	0.192	0.84	0.5300	1.0000
p.V282I	53	14	1	0.118	142	37	3	0.118	1.00	1.0000	1.0000
p.T275M	68	0	0	0.000	176	6	0	0.016	–	0.1966	0.7374
p.N281H	65	3	0	0.022	173	9	0	0.025	1.12	1.0000	1.0000
p.P350L	15	35	18	0.522	39	88	55	0.544	1.9	0.6875	1.0000
p.A17T	65	0	3	0.044	157	0	25	0.137	3.44	0.0023	0.0432
p.L108L	0	17	51	0.875	7	31	144	0.876	1.1	1.0000	1.0000
p.T125T	0	20	48	0.853	10	38	134	0.841	0.91	0.7834	1.0000
p.I168I	66	2	0	0.015	171	11	0	0.030	2.9	0.5291	1.0000
p.L189L	0	15	53	0.890	7	32	143	0.874	0.86	0.7589	1.0000
p.S515S	68	0	0	0.000	175	7	0	0.019	–	0.1978	0.7374
c.150+24A>G	66	2	0	0.015	175	7	0	0.019	1.31	1.0000	1.0000
c.150+65C>T	66	2	0	0.015	173	9	0	0.025	1.70	0.7351	1.0000
c.151-60T>C	4	17	47	0.816	40	35	107	0.684	0.49	0.0035	0.0432
c.249+35C>T	4	15	49	0.831	38	31	113	0.706	0.49	0.0042	0.0432
c.249+119G>A	4	10	54	0.868	41	15	126	0.734	0.42	0.0012	0.0432
c.250-40A>G	0	17	51	0.875	8	31	143	0.871	0.96	1.0000	1.0000
c.410+29G>T	67	1	0	0.007	179	3	0	0.008	1.12	1.0000	1.0000

Table 4. *Cont.*

Variant	Individuals with Hyperuricemia				Patients with Gout				OR	Fisher Test *p*-Value	Benjamini -Hochberg Method: *p*-Value Adjusted
	Wild Type Homozygotes (Number)	Wild Type/Variant Heterozygotes (Number)	Variant Allele Homozygotes (Number)	Variant Allele MAF	Wild Type Homozygotes (Number)	Wild Type/Variant Heterozygotes (Number)	Variant Allele Homozygotes (Number)	Variant Allele MAF			
c.410+49A>G	68	0	0	0.000	181	1	0	0.003	–	1.0000	1.0000
c.535+67A>G	65	3	0	0.022	171	11	0	0.030	1.38	0.7676	1.0000
c.681+25G>A	8	34	26	0.632	42	75	65	0.563	0.75	0.1855	0.7374
c.681+13C>T	68	0	0	0.000	180	2	0	0.005	–	1.0000	1.0000
c.682-31C>T	43	23	2	0.199	99	74	9	0.253	1.36	0.2383	0.8141
c.1002+68C>T	68	0	0	0.000	181	1	0	0.003	–	1.0000	1.0000
c.1002+72G>A	68	0	0	0.000	181	1	0	0.003	–	1.0000	1.0000
c.1002+78A>G	37	17	14	0.331	91	54	37	0.352	1.10	0.7514	1.0000
c.1113+9A>C	53	15	0	0.110	143	33	6	0.124	1.14	0.7585	1.0000
c.1114-89G>C	68	0	0	0.000	181	1	0	0.003	–	1.0000	1.0000
c.63+18delT	0	66	2	0.515	1	170	11	0.527	1.5	0.8407	1.0000
c.-40-13T>C	66	0	2	0.029	166	0	16	0.088	3.18	0.0306	0.2510
c.-40-45G>A	68	0	0	0.000	181	0	1	0.005	–	1.0000	1.0000
p.N82N	67	1	0	0.007	181	1	0	0.003	0.37	0.4704	1.0000
p.H86H	8	24	36	0.706	29	82	71	0.615	0.67	0.0749	0.4385
p.H142H	7	25	36	0.713	29	81	72	0.618	0.65	0.0586	0.4006
p.A416A	0	0	68	1.000	0	0	182	1.000	0.00	1.0000	1.0000
p.L437L	45	16	7	0.221	109	61	12	0.234	1.8	0.8119	1.0000
c.662-7C>T	67	1	0	0.007	178	4	0	0.011	1.50	1.0000	1.0000
c.1598+18C>T	44	17	7	0.228	108	62	12	0.236	1.5	0.9058	1.0000
c.955-38G>A	67	1	0	0.007	181	1	0	0.003	0.37	0.4704	1.0000

OR, odds ratio. In cases without a variant allele among hyperuricemic patients, the OR could not be enumerated (shown as a '–' sign in the cell).

The results of the statistical evaluation of the associations between variants of the genes and serum uric acid levels and fractional excretion of uric acid are shown in Table 5. After adjustment for multiple comparisons, there were no statistically significant associations. We also evaluated the relationship between genetic variants and creatinine, hypertension, age of onset of hyperuricemia or gout, but no associations were detected.

Since we already knew the *ABCG2* gene sequencing results for the investigated cohort, we also focused on comparing the mutual occurrence of variants in the *ABCG2*, *SLC2A9,* and *SLC22A12* genes. As for the *ABCG2* gene, we focused on dysfunctional variants p.Q141K (*rs2231142*), p.R147W (*rs372192400*), p.T153M (*rs753759474*), p.F373C (*rs752626614*), p.T434M (*rs769734146*), p.S476P, and p.S572R (*rs200894058*) [13]. Concerning the *SLC2A9* and *SLC22A12* genes, we were particularly interested in nonsynonymous variants (p.G25R, p.T275M, p.D281H, p.V282I, p.R294H, p.P350L) and other variants known from the literature to be associated with hyperuricemia and gout, or vice versa, i.e., to reduce the risk of gout, namely p.N82N, p.H86H, p.H142H, p.L108L, p.I168I, c.1002+78A>G, and c.535+67A>G [18–23]. We found that individuals with any of the above-mentioned dysfunctional variants of *ABCG2* (except p.Q141K) were more likely to have the p.D281H allele in *SLC2A9* ($p = 0.0389$). An interesting finding was that individuals with any of the dysfunctional variants of *ABCG2* were less likely to have the homozygous variant p.P350L of *SLC2A9*. Furthermore, we found that individuals with the intronic variant c.1002+78A>G of *SLC2A9* were less likely to have dysfunctional variants of *ABCG2* ($p = 0.014$). Comparisons of the mutual occurrence of other variants did not show any statistically significant results, so only results for variants p.D281H, p.P350L, and c.1002+78A>G are summarized in Tables 6–9.

Table 5. The relationship between the detected variants and serum uric acid levels and fractional excretion of uric acid.

Variant	Median of Serum Uric Acid Levels [µmol/L]			Kruskal-Wallis ANOVA	Benjamini-Hochberg Method: *p*-Value Adjusted	Median of FEUA [%]			Kruskal-Wallis ANOVA	Benjamini-Hochberg Method: *p*-Value Adjusted
	Wild Type Homozygotes	Wild Type/Variant Heterozygotes	Variant Allele Homozygotes			Wild Type Homozygotes	Wild Type/Variant Heterozygotes	Variant Allele Homozygotes		
p.G25R	470	446	448	0.2114	0.653	3.6	3.5	3.7	0.5943	0.933
p.R294H	461	442	430	0.9211	0.921	3.6	3.6	4.2	0.2394	0.933
p.V282I	451	464	395	0.6131	0.735	3.7	3.3	3.1	0.2560	0.933
p.T275M	461	408	NA	0.1623	0.622	3.6	3.4	NA	0.9092	0.937
p.N281H	458	463	NA	0.4241	0.728	3.6	3.7	NA	0.6986	0.933
p.P350L	467	458	440	0.7566	0.830	3.6	3.6	3.7	0.9675	0.968
p.A17T	454	NA	476	0.3181	0.728	3.7	NA	3.6	0.5109	0.933
p.L108L	464	468	451	0.5179	0.734	3.3	3.6	3.6	0.5890	0.933
p.T125T	485	462	452	0.8198	0.867	3.6	3.6	3.6	0.6707	0.933
p.I168I	460	333	NA	0.1300	0.622	3.6	3.9	NA	0.5167	0.933
p.L189L	464	472	455	0.5865	0.735	3.3	3.2	3.7	0.3902	0.933
p.S515S	456	464	NA	0.6484	0.735	3.6	4.4	NA	0.5417	0.933
c.150+24A>G	460	312	NA	0.0215	0.622	3.6	3.9	NA	0.7131	0.933
c.150+65C>T	460	333	NA	0.1300	0.622	3.6	3.9	NA	0.4553	0.933
c.151-60T>C	473	444	446	0.0732	0.622	3.6	4.0	3.6	0.0656	0.736
c.249+35C>T	478	458	444	0.1310	0.622	3.6	4.0	3.6	0.0803	0.736
c.249+119G>A	482	458	444	0.0829	0.622	3.6	4.5	3.5	0.0037	0.127
c.250-40A>G	464	468	451	0.5179	0.734	3.2	3.6	3.6	0.5163	0.933
c.410+29G>T	460	568	NA	0.1830	0.622	3.6	4.2	NA	0.2784	0.933
c.410+49A>G	460	NA	NA	NA	NA	3.6	14.3	NA	NA	NA
c.535+67A>G	460	401	NA	0.3475	0.728	3.6	4.2	NA	0.1387	0.893
c.681+25G>A	477	451	442	0.0679	0.622	3.4	3.7	3.7	0.8218	0.933
c.681+13C>T	460	482	NA	0.6450	0.735	3.6	2.8	NA	0.1577	0.893
c.682-31C>T	442	462	482	0.2579	0.728	3.7	3.5	3.7	0.8507	0.933
c.1002+68C>T	460	600	NA	NA	NA	3.6	5.5	NA	NA	NA
c.1002+72G>A	460	437	NA	NA	NA	3.6	3.1	NA	NA	NA
c.1002+78A>G	450	451	469	0.3498	0.728	3.6	3.4	3.9	0.0865	0.736
c.1113+9A>C	462	441	385	0.6242	0.735	3.7	3.6	3.8	0.8498	0.933
c.1114-89G>C	460	NA	NA	NA	NA	3.6	2.9	NA	NA	NA
c.63+18delT	462	455	495	0.4499	0.728	3.1	3.6	3.8	0.6716	0.933
c.-40-13T>C	454	NA	477	0.1799	0.622	3.6	NA	3.6	0.9065	0.937
c.-40-45G>A	460	NA	548	NA	NA	3.6	NA	4.3	NA	NA
p.N82N	460	430	NA	0.8417	0.867	3.6	4.4	NA	0.3696	0.933
p.H86H	415	470	451	0.3873	0.728	3.6	3.6	3.7	0.4343	0.933
p.H142H	418	470	450	0.4299	0.728	3.6	3.6	3.7	0.4044	0.933
p.A416A	NA	NA	460	NA	NA	NA	NA	3.6	NA	NA
p.L437L	460	464	406	0.4105	0.728	3.6	3.6	3.4	0.7912	0.933
c.662-7C>T	460	414	NA	0.5885	0.735	3.6	2.7	NA	0.7552	0.933
c.1598+18C>T	460	468	406	0.3664	0.728	3.6	3.6	3.4	0.7872	0.933
c.955-38G>A	460	492	NA	0.4847	0.734	3.6	4.2	NA	0.4767	0.933

Table 6. Comparison of mutual occurrence of dysfunctional variants of *ABCG2* (p.R147W, p.T153M, p.F373C, p.T434M, p.S476P, and p.S572R) and the variant p.D281H in a cohort of individuals with hyperuricemia and gout.

		Without *ABCG2* Variants (Number)	**Without *ABCG2* Variants (%)**	**Occurrence of Variants of *ABCG2* (Number)**	**Occurrence of Variants of *ABCG2* (%)**	**Total Number (without Distinction of Alleles in *ABCG2*)**	**Portion of the Whole Cohort (%)**
p.D281H	wild type	233	95.9	5	71.4	238	95.2
	heterozygotes + homozygotes	10	4.1	2	28.6	12	4.8
total in the given column		243	100.0	7	100.0	250	100.0

Fisher's Exact Test: p-value = 0.0389, odds ratio 9.11.

Table 7. Comparison of mutual occurrence of dysfunctional variants of *ABCG2* (p.R147W, p.T153M, p.F373C, p.T434M, p.S476P, and p.S572R) and the variant p.D281H in a cohort of individuals with gout.

		Without *ABCG2* Variants (Number)	**Without *ABCG2* Variants (%)**	**Occurrence of Variants of *ABCG2* (Number)**	**Occurrence of Variants of *ABCG2* (%)**	**Total Number (without Distinction of Alleles of *ABCG2*)**	**Portion in the Whole Cohort (%)**
p.D281H	wild type	169	96	4	66.7	173	95.1
	heterozygotes + homozygotes	7	4	2	33.3	9	4.9
total in the given column		176	100	6	100.0	182	100.0

Fisher's Exact Test: p-value = 0.0295, odds ratio 11.6.

Table 8. Comparison of mutual occurrence of dysfunctional variants of *ABCG2* (p.Q141K, p.R147W, p.T153M, p.F373C, p.T434M, p.S476P, and p.S572R) and the variant p.350L in a cohort of individuals with hyperuricemia and gout.

		Without *ABCG2* Variants (Number)	**Without *ABCG2* Variants (%)**	**Occurrence of Variants of *ABCG2* (Number)**	**Occurrence of Variants of *ABCG2* (%)**	**Total Number (without Distinction of Alleles of *ABCG2*)**	**Portion in the Whole Cohort (%)**
p.P350L	wild type + heterozygotes	175	72	2	28.6	177	70.8
	homozygotes	68	28	5	71.4	73	29.2
total in the given column		243	100	7	100.0	250	100.0

Fisher's Exact Test: p-value = 0.0239, odds ratio 6.38.

Table 9. Comparison of mutual occurrence of dysfunctional variants of *ABCG2* (p.Q141K, p.R147W, p.T153M, p.F373C, p.T434M, p.S476P, and p.S572R) and the variant c.1002+78A>G in a cohort of individuals with hyperuricemia and gout.

		Without *ABCG2* Variants (Number)	**Without *ABCG2* Variants (%)**	**Occurrence of Heterozygous Variants of *ABCG2* (Number)**	**Occurrence of Heterozygous Variants of *ABCG2* (%)**	**Occurrence of Homozygous Variants of *ABCG2* (Number)**	**Occurrence of Homozygous Variants of *ABCG2* (%)**	**Total Number (without Distinction of Alleles of *ABCG2*)**	**Portion in the Whole Cohort (%)**
c.1002+78A>G	wild type	68	47.6	48	54.5	12	63.2	128	51.2
	heterozygotes	52	36.4	17	19.3	2	10.5	71	28.4
	homozygotes	23	16.1	23	26.1	5	26.3	51	20.4
total in the given column		143	100.0	88	100.0	19	100.0	250	100.0

Fisher's Exact Test: p-value = 0.014.

4. Discussion

The main aims of our single center study were to (1) identify variants of the *SLC2A9* and *SLC22A12* genes, (2) determine their frequency compared to the European population, and (3) to evaluate the variants in relation to clinical, biochemical, and genetic data of a cohort with primary hyperuricemia and gout.

No nonsynonymous variants were found of the *SLC22A12* gene, which was highly conserved. This leads to an important question about the effect of synonymous and intronic variants on the development of hyperuricemia and gout. From variants detected in our cohort, we found references in the literature to three synonymous variants. In one study comparing the effect of single nucleotide polymorphisms on uric acid levels, the p.N82N variant was found to be associated with hyperuricemia [18]. Another synonymous variant, p.H86H, was also associated with hyperuricemia and gout [19,24,25]. In contrast, variant p.H142H reduces the risk of gout, according to authors of the study carried out on the Vietnamese population [20]. However, variants p.H86H and p.H142H are common in the European population as well as in our cohort. In contrast, the p.N82N variant rarely occurs; the MAF for the European population is 0.004; in our cohort, this variant occurred in two individuals.

In the *SLC2A9* gene, the variant p.V282I was found to be significantly more frequent in the European population (0.214) than in our cohort (0.118) ($p = 0$). According to a previously published study, this variant reduces the risk of gout [21]. Results regarding intronic variant c.1002+78A>G were also interesting. We found that this variant is significantly more common in the European population compared to our cohort. Our results also seem to be consistent with other research that found c.1002+78A>G reduces the risk of gout [23].

Functional studies have already been performed for all seven nonsynonymous variants that we found of the *SLC2A9* gene. Evaluation of urate uptake and expression was performed using *Xenopus laevis* oocytes. The results did not show significant differences (i.e., expression, location, and urate uptake) between native GLUT9 and proteins with nonsynonymous variants [26].

The association between genetic variants and serum uric acid levels and fractional excretion of uric acid cannot be interpreted with certainty. However, no association was found between creatinine, hypertension, age of onset of hyperuricemia or gout, and variants of the genes.

It is worth mentioning that in patients with primary gout, variants of the *ABCG2* gene occur more frequently than SNPs in *SLC2A9*, *SLC22A12*, and the other genes coding urate transporters. This matches our earlier observations, which showed that, in our cohort of 250 individuals with primary hyperuricemia and gout, the p.Q141K variant of *ABCG2* has a higher allele frequency relative to its allele frequency in the European population (0.24 vs. 0.09). Interestingly, the p.Q141K variant reduces urate transport capacity by up to 53% [16]. This variant also appears to be associated with a lower body mass index and C-reactive protein value [27].

Since different urate transporters are involved in the regulation of uric acid, it was interesting to compare the mutual occurrence of dysfunctional variants of the *ABCG2* gene with variants of the *SLC2A9* and *SLC22A12* genes. One study has already focused on the co-occurrence of selected variants of these genes, i.e., which variants p.H142H (*SLC22A12*), p.V282I (*SLC2A9*) or p.G141K (*ABCG2*) were associated with reduced uric acid excretion [28]. According to our results, variant p.D281H appears to occur more frequently along with the dysfunctional variants of the *ABCG2* gene, so this allele could contribute, together with other variants of *ABCG2*, to increased levels of uric acid. Results regarding two other variants, p.P350L and c.1002+78A>G, are also noteworthy, i.e., they occur more frequently in individuals who do not have dysfunctional variants of *ABCG2*. Taking into account that, according to the conclusion of another study, variant c.1002+78A>G reduces the risk of gout, our results suggest that c.1002+78A>G and p.P350L could reduce the risk of hyperuricemia and gout [23].

It is also important to mention that GLUT9 and URAT1 are referred to as proteins that are associated not only with hyperuricemia and gout, but also with hypouricemia since they are urate reuptake transporters. Figure 1 provides an overview of selected variants of the *SLC2A9* and *SLC22A12*

genes that are associated with hyperuricemia and gout, or vice versa, with hypouricemia. None of the variants found in our cohort were associated with hypouricemia, which is not surprising in light of the characteristics of our cohort and also because renal hypouricemia is a very rare disease [29,30].

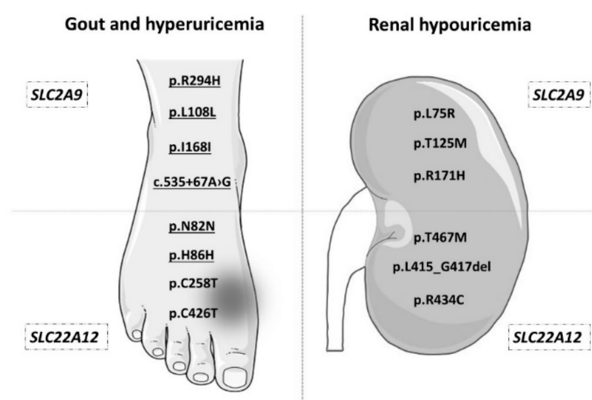


Figure 1. Genetic variants of *SLC2A9* and *SLC22A12* associated with hyperuricemia, gout, and renal hypouricemia. The picture shows some of the genetic variants that are, according to various studies, associated with elevated uric acid levels and increased risk of gout (on the left), or with rare renal hypouricemia (on the right). In the upper two quadrants, SNPs in the *SLC2A9* gene are shown, while in the lower quadrants, SNPs in *SLC22A12* are listed. The underlined genetic variants were found in our cohort. References to studies relating to genetic variants in this figure: p. R294H and p.I168I [22], p.L108L [31], c.535 + 67A > G [23], p.N82N [18], p.H86H [19], p.C258T and p.C426T [32], p.L75R [33], p.T125M [34], p.R171H [35], p.T467M and p.L415_G417del [29], p.R434M [36]. Foot and kidney images were copied from Servier Medical Art, by Servier (https://smart.servier.com; kidney image: https://smart.servier.com/smart_image/kidney-2/; foot image: https://smart.servier.com/smart_image/pied/) and adapted for the purposes of this article. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.

The lack of nonsynonymous variants of *SLC22A12* in our cohort was not so surprising since it is likely that these variants act as gout suppressors based on the reabsorption function of the URAT1 protein. This possibility is supported by a study that focused on nonsynonymous variant p.G774A, which is known to lead to the development of idiopathic renal hypouricemia in the Japanese population. In a cohort of 185 individuals with gout, the authors did not find p.G774A in any patient, while in healthy control subjects, it was present with a frequency of 2.3% [37]. Another study, which focused on two nonsynonymous variants p.R90H and p.W258X of *SLC22A12*, had very similar findings. These variants were also associated with renal hyperuricemia and were not detected in a large cohort of 1993 gout patients. In the group of healthy controls, these variants occur and reduce the risk of hyperuricemia [38]. However, the authors of another study came to different conclusions; they found nonsynonymous variants of the *SLC22A12* gene in 16 patients from a cohort of 69 individuals with gout. The p.C850G variant was detected in 11 patients from the cohort, while no nonsynonymous variants were found in the healthy controls. The unexpected results of this study can be explained by the different frequencies of the variants in diverse populations, i.e., the research was done in the Mexican population. Insight into this issue could provide useful information on the functional impact of the variants detected in this study, which is a question for further research [39].

The main advantage of our study was primarily its detailed genetic analysis of urate transporters GLUT9, URAT1, and the previously analyzed ABCG2 in a clinically and biochemically characterized cohorts of Czech patients with primary hyperuricemia and gout. However, our study has some limitations. A larger cohort would provide a clearer view of the effects of the variants of the *SLC22A12* and *SLC2A9* genes on the development of hyperuricemia and gout. This would also facilitate a more accurate statistical evaluation of less frequent variants in terms of uric acid levels. We also do

not have data on the possible occurrence of asymptomatic urate crystal deposition in individuals with hyperuricemia, which could explain the association with genetic variants of the examined genes. It should also be noted that other urate transporters are involved in the transport of uric acid. Collectively these proteins act as a complex mechanism in the proximal kidney tubules, and it is very likely that the impaired function of one transporter could be compensated for by one or more of the other proteins.

However, more research on this topic needs to be done before the complexities of uric acid transport are fully understood, and other genes that encode urate transporters need to be examined.

Author Contributions: Conceptualization, B.S.; Formal analysis, K.P. (Katerina Pavelcova), J.B., M.P. and E.B.; Investigation, K.P. (Katerina Pavelcova) and J.B.; Writing—original draft, K.P. (Katerina Pavelcova); Writing—review & editing, J.B., M.P., E.B., K.P. (Karel Pavelka) and B.S. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the project for the conceptual development of research organization 00023728 (Institute of Rheumatology).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bobulescu, I.A.; Moe, O.W. Renal transport of uric acid: Evolving concepts and uncertainties. *Adv. Chronic Kidney Dis.* **2012**, *19*, 358–371. [CrossRef] [PubMed]
2. So, A.; Thorens, B. Uric acid transport and disease. *J. Clin. Investig.* **2010**, *120*, 1791–1799. [CrossRef] [PubMed]
3. Nath, S.D.; Voruganti, V.S.; Arar, N.H.; Thameem, F.; Lopez-Alvarenga, J.C.; Bauer, R.; Blangero, J.; MacCluer, J.W.; Comuzzie, A.G.; Abboud, H.E. Genome scan for determinants of serum uric acid variability. *J. Am. Soc. Nephrol.* **2007**. [CrossRef] [PubMed]
4. Sluijs, I.; Beulens, J.W.J.; van der A, D.L.; Spijkerman, A.M.W.; Schulze, M.B.; van der Schouw, Y.T. Plasma uric acid is associated with increased risk of type 2 diabetes independent of diet and metabolic risk factors. *J. Nutr.* **2013**. [CrossRef]
5. Feig, D.I. Hyperuricemia and hypertension. *Adv. Chronic Kidney Dis.* **2012**, *19*, 377–385. [CrossRef]
6. Reginato, A.M.; Mount, D.B.; Yang, I.; Choi, H.K. The genetics of hyperuricaemia and gout. *Nat. Rev. Rheumatol.* **2012**, *8*, 610–621. [CrossRef]
7. Yang, Q.; Guo, C.Y.; Cupples, L.A.; Levy, D.; Wilson, P.W.F.; Fox, C.S. Genome-wide search for genes affecting serum uric acid levels: The Framingham Heart Study. *Metabolism* **2005**, *54*, 1435–1441. [CrossRef]
8. Matsuo, H.; Yamamoto, K.; Nakaoka, H.; Nakayama, A.; Sakiyama, M.; Chiba, T.; Takahashi, A.; Nakamura, T.; Nakashima, H.; Takada, Y.; et al. Genome-wide association study of clinically defined Gout identifies multiple risk loci and its association with clinical subtypes. *Ann. Rheum. Dis.* **2016**, *75*, 652–659. [CrossRef]
9. Nakayama, A.; Nakaoka, H.; Yamamoto, K.; Sakiyama, M.; Shaukat, A.; Toyoda, Y.; Okada, Y.; Kamatani, Y.; Nakamura, T.; Takada, T.; et al. GWAS of clinically defined gout and subtypes identifies multiple susceptibility loci that include urate transporter genes. *Ann. Rheum. Dis.* **2017**, *76*, 869–877. [CrossRef]
10. Stiburkova, B.; Taylor, J.; Marinaki, A.M.; Sebesta, I. Acute kidney injury in two children caused by renal hypouricaemia type 2. *Pediatr. Nephrol.* **2012**, *27*, 1411–1415. [CrossRef]
11. Mancikova, A.; Krylov, V.; Hurba, O.; Sebesta, I.; Nakamura, M.; Ichida, K.; Stiburkova, B. Functional analysis of novel allelic variants in URAT1 and GLUT9 causing renal hypouricemia type 1 and 2. *Clin. Exp. Nephrol.* **2016**, *20*, 578–584. [CrossRef]
12. Hosomi, A.; Nakanishi, T.; Fujita, T.; Tamai, I. Extra-renal elimination of uric acid via intestinal efflux transporter BCRP/ABCG2. *PLoS ONE* **2012**, *7*, 2–9. [CrossRef] [PubMed]
13. Toyoda, Y.; Mančíková, A.; Krylov, V.; Morimoto, K.; Pavelcová, K.; Bohatá, J.; Pavelka, K.; Pavlíková, M.; Suzuki, H.; Matsuo, H.; et al. Functional characterization of clinically-relevant rare variants in abcg2 identified in a gout and hyperuricemia cohort. *Cells* **2019**, *8*, 363. [CrossRef] [PubMed]
14. Stiburkova, B.; Pavelcova, K.; Pavlikova, M.; Ješina, P.; Pavelka, K. The impact of dysfunctional variants of ABCG2 on hyperuricemia and gout in pediatric-onset patients. *Arthritis Res. Ther.* **2019**, *21*. [CrossRef] [PubMed]

15. Zhu, W.; Deng, Y.; Zhou, X. Multiple membrane transporters and some immune regulatory genes are major genetic factors to gout. *Open Rheumatol. J.* **2018**, *12*, 94–113. [CrossRef] [PubMed]
16. Stiburkova, B.; Pavelcova, K.; Zavada, J.; Petru, L.; Simek, P.; Cepek, P.; Pavlikova, M.; Matsuo, H.; Merriman, T.R.; Pavelka, K. Functional non-synonymous variants of ABCG2 and gout risk. *Rheumatology* **2017**, *56*, 1982–1992. [CrossRef] [PubMed]
17. Neogi, T.; Jansen, T.L.T.A.; Dalbeth, N.; Fransen, J.; Schumacher, H.R.; Berendsen, D.; Brown, M.; Choi, H.; Edwards, N.L.; Janssens, H.J.E.M.; et al. 2015 Gout classification criteria: An American College of Rheumatology/European League against Rheumatism Collaborative Initiative. *Arthritis Rheumatol.* **2015**, *67*, 2557–2568. [CrossRef]
18. Cho, S.K.; Kim, S.; Chung, J.Y.; Jee, S.H. Discovery of URAT1 SNPs and association between serum uric acid levels and URAT1. *BMJ Open* **2015**, *5*. [CrossRef]
19. Sun, H.; Qu, Q.; Qu, J.; Lou, X.Y.; Peng, Y.; Zeng, Y.; Wang, G. URAT1 gene polymorphisms influence uricosuric action of losartan in hypertensive patients with hyperuricemia. *Pharmacogenomics* **2015**, *16*, 855–863. [CrossRef]
20. Duong, N.T.; Ngoc, N.T.; Thang, N.T.M.; Phuong, B.T.H.; Nga, N.T.; Tinh, N.D.; Quynh, D.H.; Ton, N.D.; van Hai, N. Polymorphisms of ABCG2 and SLC22A12 genes associated with gout risk in Vietnamese population. *Medicina* **2019**, *55*, 8. [CrossRef]
21. Meng, Q.; Yue, J.; Shang, M.; Shan, Q.; Qi, J.; Mao, Z.; Li, J.; Zhang, F.; Wang, B.; Zhao, T.; et al. Correlation of GLUT9 polymorphisms with gout risk. *Medicine* **2015**, *94*, e1742. [CrossRef] [PubMed]
22. Tu, H.P.; Chen, C.J.; Tovosia, S.; Ko, A.M.S.; Lee, C.H.; Ou, T.T.; Lin, G.T.; Chang, S.J.; Chiang, S.L.; Chiang, H.C.; et al. Associations of a non-synonymous variant in SLC2A9 with gouty arthritis and uric acid levels in Han Chinese subjects and Solomon Islanders. *Ann. Rheum. Dis.* **2010**, *69*, 887–890. [CrossRef] [PubMed]
23. Zhang, D.; Yang, M.; Zhou, D.; Li, Z.; Cai, L.; Bao, Y.; Li, H.; Shan, Z.; Liu, J.; Lv, D.; et al. The polymorphism rs671 at ALDH2 associated with serum uric acid levels in Chinese Han males: A genome-wide association study. *Gene* **2018**, *651*, 62–69. [CrossRef] [PubMed]
24. Tu, H.P.; Ko, A.M.S.; Lee, S.S.; Lee, C.P.; Kuo, T.M.; Huang, C.M.; Ko, Y.C. Variants of ALPK1 with ABCG2, SLC2A9, and SLC22A12 increased the positive predictive value for gout. *J. Hum. Genet.* **2018**, *63*, 63–70. [CrossRef] [PubMed]
25. Zou, Y.; Du, J.; Zhu, Y.; Xie, X.; Chen, J.; Ling, G. Associations between the SLC22A12 gene and gout susceptibility: A meta-analysis. *Clin. Exp. Rheumatol.* **2018**, *36*, 0442–0447.
26. Hurba, O.; Mancikova, A.; Krylov, V.; Pavlikova, M.; Pavelka, K.; Stiburková, B. Complex analysis of urate transporters SLC2A9, SLC22A12 and functional characterization of non-synonymous allelic variants of GLUT9 in the Czech population: No evidence of effect on hyperuricemia and gout. *PLoS ONE* **2014**, *9*. [CrossRef]
27. Horváthová, V.; Bohatá, J.; Pavlíková, M.; Pavelcová, K.; Pavelka, K.; Šenolt, L.; Stibůrková, B. Interaction of the p.Q141K variant of the ABCG2 gene with clinical data and cytokine levels in primary hyperuricemia and gout. *J. Clin. Med.* **2019**, *8*, 1965. [CrossRef]
28. Torres, R.J.; De Miguel, E.; Bailén, R.; Banegas, J.R.; Puig, J.G. Tubular urate transporter gene polymorphisms differentiate patients with gout who have normal and decreased urinary uric acid excretion. *J. Rheumatol.* **2014**, *41*, 1863–1870. [CrossRef]
29. Stiburkova, B.; Gabrikova, D.; Čepek, P.; Šimek, P.; Kristian, P.; Cordoba-Lanus, E.; Claverie-Martin, F. Prevalence of URAT1 allelic variants in the Roma population. *Nucleosides Nucleotides Nucleic Acids* **2016**. [CrossRef]
30. Matsuo, H.; Chiba, T.; Nagamori, S.; Nakayama, A.; Domoto, H.; Phetdee, K.; Wiriyasermkul, P.; Kikuchi, Y.; Oda, T.; Nishiyama, J.; et al. Mutations in glucose transporter 9 gene SLC2A9 cause renal hypouricemia. *Am. J. Hum. Genet.* **2008**, *83*, 744–751. [CrossRef]
31. Sarzynski, M.A.; Jacobson, P.; Rankinen, T.; Carlsson, B.; Sjöström, L.; Bouchard, C.; Carlsson, L.M.S. Changes in uric acid levels following bariatric surgery are not associated with SLC2A9 Variants in the swedish obese subjects study. *PLoS ONE* **2012**, *7*. [CrossRef] [PubMed]
32. Graessler, J.; Graessler, A.; Unger, S.; Kopprasch, S.; Tausche, A.K.; Kuhlisch, E.; Schroeder, H.E. Association of the human urate transporter 1 with reduced renal uric acid. Excretion and hyperuricemia in a German caucasian population. *Arthritis Rheum.* **2006**, *54*, 292–300. [CrossRef] [PubMed]

33. Dinour, D.; Gray, N.K.; Campbell, S.; Shu, X.; Sawyer, L.; Richardson, W.; Rechavi, G.; Amariglio, N.; Ganon, L.; Sela, B.A.; et al. Homozygous SLC2A9 mutations cause severe renal hypouricemia. *J. Am. Soc. Nephrol.* **2010**. [CrossRef] [PubMed]
34. Claverie-Martin, F.; Trujillo-Suarez, J.; Gonzalez-Acosta, H.; Aparicio, C.; Justa Roldan, M.L.; Stiburkova, B.; Ichida, K.; Martín-Gomez, M.A.; Herrero Goñi, M.; Carrasco Hidalgo-Barquero, M.; et al. URAT1 and GLUT9 mutations in Spanish patients with renal hypouricemia. *Clin. Chim. Acta* **2018**, *481*, 83–89. [CrossRef]
35. Windpessl, M.; Ritelli, M.; Wallner, M.; Colombi, M. A novel homozygous SLC2A9 mutation associated with renal-induced hypouricemia. *Am. J. Nephrol.* **2016**. [CrossRef]
36. Tasic, V.; Hynes, A.M.; Kitamura, K.; Cheong, H., Il; Lozanovski, V.J.; Gucev, Z.; Jutabha, P.; Anzai, N.; Sayer, J.A. Clinical and functional characterization of URAT1 variants. *PLoS ONE* **2011**. [CrossRef]
37. Taniguchi, A.; Urano, W.; Yamanaka, M.; Yamanaka, H.; Hosoyamada, M.; Endou, H.; Kamatani, N. A common mutation in an organic anion transporter gene, SLC22A12, is a suppressing factor for the development of gout. *Arthritis Rheum.* **2005**, *52*, 2576–2577. [CrossRef]
38. Sakiyama, M.; Matsuo, H.; Shimizu, S.; Nakashima, H.; Nakamura, T.; Nakayama, A.; Higashino, T.; Naito, M.; Suma, S.; Hishida, A.; et al. The effects of URAT1/SLC22A12 nonfunctional variants, R90H and W258X, on serum uric acid levels and gout/hyperuricemia progression. *Sci. Rep.* **2016**, *6*, 1–6. [CrossRef]
39. Vázquez-Mellado, J.; Jiménez-Vaca, A.L.; Cuevas-Covarrubias, S.; Alvarado-Romano, V.; Pozo-Molina, G.; Burgos-Vargas, R. Molecular analysis of the SLC22A12 (URAT1) gene in patients with primary gout. *Rheumatology* **2007**, *46*, 215–219. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

RHEUMATOLOGY

Rheumatology 2017;56:1982–1992
doi:10.1093/rheumatology/kex295
Advance Access publication 14 August 2017

Original article

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]

Downloaded from https://academic.oup.com/rheumatology/article/56/11/1982/4082557 by Revmatologicky ustav user on 30 April 2021

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

Submitted 15 March 2017; revised version accepted 21 June 2017

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 identified [2]. However, detailed knowledge of the

© The Author 2017. Published by Oxford University Press on behalf of the British Society for Rheumatology. All rights reserved. For Permissions, please email: journals.permissions@oup.com

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

Downloaded from https://academic.oup.com/rheumatology/article/56/11/1982/4082557 by Revmatologicky ustav user on 30 April 2021

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 concentration, or received treatment other than allopurinol.

Downloaded from https://academic.oup.com/rheumatology/article/56/11/1982/4082557 by Revmatologicky ustav user on 30 April 2021

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.

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%), while patients without non-synonymous variants had

Downloaded from https://academic.oup.com/rheumatology/article/56/11/1982/4082557 by Revmatologicky ustav user on 30 April 2021

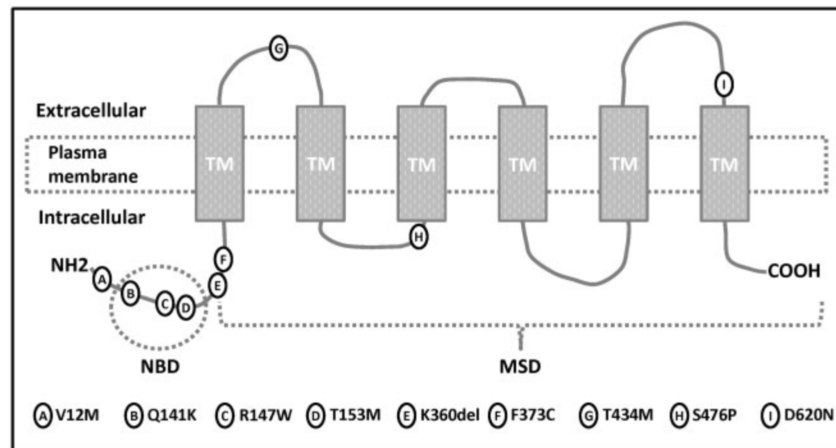
Table 2 Identified non-synonymous *ABCG2* allelic variants, genotype distribution and alternative allele frequency in the gout cohort and database in 145 subjects with primary gout

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.

Downloaded from https://academic.oup.com/rheumatology/article/56/11/1982/4082557 by Revmatologicky ustav user on 30 April 2021

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



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 $\geqslant$ 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,

Downloaded from https://academic.oup.com/rheumatology/article/56/11/1982/4082557 by Revmatologicky ustav user on 30 April 2021

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.

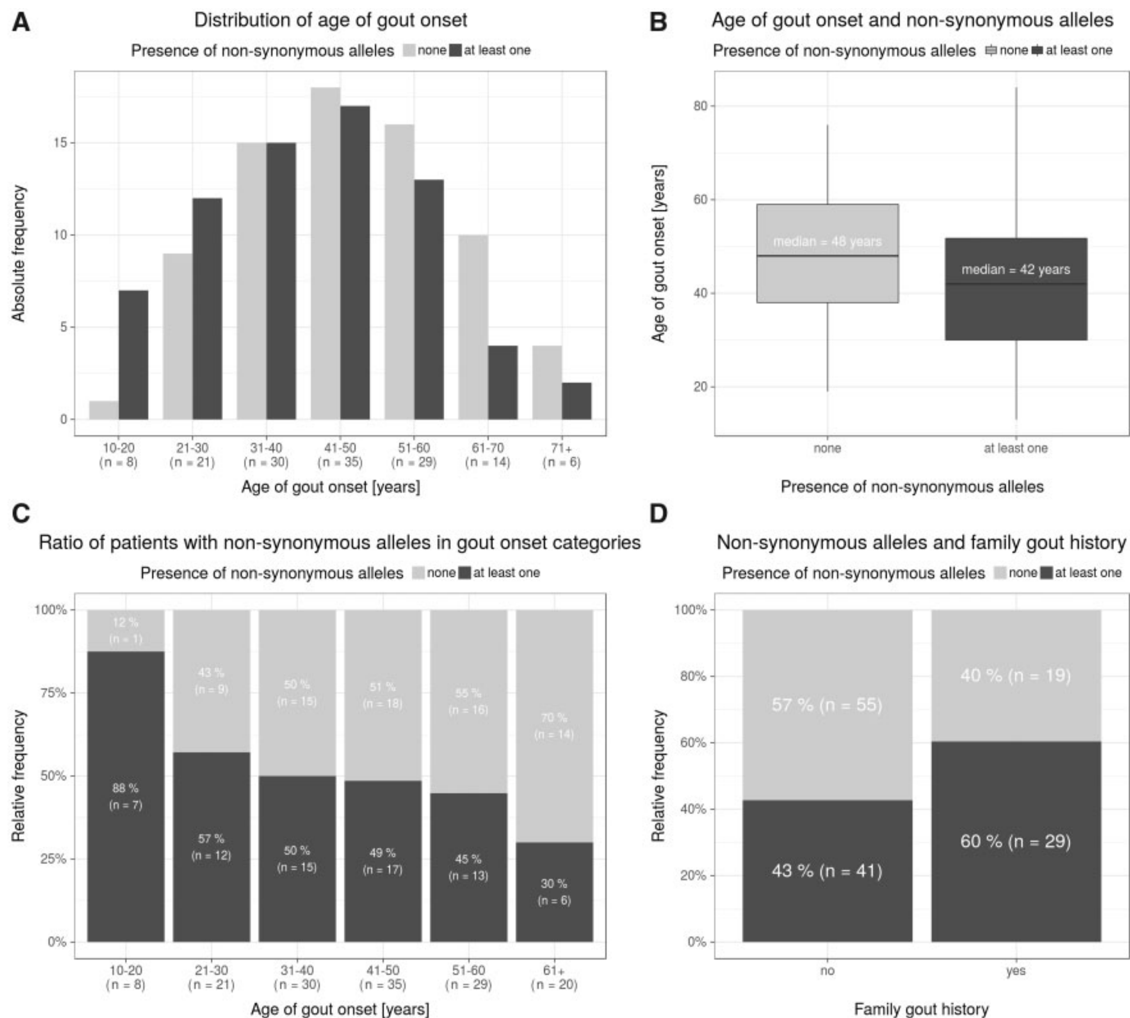
In the Czech cohort, the p.Q141K variant was present with a significantly higher allele frequency, almost triple the frequency in normouricaemic controls (23% *vs* 8%, $P < 0.0001$) and higher than in the European-origin population (9%, $P < 0.0001$). p.Q141K is located in the nucleotide binding domain, 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

Downloaded from https://academic.oup.com/rheumatology/article/56/11/1982/4082557 by Revmatologicky ustav user on 30 April 2021

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



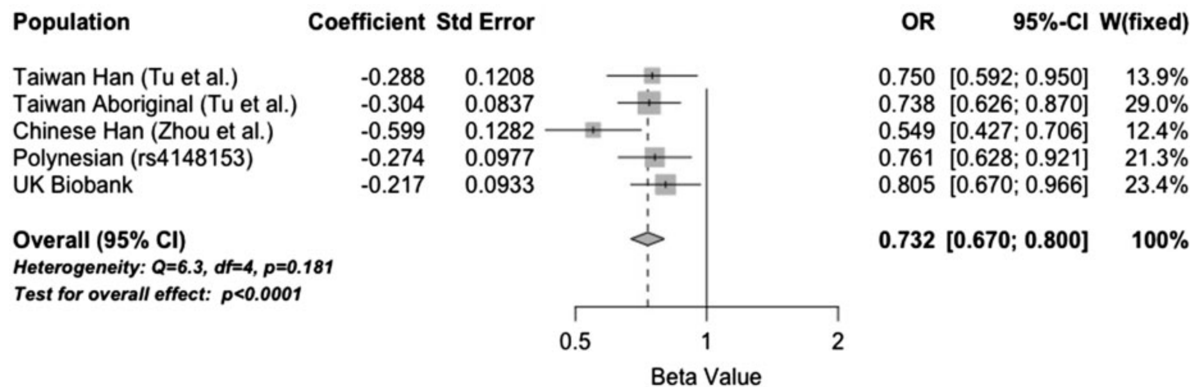
(**A**) Histogram of age of gout onset among patients with and without any of the nine exon non-synonymous alleles. (**B**) Box-and-whiskers plot of age of gout onset among patients with and without any of the nine exon non-synonymous alleles. (**C**) Proportion of patients with and without any of the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

Downloaded from https://academic.oup.com/rheumatology/article/56/11/1982/4082557 by Revmatologicky ustav user on 30 April 2021

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.

Acknowledgements

We would like to thank Tereza Kovacikova (Institute of Inherited Metabolic Disorders, Czech Republic) for her assistance with high performance liquid chromatography determination of purine metabolites). We are grateful to all the patients who took part in this study as well as our colleagues at the Institute of Rheumatology for their help in recruiting patients for the study.

Author contributions: All authors were involved in drafting the manuscript or revising it critically for content. All the authors approved the final version for publication.

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.

Funding: Supported by a grant from the Czech Republic Ministry of Health AZV 15-26693 A.

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

References

1 Köttgen A, Albrecht E, Teumer A *et al*. Genome-wide association analyses identify 18 new loci associated with serum urate concentrations. Nat Genet 2013;45:145–54.

Downloaded from https://academic.oup.com/rheumatology/article/56/11/1982/4082557 by Revmatologicky ustav user on 30 April 2021

2 Nakayama A, Nakaoka H, Yamamoto K *et al*. GWAS of clinically defined gout and subtypes identifies multiple susceptibility loci that include urate transporter genes. Ann Rheum Dis 2016;76:869–77.

3 Mandal AK1, Mount DB. The molecular physiology of uric acid homeostasis. Annu Rev Physiol 2015;77:323–45.

4 Sorensen LB. Role of the intestinal tract in the elimination of uric acid. Arthritis Rheum 1965;8:694–706.

5 Tang W, Miller MB, Rich SS *et al*. Linkage analysis of a composite factor for the multiple metabolic syndrome: the National Heart, Lung, and Blood Institute Family Heart Study. Diabetes 2003;52:2840–7.

6 Wilk JB, Djousse L, Borecki I *et al*. Segregation analysis of serum uric acid in the NHLBI Family Heart Study. Hum Genet 2000;106:355–9.

7 Nath SD, Voruganti VS, Arar NH *et al*. Genome scan for determinants of serum uric acid variability. J Am Soc Nephrol 2007;18:3156–63.

8 Yang Q, Guo CY, Cupples LA *et al*. Genome-wide search for genes affecting serum uric acid levels: the Framingham Heart Study. Metabolism 2005;54:1435–41.

9 Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, Dean M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res 1998;58:5337–9.

10 Mao Q, Unadkat JD. Role of the breast cancer resistance protein (ABCG2) in drug transport. AAPS J 2005;7:E118–33.

11 Takada T, Ichida K, Matsuo H *et al*. ABCG2 dysfunction increases serum uric acid by decreased intestinal urate excretion. Nucleosides Nucleotides Nucleic Acids 2014;33:275–81.

12 Matsuo H, Tsunoda T, Ooyama K *et al*. Hyperuricemia in acute gastroenteritis is caused by decreased urate excretion via ABCG2. Sci Rep 2016;6:31003.

13 Kolz M, Johnson T, Sanna S *et al*. Meta-analysis of 28,141 individuals identifies common variants within five new loci that influence uric acid concentrations. PLoS Genet 2009;5:e1000504.

14 Matsuo H, Takada T, Ichida K *et al*. Common defects of ABCG2, a high-capacity urate exporter, cause gout: a function-based genetic analysis in a Japanese population. Sci Transl Med 2009;1:5ra11.

15 Woodward OM, Köttgen A, Coresh J *et al*. Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout. Proc Natl Acad Sci U S A 2009;106:10338–42.

16 Ichida K, Matsuo H, Takada T *et al*. Decreased extra-renal urate excretion is a common cause of hyperuricemia. Nat Commun 2012;3:764.

17 Roberts RL, Wallace MC, Phipps-Green AJ *et al*. ABCG2 loss-of-function polymorphism predicts poor response to allopurinol in patients with gout. Pharmacogenomics J 2016;17:201–3.

18 Wen CC, Yee SW, Liang X *et al*. Genome-wide association study identifies ABCG2 (BCRP) as an allopurinol transporter and a determinant of drug response. Clin Pharmacol Ther 2015;97:518–25.

19 Petru L, Pavelcova K, Sebesta I, Stiburkova B. Genetic background of uric acid metabolism in a patient with severe chronic tophaceous gout. Clin Chim Acta 2016;460:46–9.

20 Wallace SL, Robinson H, Masi AT *et al*. Preliminary criteria for the classification of the acute arthritis of primary gout. Arthritis Rheum 1977;20:895–900.

21 Rasheed H, Phipps-Green AJ, Topless R *et al*. Replication of association of the apolipoprotein A1-C3-A4 gene cluster with the risk of gout. Rheumatology 2016;55:1421–30.

22 Stiburkova B, Krijt J, Vyletal P *et al*. Novel mutations in xanthine dehydrogenase/oxidase cause severe hypouricemia: biochemical and molecular genetic analysis in two Czech families with xanthinuria type I. Clin Chim Acta 2012;413:93–9.

23 Cadzow M, Merriman TR, Dalbeth N. Precision of gout definitions for population-based genetic studies: analysis of the UK Biobank. Arthritis Rheumatol 2016;68(Suppl 10): abstract number: 2278.

24 Stiburkova B, Miyata H, Závada J *et al*. Novel dysfunctional variant in ABCG2 as a cause of severe tophaceous gout: biochemical, molecular genetics and functional analysis. Rheumatology 2016;55:191–4.

25 Tu H-P, Ko AM-S, Chiang S-L *et al*. Joint effects of alcohol consumption and ABCG2 Q141K on chronic tophaceous gout risk. J Rheumatol 2014;41:749–58.

26 Zhou D, Liu Y, Zhang X *et al*. Functional polymorphisms of the ABCG2 gene are associated with gout disease in the Chinese Han male population. Int J Mol Sci 2014;15:9149–59.

27 Matsuo H, Nakayama A, Sakiyama M *et al*. ABCG2 dysfunction causes hyperuricemia due to both renal urate underexcretion and renal urate overload. Sci Rep 2014;4:3755.

28 Telbisz Á, Özvegy-Laczka C, Hegedű s T, Váradi A, Sarkadi B. Effects of the lipid environment, cholesterol and bile acids on the function of the purified and reconstituted human ABCG2 protein. Biochem J 2013;450:387–95.

29 Stibůrková B, Pavlíková M, Sokolová J, Kožich V. Metabolic syndrome, alcohol consumption and genetic factors are associated with serum uric acid concentration. PLoS One 2014;9:e97646.

30 Dehghan A, Kottgen A, Yang Q *et al*. Association of three genetic loci with uric acid concentration and risk of gout: a genome-wide association study. Lancet 2008;372:1953–61.

31 Yamagishi K, Tanigawa T, Kitamura A *et al*. The rs2231142 variant of the ABCG2 gene is associated with uric acid levels and gout among Japanese people. Rheumatology 2010;49:1461–5.

32 Phipps-Green AJ, Hollis-Moffatt JE, Dalbeth N *et al*. A strong role for the ABCG2 gene in susceptibility to gout in New Zealand Pacific Island and Caucasian, but not Maori, case and control sample sets. Hum Mol Genet 2010;19:4813–9.

33 László L, Sarkadi B, Hegedű s T. Jump into a new fold-a homology based model for the ABCG2/BCRP multidrug transporter. PLoS One 2016;11:e0164426.

34 Stahl E, Choi H, Cadzow M *et al*. Conditional analysis of 30 serum urate loci identifies 25 additional independent effects. Arthritis Rheumatol 2014;66:S1294.

35 Nakayama A, Matsuo H, Nakaoka H *et al*. Common dysfunctional variants of ABCG2 have stronger impact on

Downloaded from https://academic.oup.com/rheumatology/article/56/11/1982/4082557 by Revmatologicky ustav user on 30 April 2021

hyperuricemia progression than typical environmental risk factors. Sci Rep 2014;4:5227.

36 Rothenbacher D, Primatesta P, Ferreira A, Cea-Soriano L, Rodríguez LA. Frequency and risk factors of gout flares in a large population-based cohort of incident gout. Rheumatology 2011;50:973–81.

37 López López CO, Lugo EF, Alvarez-Hernández E et al. Severe tophaceous gout and disability: changes in the past 15 years. Clin Rheumatol 2017;36:199–204.

38 Matsuo H, Ichida K, Takada T et al. Common dysfunctional variants in ABCG2 are a major cause of early-onset gout. Sci Rep 2013;3:2014.

Clinical Vignette

Rheumatology 2017;56:1992
doi:10.1093/rheumatology/kex243
Advance Access publication 30 June 2017

Large-vessel vasculitis in positron emission tomography and ultrasound fusion imaging

A 65-year-old female was referred with loss of weight, dyspnoea and night sweats. Laboratory tests revealed elevated inflammatory markers. Imaging with ^{18}F-labelled fluorodeoxyglucose (^{18}F-FDG) positron emission tomography/computed tomography (PET/CT) showed increased ^{18}F-FDG-uptake in the axillary arteries and the aorta (Fig. 1A, blue and green arrows). Large-vessel vasculitis was suspected, given the high specificity (89%) and sensitivity (80%) of PET/CT for this diagnosis [1]. The limited soft-tissue contrast of CT images impedes a detailed assessment of morphological vascular pathologies. These can be better examined using US, which depicts vascular wall thickening as a sign of inflammatory processes (Fig. 1B, blue arrow).

PET/US fusion was performed, combining the advantages of metabolic (PET) and morphological (US) examinations [2]. The Volume Navigation technology of GE Healthcare's LOGIQ™ E9 US device allows the merging of previously acquired PET datasets with real-time US. Figure 1C shows the accurate correlation of PET and US images in the left-sided axillary artery. Additional Doppler sonography was applied to assess the perfused vessel lumen (Fig. 1D). Sagittal images of PET, CT and US in Fig. 1E–G demonstrate the co-registration of PET and US images, using spine and liver as morphological landmarks. The increased ^{18}F-FDG-uptake correlates with the pathologically thickened wall of the abdominal aorta (Fig. 1E–G, green arrows and videos of transversal and sagittal PET/US fusion imaging of the abdomen, available as supplementary data at *Rheumatology* Online).

Funding: No specific funding was received from any funding bodies in the public, commercial or not-for-profit sectors to carry out the work described in this manuscript.

Disclosure statement: The authors have declared no conflicts of interest.

Philipp Seifert[1], Robert Drescher[1], Alexander Pfeil[2] and Martin Freesmeyer[1]

[1]Clinic of Nuclear Medicine and [2]Department of Rheumatology, Clinic of Internal Medicine, Jena University Hospital, Jena, Germany

Correspondence to: Martin Freesmeyer, Clinic of Nuclear Medicine, Jena University Hospital, Am Klinikum 1, Gebäude A4 Ebene U1, 07747 Jena Lobeda-Ost, Germany.
E-mail: martin.freesmeyer@med.uni-jena.de

Supplementary data

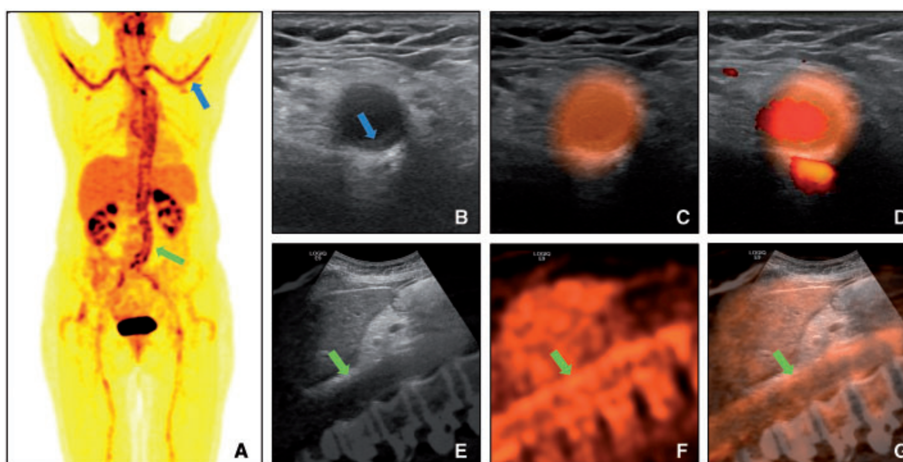
Supplementary data are available at *Rheumatology* Online.

References

1 Danve A, O'Dell J. The role of ^{18}F fluorodeoxyglucose positron emission tomography scanning in the diagnosis and management of systemic vasculitis. Int J Rheum Dis 2015;18:714–24.

2 Drescher R, Müller A, Lesser T, Freesmeyer M. Differential diagnosis of thyroid nodules via real-time PET/ultrasound (US) fusion in a case of co-existing medullary thyroid cancer and adenoma. J Clin Endocrinol Metab 2013;98:4250–1.

Fig. 1 PET/CT/US fusion images of axillary artery and abdominal aorta in large-vessel vasculitis



© The Author 2017. Published by Oxford University Press on behalf of the British Society for Rheumatology. All rights reserved. For Permissions, please email: journals.permissions@oup.com

Downloaded from https://academic.oup.com/rheumatology/article/56/11/1982/4082557 by Revmatologicky ustav user on 30 April 2021

Arthritis Research & Therapy

RESEARCH ARTICLE Open Access

The impact of dysfunctional variants of ABCG2 on hyperuricemia and gout in pediatric-onset patients

Blanka Stiburkova[1,3*], Katerina Pavelcova[1,2], Marketa Pavlikova[1], Pavel Ješina[3] and Karel Pavelka[1]

Abstract

Background: ABCG2 is a high-capacity urate transporter that plays a crucial role in renal urate overload and extra-renal urate underexcretion. Previous studies have suggested an association between hyperuricemia and gout susceptibility relative to dysfunctional ABCG2 variants, with rs2231142 (Q141K) being the most common. In this study, we analyzed the *ABCG2* gene in a hyperuricemia and gout cohort focusing on patients with pediatric-onset, i.e., before 18 years of age.

Method: The cohort was recruited from the Czech Republic ($n = 234$) and consisted of 58 primary hyperuricemia and 176 gout patients, with a focus on pediatric-onset patients ($n = 31$, 17 hyperuricemia/14 gouts); 115 normouricemic controls were used for comparison. We amplified, sequenced, and analyzed 15 *ABCG2* exons. The chi-square goodness-of-fit test was used to compare minor allele frequencies (MAF), and the log-rank test was used to compare empirical distribution functions.

Results: In the pediatric-onset cohort, two common (p.V12M, p.Q141K) and three very rare (p.K360del, p.T421A, p.T434M) allelic ABCG2 variants were detected. The MAF of p.Q141K was 38.7% compared to adult-onset MAF 21.2% (OR = 2.4, $P = 0.005$), to the normouricemic controls cohort MAF 8.5% (OR = 6.8, $P < 0.0001$), and to the European population MAF 9.4% (OR = 5.7, $P < 0.0001$). The MAF was greatly elevated not only among pediatric-onset gout patients (42.9%) but also among patients with hyperuricemia (35.3%). Most (74%) of the pediatric-onset patients had affected family members (61% were first-degree relatives).

Conclusion: Our results show that genetic factors affecting ABCG2 function should be routinely considered in a hyperuricemia/gout diagnosis, especially in pediatric-onset patients. Genotyping of *ABCG2* is essential for risk estimation of gout/hyperuricemia in patients with very early-onset and/or a family history.

Keywords: Gout, Hyperuricemia, Urate transport, ABCG2

Background

Serum urate concentration is a complex phenotype influenced by both genetic and environmental factors, as well as their interactions. Hyperuricemia results from an imbalance between endogenous production and excretion of urate. The most common mechanism leading to hyperuricemia is decreased excretion of urate. Hyperuricemia is a central feature in the pathogenesis of gout. Gout is a metabolic disorder caused by an inflammatory reaction to the deposit of urate crystals in joints and soft tissues. The disorder progresses through several degrees, and chronic hyperuricemia is a necessary condition for gout to develop. Prevalence of gout is higher in men [1], and women with gout are more likely to be older (link with menopause) [2], have co-morbidities, and be on diuretics compared with men with gout [3, 4]. Gout usually occurs between the fourth and sixth decade of life. Pediatric-onset of hyperuricemia and gout in clinical practice is rare and suggestive of a genetic disorder as PRPS1 superactivity [5] and hypoxanthine-guanine

* Correspondence: stiburkova@revma.cz
[1]Institute of Rheumatology, Na Slupi 4, 128 50 Prague 2, Czech Republic
[3]Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic
Full list of author information is available at the end of the article

© The Author(s). 2019 **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

phosphoribosyltransferase deficiency [6], especially when a strong family history is obtained.

Serum urate (SU) concentrations are heritable (0.38–0.63) [7–9], which is consistent with a significant genetic component. Over the past decade, genome-wide association studies (GWAS) and meta-analyses have led to an increase in our knowledge of the common genetic variants that influence SU concentrations. To date over 30 common sequence variants, which can affect hyperuricemia/gout have been revealed, most of which are in urate transporters [10, 11]. Urate transport is a complex process involving several transmembrane proteins that provide reabsorption (e.g., URAT1, GLUT9) and secretion (ABCG2). They are located on the apical and basolateral membrane of proximal tubule cells. ABCG2 also plays a significant role in regulating uric acid transport in the gastrointestinal tract [12]. The genetic predisposition to hyperuricemia is evidenced by monogenic diseases and population-based studies [13]. However, detailed knowledge of the degree to which genetic variants predict SU concentrations remains limited.

ABCG2 is a high-capacity urate transporter that plays a crucial role in renal urate overload and extra-renal urate underexcretion. Many previous studies have indicated that the common dysfunctional variants rs72552713 (p.Q126X) and rs2231142 (p.Q141K) increase the risk of gout and hyperuricemia, significantly influence the age of onset of gout, and are highly associated with a familial gout history [14, 15]. Variant p.Q126X, a common variant in the Japanese population, is a rare variant in European and African-American populations, whereas p.Q141K is a common variant in all these populations [16]. The ABCG2 population-attributable percent risk for hyperuricemia has been reported to be 29.2%, which is much higher than those with more typical environmental risks, i.e., BMI ≥ 25.0 (18.7%), heavy drinking (15.4%), and age (≥ 60 years old, 5.74%) [17]. In a GWAS of clinically defined gout, the ABCG2 locus showed the most significant association with gout susceptibility [11, 18, 19]. These findings indicate that common variants of ABCG2 are extremely important in gout pathogenesis. However, some variants of increased penetrance that are associated with gout are population specific and/or uncommon. Approximately 80% of Japanese patients with gout have been reported to possess either the p.Q126X or p.Q141K variant of ABCG2 [20], and these variants increased the risk of gout conferring an OR of more than 3 [14]. On the other hand, the 19 rare non-synonymous variants of ABCG2 identified in Japanese gout cohort [21] were not shared among the population samples that were tested in Czech gout cohort where eight rare non-synonymous ABCG2 variants were identified [15, 21]. These populations, with a specific distribution of dysfunctional ABCG2 variants, thus increase the risk of gout.

In our previous study, we analyzed the *ABCG2* gene [15] in a cohort of 145 subjects with gout. Our results showed a higher minor allele frequency of the p.Q141K variant in the gout patients (0.23) compared with the European-origin population (0.09) and were significantly more common among gout patients than among normouricemic controls (odds ratio = 3.26, $P < 0.0001$). Our analysis shows also an apparent shift in proportions of patients with non-synonymous alleles who are over-represented in an earlier age of onset categories and under-represented in older age of onset categories (χ2-test for the trend in proportions, $P = 0.010$). Such over-representation merited a detailed exploration. We therefore expanded the cohort by including both newly recruited gout patients (five pediatric-onset) and the hyperuricemic patients (17 pediatric-onset).

Until now, no study of individual variants of the ABCG2 transporter, using a well-characterized pediatric-onset cohort suffering from primary hyperuricemia/gout (i.e., considering clinical data on purine metabolism, the occurrence of associated diseases, familiar anamnesis, medication), has been performed. In this study, we analyzed the *ABCG2* gene in a Czech hyperuricemia and gout cohort focusing on pediatric-onset (before 18 years of age) patients. Our data show, for the first time, that ABCG2 dysfunction is a strong independent risk for pediatric-onset of hyperuricemia and gout.

Methods

The definition of hyperuricemia was as follows: (1) men > 420 μmol/l on two repeated measurements and (2) women and children under 15 years > 360 μmol/l on two repeated measurements, taken at least 4 weeks apart. Gouty arthritis was diagnosed according to the American College of Rheumatology criteria, i.e., (1) the presence of sodium urate crystals seen in the synovial fluid using a polarized microscope or (2) subjects meet 6 of 12 clinical criteria [22].

A cohort of 145 previously described gout subjects (9 with pediatric-onset) was enlarged to 176 gout patients (5 more with pediatric-onset), and a group of 58 hyperuricemic patients (17 pediatric-onset) was added. In total, 234 hyperuricemic or gout patients were recruited, 31 with a pediatric-onset (22 newly recruited patients). The age of ascertainment (hyperuricemia) and onset (gout) was determined as the age of laboratory diagnosis in case of asymptomatic hyperuricemia, or as the first symptoms of gout. For the sake of shortness, the term "onset" is used for both situations.

Asymptomatic hyperuricemic patients with pediatric-onset were identified through a random finding in a routine laboratory examination (e.g., for infectious diseases). In one case, the patient was ascertained based on a positive family history.

The pediatric-onset group of 31 patients from 30 families (one pair of siblings) consists of two separate sets. The first part consisted of 15 pediatric patients with hyperuricemia (10 subjects) and gout (5 subjects), mostly from the Department of Pediatrics and Adolescent Medicine, which includes the Metabolic Center (the only one in the Czech Republic involved in the Metabolic European Reference Network MetabERN). This set is complemented with 16 adult patients with pediatric-onset of hyperuricemia (7 subjects) and gout (9 subjects) from the Institute of Rheumatology (super-conciliar institute for the Czech Republic). All patients were residents of the Czech Republic, Central-European population, with no history or signs of renal diseases.

To explore the cause of hyperuricemia and gout, we performed a detailed metabolic investigation. The biochemical tests were performed using morning urine samples; 24-h urine collections were not available. Patients suffering from secondary gout and other purine metabolic disorders associated with pathological concentrations of SU (such as the reduced activity of hypoxanthine-guanine phosphoribosyltransferase and superactivity of phosphoribosyl pyrophosphate synthetase 1, i.e., resulting in increased excretion of xanthine and hypoxanthine in urine) were excluded. Pediatric subjects were specifically screened for kidney disorders (Fanconi syndrome and uromodulin-associated disorders) and for metabolic genetic disorders (glycogen storage disease, hereditary fructose intolerance, and mitochondrial disorders). Patients with such disorders were excluded from the study. The diagnostic algorithm and appropriate examinations are summarized in Fig. 1.

The analysis of *ABCG2* was performed from genomic DNA, as reported previously [8]. All tests were performed in accordance with standards set by the institutional ethics committees (no. 6181/2015). All statistical analyses were performed in the statistical language and environment R, v. 3.5. The Wilcoxon two-sample test and the Fisher exact test were used to compare group characteristics, the χ^2 goodness-of-fit test was used to compare minor allele frequencies, and the log-rank test was used to compare empirical distribution functions.

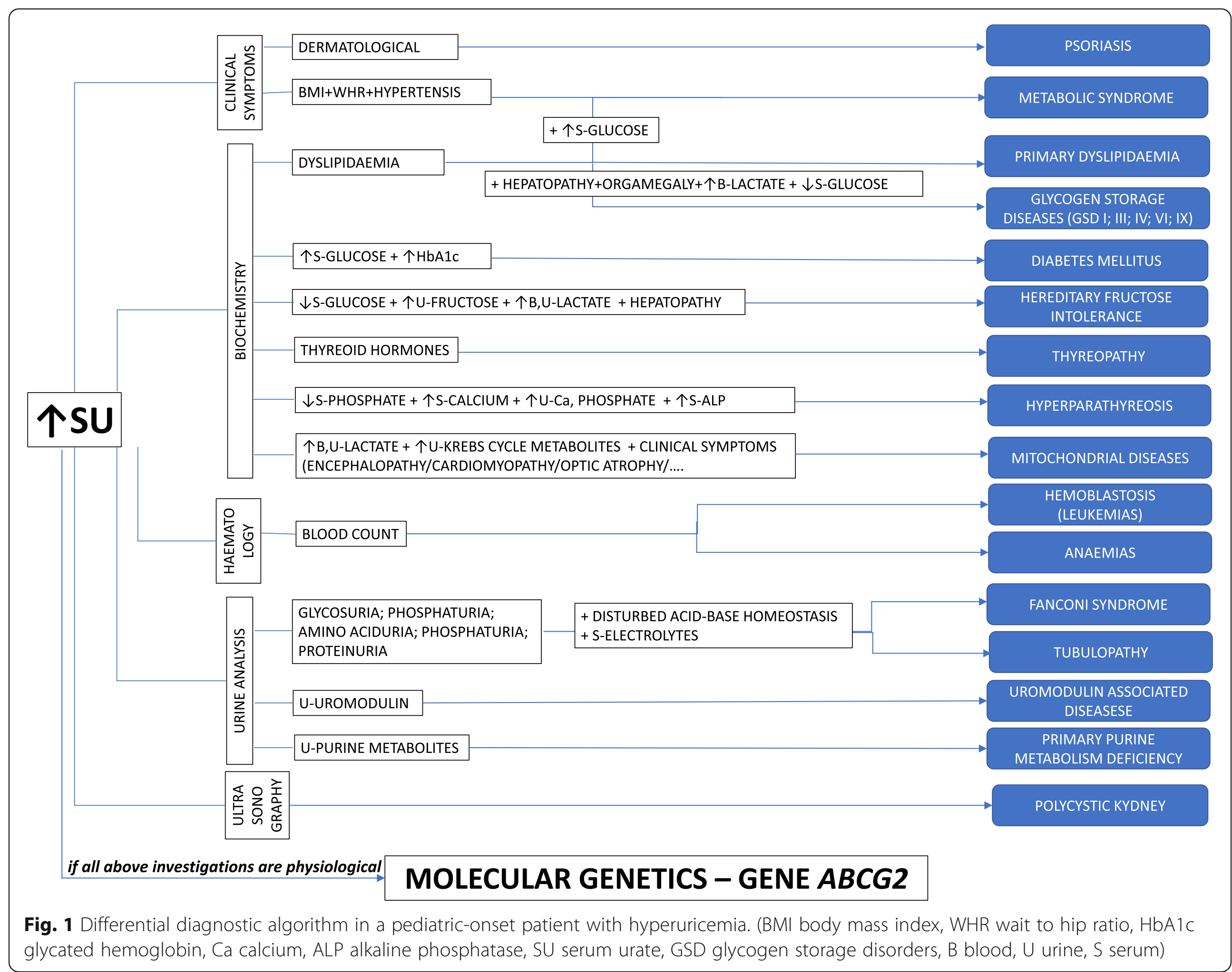


Fig. 1 Differential diagnostic algorithm in a pediatric-onset patient with hyperuricemia. (BMI body mass index, WHR wait to hip ratio, HbA1c glycated hemoglobin, Ca calcium, ALP alkaline phosphatase, SU serum urate, GSD glycogen storage disorders, B blood, U urine, S serum)

The role of possible confounders was checked through the Mantel-Haenszel test and logistic regression.

Results

In the cohort of 234 Caucasians suffering from hyperuricemia ($N = 58$) or primary gout ($N = 176$), we focused on 31 individuals with pediatric-onset of either hyperuricemia or gout. The main characteristics of both pediatric-onset and adult-onset groups are summarized in Table 1. Of the 31 pediatric-onset subjects, 19 non-consanguinity patients had at least of one non-synonymous allelic variant in the coding region of the *ABCG2* gene. Their main characteristics are summarized in Table 2.

Although pediatric-onset patients were consecutively included in the study, and even though they often did not show renal function impairment and were not taking medications known to change renal handling of uric acid (such as diuretics, aspirin, cyclosporine, pyrazinamide), the excretion fraction of urate (FE-U) was under the reference range in 14 of 19 patients with allelic *ABCG2* variants, while another two patients were at the lower border of the reference range. Only one patient has decreased urate levels in their urine (the lower limit was ≤

Table 1 Demographic, biochemical, and genetic characteristics of pediatric-onset ($N = 31$) and adult-onset ($N = 203$) cohorts

	Pediatric-onset ($N = 31$)		Adult-onset ($N = 203$)		Fisher's test *P* value
	N	**%**	***N***	**%**	
Sex M/F	26/5	83.9/16.1	174/29	85.7/14.3	0.786
Hyperuricemia/primary gout	17/14	58.1/41.9	41/162	79.8/21.2	< 0.0001
Familial occurrence	23	74.2	64	31.5	< 0.0001
Familial occurrence,					
1st degree	19	61.3	Not specified		–
2nd degree	4	12.9			
No treatment	15	48.4	36	17.7	< 0.0001
Allopurinol treatment	12	38.7	154	75.9	
Febuxostat treatment	4	12.9	13	6.4	
rs2231142					
GG	14	45.2	124	61.1	0.001
GT	10	32.3	72	35.5	
TT	7	22.6	7	3.4	
rs2231142, MAF	24	38.7	86	21.2	0.005
rs2231137, MAF **	1	1.6	7	1.7	1.000
rs769734146, MAF	1	1.6	1	0.2	0.623
rs750972998, MAF	1	1.6	0	0.0	0.278
rs199854112, MAF	1	1.6	0	0.0	0.278
	Median (IQR)	Range	Median (IQR)	Range	Wilcoxon's test *P* value
Age of onset, years	15.0 (4.0)	1–18	43.5 (24.2)	18–84	< 0.0001
Age now, years	19.0 (19.5)	3–59	55.0 (22.0)	19–90	< 0.0001
BMI now	25.1 (7.6)	16.0–41.0	29.0 (5.1)	19.5–50.0	< 0.0001
Max recorded SU, μmol/l ($N = 34/155$) #	522.0 (144.0)	314–796	481.0 (101.0)	252–770	0.021
SU on treatment, μmol/l ($N = 17/176$) #	419.0 (96.0)	300–608	371.5 (132.5)	252–770	0.091
FE-U on treatment ($N = 15/173$) #	3.2 (1.7)	1.6–5.5	3.2 (1.7)	0.9–14.3	0.403
Treatment dose, mg * ($N = 15/168$)	100 (200)	80–500	200 (200)	0–800	0.181

#For some parameters, there were missing data; in case missing data amounted to 5% or more, the real *N* is mentioned in parentheses in the form $N_{adolescent}/N_{adult}$
*Febuxostat dose was recomputed so that 40 mg febuxostat = 300 mg allopurinol
**Of the functional ABCG2 variants explored in [15], the five mentioned in the table were present among adolescent-onset patients. The variants rs372192400, rs753759474, rs752626614, and p.S476P (not annotated) had MAF 0.0025 among adult-onset patients, and rs34783571 had MAF 0.0049 among adult-onset patients. Neither of them appeared among adolescent-onset patients (*P* values of the test for difference were equal to 1.000)

Table 2 Demographic, biochemical, and genetic characteristics of pediatric-onset patients with non-synonymous ABCG2 variants ($N = 19$) and reference sequence of ABCG2 ($N = 12$)

Age of onset/ measurement	Sex	Hyperuricemia	Gout	ABCG2	Familiarity	BMI	Metabolic syndrome	SU	FE-U	U-U	u-Hypoxanthine	u-Xanthine
Year		Evidence		aa	Affected family members		Number of criteria				Ref. ranges ≤ 30	Ref. ranges ≤ 25
								μmol/L	%	mmol/mol creatinine		
17/21	M	Yes	No	p.[Q141K];[Q141K]	–	23	0	507	*4.7	0.2	3.5	2.1
16/31	M	Yes	Yes	p.[Q141K];[Q141=]	Pat. grandfather	39**	2	796	*2.0	0.16	2.5	9.8
18/40	F	Yes	Yes	p.[V12M];[V12=]	Father	21	0	546	*2.6	0.17	4.1	14.2
18/59	M	Yes	Yes	p.[Q141K];[Q141=]	Mother	30**	1	492	5.2	0.33	15.4	10.2
16/17	M	Yes	Yes	p.[Q141K];[Q141=]	Two brothers, pat. grandfather	22	0	540	*2.5	0.2	11.7	8.6
14/15	F	Yes	No	p.(Q141K)(;)(T434M)	–	24	0	495	*3.2	0.28	2.1	3.2
8/54	M	Yes	Yes	p.[Q141K];[Q141K]	Brother, father, pat. grandfather	41**	2	514	5	0.31	2.9	2.3
18/37	M	Yes	Yes	p.(Q141K)(;)(K360del)	–	30**	2	627	5	0.3	5.5	Under limit
13/14	M	Yes	Yes	p.[Q141K];[Q141=]	Father, pat. grandfather	24	1	621	*4.8	0.48	13.5	3.6
15/15	M	Yes	No	p.[Q141K];[Q141K]	Father	18	0	522	*3.9	0.3	13.5	8.2
15/39	M	Yes	Yes	p.[Q141K];[Q141=]	n/a	30**	2	670	*2.5	*0.09	24.3[a]	49.9*[a]
17/18	M	Yes	No	p.[T421A];[T421=]	Father	24	1	420	*3.9	0.15	2.4	Under limit
16/18	M	Yes	Yes	p.[Q141K];[Q141K]	–	27*	2	655	*4.3	0.35	1.2	2
6/11	F	Yes	No	p.[Q141K];[Q141=]	Mother, mat. grandmother	26*	1	430	*2.1	0.19	6.9	6.6
18/19	M	Yes	No	p.[Q141K];[Q141=]	Mother, mat. uncle	25	1	439	*4.9	0.27	5.4	2.9
12/12	F	Yes	No	p.[Q141K];[Q141=]	Brother, mother	30**	1	473	*2.3	0.29	5.4	6.5
14/14	M	Yes	No	p.[Q141K];[Q141K]	Father	22	0	465	6	0.31	15.3	27.5
13/14	M	Yes	Yes	p.[Q141K];[Q141K]	Mother	21	NA	506	*3.0	0.23	3.2	3.1
18/19	M	Yes	No	p.[Q141K];[Q141K]	Pat. grandmother	26*	1	730	5.5	0.35	3.4	2.8
11/20	M	Yes	No	No allelic variants	Mother	28*	0	662	*2.6	0.18	17	11.2
14/19	M	Yes	No	No allelic variants	–	27*	2	631	*2.0	0.17	13.1	18.4
1/3	F	Yes	No	No allelic variants	Mother	16	0	522	*2.6	0.47	9.4	8.7
15/21	M	Yes	No	No allelic variants	Pat. grandmother	35**	1	524	*3.8	0.17	20.1[b]	26.1*[b]
10/11	M	Yes	No	No allelic variants	Brother, father	20	0	384	*4.0	0.56	79*[a]	120*[a]
13/14	M	Yes	No	No allelic variants	Brother, father	24	0	435	*3.7	0.23	7.8	4.7
12/57	M	Yes	Yes	No allelic variants	Son, father, pat. grandfather	37**	2	442	*3.5	0.18	2.9	1.1
13/24	M	Yes	No	No allelic variants	Father, pat. grandfather and great-gr.	24	0	576	5.1	0.32	2	Under limit
17/18	M	Yes	Yes	No allelic variants	–	25	0	436	7.3	0.28	10	2.5
18/48	M	Yes	Yes	No allelic variants	–	26*	1	487	7.2	0.44	9.9	2.6
13/15	M	Yes	No	No allelic variants	Pat. and mat. grandfather	18	0	630	*2.9	0.46	3	4.3
18/45	M	Yes	Yes	No allelic variants	Brother, father	30**	3	610	5.0	0.35	1.7	1.2

*>< ref. range; [a]measurement with febuxostat therapy 80 mg/per day; [b]measurement with allopurinol therapy 150 mg/per day; SU < 15 years and female 120–340 μmol/l, male 120–416 μmol/l; FE-U < 13 years 5–20%, male 5–12%, female 5–15%; U-U < 15 years 0.1–1.0 mmol/mol creatinine, > 15 years 0.1–0.8 mmol/mol creatinine

0.1 mmol/L of UA per mmol/L of creatinine, which represents the lower 2.5th percentile of the entire reference range). In terms of percentiles, 7 patients were below the 5th percentile of the reference range (≤ 0.2 mmol/L of urate per mmol/L of creatinine), 13 patients were below the 20th percentile (≤ 0.3 mmol/L of urate per mmol/L of creatinine), and 18 patients were below the 40th percentile (≤ 0.4 mmol/L of urate per mmol/L of creatinine).

The analysis of *ABCG2* in the pediatric-onset cohort revealed two common non-synonymous variants (rs2231137 (p.V12M), rs2231142 (p.Q141K)) and three rare heterozygous non-synonymous variants (in-frame deletion rs750972998 (p.K360del), missense variant rs199854112 (p.T421A), and rs769734146 (p.T434M)). Seven of the 31 pediatric-onset patients were homozygous, and 10 were heterozygous for p.Q141K. This makes the minor allele frequency (MAF) of p.Q141K 38.7% compared to (1) the adult-onset MAF = 21.2% (OR = 2.4, P = 0.005), (2) 115 normouricemic controls MAF = 8.5% (OR = 6.8, $P <$ 0.0001 [15]), and (3) the European population (source 1000 Genomes Project Phase 3) MAF = 9.4% (OR = 5.7, $P <$ 0.0001, Fig. 2). To add to the picture, the MAF of p.Q141K was 35.3% (4 homozygotes, 4 heterozygotes) among the 17 pediatric-onset hyperuricemic patients and 42.9% (3 homozygotes, 6 heterozygotes) among the 14 pediatric-onset gout patients; meaning that a high frequency of p.Q141K was detected not only among symptomatic gout patients but among asymptomatic hyperuricemic patients as well.

As for other non-synonymous variants, one pediatric-onset gout patient was heterozygous for p.Q141K, and the rare p.K360del variant and another gout patient were heterozygous for the rare p.T421A variant. One hyperuricemic pediatric-onset patient was heterozygous for p.Q141K and the rare p.T434M variant. Those rare variants were not found among the adult-onset patients. One pediatric-onset hyperuricemic patient was heterozygous for p.V12M with a MAF of 1.6%, which was similar to the adult-onset MAF of 1.7%.

The in-frame three nucleotide deletion p.K360del (European MAF = 0.007) was located in the intracellular membrane-spanning domain. Variants p.T421A (European MAF < 0.0001) and p.T434M (European MAF < 0.0001) were located in the transmembrane domain 2. The in silico analysis (PROVEAN and SIFT) predicted a neutral impact for p.K360del and p.T421A and a deleterious impact for p.T434M.

In our pediatric-onset cohort, the mean age of hyperuricemia onset was 13.0 years, for gout the onset it was 15.4 years. Figure 3a shows the major role of p.Q141K homozygosity in the onset of gout/hyperuricemia from another point of view, while the median age of hyperuricemia/gout onset was around 40 years for heterozygotes and wild-type homozygotes, it was significantly lower, i.e., 21 years, for p.Q141K homozygotes (P = 0.005).

Among the 31 pediatric-onset patients, we found that 23 (74%) had affected family members (in 19 cases, 61% were first degree relatives). This was more than twice that seen in the adult-onset group (31%, $P <$ 0.0001, Table 1). The trend was even stronger for hyperuricemic patients (82%) compared to (62%) for adolescent-onset gout patients. Alternatively, while patients without a family history of hyperuricemia/gout had a median onset age of 47 years, patients with affected family members had a median onset age of 28 years ($P <$ 0.0001, Fig. 3b).

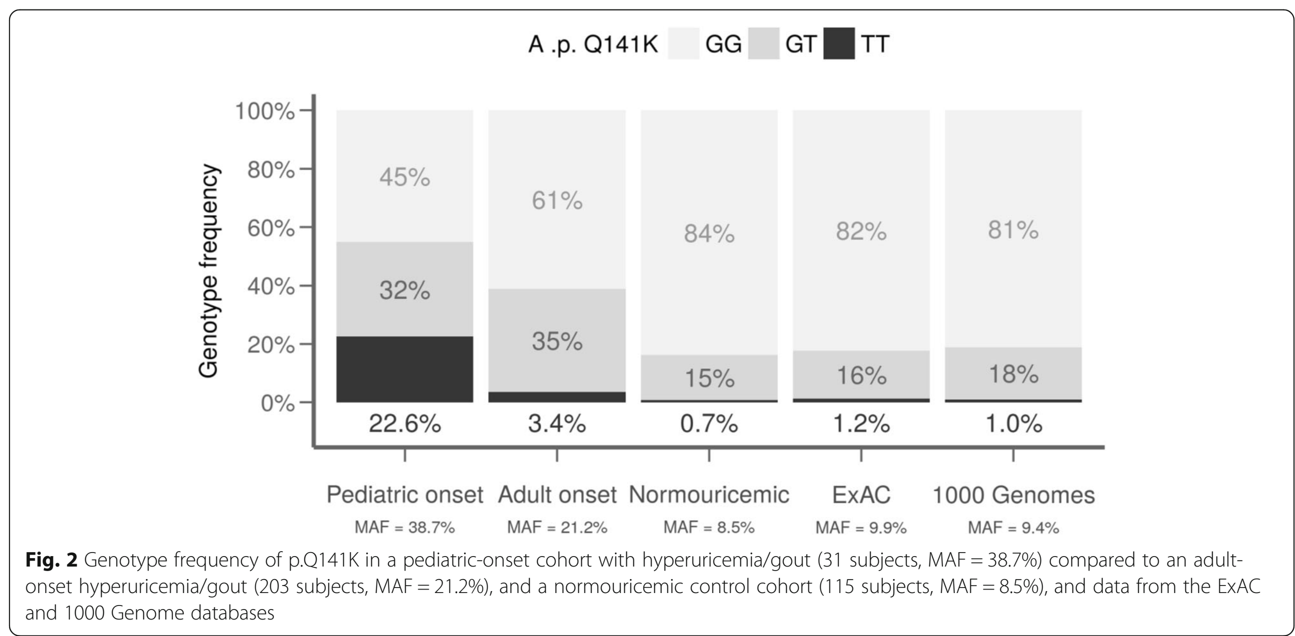


Fig. 2 Genotype frequency of p.Q141K in a pediatric-onset cohort with hyperuricemia/gout (31 subjects, MAF = 38.7%) compared to an adult-onset hyperuricemia/gout (203 subjects, MAF = 21.2%), and a normouricemic control cohort (115 subjects, MAF = 8.5%), and data from the ExAC and 1000 Genome databases

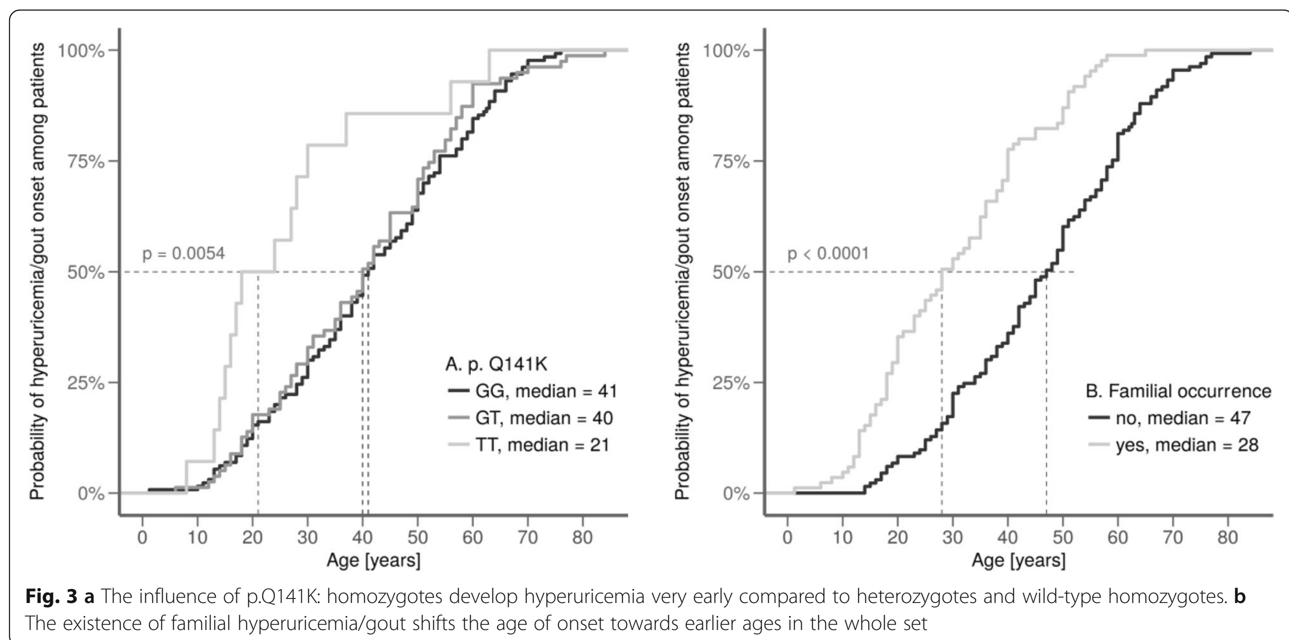


Fig. 3 **a** The influence of p.Q141K: homozygotes develop hyperuricemia very early compared to heterozygotes and wild-type homozygotes. **b** The existence of familial hyperuricemia/gout shifts the age of onset towards earlier ages in the whole set

Discussion

Elevated urate concentration is central to the pathogenesis of gout. Renal underexcretion of urate, due to the dysfunction of the ABCG2 high-capacity urate exporter, is a major contributor to hyperuricemia. This study identified a high frequency of ABCG2 variants, common and rare, in a cohort of pediatric-onset primary hyperuricemia and gout patients.

The common dysfunctional variant, p.Q141K, results in a 53% reduction in urate transport and has been reported to be a major genetic cause of gout in the European population [23]. The second most common variant in European, p.V12M (rs2231137, MAF 0.06), does not appear to affect urate transport [13] and a previously reported meta-analysis indicates that p.V12M has a gout protective effect (OR = 0.73, $P < 0.0001$) [15]. The contribution of ABCG2 variants in predicting primary hyperuricemia/gout seems to be limited mainly to the absence of a functional characterization of rare variants. However, the allelic variants can be easily analyzed, and their identification can suggest a hyperuricemia/gout risk prognosis. Functional characterizations of the rare variants p.K360del, p.T421A, and p.T434M are not available. However, an in silico analysis predicts a neutral impact for p.K360del and p.T421A and damaging impact for p.T434M. Future function studies regarding the impact of those variants are necessary to determine the correlation between functional studies and scores from prediction algorithms. For example, this relationship does not exist for the most frequent dysfunctional variant p.Q141K: a number of functional analyses in *Xenopus laevis* oocytes and HEK cells showed significantly decreased protein expression and function. However, p.Q141K is classified as tolerated by PolyPhen, SIFT, Provean, Mutation Taster, MutPred, and Human Splicing Finder software.

Our data showed that ABCG2 dysfunction was a strong independent risk for pediatric-onset hyperuricemia/gout: the MAF of p.Q141K was 38.7% compared to adult-onset with a MAF of 21.2%, compared to the normouricemic controls cohort with a MAF of 8.5%, and to the European population with a MAF of 9.4%. Compared to the whole hyperuricemia/gout cohort, there is an apparent shift in the proportion of patients with non-synonymous alleles who are over-represented in pediatric-onset patients (not only for gout but also for hyperuricemia) and under-represented in older age of onset categories. A hereditary component for pediatric-onset hyperuricemia/gout was further supported by the observation of a statistically significant association of familial hyperuricemia/gout in the pediatric-onset cohort: 74% in the pediatric-onset cohort and 33% in adult-onset ($P < 0.0001$). Taken together, our data suggested that ABCG2 genetic variants have a strong impact on the progression of hyperuricemia and gout in pediatric-onset patients and imply the importance of ABCG2 genotyping for the screening of high-risk individuals.

In summary, our findings confirmed a heritability component for hyperuricemia and gout. This relationship was recently demonstrated in a cohort of asymptomatic male offspring of parents with gout, in which the male offspring had a significantly higher frequency of hyperuricemia, urate under-excretion, and prevalence of monosodium urate crystal deposits [24]. A positive family history is an important diagnostic clue; however, it may be absent in de novo mutations and where

hyperuricemia/gout was either not diagnosed or not communicated to the rest of the family.

Detailed studies of SU in children are very rare. The first GWAS of SU was performed in the Viva La Familia Study [25]. This study found (1) SU concentrations were significantly heritable and (2) strong associations with genetic variants of the *SLC2A9* urate transporter. However, this GWAS did not extend the association of variants in the ABCG2 genetic locus with SU concentrations to children in a family-based study. Our analysis of *SLC2A9* coding regions in a pediatric-onset cohort revealed three synonymous variants in exon regions and three common heterozygous non-synonymous variants rs2276961 (p.G25R), rs3733591 (p.R294H), and rs2280205 (p.P350L). The MAF of these variants were similar to those in the adult-onset hyperuricemia/gout cohort and in the European population. Moreover, our previous study, which used association analysis together with functional and immunohistochemical characterization of these variants identified in the adult population, did not show any influence of these allelic variants on expression, subcellular localization, or urate uptake of GLUT9 transporters [26]. These different findings can partly be attributed to the population substructure and sample size considering that the majority of the GWAS with a strong association between ABCG2 and hyperuricemia/gout were conducted in European or Asian descent populations.

ABCG2 transporters are expressed on the apical epithelium membrane of the small intestines and colon, in erythrocyte membranes, apical membranes of kidney proximal tubular cells, and the canalicular membrane of hepatocytes [27]. Reduced and loss-of-function ABCG2 variants are associated with significantly decreased extra-renal clearance of urate [28]. The animal model of Abcg2-knockout mice showed increased serum urate and renal urate excretion and decreased intestinal urate excretion [12]. Moreover, a significant association between the common variant p.Q141K and an increased risk of a poor response to allopurinol has been described [29–31].

Data about the parameters of renal handling of urate in hyperuricemia/gout patients are not frequently reported in the literature. Perez-Ruiz et al. 2002 reported that renal underexcretion is the main mechanism for the development of primary hyperuricemia in gout, but even patients showing apparently high 24-h urate output showed lower urate clearance than controls, indicating that relative, low-grade underexcretion of urate was at work [32].

Our data in a pediatric-onset cohort with allelic ABCG2 variants showed a decrease in FE-U together with decreased urinary levels of urate. The published data about the relationship between ABCG2 dysfunction and the fractional excretion of urate are inconsistent. Matsuo et al. [18] observed an increase in FE-U associated with ABCG2 dysfunction. In contrast, Köttgen et al. [10] showed that, while the ABCG2 p.Q141K allele raised SU by 13 μmol/L (0.217 mg/dL) per risk allele in Europeans, there was a small decrease of 0.076% in FE-U ($P = 9.8 \times 10^{-3}$). Kannangara et al. [33] found no association between the *ABCG2* genotype and FE-U ($r = 0.02$, $P = 0.83$). Overall, these data indicate the very little clinical effect of the p.Q141K polymorphism on FE-U. These observations are in compliance with the concept of Ichida et al. [12] that the currently considered "overproduction type" hyperuricemia should be renamed to "renal overload type," comprising two subtypes: "extra-renal urate underexcretion" and genuine "urate overproduction." The common dysfunction of ABCG2 thus can cause a decrease of urate excretion via the extra-renal pathway rather than the renal pathway.

Hyperuricemia is an important risk factor for gout and has significant associations with several other conditions including hypertension, cardiovascular disease, and chronic kidney disease. The potential role of urate-lowering therapy, in the management of these "non-gout diseases," has been raised [34]. However, according to the American College of Rheumatology guidelines, therapy is not recommended for people with asymptomatic hyperuricemia. Our data showed, for the first time, that ABCG2 dysfunction is a strong independent risk in pediatric-onset hyperuricemia and gout where other factors that appear in adulthood, such as alcohol consumption, diuretic use, and increase in BMI, may further increase the risk of elevated serum urate levels. The high frequency of p.Q141K, which was detected not only among pediatric-onset gout patients but also among asymptomatic hyperuricemic pediatric-onset patients, confirmed the powerful effect of ABCG2 dysfunction on the early development of hyperuricemia and gout. Further studies into the progress of pediatric-onset hyperuricemia and its development into gout later in adult life are badly needed. Taken together, our findings strongly suggest the need for a discussion about the potential benefits of urate-lowering therapy after a diagnosis of hyperuricemia in pediatric-onset patients with ABCG2 dysfunction.

Our study has two important strengths: (1) our cohort included pediatric-onset hyperuricemia and gout patients, which are relatively rare, and includes detailed biochemical characteristics and family information and (2) we controlled for several potential confounders, such as kidney diseases and metabolic diseases that might have influenced measured SU concentrations. Limitations of this study must also be acknowledged: (1) the size of the studied group may not have been sufficiently large, and it is possible that some very rare ABCG2 and SLC2A9 associated variants may have gone undetected and (2) the number of frequent genetic variants of genes

encoding urate transporters was limited to the transcription regions and exon-intron boundaries.

Conclusion

The *ABCG2* gene is a well-established hyperuricemia/gout risk locus. In this work, we present the first study of ABCG2 allelic variants in a pediatric-onset hyperuricemia and gout cohort. The high frequency of genetic variants, common and rare, among patients with pediatric-onset hyperuricemia and gout needs to be kept in mind during differential diagnostic procedures and during therapy. Further analysis of the progress of asymptomatic hyperuricemia to gout is necessary: our data suggest a high frequency of the dysfunctional p.Q141K variant in both pediatric-onset subgroups (42.9% in gout, 35.3% in hyperuricemic) compared with adult gout onset (21.2%) and normouricemic controls (8.5%).

When working with patients, genetic data can contribute to more accurate disease prognoses, help personalized lifestyle advice, and improve therapy (urate-lowering therapy choice). The benefits of early initiation of urate-lowering therapy in pediatric-onset patients with a strong genetic risk require careful analysis. Additionally, a discussion regarding the value of a personalized approach to the management of hyperuricemia in clinical practice is necessary.

Abbreviations

BMI: Body mass index; FE-U: Excretion fraction of urate; GWAS: Genome-wide association studies; MAF: Minor allele frequencies; SU: Serum urate

Acknowledgements

We are grateful to all patients who took part in this study as well as our colleagues at the Institute of Rheumatology and Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and the General University Hospital in Prague, for their help in recruiting patients for the study (namely J. Zavada, T. Honzík, M. Magner). We would like to thank Jana Bohata (Institute of Rheumatology) for her assistance with the sequencing analysis of *SLC2A9*.

Funding

Supported by the Czech Ministry of Health: AZV 15-26693 A and projects for conceptual development of research organization 00023728 (Institute of Rheumatology), RVO VFN64165.

Availability of data and materials

Original data on sequencing analysis are available upon request.

Authors' contributions

BS contributed to the study conception and design of the study. PJ and KP contributed to the clinical observation of the study. BS, KatP, and MP contributed to the acquisition of data of the study. BS and MP contributed to the analysis and interpretation of data of the study. All authors were involved in drafting the manuscript or revising the content. All authors approved the final version for publication.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Institute of Rheumatology (reference number 6181/2015). All patients and healthy controls were fully informed of the aim of the study, and written informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

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

Received: 19 December 2018 Accepted: 5 March 2019
Published online: 20 March 2019

References

1. Zhu Y, Pandya BJ, Choi HK. Prevalence of gout and hyperuricemia in the US general population: the National Health and Nutrition Examination Survey 2007–2008. Arthritis Rheum. 2011;63:3136–41.
2. Hak AE, Curhan GC, Grodstein F, et al. Menopause, postmenopausal hormone use and risk of incident gout. Ann Rheum Dis. 2010;69(7):1305–9.
3. Harrold LR, Yood RA, Mikuls TR, et al. Sex differences in gout epidemiology: evaluation and treatment. Ann Rheum Dis. 2006;65(10):1368–72.
4. Harrold LR, Etzel CJ, Gibofsky A, et al. Sex differences in gout characteristics: tailoring care for women and men. BMC Musculoskelet Disord. 2017;18(1):108.
5. Zikánová M, Wahezi D, Hay A, et al. Clinical manifestations and molecular aspects of phosphoribosylpyrophosphate synthetase superactivity in females. Rheumatology (Oxford). 2018;57(7):1180–5.
6. Kostalova E, Pavelka K, Vlaskova H, et al. Hyperuricemia and gout due to deficiency of hypoxanthine-guanine phosphoribosyltransferase in female carriers: new insight to differential diagnosis. Clin Chim Acta. 2015;440:214–7.
7. Tang W, Miller MB, Rich SS, et al. Linkage analysis of a composite factor for the multiple metabolic syndrome: the National Heart, Lung, and Blood Institute Family Heart Study. Diabetes. 2003;52(11):2840–7.
8. Wilk JB, Djousse L, Borecki I, et al. Segregation analysis of serum uric acid in the NHLBI Family Heart Study. Hum Genet. 2000;106(3):355–9.
9. Yang Q, Guo CY, Cupples LA, et al. Genome-wide search for genes affecting serum uric acid levels: the Framingham Heart Study. Metabolism. 2005; 54(11):1435–4.
10. Köttgen A, Albrecht E, Teumer A, et al. Genome-wide association analyses identify 18 new loci associated with serum urate concentrations. Nat Genet. 2013;45(2):145–54.
11. Nakayama A, Nakaoka H, Yamamoto K, et al. GWAS of clinically defined gout and subtypes identifies multiple susceptibility loci that include urate transporter genes. Ann Rheum Dis. 2017;76(5):869–77.
12. Ichida K, Matsuo H, Takada T, et al. Decreased extra-renal urate excretion is a common cause of hyperuricemia. Nat Commun. 2012;3(3):764.
13. Vivante A, Hildebrandt F. Exploring the genetic basis of early-onset chronic kidney disease. Nat Rev Nephrol. 2016;12(3):133–46.
14. Matsuo H, Ichida K, Takada T, et al. Common dysfunctional variants in ABCG2 are a major cause of early-onset gout. Sci Rep. 2013;3:2014.
15. Stiburkova B, Pavelcova K, Zavada J, et al. Functional non-synonymous variants of ABCG2 and gout risk. Rheumatology (Oxford). 2017;56(11): 1982–92.
16. Sakiyama M, Matsuo H, Takada Y, et al. Ethnic differences in ATPbinding cassette transporter, sub-family G, member 2 (ABCG2/BCRP): genotype combinations and estimated functions. Drug Metab Pharmacokinet. 2014; 29(6):490–2.
17. Nakayama A, Matsuo H, Nakaoka H, et al. Common dysfunctional variants of ABCG2 have stronger impact on hyperuricemia progression than typical environmental risk factors. Sci Rep. 2014;4:5227.
18. Matsuo H, Yamamoto K, Nakaoka H, et al. Genome-wide association study of clinically defined gout identifies multiple risk loci and its association with clinical subtypes. Ann Rheum Dis. 2016;75(4):652–9.

19. Li C, Li Z, Liu S, et al. Genome-wide association analysis identifies three new risk loci for gout arthritis in Han Chinese. Nat Commun. 2015;13(6):7041.
20. Matsuo H, Takada T, Ichida K, et al. Common defects of ABCG2, a high-capacity urate exporter, cause gout: a function-based genetic analysis in a Japanese population. Sci Transl Med. 2009;1:5ra11.
21. Higashino T, Takada T, Nakaoka H, et al. Multiple common and rare variants of ABCG2 cause gout. RMD Open. 2017;3(2):e000464.
22. Wallace SL, Robinson H, Masi AT, et al. Preliminary criteria for the classification of the acute arthritis of primary gout. Arthritis Rheum. 1977; 20(3):895–900.
23. Woodward OM, Köttgen A, Coresh J, et al. Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout. Proc Natl Acad Sci U S A. 2009;106(25):10338–42.
24. Abhishek A, Courtney P, Jenkins W, et al. Monosodium urate crystal deposits are common in asymptomatic sons of people with gout - the sons of gout study. Arthritis Rheumatol. 2018;70(11):1847–52.
25. Voruganti VS, Laston S, Haack K, et al. Serum uric acid concentrations and SLC2A9 genetic variation in Hispanic children: the Viva La Familia study. Am J Clin Nutr. 2015;101(4):725–32.
26. Hurba O, Mancikova A, Krylov V, et al. Complex analysis of urate transporters SLC2A9, SLC22A12 and functional characterization of non-synonymous allelic variants of GLUT9 in the Czech population: no evidence of effect on hyperuricemia and gout. PLoS One. 2014;9(9):e107902.
27. Maliepaard M, Scheffer GL, Faneyte IF, et al. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. Cancer Res. 2001;61(8):3458–64.
28. Matsuo H, Nakayama A, Sakiyama M, et al. ABCG2 dysfunction causes hyperuricemia due to both renal urate underexcretion and renal urate overload. Sci Rep. 2014;4:3755.
29. Roberts RL, Wallace MC, Phipps-Green AJ, et al. ABCG2 loss-of-function polymorphism predicts poor response to allopurinol in patients with gout. Pharmacogenomics J. 2016;17(2):201–3.
30. Wen CC, Yee SW, Liang X, et al. Genome-wide association study identifies ABCG2 (BCRP) as an allopurinol transporter and a determinant of drug response. Clin Pharmacol Ther. 2015;97(5):518–25.
31. Petru L, Pavelcova K, Sebesta I, Stiburkova B. Genetic background of uric acid metabolism in a patient with severe chronic tophaceous gout. Clin Chim Acta. 2016;460:46–9.
32. Perez-Ruiz F, Calabozo M, Erauskin GG, Ruibal A, Herrero-Beites AM. Renal underexcretion of uric acid is present in patients with apparent high urinary uric acid output. Arthritis & Rheum. 2002;47(6):610–3.
33. Kannangara DRW, Phipps-Green AJ, Dalbeth N, et al. Hyperuricaemia: contributions of urate transporter ABCG2 and the fractional renal clearance of urate. Ann Rheum Dis. 2016;75(7):1363–6.
34. Stamp L, Dalbeth N. Urate-lowering therapy for asymptomatic hyperuricaemia: a need for caution. Semin Arthritis Rheum. 2017;46(4): 457–64.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

cells

MDPI

Article

Functional Characterization of Clinically-Relevant Rare Variants in *ABCG2* Identified in a Gout and Hyperuricemia Cohort

Yu Toyoda [1], Andrea Mančíková [2], Vladimír Krylov [2], Keito Morimoto [1], Kateřina Pavelcová [3], Jana Bohatá [3], Karel Pavelka [3], Markéta Pavlíková [4], Hiroshi Suzuki [1], Hirotaka Matsuo [5], Tappei Takada [1] and Blanka Stiburkova [3,6,*]

1 Department of Pharmacy, The University of Tokyo Hospital, Tokyo 113-8655, Japan; ytoyoda-tky@umin.ac.jp (Y.T.); kmorimoto-tky@umin.ac.jp (K.M.); suzukihi-tky@umin.ac.jp (H.S.); tappei-tky@umin.ac.jp (T.T.)
2 Department of Cell Biology, Faculty of Science, Charles University, 128 00 Prague 2, Czech Republic; andrea.mancikova@email.cz (A.M.); vladimir.krylov@natur.cuni.cz (V.K.)
3 Institute of Rheumatology, 128 50 Prague 2, Czech Republic; pavelcova@revma.cz (K.P.); bohata@revma.cz (J.B.); pavelka@revma.cz (K.P.)
4 Department of Probability and Mathematical Statistics, Faculty of Mathematics and Physics, Charles University, 121 16 Prague 2, Czech Republic; pavlikova@karlin.mff.cuni.cz
5 Department of Integrative Physiology and Bio-Nano Medicine, National Defense Medical College, Saitama 359-8513, Japan; hmatsuo@ndmc.ac.jp
6 Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital, 121 08 Prague 2, Czech Republic
* Correspondence: stiburkova@revma.cz; Tel.: +420-234-075-319

Received: 20 March 2019; Accepted: 15 April 2019; Published: 18 April 2019

Abstract: ATP-binding cassette subfamily G member 2 (ABCG2) is a physiologically important urate transporter. Accumulating evidence demonstrates that congenital dysfunction of ABCG2 is an important genetic risk factor in gout and hyperuricemia; recent studies suggest the clinical significance of both common and rare variants of ABCG2. However, the effects of rare variants of *ABCG2* on the risk of such diseases are not fully understood. Here, using a cohort of 250 Czech individuals of European descent (68 primary hyperuricemia patients and 182 primary gout patients), we examined exonic non-synonymous variants of *ABCG2*. Based on the results of direct sequencing and database information, we experimentally characterized nine rare variants of ABCG2: R147W (rs372192400), T153M (rs753759474), F373C (rs752626614), T421A (rs199854112), T434M (rs769734146), S476P (not annotated), S572R (rs200894058), D620N (rs34783571), and a three-base deletion K360del (rs750972998). Functional analyses of these rare variants revealed a deficiency in the plasma membrane localization of R147W and S572R, lower levels of cellular proteins of T153M and F373C, and null urate uptake function of T434M and S476P. Accordingly, we newly identified six rare variants of ABCG2 that showed lower or null function. Our findings contribute to deepening the understanding of *ABCG2*-related gout/hyperuricemia risk and the biochemical characteristics of the ABCG2 protein.

Keywords: ABCG2/BCRP; common disease; European cohort; exon sequence; functional study; gout susceptibility; heritability of serum uric acid; multiple rare variant; urate transporter; WGA

1. Introduction

Gout—a common disease with increasing global occurrence, typically causing severe pain and physical disability—results from hyperuricemia characterized by elevated serum uric acid (SUA)

concentrations [1]. Indeed, uric acid accumulation in the body causes hyperuricemia and subsequently increases the risk of gout. Although not all individuals with hyperuricemia develop symptomatic gout, the risk of gout increases in proportion to the elevation of urate in circulation. Humans do not have a functional uricase (urate-degrading enzyme) [2], so uric acid is the final metabolite in the purine catabolic pathway in humans. Hence, urate excretion from the body is necessary for the maintenance of uric acid homeostasis. Moreover, accumulating evidence suggests that the net amount of excreted uric acid is regulated mainly by urate transporters, such as urate transporter 1 (URAT1, a renal urate re-absorber) [3], solute carrier family 2 member 9 (SLC2A9, also known as glucose transporter member 9) [4–6], and ATP-binding cassette subfamily G member 2 (ABCG2, a high capacity urate exporter expressed in the kidney and intestine) [7–9]. Importantly, we recently reported that decreased extra-renal urate excretion caused by ABCG2 dysfunction is a common mechanism of hyperuricemia [7,10,11].

As a 655-amino acid *N*-linked glycoprotein expressed on the renal and intestinal brush border membranes, ABCG2 transports its substrates, such as urate, from the cytosol to the extracellular space in an ATP-dependent manner [12]. The human *ABCG2* gene consists of 16 exons and 15 introns and is located on chromosome 4q21–q22 [13], a region that showed one of the most significant associations with gout susceptibility in a series of Genome-wide association studies (GWASs) [8,14–19]. Hitherto, 45 allelic variants have been found in the *ABCG2* gene with most of the variants having been reported to have wide ethnic differences in allele frequency. In contrast, two single nucleotide polymorphisms have been reported in *ABCG2*: c.34G>A (V12M) and c.421C>A (Q141K) are recognized as common variants in a relatively large number of ethnic groups [20]. Biochemical characterizations revealed that the V12M variant has no effects on the expression and urate transport activity of ABCG2, whereas the Q141K variant decreases the cellular function of ABCG2 through reduction of its protein levels [12]. In addition to Q141K, ABCG2 Q126X (c.376C>T), which is common in the Japanese population but rare in other populations, has additionally been identified as a hyperuricemia- and gout-risk allele [9–11,21]. Given that these two variants are associated with a significantly increased risk of gout (odds ratio > 3) [9,21], the effects of common variants of *ABCG2* on gout susceptibility are likely to be genetically strong. However, although some information is available [22–24], the effects of rare variants are not fully understood.

In our previous study employing genetic analyses for gout patients in Japan and functional analyses of some non-synonymous rare variants of *ABCG2*, we found that multiple rare and common variants of *ABCG2* are independently associated with gout risk [22]. Nevertheless, this "Common Disease, Multiple Common and Rare variant" model for the association between *ABCG2* and gout needs to be further validated, especially in other populations. To this point, we recently identified ten non-synonymous variants of *ABCG2*, including two common variants and eight rare variants, using a cohort of 145 patients with gout in the Czech Republic [23,24]. Among the rare variants, only an intron splicing variant c.689+1G>A that causes a frameshift-dependent premature stop codon was identified as an *ABCG2* null variant via functional assays [24]. However, regarding the other rare variants, whether each one is associated with gout risk in the Czech population remains to be elucidated. Moreover, owing to lack of molecular analyses, the previous study could not determine the effects of each rare variant on the urate transport activity of ABCG2 [23]. Importantly, except for a rare variant K360del (c.1079_1081delAGA), the rare variants identified in the Czech population [23] were not found in the Japanese population [22]. Therefore, further studies on clinical risk prediction for gout in terms of rare *ABCG2* variants as well as the functional validation of such variants are required.

In the present study, we employed an enlarged cohort of 250 patients with hyperuricemia or gout to determine non-synonymous allelic variants of *ABCG2* related to the risk of such diseases. Based on the results of the sequence analysis of *ABCG2* and database information, nine rare exonic variants of ABCG2 (R147W, T153M, K360del, F373C, T421A, T434M, S476P, S572R, D620N) were subjected to functional analyses. Here, we demonstrate the novel effects of these rare exonic variants on the expression, cellular localization, and function of ABCG2 protein as a urate transporter via molecular analyses. Our

findings might support a "Common Disease, Multiple Common and Rare variant" hypothesis for the association between *ABCG2* and gout susceptibility in a European population. Additionally, these findings about population-specific genetic variants could deepen our understanding of the heritability of SUA levels and gout, which were previously estimated to be 27–41% and approximately 30%, respectively, in Europeans [14].

2. Materials and Methods

2.1. Clinical Subjects

The analyzed set consists of two groups: a hyperuricemic group consisting of 68 subjects and a gout group consisting of 182 subjects, which was an enlarged cohort compared to that containing 145 gout subjects described previously [23]. The main cohort of 58 primary hyperuricemia subjects and 177 subjects with gout was selected from patients of the Institute of Rheumatology, Prague, the Czech Republic. The 10 pediatric subjects with hyperuricemia and five with gout was selected from patients of the Department of Pediatrics and Adolescent Medicine, Charles University, Prague as previously described [25].

In terms of SUA, the definition of hyperuricemia was as follows: (1) men > 420 μmol/L on two repeated measurements taken at least 4 weeks apart and (2) women and children under 15 years > 360 μmol/L on two repeated measurements taken at least 4 weeks apart. Gouty arthritis was diagnosed according to the American College of Rheumatology criteria: (1) the presence of sodium urate crystals seen in the synovial fluid using a polarized microscope or (2) at least six of 12 clinical criteria being met [26]. Patients suffering from secondary gout and other purine metabolic disorders associated with pathological concentrations of SUA were excluded. The control group consisted of 132 normouricemic subjects, which was an enlarged control population compared to that containing 115 subjects described previously [23], was selected from among the personnel of the Institute of Rheumatology. All tests were performed in accordance with standards set by the institutional ethics committees, which approved the project in Prague (no.6181/2015).

2.2. PCR Amplification of ABCG2 and Sequence Analysis

ABCG2 coding regions were analyzed from genomic DNA, as described previously [23]. The reference sequence was defined as version ENST00000237612.7 (location: 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).

2.3. Materials

ATP, AMP, creatine phosphate disodium salt tetrahydrate, and creatine phosphokinase type I from rabbit muscle were purchased from Sigma-Aldrich (St. Louis, MO, USA) and [8-^{14}C]-uric acid (53 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). All other chemicals used were commercially available and of analytical grade.

2.4. Preparation of ABCG2 Mutants' Expression Vectors

To express human ABCG2 (NM_004827.3) fused with the EGFP-tag at its N-terminus (EGFP-ABCG2) and EGFP (control), we used an ABCG2/pEGFP-C1 plasmid that was generated in our previous study [27]. Using a site-directed mutagenesis technique, vectors expressing different ABCG2 variants were generated from an ABCG2 wild-type (WT)/pEGFP-C1 plasmid. To confirm the introduction of mutations, each variant cDNA of ABCG2 fused with EGFP generated in the plasmid was subjected to full sequencing using the BigDye Terminator v3.1 (Applied Biosystems Inc., Foster City, CA, USA) and an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems Inc.) according to the methods described in our previous study [27].

2.5. Cell Culture

Human embryonic kidney 293 cell-derived 293A cells were purchased from Life Technologies (Carlsbad, CA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France), 1% penicillin/streptomycin, 2 mM L-glutamine (Nacalai Tesque), and 1 × Non-Essential Amino Acid (Life Technologies) at 37 °C in an atmosphere of 5% CO_2 as described previously [27]. All experiments were carried out with 293A cells at passages 10–16.

Each vector plasmid for ABCG2 WT or its mutants was transfected into 293A cells by using polyethyleneimine MAX (PEI-MAX; 1 mg/mL in milliQ water, pH 7.0; Polysciences Inc., Warrington, PA, USA) as described previously [27] with some modifications. The amount of plasmid DNA used for transfection was adjusted to be the same for ABCG2 WT and its mutants. In brief, each plasmid was mixed with PEI-MAX (1 μg of plasmid/5 μL of PEI-MAX for 5×10^5 293A cells) in Opti-MEM™ (Thermo Fisher Scientific K.K., Kanagawa, Japan) and incubated for 20 min at room temperature. 293A cells were collected after treatment with a 2.5 g/L-Trypsin and 1 mmol/L-EDTA solution (Nacalai Tesque), followed by centrifugation at 1000× *g* for 5 min. The cell pellet was re-suspended in fresh DMEM, and the resulting suspension was mixed with plasmid/PEI-MAX mixture (50:50, v/v). Then, the cells were re-seeded at a concentration of 1.4×10^5 cells/cm^2 onto a collagen-coated glass bottom dish (cover size 22 × 22 mm and 0.16–0.19 mm thick; Matsunami Glass Inc., Tokyo, Japan) for confocal microscopy or cell culture plates for whole cell lysate preparation. The medium was replaced with a fresh medium after the first 24 h of incubation.

2.6. Preparation of Whole Cell Lysates

At indicated periods after the plasmid transfection, whole cell lysates were prepared in an ice-cold lysis buffer A containing 50 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol, 1% (w/v) Triton X-100, and a protease inhibitor cocktail for general use (Nacalai Tesque) as described previously [28]. Protein concentration of the whole cell lysate was quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard according to the manufacturer's protocol. For glycosidase treatment, the whole cell lysate samples were incubated with PNGase F (New England Biolabs Japan Inc., Tokyo, Japan) (1.25 U/μg of protein) at 37 °C for 10 min as described previously [29,30], and then subjected to immunoblotting.

2.7. Preparation of ABCG2-Expressing Plasma Membrane Vesicles

The membrane vesicles were prepared from ABCG2-expressing 293A cells as described previously [24] with minor modifications. In brief, 293A cells seeded on 145-mm tissue culture dishes (CELLSTAR, 145 × 20 mm; Greiner Japan, Tokyo, Japan; 10 dishes/variant) at approximately 80% confluency were transiently transfected with each plasmid using PEI-MAX (24 μg of plasmid/120 μL of PEI-MAX/145-mm dish). Forty-eight hours after the transfection, the cells were collected and subjected to plasma membrane isolation. The 293A cells were suspended in a hypotonic buffer (1 mM Tris/HCl, 0.1 mM EDTA, pH 7.4, and the protease inhibitor cocktail for general use) and gently stirred for 1 h on ice. Then, the solution was ultra-centrifuged at 100,000× *g* for 30 min at 4 °C, and the pellet was diluted with an ice-cold isotonic buffer (10 mM Tris/HCl, 250 mM sucrose, pH 7.4), then homogenized with a dounce tissue homogenizer (Cat#: 2-4527-03; ASONE, Osaka, Japan) on ice. The crude membrane fraction was carefully layered over a 38% sucrose solution (5 mM Tris/HEPES, pH 7.4). After ultra-centrifugation at 280,000× *g* for 45 min at 4 °C, the turbid layer at the interface was collected, suspended in the isotonic buffer, and ultra-centrifuged at 100,000× *g* for 30 min at 4 °C. The membrane fraction was collected and re-suspended in an isotonic buffer and then passed through a 25-gauge needle. The resulting plasma membrane vesicles were rapidly frozen in liquid N_2 and kept at −80 °C until used. The protein concentration was measured using the BCA Protein Assay Kit.

2.8. Immunoblotting

Expression of ABCG2 protein in whole cell lysate and plasma membrane vesicles was examined by immunoblotting according to the methods reported in our previous study [31]. In brief, the prepared samples were mixed with a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer solution containing 10% 2-mercaptoethanol, separated by electrophoresis on poly-acrylamide gels, and then transferred to Polyvinylidene Difluoride membranes (Immobilon; Millipore Corporation, Billerica, MA, USA) by electroblotting at 15 V for 60 min.

For blocking, the membrane was incubated in Tris-buffered saline containing 0.05% Tween 20 and 3% BSA (Nacalai Tesque) (TBST-3% BSA) for 1 h at room temperature. Blots were probed with a rabbit anti-EGFP polyclonal antibody (A11122; Life Technologies; diluted 1000 fold in TBST 0.1% BSA), a rabbit anti-α-tubulin antibody (ab15246; Abcam Inc., Cambridge, MA, USA; diluted 1000 fold), or a rabbit anti-Na^+/K^+-ATPase α antibody (sc-28800; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; diluted 1000 fold) followed by incubation with a donkey anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP)-conjugated antibody (NA934V; diluted 3000 fold). HRP-dependent luminescence was developed using the ECL™ Prime Western Blotting Detection Reagent (GE Healthcare UK Ltd., Buckinghamshire, UK) and detected using a multi-imaging Analyzer Fusion Solo 4™ system (Vilber Lourmat, Eberhardzell, Germany). The band density was quantified using the Fusion software (Vilber Lourmat) to assess protein expression levels.

2.9. Confocal Laser Scanning Microscopic Observation

For confocal laser scanning microscopy, 48 h after the transfection, 293A cells were fixed with ice-cold methanol for 10 min and then washed three times with PBS (-). Then, the cells were incubated with TO-PRO-3 Iodide (Molecular Probes, Eugene, OR, USA) diluted 250 fold in PBS (-) for 10 min at room temperature. After the visualization of nuclei, the cells were washed with PBS (-) twice and then mounted in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA). To analyze the localization of EGFP-fused ABCG2 protein, fluorescence was detected using a FV10i Confocal Laser Scanning Microscope (Olympus, Tokyo, Japan).

To visualize plasma membranes, we used a fluorescent wheat germ agglutinin (WGA) conjugate (WGA, Alexa Fluor® 594 conjugate; Thermo Fisher Scientific K.K.) according to the manufacturer's protocol, with minor modifications. Specifically, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then washed three times with PBS (-). Then, the cells were treated with WGA (10 μg/mL) in PBS (-) for 10 min at room temperature. After washing with PBS (-), the cells were treated with PBS (-) containing 0.02% (w/v) Triton X-100 and then subjected to TO-PRO-3 Iodide staining as described above.

2.10. Urate Transport Assay

The urate transport assay with ABCG2-expressing plasma membrane vesicles was conducted using a rapid filtration technique [31–33]. As described in our previous report [31], we used 20 μM of radiolabeled urate in reaction mixtures (10 mM Tris/HCl, 250 mM sucrose, 10 mM $MgCl_2$, 10 mM creatine phosphate, 1 mg/mL creatine phosphokinase, 0.25 mg/mL each plasma membrane vesicle, pH 7.4, and 50 mM ATP or AMP as the absence of ATP) which were incubated for 10 min at 37°C for the evaluation of ABCG2 function as an ATP-dependent urate transporter. The urate transport activity was calculated as an incorporated clearance defined as the incorporated level of urate [DPM/mg protein/min]/urate level in the incubation mixture [DPM/μL]. ATP-dependent urate transport was calculated by subtracting the urate transport activity in the absence of ATP from that in the presence of ATP. Furthermore, ATP-dependent urate transport activities of each ABCG2 variant were described as the percent of the activity of ABCG2 WT.

In addition, because urate uptake assays for the identified ABCG2 variants were conducted using a *Xenopus leavis* oocyte expression system, the relevant information is shown in the Supplemental Data [34].

2.11. Schematic Illustration of ABCG2 Protein

According to the structure of human ABCG2, which was determined by cryo-electron microscopy [35], 2D topology of ABCG2 protein was constructed, and the obtained-topology data were plotted and modified using the T(E)Xtopo package [36]. Information of highly conserved peptide motifs among ABC transporters, such as Walker A, Walker B, and signature C, in ABCG2 protein were obtained from a previous report [37].

2.12. Statistical Analysis

Clinical data were summarized as absolute and relative frequencies for categorical variables and as medians with interquartile range (IQR) and data range for continuous variables. Hyperuricemic and gout sub-cohorts were compared using either Fisher's exact test for categorical variables or Wilcoxon sum-rank test for continuous variables. Kruskall-Wallis test (one-way non-parametric ANOVA) and pairwise Wilcoxon tests with Bonferroni correction (for post-hoc comparisons) were used to compare ages of gout/hyperuricemia onset among groups with zero, one, and two allelic variants of interest. Fisher's exact test was used to compare the family gout history and the presence of alleles of interest. For individual allelic variants, minor allele frequency (MAF) data were excreted from the public databases NCBI and ExomAc and compared to cohort MAF in this study using a binomial test. Statistical language and environment R (version 3.5.0) (R Foundation for Statistical Computing, Vienna, Austria) was used for clinical data analyses.

Regarding experimental results, all statistical analyses were performed by using EXCEL 2013 (Microsoft Corp., Redmond, WA, USA) with Statcel3 add-in software (OMS publishing Inc., Saitama, Japan) according to our previous study [38]. The specific statistical tests that were used for individual experiments are described in the figure legends. Statistical significance was defined in terms of P values less than 0.05 or 0.01.

3. Results and Discussion

3.1. Subjects

The main demographic and biochemical characteristics of the subjects are summarized in Table 1. Our cohort (a total of 250 patients recruited from the Czech Republic) consisted of 182 individuals with primary gout (166 male/16 female) among which 66 patients (36% of the cohort) had a positive family history of gout. In a sub-cohort of 68 hyperuricemia patients (48 male/20 female), 31 patients (46% of the sub-cohort) had a positive family history. With respect to medications, 58 patients (23%) did not take any urate-lowering therapy medication, 175 patients (70%) took allopurinol, and 17 patients (7%) took febuxostat (the details are summarized in Supplemental Table S1). Considering that *ABCG2* is one of the most influential genetic risk factors for gout and hyperuricemia, we next examined a relationship between non-synonymous mutations in *ABCG2* and the risk of such diseases in our gout/hyperuricemia cohort.

Table 1. Demographic, biochemical, and genetic characteristics of gout and hyperuricemia cohorts.

Characteristic		All Patients (N = 250)		Gout Patients (N = 182)		Hyperuricemia Patients (N = 68)		P-Value [#]
		N	%	N	%	N	%	
Gender	Male	214	85.6	166	91.2	48	70.6	0.0002
	Female	36	14.4	16	16.8	20	29.4	
Familial occurrence		97	38.8 (40.2 *)	66	36.3 (36.5 *)	31	45.6 (51.7 *)	0.0480

Table 1. *Cont.*

Characteristic	*N*	Median (IQR)	Range	*N*	Median (IQR)	Range	*N*	Median (IQR)	Range	*P*-Value †
Age at examination [years]	250	51.5 (25.0)	3–90	182	54.0 (21.0)	11–90	68	36.0 (42.0)	3–78	<0.0001
BMI at examination	209	28.4 (5.8)	16–50	151	28.4 (5.4)	19.5–50	58	28.1 (6.4)	16–41	0.0822
Gout/hyperuricemia onset $ [years]	236	40.0 (28.0)	1.2–84	181	40.0 (24.0)	8–84	55	27.0 (40.5)	1.2–76	0.0070
SUA at examination, with medication [µmol/L]	201	375.0 (134.0)	163–808	159	372.0 (128.0)	163–808	42	424.0 (140.0)	240–628	0.0515
FEUA at examination, with medication	194	3.4 (2.0)	0.9–14	158	3.4 (1.9)	0.9–14	36	3.8 (2.1)	1.3–8	0.5862

Fisher's exact test for categorical and † Wilcoxon two-sample sum rank test were used to compare the gout sub-group with the hyperuricemia sub-group; * relative frequencies when missing information about familial occurrence was excluded; $ onset (gout) and age of ascertainment (hyperuricemia). IQR, interquartile range; SUA, serum uric acid; FEUA, fractional excretion of uric acid.

3.2. Identification of ABCG2 Variants in a Gout/Hyperuricemia Cohort

To determine non-synonymous allelic variants in *ABCG2* relating to the risk of gout or hyperuricemia, we performed targeted exon sequencing of *ABCG2* in our cohort. The results—all identified variants and their allele frequencies—are summarized in Table 2. We identified 11 exonic non-synonymous variants including two common variants: p.V12M (rs2231137) and p.Q141K (rs2231142), which are well-characterized, nine rare variants: p.R147W (rs372192400), p.T153M (rs753759474), p.F373C (rs752626614), p.T421A (rs199854112), p.T434M (rs769734146), p.S476P (not annotated), p.S572R (rs200894058), p.D620N (rs34783571), and a three-base deletion p.K360del (rs750972998). The *ABCG2* genotype frequency of our gout/hyperuricemia cohort was compared to MAF data from the Exome Aggregation Consortium (http://exac.broadinstitute.org/) and 1000 Genomes (http://www.internationalgenome.org/), which addressed a European-origin population (Table 2). The frequency of Q141K (a common variant linked to the risk of gout/hyperuricemia [9–11,21]) in our cohort (0.238)—0.247 in the gout sub-cohort (87 heterozygotes and 16 homozygotes) and 0.213 in the hyperuricemia sub-cohort (19 heterozygotes and 5 homozygotes)—was significantly higher than that was reported in the European-origin population (0.094). Several rare variants were present at higher allele frequencies in our cohort compared with the control population as shown in Table 2. However, as a limitation in this study, we note that our cohort population was not sufficiently large for a detailed analysis of the individual effect of each rare variant, owing to very low MAF on the disease risk. To overcome this limitation, analysis of a larger data set will be needed in the future.

Next, we focused on the combination of the common and rare variants of *ABCG2*. In total, 115 gout/hyperuricemia patients (46.0% of case) harbored at least one of the identified 11 non-synonymous variants (Supplemental Table S1). Among the patients, 23 individuals harbored two non-synonymous variants: 16 patients were homozygous for Q141K while seven patients had Q141K and one of the other variants. No patients harbored three or more of the non-synonymous variants, and no participants were found to be homozygous for rare variants. Interestingly, we found an association between the number of non-synonymous variants in *ABCG2* and the age of onset of gout/hyperuricemia in our cohort (Supplemental Figure S2). The median age of onset among patients with zero, one, or two variants were 42, 40, and 22 years, respectively ($P < 0.0002$, Kruskal-Wallis test). Post-hoc analysis revealed that the group with two variants significantly differs from the other groups, suggesting that an increased number of non-synonymous alleles might cause an earlier age of onset.

Regarding family history, patients with non-synonymous variants had familial gout in 54 of 111 cases (48.6%), whereas patients who did not have non-synonymous variants had familial gout in 43 of 130 (33%) cases (Figure 1). This association was statistically significant (odds ratio = 1.91, 95% CI: 1.10, 3.34; $P = 0.0176$, Fisher's exact test). Considering that a previous study with a small cohort had found only borderline significance ($P = 0.053$) for this association [23], our result can be considered to be a strength of this study. As previously described, these findings suggest the epidemiological

importance of *ABCG2* common and rare variants for gout/hyperuricemia risk in the Czech population, supporting a "Common Disease, Multiple Common and Rare variant" hypothesis for the association between *ABCG2* and gout, which was proposed in our previous study on Japanese gout patients [22].

Table 2. Identified ABCG2 variants and their mutant allele frequency.

ABCG2 Variants (rs Number)	Gout (N = 182)		Hyperuricemia (N = 68)		All Patients (N = 250)			Normouricemia (N = 132)		Population MAF	P-Value #
	N	MAF	N	MAF	N	MAF	95% CI *	N	MAF		
Common											
V12M (rs2231137)	8	0.0220	1	0.0074	9	0.0180	0.0083, 0.0339	5	0.0189	0.0610	<0.0001
Q141K (rs2231142)	90	0.2473	29	0.2132	119	0.2380	0.2013, 0.2778	22	0.0833	0.0940	<0.0001
Rare	**N**		**N**		**N**	**MAF**		**N**		**Population MAF**	
R147W (rs372192400)	1		0		1	0.0020		1		0.0001	
T153M (rs753759474)	1		0		1	0.0020		0		0.0001	
F373C (rs752626614)	1		0		1	0.0020		0		0.0000	
T421A (rs199854112)	0		1		1	0.0020		0		0.0001	
T434M (rs769734146)	1		1		2	0.0040		1		0.0000	
S476P (Not annotated)	1		0		1	0.0020		0		*No data*	
S572R (rs200894058)	1		0		1	0.0020		0		0.0002	
D620N (rs34783571)	2		0		2	0.0040		0		0.0040	
K360del (rs750972998)	1		0		1	0.0020		0		0.0001	

For common variants: * A 95% confidence interval (CI) for minor allele frequency (MAF) was estimated; # Binomial test was used (all patients vs population control). Comparative information on the obtained MAFs for the cases in this study versus those for the 132 normouricemic subjects in the control group from the Institute of Rheumatology (Prague, Czech Republic) is shown. For rare variants: due to very small counts of each rare variant, MAF for the whole sample of 250 patients was given as well as population MAF (if available).

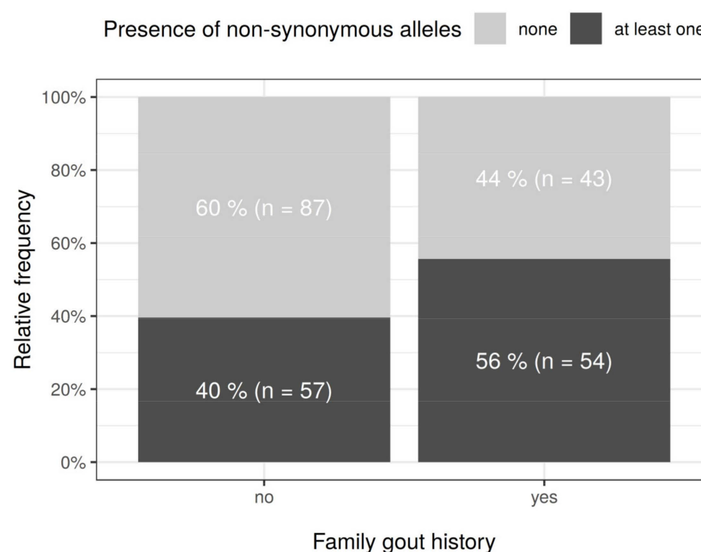


Figure 1. Family history of gout and the numbers of allelic variants in *ABCG2*. Depending on the presence or absence of family gout history, proportion of gout patients with or without any of the 11 non-synonymous alleles identified in *ABCG2* is summarized. The presence of *ABCG2* allelic variants was associated with the gout family history (odds ratio = 1.91, 95% CI: 1.10, 3.34; $P = 0.0176$, Fisher's exact test).

Importantly, regarding the identified rare variants of ABCG2, T421A was a novel variant and the effects of the other variants on the protein function of ABCG2 as a urate transporter have not yet been characterized. Thus, we conducted a series of functional analyses to assess the rare variants that we identified (Table 2); the positions of each mutant are described in Figure 2.

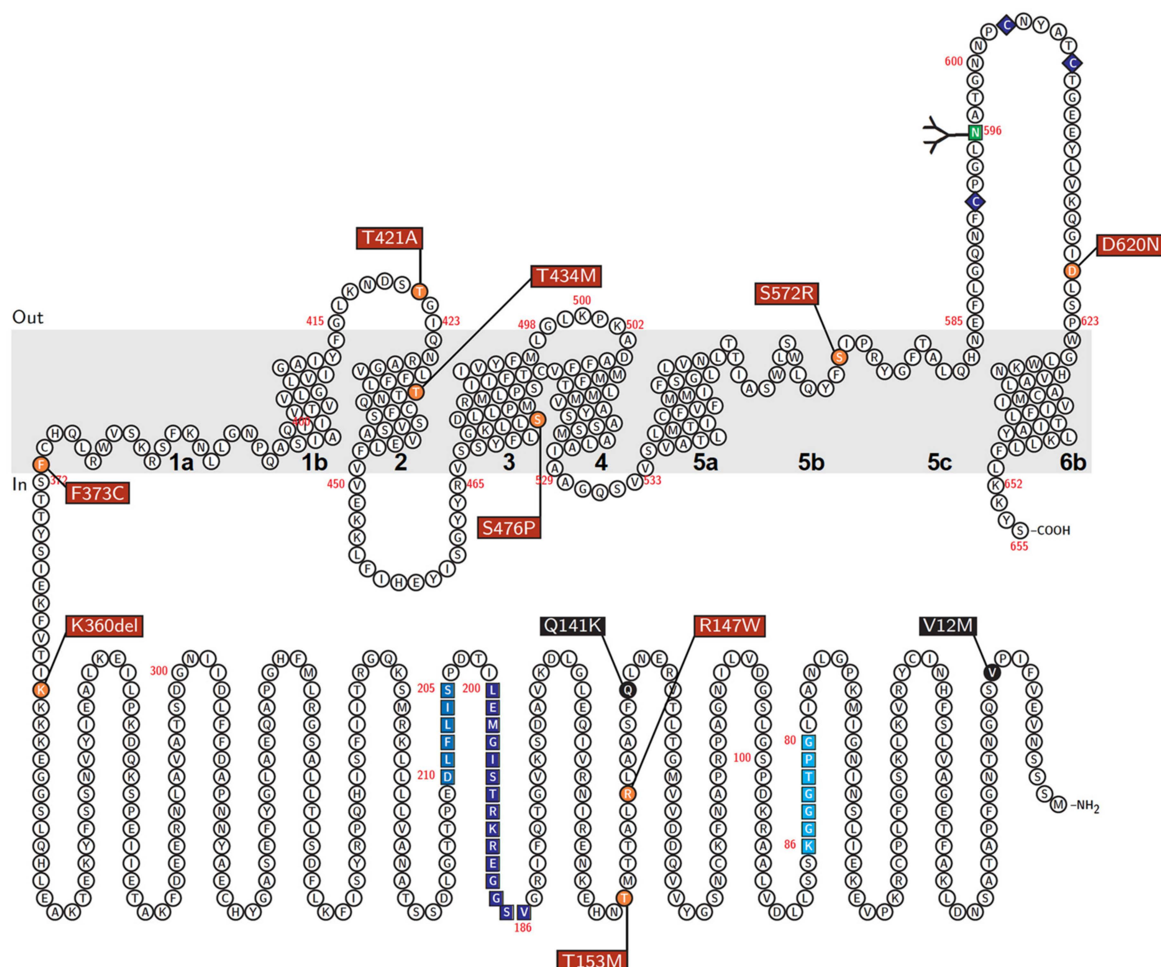


Figure 2. Schematic illustration of a putative topological model of human ABCG2 protein. Red box, rare variants analyzed in the present study; *Black box*, common variants. Helices in the transmembrane domain are numbered (1a to 6b) according to a previous study [34]. Asn596 is an *N*-linked glycosylation site. Unique motifs common to ABC proteins: Walker A (amino acids 80–86), Walker B (amino acids 205–210), and signature C (amino acids 186–200) in ABCG2 protein are indicated by colors.

3.3. Effect of Each Mutation on the Glycosylation Status of ABCG2 Protein

To investigate the effect of each non-synonymous mutation in the *ABCG2* gene on the intracellular processing and function of ABCG2 protein, we transiently expressed ABCG2 WT and its nine variants (R147W, T153M, K360del, F373C, T421A, T434M, S476P, S572R, and D620N) in 293A cells. Each expression vector was prepared using a site-directed mutagenesis technique from an ABCG2 WT/pEGFP-C1 plasmid and confirmed by DNA sequence. To address the former topic, we first performed immunoblot analyses using the anti-EGFP antibody for the detection of EGFP-tagged ABCG2. The results revealed that the R147W and S572R variants did not produce a matured glycoprotein (Figure 3A). Moreover, using *N*-glycosydase (PNGase F)-treated whole cell lysates, we compared the levels of ABCG2 protein in the cells among the variants and WT (Figure 3B). Since the Q141K variant reportedly reduces ABCG2 protein level [39–41], we employed this variant as a control in this study. The reducing effect of Q141K on the ABCG2 protein level detected in the present study

(Figure 3) was almost comparable to that in a previous study [41] which, like us, also used a similar transient expression strategy to validate *ABCG2* mutations in vitro. This consistency supports the reliability of the following results. Semi-quantitative evaluation of immunoblot signals showed that each variant decreased the levels of ABCG2 protein; the R147W and S572R variants showed significant effects (<25% of WT), the T153M and F373C variants had profound effects on reducing ABCG2 protein level (<50% of WT), and the other variants only mildly affected the protein expression of ABCG2.

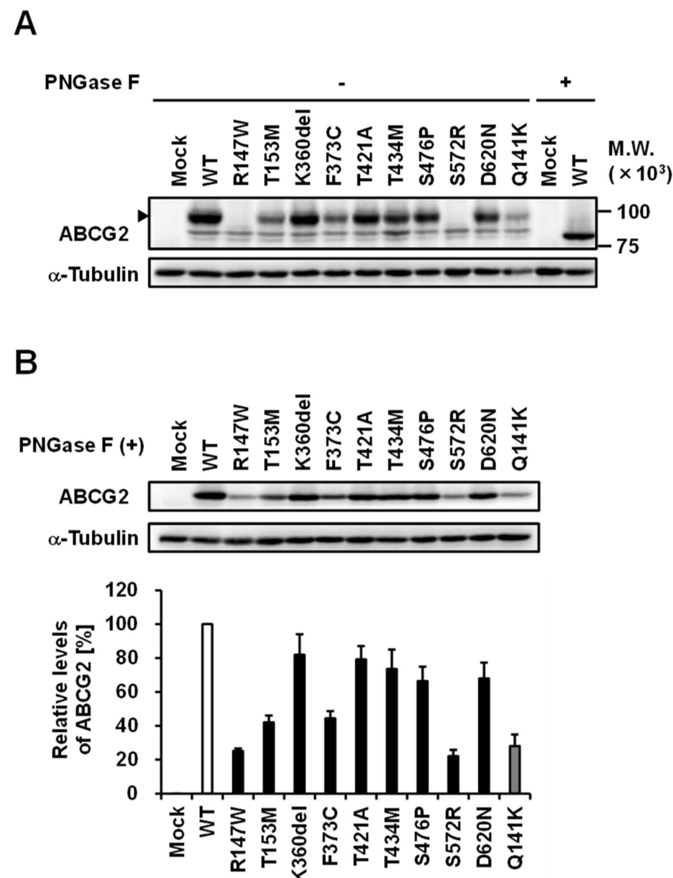


Figure 3. Effects of each mutation on the maturation status and protein levels of ABCG2 in transiently transfected 293A cells. (**A**) Immunoblot detection of ABCG2 wild-type (WT) and its variants in the whole cell lysate samples that were prepared 48 h after the transfection. Arrowhead, matured ABCG2 as a glycoprotein; α-Tubulin, a loading control. (**B**) Relative protein levels of ABCG2 WT and its variants. The signal intensity ratio (ABCG2/α-tubulin) of the immunoreactive bands was determined and normalized to that in ABCG2 WT-expressing cells. Data are expressed as the mean ± SD. $n = 3$.

3.4. *Effect of Each Mutation on the Intracellular Localization of ABCG2 Protein*

We next investigated the effect of each mutation on the intracellular localization of the ABCG2 protein. Confocal microscopic observation demonstrated that ABCG2 WT and most of the variants localized on the plasma membrane of the cells (Figure 4A). In contrast, the R147W and S572R variants that hardly experienced protein maturation processing in the cells were not localized on the plasma membrane. This result was supported by high magnification image observation under a fluorescent WGA (a plasma membrane probe)-treated condition (Figure 4B). Thus, we concluded that the R147W and S572R variants have little function as a urate transporter on the plasma membrane and we performed further analyses for the other seven variants, as described below.

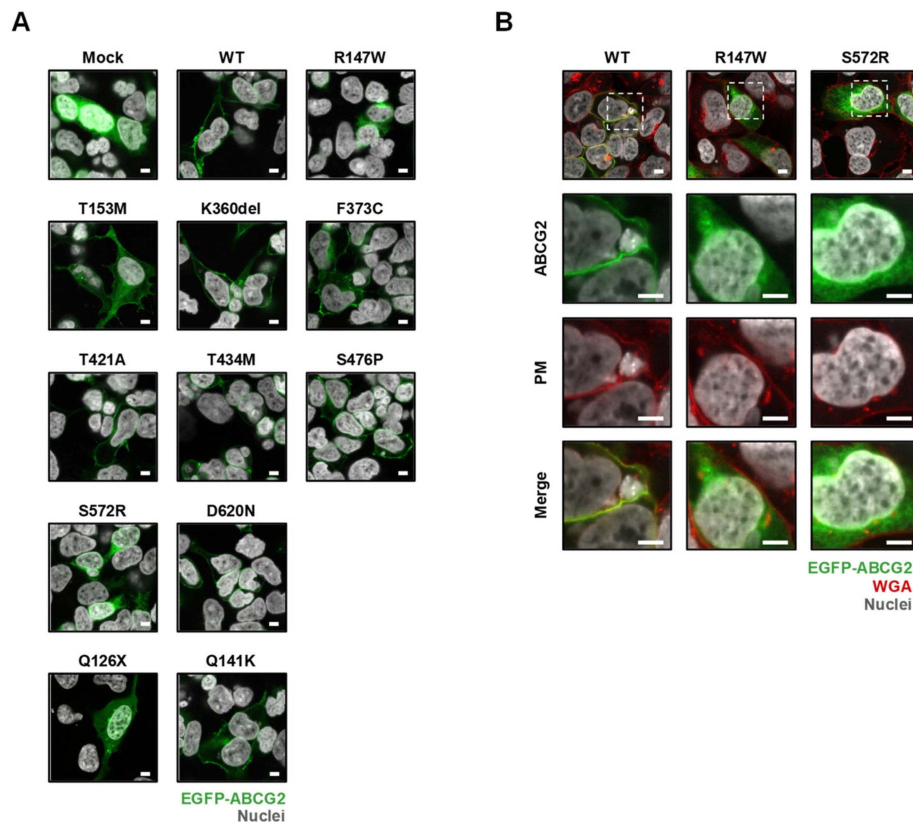


Figure 4. Effects of each mutation on the cellular localization of ABCG2 protein in transiently transfected 293A cells. (**A**) Intracellular localization of ABCG2 variants. Q126X (a stop gain variant that is deficient in the plasma membrane localization [22]) and Q141K are controls. (**B**) High magnification images of cells transfected with R147W and S572R variants indicate that these mutations impaired localization to the plasma membrane of the cells. Framed areas in the panels of top lane were observed under a higher magnification. Confocal microscopic images were obtained 48 h after the transfection. Nuclei were stained with TO-PRO-3 iodide (gray). Plasma membrane (PM) was labeled with Alexa Fluor® 594-conjugated wheat germ agglutinin (WGA) (red). Bars indicate 5 µm.

3.5. Effect of Each Mutation on the Urate Transport Activity of ABCG2 Protein

To investigate the seven ABCG2 variants that localized on the plasma membrane, we performed functional assays using ABCG2-expressing plasma membrane vesicles. Prior to the assay, the expression of each ABCG2 variant on the plasma membrane vesicles was confirmed by immunoblot analysis (Figure 5A), which supported the results obtained through confocal microscopy (Figure 4A). Then, the ABCG2 function was evaluated as an ATP-dependent urate transport into the vesicles (Figure 5B). The results show that the T434M and S476P variants diminished the function of the ABCG2 protein. Although the T153M and F373C variants-expressing plasma membrane vesicles exhibited reduced activity of urate transport as compared with ABCG2 WT (Figure 5B), this difference in the ABCG2 function between WT and these two variants was rescued by the normalization of urate transport activity by ABCG2 protein expression on plasma membrane vesicles (Supplemental Figure S3). These results suggest that the T153M and F373C variants do not affect ABCG2 function qualitatively (via alteration of its intrinsic transporter activity), but rather do so quantitatively (via decreasing its cellular protein level). The other ABCG2 variants (K360del, T421A, and D620N) had little effect on ABCG2 function. Additionally, supportive results were obtained from the *Xenopus oocyte* expression system.

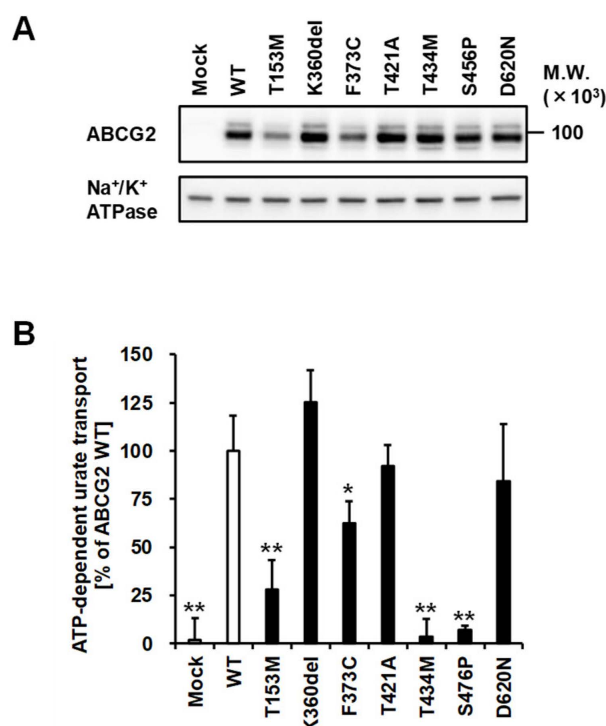


Figure 5. Functional validation of each ABCG2 variant as ATP-dependent urate transporters. (**A**) Immunoblot detection of ABCG2 WT and its variants expressed in plasma membrane vesicles prepared from 293A cells. Na^+/K^+ ATPase, a loading control. (**B**) ATP-dependent transport of urate by ABCG2 WT and its variants. The data are shown as % of WT; data are expressed as the mean ± SD. $n = 3$. Statistical analyses for significant differences were performed using Bartlett's test, followed by a Dunnett's test (*, $P < 0.05$; **, $P < 0.01$ *vs* WT).

3.6. Integration of the Obtained Data

Finally, we integrated the results of functional analyses (Figures 3–5) and classified the nine ABCG2 variants according to their effects on the intracellular processing and protein function of ABCG2 (Supplemental Figure S4). As summarized in Table 3, five of the eight rare variants of ABCG2 found in our gout/hyperuricemia cohort were less functional or null. Although the available information was limited, the allele frequencies of such less functional or null variants in the cohort tended to be higher than those in the European-origin population (Table 2). To this point, there was little inconsistency between the results of functional analyses and genetic analyses. However, a more comprehensive understanding requires further studies with a larger data set.

Table 3. Summary of the effects of each mutation on ABCG2 protein and its function.

rs Number	Nucleotide Change	AA Change	PM Localization	Protein Level on PM	Urate Transport	Effect on the Cellular ABCG2 Function
rs372192400	439C>T	R147W	−	N.D.	N.D.	Null
rs753759474	458C>T	T153M	+	+	+	Decrease to about a quarter
rs750972998	1079_1081delAGA	K360del	+	++	++	N.S.
rs752626614	1118T>G	F373C	+	+	++	Decrease to about half
rs199854112	1261A>G	T421A	+	++	++	N.S.
rs769734146	1301C>T	T434M	+	++	−	Almost null
Not annotated	1426T>C	S476P	+	++	−	Almost null
rs200894058	1714A>C	S572R	−	N.D.	N.D.	Null
rs34783571	1858G>A	D620N	+	++	++	N.S.

The effects in each column are relatively indicated by: ++, comparable to WT; +, positive in localization or less than ++; −, negative (disruption). AA, amino acid; PM, plasma membrane; N.D., not determined; N.S. not significantly different.

Regarding three rare variants—K360del, T421A, and D620N—that were functionally comparable to WT, their allele frequencies in our cohort were not significantly different from those in the European-origin population (Table 2). Thus, at least in their individual cases, these rare variants might have smaller effects on gout/hyperuricemia risk compared with the other identified rare variants. To further elucidate whether the presence of such functional rare variants could affect disease risk, future studies will be required to assess enlarged cohorts in terms of haplotype. With respect to biochemistry, our results indicated that K360, T421, and D620 are not essential for the function of the ABCG2 protein. With respect to D620N, a previous study, using an insect cell expression system, reported that this amino acid substitution had little effect on the ABCG2-mediated transport of porphyrin, which is an endogenous substrate of ABCG2 [37]; a similar result was obtained in this study, supporting the adequacy of functional validation we carried out. Considering that these three amino acid positions (K360, T421, and D620) are not predicted to be located in the transmembrane domains of ABCG2 (Figure 2), these positions could have a flexibility in the kind of amino acids and little effect on the appropriate structures of the ABCG2 transporter during its molecular function on the plasma membrane.

Additionally, our findings provide insights into the amino acid positions that are important for ABCG2 function. Interestingly, in null or approximately null variants of ABCG2, the original amino acids (R147, T434, S476, and S572) are conserved with several major mammalian species (Figure 6). Among them, R147 is close to Q141, while the others are in transmembrane domains (Figure 2). Given that the Q141K and R147W variants decreased the protein levels of ABCG2 (Figure 3), peptides around these two amino acids might be important for the stabilization of ABCG2 protein. Since the S572R variant likewise diminished ABCG2 protein levels (Figure 3), the S572 residue, which is located in the boundary region between helices 5b and 5c (Figure 2), may be important. Interestingly, a previous study reported that an artificial mutation I573A (a neighbor position of S572) disrupted maturation and reduced plasma membrane localization of ABCG2 protein [42], which could support the biochemical importance of the boundary region in the intracellular stability of the ABCG2 protein. Regarding T434 and S476, there is a possibility that these amino acids might be involved in the formation of a penetrating pathway for ABCG2 substrates or may be critical for the dynamics of the function of the ABCG2 transporter.

Before closing, we would like to discuss the necessity for the analyses of rare *ABCG2* variants. Based on the recent findings of GWASs of clinically defined gout [16,43], common variants of *ABCG2* are extremely important in gout pathogenesis. However, because the *ABCG2* gene is reportedly to be highly-polymorphic with population specificity [44], there will be population-specific rare variants of *ABCG2* that could be a genetic risk factor of gout. Indeed, such rare variants that were found in the present study with the Czech Republic population were distinct from those found in our previous study with a Japanese population [22]. Considering the theoretical limitations in GWAS analyses, which focus on only single nucleotide polymorphisms (SNPs), the clinical and experimental approaches that we employed in this study could be a reasonable way to identify pathophysiologically important rare variants. Given that many SNPs in *ABCG2* have been studied [20], identification and validation of population-specific rare variants will be important for achieving more effective and accurate prediction of ABCG2-related gout/hyperuricemia risk.

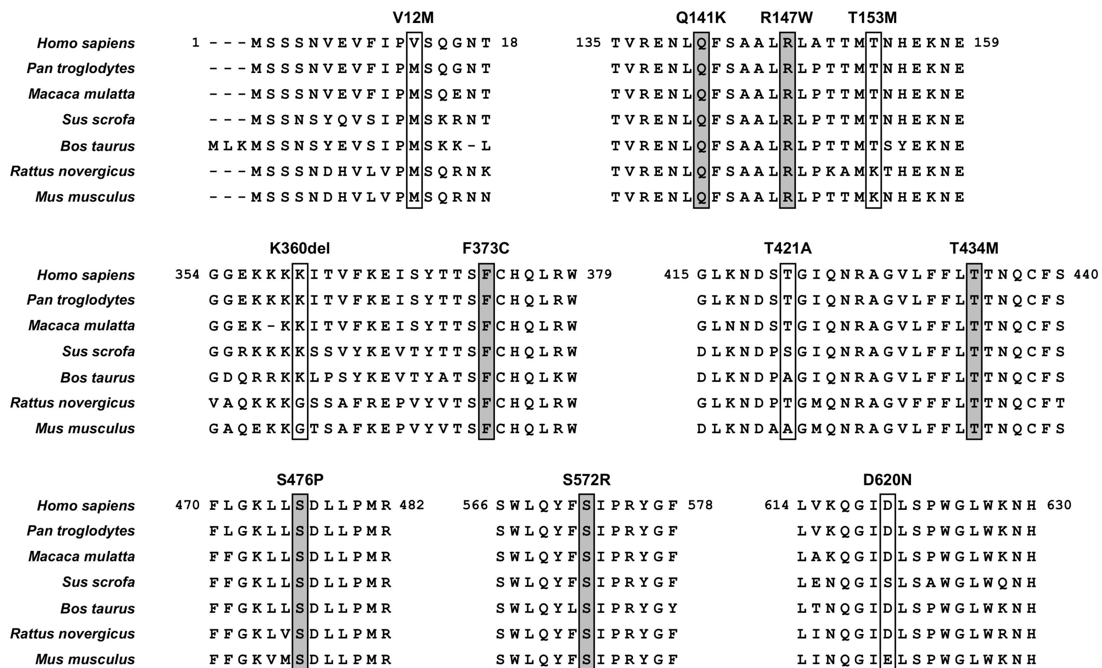


Figure 6. ABCG2 amino acids evolutionary conserved among seven mammalian species. The positions of non-synonymous substitutions conserved among seven species examined in the present study are grey labelled. Regarding Abcg2 protein in each species, NCBI Reference Sequence ID and amino acid sequence identity (*vs* human ABCG2, NM_004827.3) are summarized as below: *Pan troglodytes* (Chimpanzee, GABE01009237.1), 99%; *Macaca mulatta* (Rhesus macaque, NM_001032919.1, 96%; *Sus scrofa* (Pig, NM_214010.1), 84%; *Bos taurus* (Bovine, NM_001037478.3), 84%; *Rattus novergicus* (Rat, NM_181381.2), 81%; *Mus musculus* (Mouse, NM_011920.3), 81%. Multiple sequence alignments and homology calculations were carried out using the GENETYX software (GENETYX Co., Tokyo, Japan) with the ClustalW2.1 Windows program according to our previous study [27].

4. Conclusions

In the present study, we explored the exonic non-synonymous variants of *ABCG2* using a cohort of 250 individuals (68 primary hyperuricemia patients and 182 primary gout patients) recruited from a European descent population in the Czech Republic. Patients with non-synonymous variants showed an earlier on set of gout (Supplemental Figure S2), which is consistent with the results of previous studies [21,23]. The enlarged cohort enabled us to reveal that the numbers of non-synonymous variants of *ABCG2* could affect the frequency of familial gout (Figure 1), which was inconclusive in our previous study because of the small sample size [23]. Moreover, as summarized in Table 3, we successfully characterized the nine rare variants of ABCG2 (Figures 3–5). Additionally, given that ABCG2 is recognized to be an important determinant of the pharmacokinetic characteristics of its substrate drugs [45,46], this information will be significant for the field of pharmacogenomics.

In summary, our findings will deepen our understanding of *ABCG2*-related gout/hyperuricemia risk as well as the biochemical characteristics of the ABCG2 protein. To achieve a more accurate evaluation of an individual's risk for gout, addressing rare *ABCG2* variants is of importance. Furthermore, for effective genotyping in clinical situations, uncovering the population-specificities of such rare variants will be important.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/8/4/363/s1, Supplemental Figure S1: Urate accumulation in Xenopus oocytes expressing URAT1 WT and ABCG2 variants after a 30 min incubation in ND-96 solution containing 600 μM radiolabeled uric acid. Supplemental Figure S2: Onset of gout/age of ascertainment of hyperuricemia and the numbers of allelic variants in ABCG2, Supplemental Figure S3: Urate transport activities relative to ABCG2 protein levels, Supplemental Figure S4: Schematic illustration of

the effects of each rare mutation we studied on the intracellular processing and function of ABCG2 protein, Supplemental Table S1: Medication and number of allelic variants in the gout and hyperuricemia cohorts.

Author Contributions: Conceptualization, Y.T., T.T., and B.S.; validation, Y.T., K.M., and K.P. (Kateřina Pavelcová); formal analysis, Y.T., and A.M.; investigation, Y.T., V.K., K.P. (Karel Pavelka), J.B., K.P. (Karel Pavelka), M.P., and T.T.; i.e., Y.T. and T.T. were responsible for experimental design using mammalian cells; Y.T. performed the experimental work using mammalian cells and analyzed the data; A.M. and V.K. worked on experiments using Xenopus oocytes, and analyzed the data; K.P. (Kateřina Pavelcová) and J.B. conducted sequencing analyses for clinical samples; K.P. (Karel Pavelka) was responsible for clinical observations; M.P. performed statistical analyses; data curation, Y.T., T.T., and B.S.; writing—original draft preparation, Y.T. and B.S.; writing—review and editing, Y.Y., T.T. and B.S.; supervision, H.S., H.M., and B.S.; project administration, B.S.; funding acquisition, Y.T., T.T. and B.S; A.M. was a co-first author.

Funding: The present study was supported by the JSPS KAKENHI Grant Numbers 15H05610 (to Y.T.); 16H1808 and 18KK0247 (to T.T.) as well as a grant from the Czech Republic Ministry of Health AZV 15-26693 A to B.S. T.T. has received research grants from Gout Research Foundation, The Uehara Memorial Foundation, Mochida Memorial Foundation for Medical and Pharmaceutical Research, The Takeda Medical Foundation, and MSD Life Science Foundation, Public Interest Incorporated Foundation.

Acknowledgments: All authors are grateful to all the patients who kindly took part in this study as well as our colleagues at the Institute of Rheumatology (Prague, Czech Republic) for their help in recruiting patients for the study. We would like to thank Editage (www.editage.jp) for English language editing.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ABCG2	ATP-binding cassette subfamily G member 2
BCRP	Brest cancer resistance protein
CI	Confidence interval
GWAS	Genome-wide association study
IQR	Interquartile range
MAF	Minor allele frequency
PM	Plasma membrane
SNP	Single nucleotide polymorphism
SUA	Serum uric acid
TBST	Tris-buffered saline containing 0.05% Tween 20
WT	Wild-type

References

1. Dalbeth, N.; Merriman, T.R.; Stamp, L.K. Gout. *Lancet* **2016**, *388*, 2039–2052. [CrossRef]
2. Yeldandi, A.V.; Wang, X.D.; Alvares, K.; Kumar, S.; Rao, M.S.; Reddy, J.K. Human urate oxidase gene: Cloning and partial sequence analysis reveal a stop codon within the fifth exon. *Biochem. Biophys. Res. Commun.* **1990**, *171*, 641–646. [CrossRef]
3. Enomoto, A.; Kimura, H.; Chairoungdua, A.; Shigeta, Y.; Jutabha, P.; Cha, S.H.; Hosoyamada, M.; Takeda, M.; Sekine, T.; Igarashi, T.; et al. Molecular identification of a renal urate anion exchanger that regulates blood urate levels. *Nature* **2002**, *417*, 447–452. [CrossRef] [PubMed]
4. Vitart, V.; Rudan, I.; Hayward, C.; Gray, N.K.; Floyd, J.; Palmer, C.N.; Knott, S.A.; Kolcic, I.; Polasek, O.; Graessler, J.; et al. SLC2A9 is a newly identified urate transporter influencing serum urate concentration, urate excretion and gout. *Nat. Genet.* **2008**, *40*, 437–442. [CrossRef] [PubMed]
5. Matsuo, H.; Chiba, T.; Nagamori, S.; Nakayama, A.; Domoto, H.; Phetdee, K.; Wiriyasermkul, P.; Kikuchi, Y.; Oda, T.; Nishiyama, J.; et al. Mutations in glucose transporter 9 gene SLC2A9 cause renal hypouricemia. *Am. J. Hum. Genet.* **2008**, *83*, 744–751.
6. Caulfield, M.J.; Munroe, P.B.; O'Neill, D.; Witkowska, K.; Charchar, F.J.; Doblado, M.; Evans, S.; Eyheramendy, S.; Onipinla, A.; Howard, P.; et al. SLC2A9 is a high-capacity urate transporter in humans. *PLoS Med.* **2008**, *5*, e197. [CrossRef]
7. Ichida, K.; Matsuo, H.; Takada, T.; Nakayama, A.; Murakami, K.; Shimizu, T.; Yamanashi, Y.; Kasuga, H.; Nakashima, H.; Nakamura, T.; et al. Decreased extra-renal urate excretion is a common cause of hyperuricemia. *Nat. Commun.* **2012**, *3*, 764. [CrossRef] [PubMed]

8. Woodward, O.M.; Kottgen, A.; Coresh, J.; Boerwinkle, E.; Guggino, W.B.; Kottgen, M. Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 10338–10342. [CrossRef]
9. Matsuo, H.; Takada, T.; Ichida, K.; Nakamura, T.; Nakayama, A.; Ikebuchi, Y.; Ito, K.; Kusanagi, Y.; Chiba, T.; Tadokoro, S.; et al. Common defects of ABCG2, a high-capacity urate exporter, cause gout: A function-based genetic analysis in a Japanese population. *Sci. Transl. Med.* **2009**, *1*, 5ra11. [CrossRef]
10. Nakayama, A.; Matsuo, H.; Nakaoka, H.; Nakamura, T.; Nakashima, H.; Takada, Y.; Oikawa, Y.; Takada, T.; Sakiyama, M.; Shimizu, S.; et al. Common dysfunctional variants of ABCG2 have stronger impact on hyperuricemia progression than typical environmental risk factors. *Sci. Rep.* **2014**, *4*, 5227. [CrossRef] [PubMed]
11. Matsuo, H.; Nakayama, A.; Sakiyama, M.; Chiba, T.; Shimizu, S.; Kawamura, Y.; Nakashima, H.; Nakamura, T.; Takada, Y.; Oikawa, Y.; et al. ABCG2 dysfunction causes hyperuricemia due to both renal urate underexcretion and renal urate overload. *Sci. Rep.* **2014**, *4*, 3755. [CrossRef] [PubMed]
12. Robey, R.W.; To, K.K.; Polgar, O.; Dohse, M.; Fetsch, P.; Dean, M.; Bates, S.E. ABCG2: A perspective. *Adv. Drug Deliv. Rev.* **2009**, *61*, 3–13. [CrossRef]
13. Knutsen, T.; Rao, V.K.; Ried, T.; Mickley, L.; Schneider, E.; Miyake, K.; Ghadimi, B.M.; Padilla-Nash, H.; Pack, S.; Greenberger, L.; et al. Amplification of 4q21-q22 and the MXR gene in independently derived mitoxantrone-resistant cell lines. *Genes Chromosomes Cancer* **2000**, *27*, 110–116. [CrossRef]
14. Major, T.J.; Dalbeth, N.; Stahl, E.A.; Merriman, T.R. An update on the genetics of hyperuricaemia and gout. *Nat. Rev. Rheumatol.* **2018**, *14*, 341–353. [CrossRef]
15. Nakayama, A.; Nakaoka, H.; Yamamoto, K.; Sakiyama, M.; Shaukat, A.; Toyoda, Y.; Okada, Y.; Kamatani, Y.; Nakamura, T.; Takada, T.; et al. GWAS of clinically defined gout and subtypes identifies multiple susceptibility loci that include urate transporter genes. *Ann. Rheum. Dis.* **2017**, *76*, 869–877. [CrossRef] [PubMed]
16. Matsuo, H.; Yamamoto, K.; Nakaoka, H.; Nakayama, A.; Sakiyama, M.; Chiba, T.; Takahashi, A.; Nakamura, T.; Nakashima, H.; Takada, Y.; et al. Genome-wide association study of clinically defined gout identifies multiple risk loci and its association with clinical subtypes. *Ann. Rheum. Dis.* **2016**, *75*, 652–659. [CrossRef] [PubMed]
17. Kottgen, A.; Albrecht, E.; Teumer, A.; Vitart, V.; Krumsiek, J.; Hundertmark, C.; Pistis, G.; Ruggiero, D.; O'Seaghdha, C.M.; Haller, T.; et al. Genome-wide association analyses identify 18 new loci associated with serum urate concentrations. *Nat. Genet.* **2013**, *45*, 145–154. [CrossRef]
18. Kolz, M.; Johnson, T.; Sanna, S.; Teumer, A.; Vitart, V.; Perola, M.; Mangino, M.; Albrecht, E.; Wallace, C.; Farrall, M.; et al. Meta-analysis of 28,141 individuals identifies common variants within five new loci that influence uric acid concentrations. *PLoS Genet.* **2009**, *5*, e1000504. [CrossRef] [PubMed]
19. Dehghan, A.; Kottgen, A.; Yang, Q.; Hwang, S.J.; Kao, W.L.; Rivadeneira, F.; Boerwinkle, E.; Levy, D.; Hofman, A.; Astor, B.C.; et al. Association of three genetic loci with uric acid concentration and risk of gout: A genome-wide association study. *Lancet* **2008**, *372*, 1953–1961. [CrossRef]
20. Heyes, N.; Kapoor, P.; Kerr, I.D. Polymorphisms of the Multidrug Pump ABCG2: A Systematic Review of Their Effect on Protein Expression, Function, and Drug Pharmacokinetics. *Drug Metab. Dispos.* **2018**, *46*, 1886–1899. [CrossRef]
21. Matsuo, H.; Ichida, K.; Takada, T.; Nakayama, A.; Nakashima, H.; Nakamura, T.; Kawamura, Y.; Takada, Y.; Yamamoto, K.; Inoue, H.; et al. Common dysfunctional variants in ABCG2 are a major cause of early-onset gout. *Sci. Rep.* **2013**, *3*, 2014. [CrossRef] [PubMed]
22. Higashino, T.; Takada, T.; Nakaoka, H.; Toyoda, Y.; Stiburkova, B.; Miyata, H.; Ikebuchi, Y.; Nakashima, H.; Shimizu, S.; Kawaguchi, M.; et al. Multiple common and rare variants of ABCG2 cause gout. *RMD Open* **2017**, *3*, e000464. [CrossRef]
23. Stiburkova, B.; Pavelcova, K.; Zavada, J.; Petru, L.; Simek, P.; Cepek, P.; Pavlikova, M.; Matsuo, H.; Merriman, T.R.; Pavelka, K. Functional non-synonymous variants of ABCG2 and gout risk. *Rheumatology (Oxford)* **2017**, *56*, 1982–1992. [CrossRef] [PubMed]
24. Stiburkova, B.; Miyata, H.; Zavada, J.; Tomcik, M.; Pavelka, K.; Storkanova, G.; Toyoda, Y.; Takada, T.; Suzuki, H. Novel dysfunctional variant in ABCG2 as a cause of severe tophaceous gout: Biochemical, molecular genetics and functional analysis. *Rheumatology (Oxford)* **2016**, *55*, 191–194. [CrossRef] [PubMed]
25. Stiburkova, B.; Pavelcova, K.; Pavlikova, M.; Jesina, P.; Pavelka, K. The impact of dysfunctional variants of ABCG2 on hyperuricemia and gout in pediatric-onset patients. *Arthritis Res. Ther.* **2019**, *21*, 77. [CrossRef] [PubMed]

26. Wallace, S.L.; Robinson, H.; Masi, A.T.; Decker, J.L.; McCarty, D.J.; Yu, T.F. Preliminary criteria for the classification of the acute arthritis of primary gout. *Arthritis Rheum.* **1977**, *20*, 895–900. [CrossRef] [PubMed]
27. Toyoda, Y.; Takada, T.; Miyata, H.; Ishikawa, T.; Suzuki, H. Regulation of the Axillary Osmidrosis-Associated ABCC11 Protein Stability by N-Linked Glycosylation: Effect of Glucose Condition. *PLoS ONE* **2016**, *11*, e0157172. [CrossRef]
28. Toyoda, Y.; Sakurai, A.; Mitani, Y.; Nakashima, M.; Yoshiura, K.; Nakagawa, H.; Sakai, Y.; Ota, I.; Lezhava, A.; Hayashizaki, Y.; et al. Earwax, osmidrosis, and breast cancer: Why does one SNP (538G>A) in the human ABC transporter ABCC11 gene determine earwax type? *FASEB J.* **2009**, *23*, 2001–2013. [CrossRef]
29. Toyoda, Y.; Takada, T.; Gomi, T.; Nakagawa, H.; Ishikawa, T.; Suzuki, H. Clinical and Molecular Evidence of ABCC11 Protein Expression in Axillary Apocrine Glands of Patients with Axillary Osmidrosis. *Int. J. Mol. Sci.* **2017**, *18*, 417. [CrossRef]
30. Nakagawa, H.; Wakabayashi-Nakao, K.; Tamura, A.; Toyoda, Y.; Koshiba, S.; Ishikawa, T. Disruption of N-linked glycosylation enhances ubiquitin-mediated proteasomal degradation of the human ATP-binding cassette transporter ABCG2. *FEBS J.* **2009**, *276*, 7237–7252. [CrossRef]
31. Miyata, H.; Takada, T.; Toyoda, Y.; Matsuo, H.; Ichida, K.; Suzuki, H. Identification of Febuxostat as a New Strong ABCG2 Inhibitor: Potential Applications and Risks in Clinical Situations. *Front. Pharmacol.* **2016**, *7*, 518. [CrossRef] [PubMed]
32. Toyoda, Y.; Takada, T.; Suzuki, H. Halogenated hydrocarbon solvent-related cholangiocarcinoma risk: Biliary excretion of glutathione conjugates of 1,2-dichloropropane evidenced by untargeted metabolomics analysis. *Sci. Rep.* **2016**, *6*, 24586. [CrossRef] [PubMed]
33. Takada, T.; Yamamoto, T.; Matsuo, H.; Tan, J.K.; Ooyama, K.; Sakiyama, M.; Miyata, H.; Yamanashi, Y.; Toyoda, Y.; Higashino, T.; et al. Identification of ABCG2 as an Exporter of Uremic Toxin Indoxyl Sulfate in Mice and as a Crucial Factor Influencing CKD Progression. *Sci. Rep.* **2018**, *8*, 11147. [CrossRef] [PubMed]
34. Hurba, O.; Mancikova, A.; Krylov, V.; Pavlikova, M.; Pavelka, K.; Stiburkova, B. Complex analysis of urate transporters SLC2A9, SLC22A12 and functional characterization of non-synonymous allelic variants of GLUT9 in the Czech population: No evidence of effect on hyperuricemia and gout. *PLoS ONE* **2014**, *30*, e107902. [CrossRef]
35. Taylor, N.M.I.; Manolaridis, I.; Jackson, S.M.; Kowal, J.; Stahlberg, H.; Locher, K.P. Structure of the human multidrug transporter ABCG2. *Nature* **2017**, *546*, 504–509. [CrossRef]
36. Beitz, E. T(E)Xtopo: Shaded membrane protein topology plots in LAT(E)X2epsilon. *Bioinformatics* **2000**, *16*, 1050–1051. [CrossRef]
37. Tamura, A.; Watanabe, M.; Saito, H.; Nakagawa, H.; Kamachi, T.; Okura, I.; Ishikawa, T. Functional validation of the genetic polymorphisms of human ATP-binding cassette (ABC) transporter ABCG2: Identification of alleles that are defective in porphyrin transport. *Mol. Pharmacol.* **2006**, *70*, 287–296.
38. Toyoda, Y.; Takada, T.; Umezawa, M.; Tomura, F.; Yamanashi, Y.; Takeda, K.; Suzuki, H. Identification of hepatic NPC1L1 as an NAFLD risk factor evidenced by ezetimibe-mediated steatosis prevention and recovery. *FASEB BioAdv.* **2019**, in press. [CrossRef]
39. Kondo, C.; Suzuki, H.; Itoda, M.; Ozawa, S.; Sawada, J.; Kobayashi, D.; Ieiri, I.; Mine, K.; Ohtsubo, K.; Sugiyama, Y. Functional analysis of SNPs variants of BCRP/ABCG2. *Pharm. Res.* **2004**, *21*, 1895–1903. [CrossRef]
40. Nakagawa, H.; Toyoda, Y.; Wakabayashi-Nakao, K.; Tamaki, H.; Osumi, M.; Ishikawa, T. Ubiquitin-mediated proteasomal degradation of ABC transporters: A new aspect of genetic polymorphisms and clinical impacts. *J. Pharm. Sci.* **2011**, *100*, 3602–3619. [CrossRef]
41. Zambo, B.; Bartos, Z.; Mozner, O.; Szabo, E.; Varady, G.; Poor, G.; Palinkas, M.; Andrikovics, H.; Hegedus, T.; Homolya, L.; et al. Clinically relevant mutations in the ABCG2 transporter uncovered by genetic analysis linked to erythrocyte membrane protein expression. *Sci. Rep.* **2018**, *8*, 7487. [CrossRef] [PubMed]
42. Haider, A.J.; Cox, M.H.; Jones, N.; Goode, A.J.; Bridge, K.S.; Wong, K.; Briggs, D.; Kerr, I.D. Identification of residues in ABCG2 affecting protein trafficking and drug transport, using co-evolutionary analysis of ABCG sequences. *Biosci. Rep.* **2015**, *35*, e00241. [CrossRef] [PubMed]
43. Li, C.; Li, Z.; Liu, S.; Wang, C.; Han, L.; Cui, L.; Zhou, J.; Zou, H.; Liu, Z.; Chen, J.; et al. Genome-wide association analysis identifies three new risk loci for gout arthritis in Han Chinese. *Nat. Commun.* **2015**, *6*, 7041. [CrossRef] [PubMed]

44. Sakiyama, M.; Matsuo, H.; Takada, Y.; Nakamura, T.; Nakayama, A.; Takada, T.; Kitajiri, S.; Wakai, K.; Suzuki, H.; Shinomiya, N. Ethnic differences in ATP-binding cassette transporter, sub-family G, member 2 (ABCG2/BCRP): Genotype combinations and estimated functions. *Drug Metab. Pharmacokinet.* **2014**, *29*, 490–492. [CrossRef] [PubMed]
45. Giacomini, K.M.; Huang, S.M.; Tweedie, D.J.; Benet, L.Z.; Brouwer, K.L.; Chu, X.; Dahlin, A.; Evers, R.; Fischer, V.; Hillgren, K.M.; et al. Membrane transporters in drug development. *Nat. Rev. Drug Discov.* **2010**, *9*, 215–236. [PubMed]
46. Toyoda, Y.; Takada, T.; Suzuki, H. Inhibitors of human ABCG2: From technical background to recent updates with clinical implications. *Front. Pharmacol.* **2019**, *10*, 208. [CrossRef]

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).