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SIGNIFICANT ROLE OF
ABCG2 TRANSPORTERS IN GOUT

PhD thesis

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"Faith and reason are like two wings on which the human spirit rises to the contemplation of truth"

John Paul II.

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List of Abbreviations

ABCG2   ATP-binding cassette G2

ACR   American College of Rheumatology

EULAR   European Alliance of Associations for Rheumatology

APRT   adenine phosphoribosyltransferase

AMP   adenosine monophosphate

ATP   adenosine triphosphate

BRCP   breast cancer resistance protein

CARD   Caspase activation domain

CKD   chronic kidney disease

DAMP   danger-associated molecular pattern

EDTA   ethylene diamine tetra acetic acid

FUE   fractional urate excretion

GWAS   genome-wide association studies

GMP   guanosine monophosphate

HGPRT   hypoxanthine-guanine phosphoribosyltransferase

ICC   intraclass correlation coefficient

IMP	inosine monophosphate
LRR	Leucine rich region
MAF	minor allele frequency
MSK	musculoskeletal
MSU	monosodium urate
MXR	mitoxantrone resistance protein
NALP3	Inflammasome, NACHT, LRR and PYD containing 3 multiprotein
NACHT	NAIP, CIITA, HET-E structure domain
NBD	nucleotide binding domain
NET	neutrophil extracellular trap
NF-κB	Nuclear factor-κB
OAT	organic anion transporter
PRPP	phosphoribosyl pyrophosphate
PYD	Pyrin domain
qPCR	quantitative polymerase chain reaction
RBC	red blood cell
ROL	renal-overload
SU	Serum urate

SNP   single nucleotide polymorphism

SLC   solute carrier superfamily

TLR   toll-like membrane receptor (family)

TMD   transmembrane domain

URAT1   urate transporter 1, SLC22A12

UUE   urinary urate excretion

XO   xanthine oxidase

WGA   wheat germ agglutinin

WT   wild type

1. Introduction

Being one of the most common inflammatory arthritis, gout represents a high personal and social burden both physically and economically (1). The Global Burden of Disease project of the World Health Organisation marks gout among the five most important musculoskeletal (MSK) diseases, and estimates that the number of adults affected is over twice as high as those with rheumatoid arthritis (2). Besides the musculoskeletal complications, gout in itself and with its significant comorbidities (chronic kidney disease, cardiovascular diseases, etc.) raises morbidity and mortality rates (3,4). Meanwhile, the efficacy of urate lowering therapy and gout management compared to that of rheumatoid arthritis is still inadequate, despite the fact that the treat-to-target method with its clear principles was introduced in this field as well (5,6). The better understanding of gout's pathomechanism may take us a step ahead to overcome the challenges of such importance.

1.1. Definition of gout

Gout is a common crystal induced arthritis, characterised by intra- and/or extraarticular crystal deposition induced inflammation and the appearance of tophi in chronic cases. Besides erosive arthritis, it leads to severe systemic comorbidities if untreated. Crystallisation appears if the serum urate level chronically exceeds its threshold of solubility. Normal serum urate ranges vary between 180-360 μmol/l (3-6 mg/dl).

1.2. Epidemiology

The prevalence shows geographical differences varying from 0.5-4% (500-4000/100000) in the adult population. It can be said that the number of patients affected were on the rise in the past century, following the extent of overconsumption, obesity and other manifestations of the so-called diseases of civilization. Incidence increases with

age, resulting in a prevalence over 6-8% among men over 65 and 2-4% among women of the same age. Asymptomatic hyperuricemia is per definition not gout, but an anticipating state to it. The prevalence of hyperuricemia is 20-22% in the adult population (7–9).

1.3. Etiopathogenesis

Due to their diversified functions, purine- and pyrimidine bases represent a central role in the life of a living cell. They have distinguished tasks in preserving (DNA) and expressing (RNA) our genetic code, transferring energy (ATP) or building up coenzyme components and promoting lipid and carbohydrate metabolism. As uricase enzyme is not present in humans, the end stage of purine catabolism is uric acid (2,6,8-trihydroxy-purin), which then further forms ions and salts.

1.3.1.   Purine metabolism

The biologically active antioxidant urate may originate from endogenous (80%) or nutritional, exogenous (20%) sources. Endogenous purine nucleotides are a result of nucleic acid catabolism and de novo purine synthesis. In the synthetic process, phosphoribosyl pyrophosphate (PRPP) is made from ribose 5-phosphate with the help of PRPP-synthetase enzyme which in several steps turns into inosine monophosphate (IMP) and then into adenosine monophosphate (AMP) or guanosine monophosphate (GMP).

During purine catabolism adenosine first degrades into inosine and then into hypoxanthine. One central actor of urate metabolism is xanthine oxidase (XO). Besides its other functions, this enzyme facilitates the oxidation of hypoxanthine to xanthine and then of xanthine to uric acid. With the dephosphorylation and deamination of GMP the result is also xanthine that is also degraded into uric acid by XO. In purine metabolism, some extent of purine bases is oxidised into uric acid. In the meantime, the rest is reutilised with the help of salvage mechanisms, when the purine base reacts with PRPP to nucleoside-monophosphate. This process is catalysed by two enzymes, adenine phosphoribosyltransferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (Figure 1).
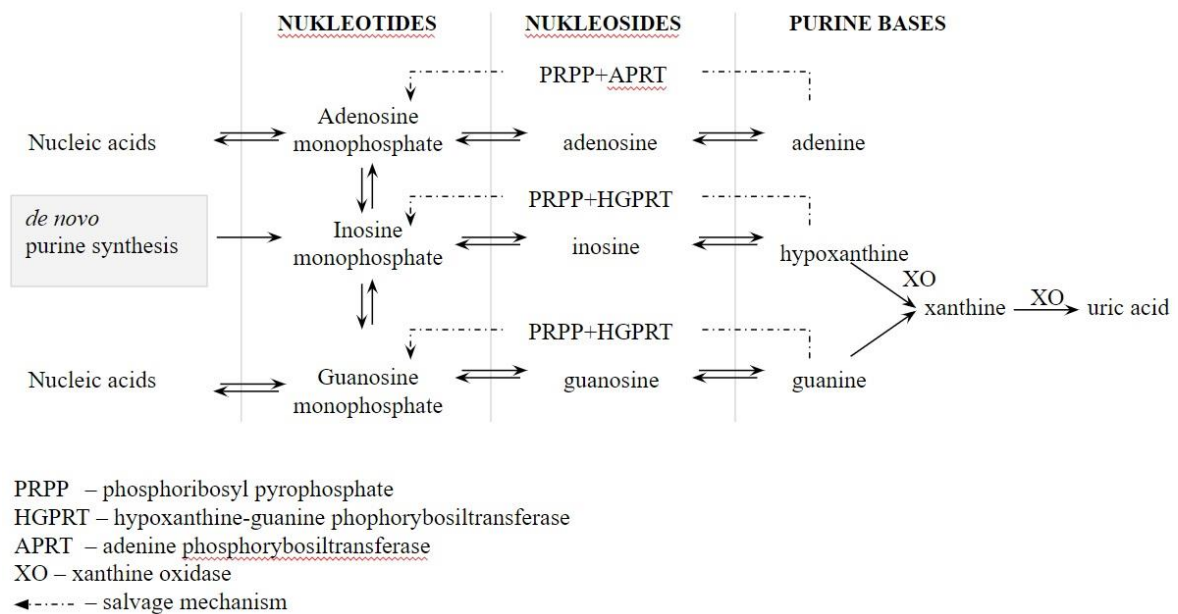
Figure 1. Purine metabolism (modified after 10)

1.3.2.   Hyperuricemia, the vestibule of gout

More than two thirds (70%) of uric acid is excreted through the kidneys, while one third (30%) through the intestines. As a first step in renal excretion, 100% of uric acid is filtrated in the glomerulus due to its small molecular weight. This is followed by a 98% reabsorption at the level of the proximal tubules. The third step is an active transport, when 50% is again secreted to the lumen only to be reabsorbed in 80-90% at the distal tubules. After the fine tuning in the third and fourth step, approximately 6-12% of uric acid is excreted in the urine (Figure 2).

		% GFR	Localisation
1.	Filtration	100	Glomerule
2.	Proximal reabsorption	98-100	Proximal tubule
3.	Secretion	50	Proximal tubule pars recta
4.	Distal reabsorption	40-44	Proximal tubule, Henle's loop, Collecting ducts (tubulus colligens)
	Excretion	6-12	
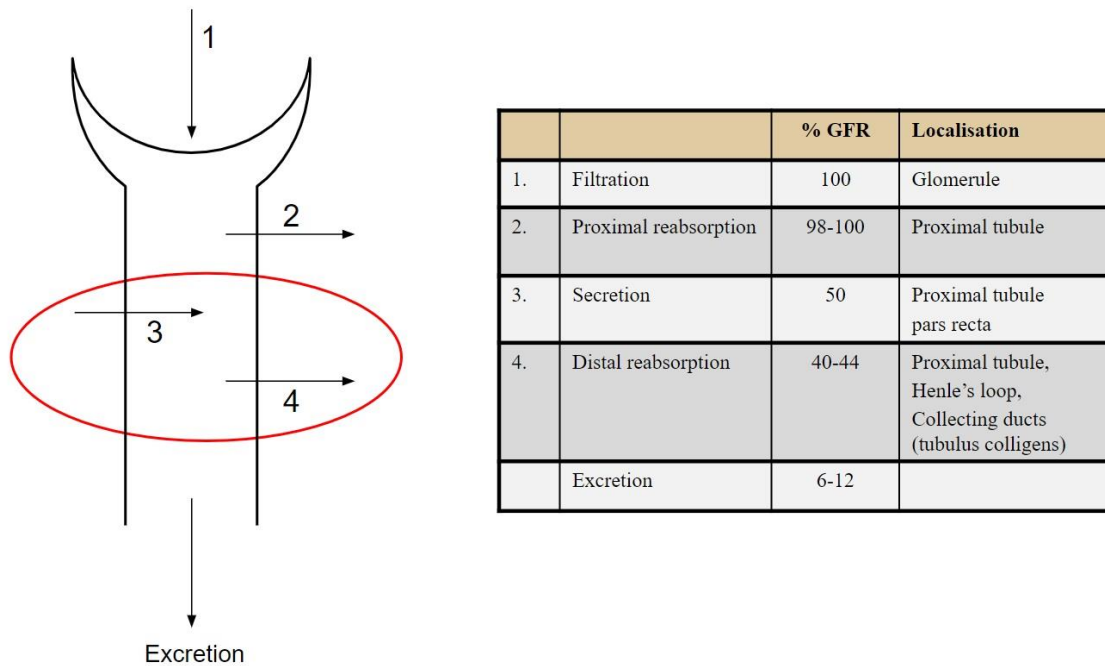
Figure 2. Renal excretion of uric acid (modified after 10)

The excretion of uric acid is implemented through specific urate transporters (Figure 3). Members of the solute carrier (SLC) superfamily, the SLC22A12 (also named URAT1 transporter) and the SLC22A11 (or OAT4) are organic anion transporters that along with SLC2A9 glucose transporter take part in reabsorption. Besides common risk factors (11), genetics play an important role in the etiopathogenesis of gout (12). Gain of function mutations within the coding sequences of these proteins may result in elevated reabsorption of uric acid. On the other hand, loss of function mutations of SLC17A3 and SLC17A1 along with ABCG2, a member of the ATP-binding cassette (ABC) transporter family (subfamily G2) are connected to impaired uric acid secretion.
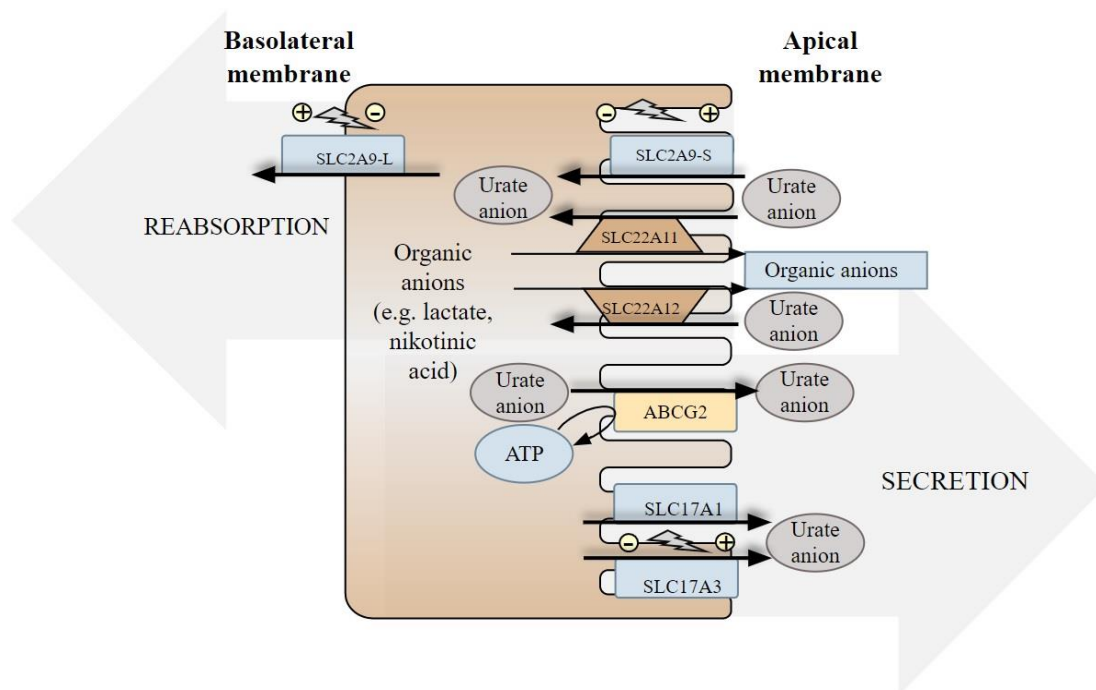
**Figure 3. Urate transport mechanisms (modified after 10)**

Transporters responsible for reabsorption and secretion of uric acid: SLC2A9, solute carrier 2A9; SLC22A11/12, solute carrier 22A11/12; ABCG2, ATP-binding cassette G2, SLC17A1/3, solute carrier 17A1/3

1.3.2.1. ABCG2 transporter and hyperuricemia

The ATP-binding cassette (ABC) type proteins form an ancient superfamily, existing from as early as prokaryotes (13). The two main subunits of ABC transporters are the transmembrane domain (TMD) and the nucleotide binding domain (NBD). The catalytic site of NBD involves conserved motifs: the Walker A, Q-loop, Walker B, H-loop and the c-signature and D-loop. The functional protein must consist of 2-2 NBDs and TMDs, to be able to bind and hydrolyze ATP. There exist seven ABC subfamilies labelled from A to G (14). Among them, the ABCG transporter subfamily member 2 (ABCG2, also known as BRCP or MXR) is characterised as "half-transporter", that must homodimerize to form a functional efflux pump. ABCG2 has an outstanding role in eliminating urate, organic anions, steroids or xeno- and endobiotics (15). Based on its location and function, the ABCG2 efflux pump is thought to act as a 'gatekeeper',

preventing harmful compounds from crossing biological barriers and entering sensitive tissues. Earlier thought to be kidney specific, it is now known to be expressed in several other locations, such as blood-brain-barrier, placenta, liver and gut. It is also present in the erythrocyte membrane, even though the precise role of ABCG2 in red blood cells (RBC) is not yet clarified. However, ABCG2 is a key urate transporter mainly expressed in the terminal ileum and proximal tubules of the kidney cortex (16,17).

Genome-wide association studies (GWAS) demonstrated that impaired function of ABCG2 resulted in hyperuricemia and may act as an independent risk factor for gout (18). The most important, relatively common polymorphism associated with hyperuricemia is the rs2231142, resulting in the ABCG2-Q141K (c.C421>A) transporter variant. This appears with a minor allele frequency (MAF) of 0.09 in the European population and that may be causally related to at least 10% of all gout cases (19). The Q141K polymorphism has been associated with impaired expression of the membrane protein ABCG2, which reduces cellular urate efflux by approximately 50% compared to wild-type ABCG2 levels. Moreover, based on the GWAS data, a 40-70% heritability of serum uric acid levels can be detected, which may be associated not only with ABCG2-Q141K, but also with other functional variants which are associated with a decrease in the expression level of the protein (20). These other, less frequent polymorphisms are the rs140207606 (R236X, MAF 0.0037) and rs560659849 (R383C) (21). The presence of a nonsense stop codon mutation (c.706C > T, p.R236X) in ABCG2 has been described to cause childhood-onset hyperuricemia and thus very early-onset gout by truncating the ABCG2 protein sequence to approximately 1/3 of the full-length protein. As a result of the missing functional transmembrane domain from the mutant protein, the presence of ABCG2 could not be detected in the plasma membrane, thus greatly impairing its urate transport activity (22). The ABCG2-R383C (c.1147C>T) mutation results in the loss of a critical salt bridge, rendering the protein inactive and unstable. This can lead to a disorder of urate metabolism due to the functional deterioration. However, it is important to note that a patient may not only have a single variant associated with decreased protein expression, but may also have multiple variants that accumulate and combine (23). The structure and most important mutations of ABCG2 transporter are shown in Figure 4.
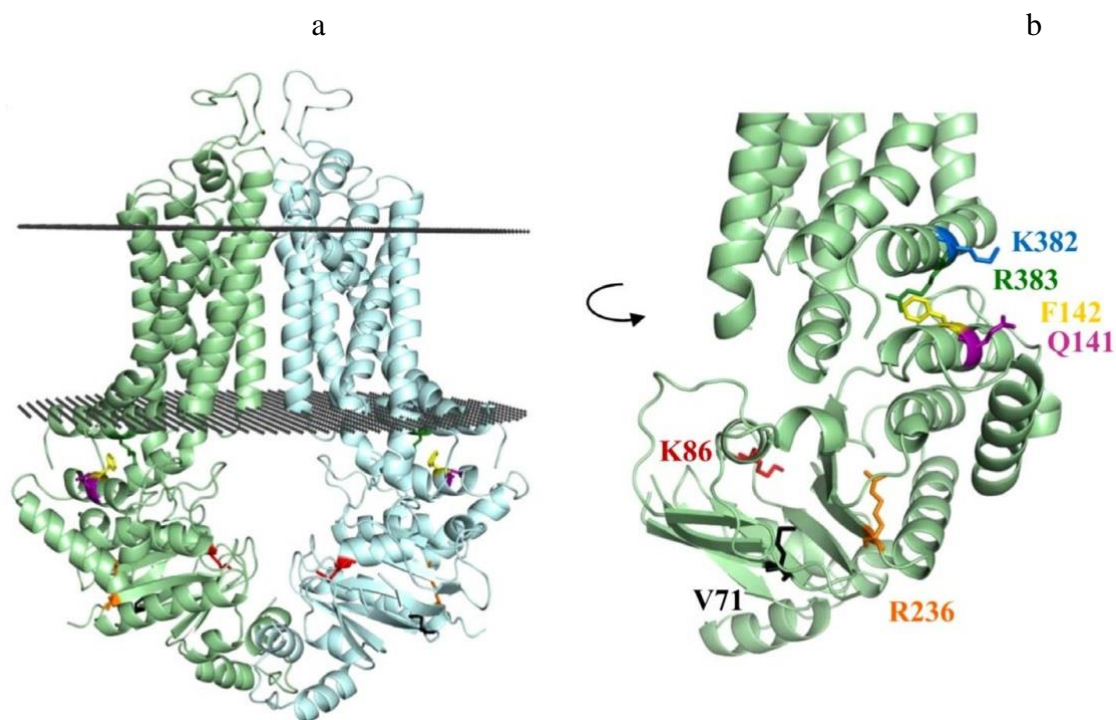
Figure 4a, b. Structure and most important mutations of ABCG2 transporter (21)
A: ABCG2 homodimer with transmembrane domain (TMD) and nucleotide binding domain (NBD). Green and blue parts represent the two polypeptide chains of ABCG2. B: The positions of polymorphisms on the conserved motifs of NBD are labelled on the magnified scheme.

1.3.3.   The turning point: crystallisation

The actual level of serum urate is determined by the balance of production and excretion. In case of chronic hyperuricemia, this balance is disturbed. As a result, the total amount of uric acid in the human body (1500 mg) may be multiplied (Figure 5).
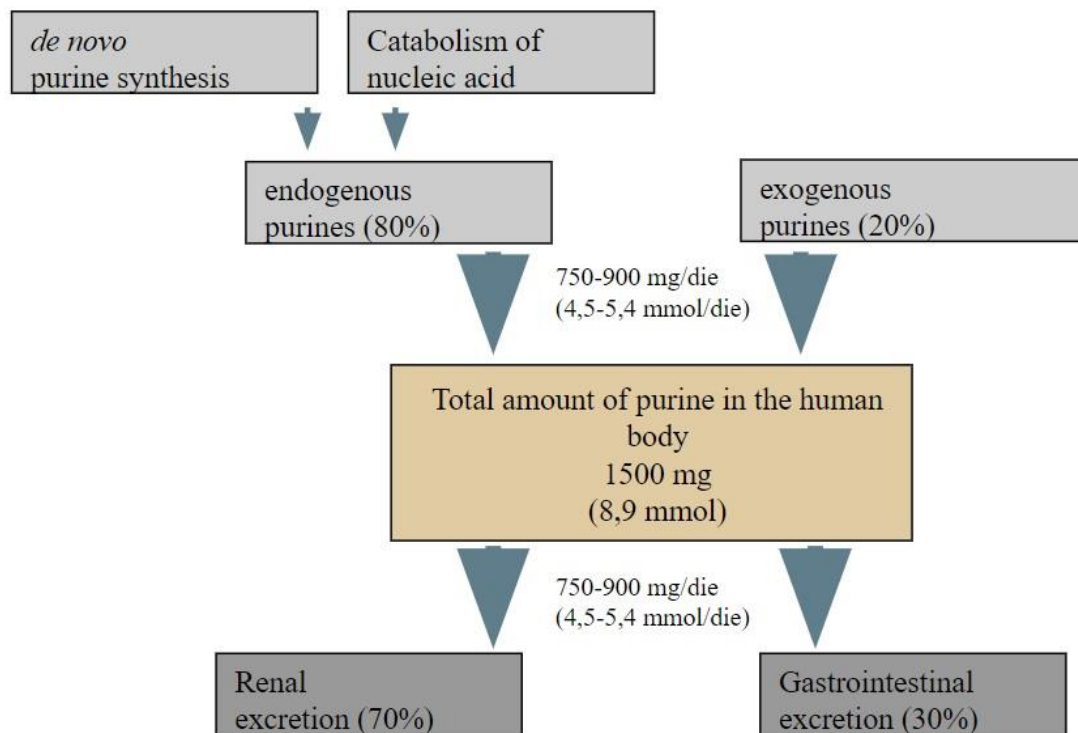
Figure 5. Production and excretion of urate (modified after 10)

However, it is important to note that hyperuricemia alone is not sufficient to cause gout. Many individuals with elevated urate levels do not develop arthritis (24). Those who do, present typical needle-like urate crystals as a result of crystallisation. These crystals can typically be found in the intra- and periarticular tissue, but they may also be present along the vascular system and in various other parts of the body. Even though the presence of a crystal is obligatory in gouty inflammation, not all crystals induce arthritis. These depositions of MSU crystals are called silent crystals.

The average uric acid saturation point in adult men is at 420 μmol/l (7 mg/dl). However, lower outer temperature, dehydration, change in pH and cation concentration (Na^+, Ca^{2+}) may reduce solubility, thus lower the saturation limit. Cartilage and synovial debris caused by trauma or as a result of osteoarthritis may also promote crystallisation.

1.3.4.   Gouty inflammation

Monosodium urate is a common form of urate crystals. It acts as a danger-associated molecular pattern (DAMP) for cells of the innate immune system (monocytes, macrophages) that promotes further inflammatory action by activating an intracellular Nod-like receptor, the NACHT, LRR and PYD containing 3 (NALP3) inflammasome. This multiprotein complex consists of several domains (details in Figure 6). The activation of the complex then results in the activation of caspase-1 which further promotes pro-IL1β activation. However, gouty inflammation also requires another, NF-κB driven signal from the Toll-like membrane receptor family (TLR), mostly TLR 2 and 4, resulting in enhanced production of pro-IL1β. As a result of the two pathways, an IL1β efflux appears inducing the typical gouty inflammation (25).

Gouty arthritis is known to be self-limiting. This is largely due to a unique apoptosis of neutrophils, where as a result, neutrophils form special neutrophil extracellular traps (NET). During NETosis, the projection of the cell's chromatin can prevent other inflammatory cells from reacting with MSU crystals, while also trapping proinflammatory mediators (26). Besides NETosis, a change in the local apolipoprotein (ApoB and ApoE) concentration, and elevated TGF-β expression of the newly differentiated macrophages can be observed during flare resolution (27).
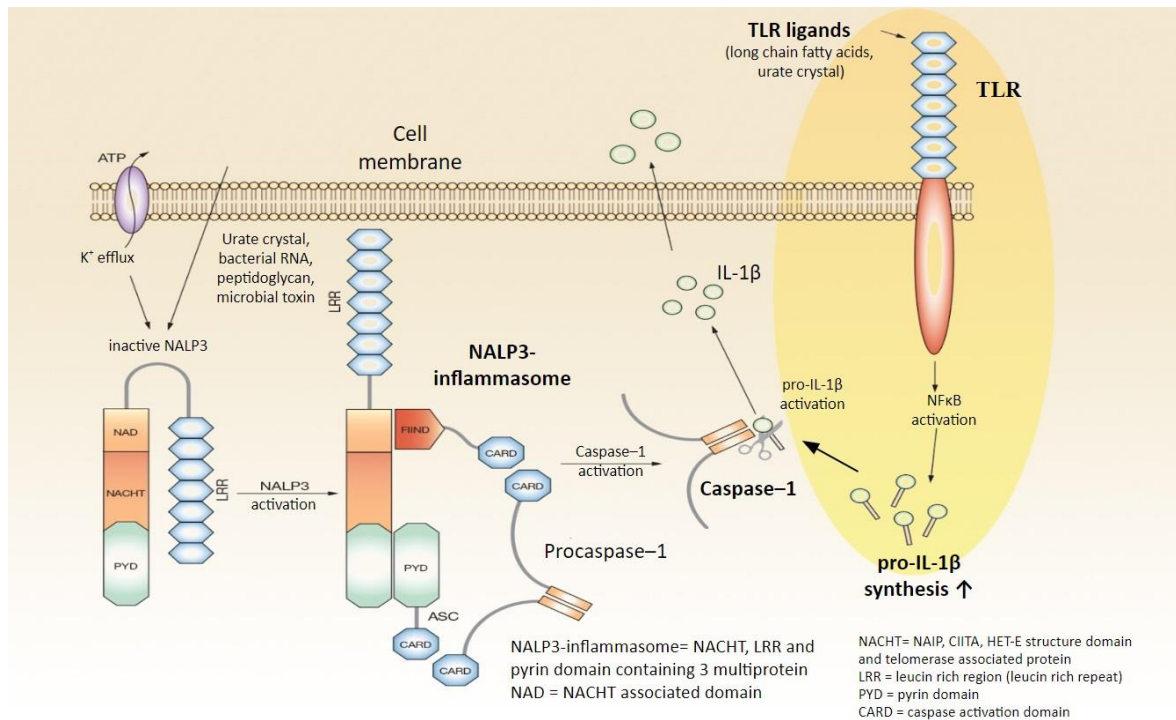
Figure 6. Gouty inflammation (modified after 10)
Activation of TLR pathway leading to increased pro-IL1β synthesis (highlighted). Activation of NALP3 inflammasome and Caspase-1 resulting in an increased IL1β activation and efflux responsible for typical characteristics of a gouty flare.

1.3.5.   Clinical appearance of gout

The clinical appearance of gout may be divided into four stages.

Stage of asymptomatic hyperuricemia. At this stage one observes an elevated uric acid level (>420 μmol/l in men, >360 in women) but no clinical symptoms. As a result of complex environmental effects and genetic predisposition, gout appears in a proportion of the hyperuricemic population. The dual activation of gouty inflammation may partially explain the phenomenon that not all urate crystals lead to arthritis (28). However, silent crystals may be of high importance in regards to endothelial dysfunction and comorbidities (29).

Stage of the first flare. A gout flare is characterised by all typical signs of inflammation. Usually it shows a rapid onset with high intensity of pain (dolor), swelling (tumour), redness (rubor) and heat of the affected joint (calor) resulting in the loss of function of the limb (functio laesa). The inflammation reaches its peak in 24 hours and in some cases fever may be present. It is most common during nights and after excessive use of alcohol, drugs (diuretics), high dietary purine intake or trauma. Gout usually appears in monoarticular form, most commonly in the first metatarsophalangeal joint (also known as podagra), but it may also affect the ankle, knee or the small joints of the hand. However, gouty flare may occur extra articularly (bursitis) or less often in other organs (eye, abdomen, larynx, ear, etc.). An acute gouty flare generally resolves in about 10-14 days. At this stage, the musculoskeletal system has not yet suffered irreversible damage (Figure 7).

Regarding the differential diagnosis, infectious arthritis, psoriatic- and rheumatoid arthritis or inflamed osteoarthritis should be considered at the first place. In the past years, the use of dual-energy CT (DECT) and ultrasound (US) became a useful and reliable aid in the process of identifying MSU crystals (30,31).

Figure 7. Acute gouty inflammation of the left foot (modified after 10)

Stage of recurrent flares. After the first flare, an intercritical period is usually experienced that is again followed by a recurrent flare. With each successive flare, the time between flares tends to shorten, and additional joints may become affected.

Stage of chronic gout. In chronic gout, inflammation is more driven by a foreign-body granulomatous inflammation with both the innate and adaptive immune cells present (32). At this stage calcium deposition appears in the slow growing tophi and erosive cartilage and bone damage is frequent. Radiologic progression is typical, as the bone tophi damages the affected joints, leading even to mutilation (Figure 8).

a   b   c
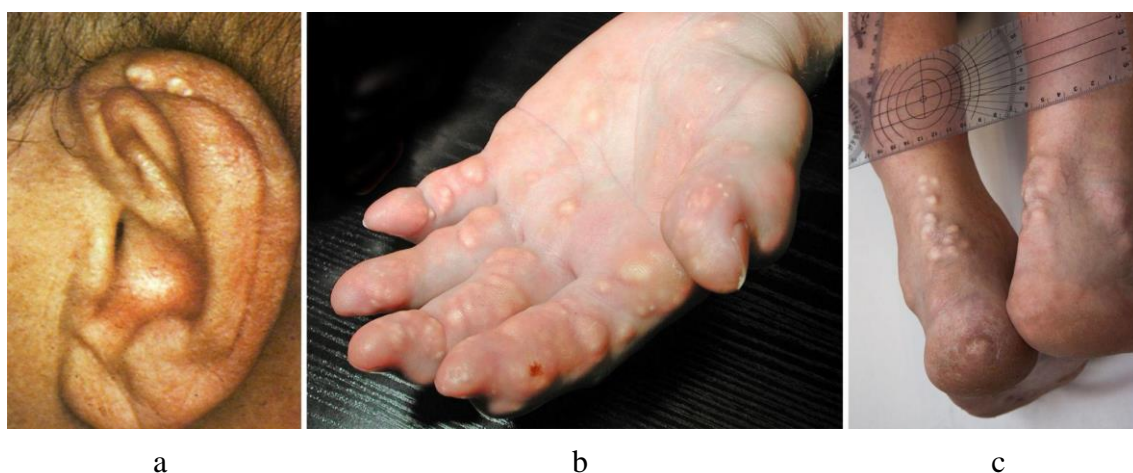
Figure 8. Subcutaneous tophi in chronic gout in the helix of the ear (a), hand (b) and above the Achilles- tendon (c) (10)

1.3.6.   Classification of hyperuricemia and gout

Hyperuricemia and gout are classically categorised into primer and seconder forms. Behind primer hyperuricemia the less frequent reasons are genetic abnormalities leading to overproduction (10 %) of uric acid. This overproduction may most commonly be a result of the hyperactivity of PRPP synthetase or XO enzymes. In rare cases (1-2%) HGPRT impaired function causes hyperuricemia, or in total enzyme defect even Lesch-Nyhan syndrome. The most frequent cause of primer hyperuricemia in classical categorisation is the underexcretion of serum urate due to urate transporter deficiencies (90 %).

Secondary hyperuricemia is in most cases a result of a well-defined disorder. Secondary overproduction appears in all states of high nucleotide turnover (e.g. myelo- and lymphoproliferative disorders, haemolytic anaemia, active psoriasis, etc.). Secondary renal underexcretion may appear in chronic kidney disease (CKD), diabetic ketoacidosis, or simply as a result of abusive use of such basic medications as loop or thiazide diuretics.

Based on all these, the classical categories of hyperuricemia and gout are overproducer type, underexcretion type and combined type as shown in Figure 9.
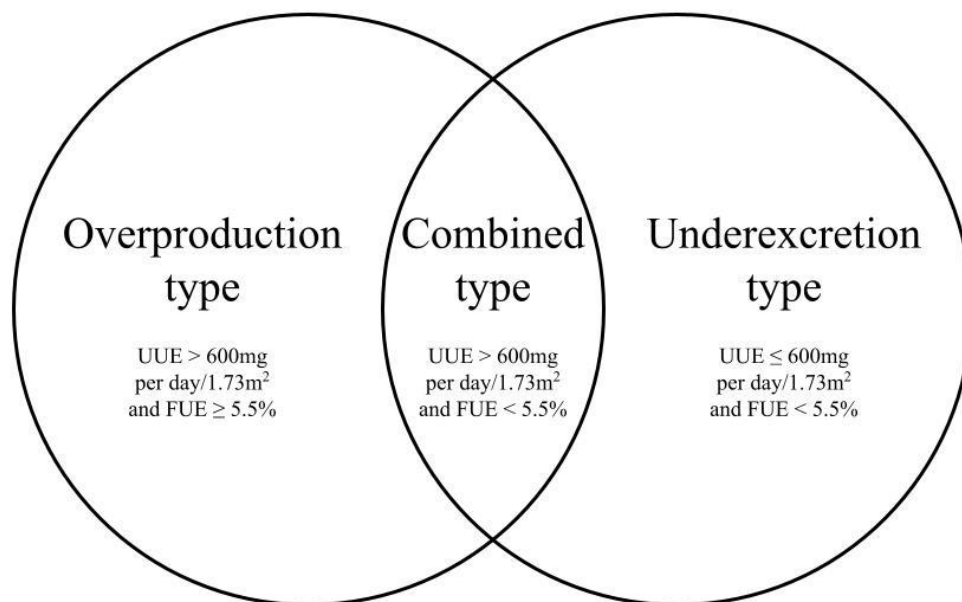
Figure 9. Earlier classification of hyperuricemia and gout (33)

UUE, urinary urate excretion; FUE, fractional urate excretion

2. Objectives

The primary objective of this dissertation was to investigate the role of the ABCG2 urate transporter in gout, providing a comprehensive examination of the topic, ranging from ABCG2 genetic polymorphisms, to protein expression and clinical parameters in gouty patients.

According to genome-wide association studies, functional genetic variations of the ABCG2 transporter are highly associated with gout (34,35). The first objective of this thesis was to investigate and establish a stronger link between functional polymorphisms of the ABCG2 urate transporter and gout susceptibility.

The evaluation of ABCG2 urate transporter protein is challenging, as it typically requires invasive sampling. Despite being well-studied, our understanding of the protein's function in hyperuricemia and gout remains limited. ABCG2 is expressed in the kidney, in the gut, and among several other tissues, also in the erythrocyte membrane. To overcome these sampling difficulties, the second objective of the thesis was to introduce a recently developed and unique method called the Ery-test (36), that allows one to analyse ABCG2 transporter from a single drop of blood as described in the Methods section. With the evaluation of ABCG2 protein, Ery-test makes it also possible to identify cases that should undergo further genetic analyses.

Besides the considerable amount of information on genetic background, our knowledge on ABCG2 protein expression is mostly based on mouse models (33). Therefore, our third objective was to demonstrate this missing link, and analyse the most common and clinically relevant functional single nucleotide polymorphisms (SNP) of ABCG2 gene along with protein expression levels in a clinically defined gouty population.

As ABCG2 loss of function mutations may be associated with an early, even paediatric onset of gout, (37,38) a fourth objective of the thesis was to evaluate whether ABCG2 polymorphisms are associated with the general characteristics of severe gout such as early onset of the disease, high number of occurring flares (flare/ past 12 months) and presence of subcutaneous tophi (6,39).

The classification of hyperuricemia and gout has been challenged in the past years. With better understanding of renal and extrarenal uric acid transport mechanisms, the classical overproduction-underexcretion approach is about to change into renal-overload (ROL) hyperuricemia (containing overproduction and extrarenal underexcretion types), and renal underexcretion type hyperuricemia. Approximately 30% of urate excretion is conducted through the intestines (40) and to our knowledge, this extrarenal underexcretion refers mostly to impaired ABCG2 urate excretion in the terminal ileum (41). Therefore, the fifth and last objective was to assess how mutations correlate to altered protein levels and whether these changes lead to a ROL- type hyperuricemia in gouty population. Based on all these, the thesis is looking for answers to the questions in Table 1.

Table 1. Objectives of the thesis

1. Study and further strengthen the linkage between ABCG2 urate transporter polymorphisms and gout **susceptibility**.

2. By testing and using the lately described **Ery-test**, one may gain information on ABCG2 protein expression levels through such an easily accessible human tissue as peripheral blood. This method could also make it possible to identify cases that should be put to further genetic analyses.

3. Evaluate the connection between ABCG2 **genetic changes** and **protein expression** in a clinically defined gouty population.

4. Investigate whether ABCG2 mutations are associated with higher **disease severity,** characterised by early onset disease (*years prior to wild type group*), frequent flares (*flare/past 12 months*) and tophi formation (*subcutaneous tophi present/absent*).

5. Assess how genetic changes and protein expression correlate with **clinical parameters** (se. urate level, UUE, FUE) and if the altered protein levels strengthen the concept of the new hyperuricemia classification based mostly on ABCG2 dysfunction caused **renal-overload hyperuricemia**.

3. Methods

3.1. Study design and patients

The dissertation is based on a prospectively recruited, non-interventional study of Hungarian gouty patients taken care of at the National Institute of Musculoskeletal Diseases, Budapest, Hungary. Informed consent was obtained from all participants and the work was conducted in accordance with the Declaration of Helsinki and with the permission of the national Scientific and Research Ethic Committee of the Medical Research Council, Hungary (study number 41006-1/2013/EKU).

Clinical classification of gout was based on the EULAR criteria (42). Overproducer type patients and those taking colchicine three months prior to enrolment were not included. Healthy volunteers with no history of hyperuricemia or gout were enrolled as a control group.

3.2. Urate and clinical parameters of participants

Patient's blood tests were taken within the frame of their general examination, therefore no extra intervention was needed. Both serum and urine uric acid levels were determined by Siemens Expansion Plus clinical chemistry analyser. The method used, is a modified form of uricase method described by Bulger and Johns (43) and as well as by Kalckar (44). The level of hyperuricemia was set at 420 μmol/l (7 mg/dl) or over. Hyperuricosuria was defined as urinary urate excretion of 600 mg/die or over. In order to categorise hyperuricemia, serum urate level, fractional urate excretion (FUE) [urate clearance/creatinine clearance×100] and urinary urate excretion (UUE) [mg urinary urate/24 hour] were measured on the same day. Hyperuricemic (gouty) patients were considered renal underexcretion type if FUE was 5.5% or below. Overproducer type was defined if FUE was >10% and UUE was >600 mg/die. We performed a 24-hour urine collection after five days of low-purine diet, adequate hydration, and suspension of xanthine-oxidase inhibitors and diuretics. Urine collection was launched from the second

urination and consisted of the first sample the morning 24 hours later. All samples were saved and kept cold in a single container.

3.3. Genetic analysis from genomic DNA

Genomic DNA was purified from 300 µl of EDTA-anticoagulated blood samples with Puregene Blood Kit (Qiagen). TaqMan-based qPCR reactions for SNP detection were performed in a StepOnePlus device (Applied Biosystems) with premade assay mixes, or with custom-designed assay mixes (R383C) (details see in Table 2), and master mix (cat. 4371353) from Thermo Fisher. TaqMan probe specificities were verified by sequencing (21).

Table 2. Details of genotyped SNPs and probes set

Amino acid change	nucleotide change	rs number	Probe ID number	cat number
Q141K	c.421C>A	rs2231142	C__15854163_70	4362691
M71V	c.211A>G	rs148475733	C_170723361_10	4351379
R236X	c.706C>T	rs140207606	C_170199283_10	4351379
R383C	c.1147C>T	rs560659849	custom-designed	-

3.4. Evaluation of ABCG2 protein expression

The measuring of ABCG2 levels in RBCs were carried out according to the recently developed flow cytometry-based Ery-test method (21,36,45). EDTA-anticoagulated whole blood samples were freshly collected (2-6 hours before the flow cytometry analysis). We mildly fixed the RBC membranes by using 1% formaldehyde solution, resulting in RBC membranes, also known as "ghosts". Wheat germ agglutinin- (WGA-) Alexa Fluor 647 (Thermo Fisher, cat. W32466, final cc. 1 µg/ml) were added to the fixed and washed RBC ghosts. Due to their small size and low light refraction, ghosts can be found in the so-called debris zone. WGA is able to non-specifically bind to the sugar chains of the membranes, thereby labelling both ghosts and cells in the sample, so they can be separated from the debris zone during flow cytometry. The WGA-labelling ghosts were incubated with the ABCG2-specific primary antibody (Bxp-34, mouse monoclonal antibody, Abcam, cat. ab3379) followed by a secondary, Alexa Fluor 488-labelled goat anti-mouse (H+L) antibody (Thermo Fisher, A-11001), in 96 well plates. Cellular fluorescence was measured twice each case by a FACSCanto II flow cytometer (excitation wavelengths: 488 nm, emission filters: 530/30 nm for Alexa Fluor 488 and excitation wavelengths: 633 nm, emission filters: 660/20, for Alexa Fluor 647) equipped with a plate loader. The agreement between the two measurements were tested by intraclass correlation coefficient (ICC). ICC estimates and their 95% confidence intervals were calculated based on an absolute-agreement, 2-way model (package irr in R). ICC showed strong significant agreement between the two measurements of Bxp34 (ICC=0.988, 95% CI: 0.982 – 0.991, $p<0.001$). Therefore, we used the average of the two results for further analysis.

3.5. Statistical analysis

Statistical analysis was conducted in R 4.0.3 and in Statistica 13.1 (Dell) softwares (46). The significance level was set at $p<0.05$. When comparing two categorical variables, the Fisher tests were used, e.g. when differences in ratio of males/females or the presence/absence of at least one ABCG2 mutation were compared between gouty and control patients, or when the presence of tophi and more than one flares were compared among gouty patients having or not having mutation. The results of the Fisher tests gave us the odds ratios (OR) with 95% confidence intervals (CI).

Separate general linear models in R were used to test the differences between gouty and control patients, or other categorical variables (such as gender, or presence/absence of at least one ABCG2 mutation) in regards to the following variables: age at the time of study, age at diagnosis, serum urate level, urinary urate excretion, fractional urate excretion and RBC-ABCG2 protein expression. For multiple comparison the Dunnett test was used.

Pearson correlation was used to test correlation among gouty patients UUE and FUE along with RBC-ABCG2 expression; the lines on the correlation plots were fitted by using general linear models.

4. Results

The results of the thesis contain the data of 151 patients, 78 gouty cases and 73 matched healthy controls. The ratio of males was 91.0% among gouty and 86.3% among control patients, the difference was not significant. Mean (SD) age (age at the time of study) for control and gouty patients were 55.0 (13.3) and 57.4 (11.3) years, respectively. Age did not differ significantly among control and gouty patients or among males and females. The average (mean, SD) serum urate level was significantly higher in gouty patients compared to age-matched controls, 8.4 (1.6) vs 5.3 (1.0) mg/dl, (p<0.001). Age and gender did not have a significant effect on serum urate level.

4.1. Genetic background: old and new mutations of ABCG2

ABCG2 plays a crucial role in hyperuricemia and gout, and being a well-studied transporter, several polymorphisms were already known. However, there may be less frequent SNPs or mutations that may have a significant effect on presenting the disease.

We came to the identification and description of a new functional mutation, c.211A>G (rs148475733), that results in an M71V variant of the protein. The M71V variant turned out to reduce the expression of the ABCG2 protein by about 50% compared to the wild type (21). Moreover, in *in vitro* functional tests, collaborating partners found that the protein is active, but unstable which is why it degrades quickly. As a result of treatment with colchicine, an agent that inhibits microtubule polymerization and is used in treating gout flares, a functional, stable protein reached the membrane by preventing the retrograde transport of the ABCG2 mutant protein into the aggresome. The novel identification of M71V ABCG2 augmented the important SNPs number that were included in the further analysis.

After screening all participants, we found that the most common polymorphism was Q141K (rs2231142) affecting, in either hetero- and homozygous form, 28.2% (n=23) of gouty and 13.7% (n=10) of control populations. Among the gouty population, we also identified one R236X (rs140207606) and one R383C (rs560659849) mutations. In the case of the newly described M71V, Q141K polymorphism was also present. Taken together, 23.2% (35/151) of the total population had at least one ABCG2 mutation (Table 3).

Out of 78 gouty patients, 25 (32.1%) had at least one ABCG2 polymorphism, while only 10 out of 73 (13.7%) healthy controls had a polymorphism. The results showed that gouty patients were three times more likely to have an ABCG2 SNP than the control group (OR= 3.0, 95% CI:1.2-7.5, p=0.012).

Table 3. Prevalence and most common types of ABCG2 polymorphisms in gouty and control patients (19)

Note: one patient had Q141K/M71V form of mutation.

MAF, minor allele frequency (47); Homozyg., homozygous; Heterozyg., heterozygous; WT, wild type

	Gouty patients, n=78				Control, n=73			
	MAF	Homozyg.	Heterozyg.	WT	MAF	Homozyg.	Heterozyg.	WT
Q141K	0.16	2	21	55	0.08	1	9	63
M71V	0.01	0	1	77	0	0	0	73
R236X	0.01	0	1	77	0	0	0	73
R383C	0.01	0	1	77	0	0	0	73

4.2. Decreased ABCG2 protein expression in erythrocyte membrane

As a next step, we compared how the presence of polymorphisms and RBC-ABCG2 protein expression were related. We found that in case of functional ABCG2 SNPs being present, the transporter density was significantly lower. Different polymorphisms, either in hetero- or homozygous form, resulted in different extent of protein change. The newly described M71V/Q141K had the most serious impact, further highlighting its importance (Figure 10 a, b).

a

b
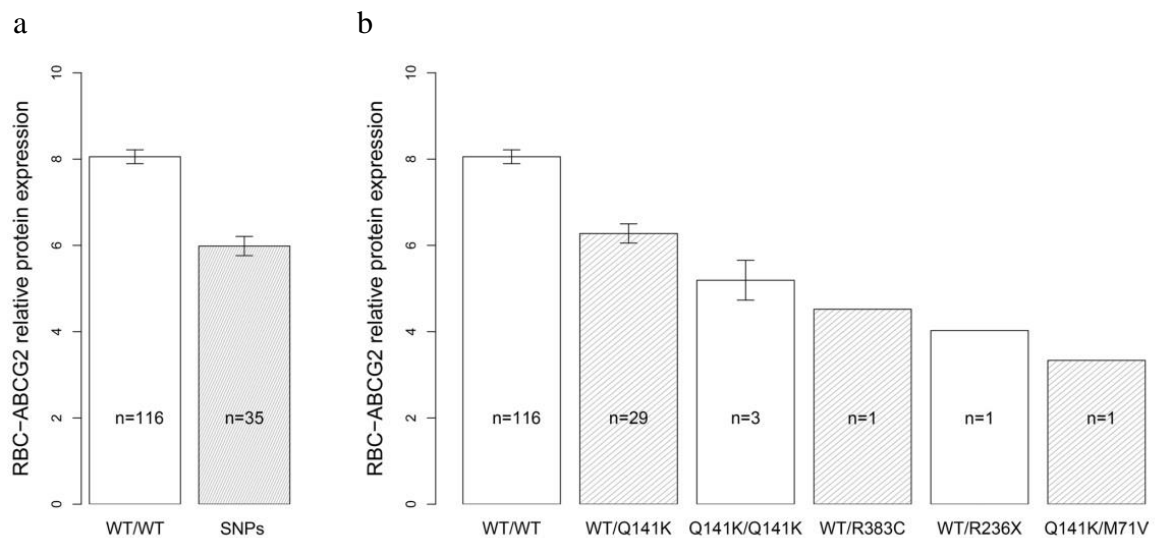
Figure 10a, b. Correlation of functional ABCG2 polymorphisms and protein expression levels (19)

Mean values ± SD of erythrocyte ABCG2 relative protein expression levels in all wild type and mutant individuals (a) or wild type and individuals with specific mutations (b)

4.3. Disease severity

In order to further analyse disease severity, the thesis also examined the potential effect of the ABCG2 variant in gout groups compared to wild-type patients. As shown in Table 4, we analysed the statistics in relation the participants' age at diagnosis, the presence or absence of tophi, the frequency of gouty flares, serum urate level and the levels of the clinical indices UUE and FUE. The mean protein expression level is also indicated in Table 4 in regard to the presence or absence of the studied SNPs.

Table 4. Clinical and laboratory parameters of gouty patients with or without the studied ABCG2 mutation (19)

UUE, urinary urate excretion; FUE, fractional urate excretion; RBC, red blood cell; ABCG2, ATP-binding cassette (ABC) transporter family (subgroup G2); AU, arbitrary units

	Presence of studied ABCG2 mutation in gouty patients, n=25	Absence of studied ABCG2 mutation in gouty patients, n=53	p value
Mean ±SD age at diagnosis (years)	37.6±11.8	45.7±12.3	0.008
Presence of two or more flares during the past 12 months	20/25 (80.0%)	28/53 (52.8%)	0.026
Presence of tophi	10/25 (40.0%)	16/53 (30.2%)	0.445
Mean ±SD UUE (mg/24h)	663.0±190.1	522.7 ±171.2	0.002
Mean ±SD FUE (%)	6.1 ±1.8	4.6 ±1.4	<0.001
Mean ±SD serum urate level (mg/dl)	8.9±1.9	8.3±1.6	0.140
Mean ±SD relative RBC-ABCG2 protein expression (AU)	6.1±1.5	7.8±1.7	<0.001

Hyperuricemic patients were considered renal underexcretion type if FUE was 5.5% or below and overproducer type if FUE was >10% and UUE was >600 mg/die.

4.4. Renal-overload hyperuricemia

As a last step, the special clinical indices FUE, UUE were used to classify gouty cases. This was later compared to the extent of ABCG2 protein expression, indicated with Bxp34 level as described in the Methods section. We have found that ABCG2 (Bxp34) levels showed significant negative correlation with FUE (Pearson correlation coefficient=−0.320, p=0.005) and UUE (Pearson correlation coefficient=−0.241, p=0.038). FUE and UUE also showed strong positive correlation (Pearson correlation coefficient=0.568, p<0.001) as shown in Figure 11a, b.

a

b
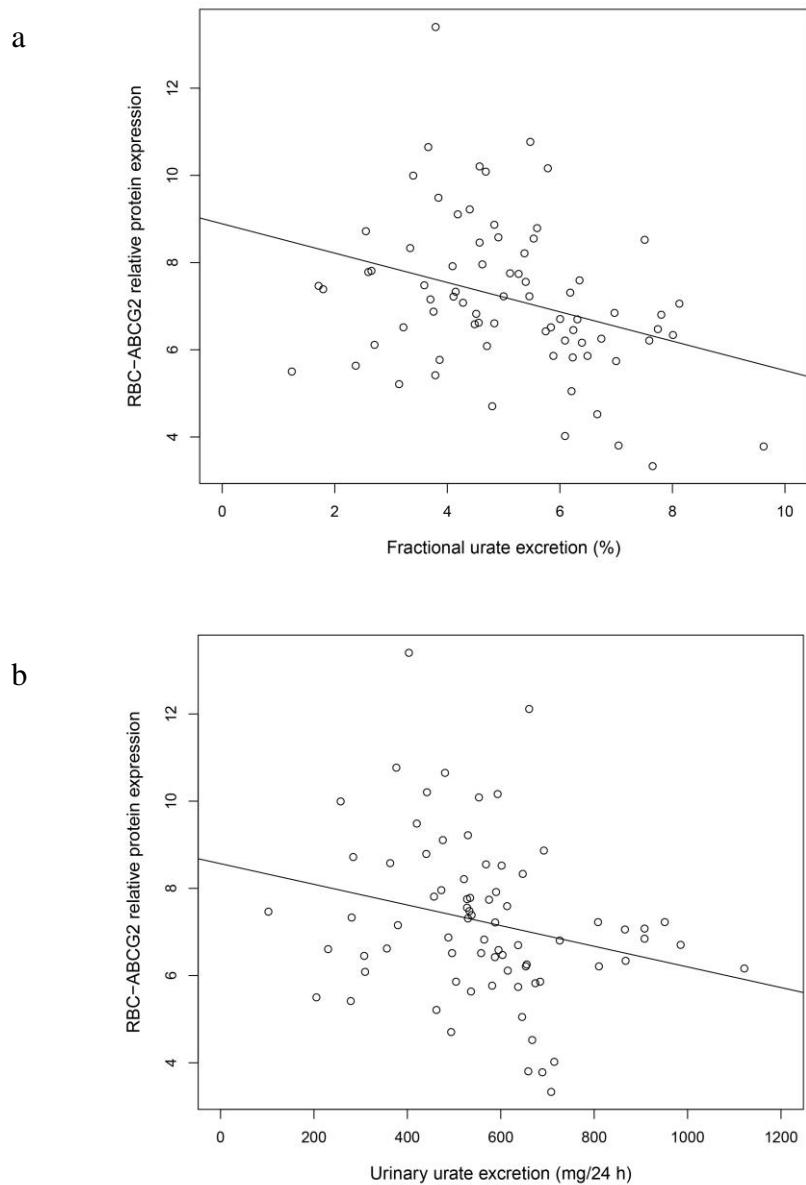
Figure 11a, b. Correlation of renal-overload hyperuricemia clinical parameters and ABCG2 protein expression (19)

Correlation plot of fractional urate excretion (a) and urinary urate excretion (b) in regards to the relative protein expression of RBC-ABCG2

5. Discussion

The thesis aimed to evaluate the significance of ABCG2 urate transporter in gout. While the topic has received significant attention in recent years, to the best of our knowledge, previous literature has not yet provided a comprehensive examination of the subject, spanning from genetic foundations to protein expression and clinical manifestation, particularly with regard to classification. In addition, this thesis also addresses the crucial issue of disease severity.

5.1. Gout susceptibility and ABCG2 genetic polymorphisms

The variants of membrane transporter ABCG2, which plays a significant role in the urate transport, among other things, has already been associated with the development of gout in several genetic studies and GWAS (33,34). Therefore, we first attempted to analyse and further interpret the relationship between ABCG2 functional polymorphisms and susceptibility to gout. As an initial step, we aimed to identify the most functional mutations possible. In order to achieve this goal, we first used the recently developed Ery-test method in a gouty population (21). This process, described in detail in the relevant Methods section, made it possible to study ABCG2 protein levels even from a few drops of peripheral blood (36). With this method, we were able to quickly and efficiently identify those individuals whose ABCG2 protein level was reduced (75% of the average or less), which is also accompanied by a reduction in the transport function. As part of the research, we also examined the genetic background of lower ABCG2 expression levels, where no known mutation could be detected, in these cases the ABCG2 gene was subsequently sequenced.

In addition to previously identified SNPs, this approach allowed us to discover a new functional polymorphism c.211A>G (rs148475733), which results in the M71V variant of the ABCG2 protein. This M71V polymorphism was found to be a functional SNP that results in an active but structurally unstable protein, thereby reducing protein expression at the cell membrane, resulting in impaired ABCG2-mediated urate excretion.

However, the function of this variant could be restored *in vitro* by treatment with small molecule chemical compounds, such as colchicine (21). Our results also demonstrate that the Ery-test is a suitable tool for evaluating ABCG2 protein expression in gout populations.

As a subsequent step, we examined four functional polymorphisms, including the relatively common Q141K and the recently identified M71V, as well as R236X and R383C, in both gouty and control populations. While the ABCG2-Q141K variant has been shown in several GWAS to cause significantly elevated serum uric acid levels and to occur more frequently among gouty patients than in the control population, we do not have enough information about the role of the R236X stop mutant, or on the unstable variant of R383C in gout. Our data significantly proved that the prevalence of SNPs was higher in the gouty population (32.1%) compared to the control population (13.7%). These results emphasised the importance of functional SNPs in regards to susceptibility to gout. According to our data, the possibility of developing the disease was three times higher if any of the studied polymorphisms were present (OR=3.0, 95% CI:1.2-7.5) (18,48,49).

5.2. Filling the gap: protein expression in regards to genetic polymorphisms

Previous analysis of ABCG2 urate transporter proteins has been hindered by the invasive nature of sample collection. As a result, much of the available data has come from animal studies (such as those using mice) and has lacked clinically relevant *in vivo* information (33,50). The implementation of the Ery-test enabled us to study ABCG2 transporter proteins in a well-controlled gout population, thus filling a previously existing gap in research.

Our work clearly demonstrated through statistical analysis that the presence of specific functional SNPs led to a marked reduction in expression levels of the ABCG2 transporter protein. The most common was the Q141K variant, but the most pronounced effect was observed when M71V/Q141K was present, further highlighting the important

role of this recently identified loss-of-function mutation. As a cumulative mutation, the effect of the two variants was additive, in accordance with previous *in vitro* data.

5.3. Disease severity

The significance of disease severity in gout has been acknowledged and emphasised in recent leading international and Hungarian guidelines (6,51). The key indicators of a severe disease can be characterised by early onset gout (before the age of 40), particularly with frequent flares (≥2/year) and/or tophi formation (6,39,52).

To prove the fourth objective of the thesis, our results demonstrate that patients with ABCG2 mutations developed gout on average 8 years earlier than wild-type gout patients, at an average age of 37.6. Furthermore, our data indicate that patients carrying ABCG2 mutations had significantly higher chances to experience frequent flares and had a higher number of tophaceous cases, although this difference did not reach statistical significance. These findings suggest that functional ABCG2 mutations may be a risk factor for early onset, severe gout.

5.4. New classification: renal-overload hyperuricemia

About 30% of urate is excreted through the intestines, mostly through the terminal ileum (40). Due to impairment of extrarenal, mostly intestinal urate excretion a renal-overload of urate may appear (ROL-type hyperuricemia) that could be clinically characterised by the elevated values of FUE and UUE (33,53).

As the last step of this thesis, we described that both the altered protein levels and the genetic changes of ABCG2 transporter correlated with an inversely proportional shift of the studied FUE and UUE parameters, showing elevated (near normal) FUE and increased UUE levels. These results *in vivo* confirm previous data (33) that impaired ABCG2 transport function acted as an extra-renal cause of urate excretion deficiency leading to a renal-overload type hyperuricemia.

This thesis has made significant contributions by linking the various stages of ABCG2 genetic, protein and functional impairment, paving the way for potential therapeutic and diagnostic advancements in the clinical field.

5.5. Limitations and future aims

We acknowledge that the thesis also has limitations.

The Ery-test method proved to be a significant breakthrough in the study of ABCG2 proteins. In addition, its application can be extended to other proteins that can be accurately measured on the surface of red blood cells, since more than 300 membrane proteins can be found on them based on mass spectrometry data. However, it is important to note that there may be proteins that are not expressed on the RBC membrane, which limits the use of this unique method (54).

Additionally, patients who are treated with previous colchicine are not suitable for investigation until the turnover of red blood cells as the compound has the ability to restore the function of the most frequent Q141K and M71V ABCG2 cases.

Proper diet and hydration are highly important in gout and were reported by the patients themselves. While hydration was checked during the physical examination, it is difficult to fully eliminate all dietary bias.

Due to strict sample management and high human resource requirements, the study population was relatively modest, and it would be beneficial to expand the sample size in future studies.

The findings of this thesis open up multiple opportunities for future research. As previously mentioned, *in vitro* studies have shown that the impaired expression caused by Q141K and M71V can be successfully corrected with colchicine treatment. However, current national and international (EULAR or American College of Rheumatology, ACR) therapeutic guidelines do not distinguish between drugs recommended to treat gouty flares (55). According to MAF data the ABCG2 affected amount in Caucasian (European) population may range from 10% to as high as 20% of all gouty cases (and even higher in Asian population), therefore further research on the use of colchicine in cases of impaired ABCG2 expression would be beneficial.

According to the current guidelines screening and treating comorbidities is a priority in patient management (56). It is also among our future aims to further evaluate disease severity in conjunction with comorbidity data to improve patient outcomes in this

serious matter.

Despite the imperative need to reach target serum urate level (T2T approach), current EULAR and ACR guidelines do not differentiate between renal underexcretion type and renal-overload type gouty patients (57). Currently, it is believed that renal-overload hyperuricemia is primarily caused by dysfunction of the ABCG2 transporter. However, in these cases allopurinol may not be the optimal choice as it competes with the transporter as a substrate (5,58–61). Therefore, it would be beneficial to investigate alternative (non-allopurinol) treatment options specifically for ROL-hyperuricemia caused by ABCG2 dysfunction.

Taking all these into account, we believe that these findings and future considerations could lead us towards a more personalised, efficient and safer therapy for gouty arthritis.

6. Conclusions

The thesis demonstrates that ABCG2 plays a crucial role in urate transport and the development of gout. It aims to trace its path from genetic polymorphisms to protein expression and clinical manifestations in the studied gout patients.

The findings show that functional mutations in ABCG2 are associated with an increased risk of gout and a clinically severe, early onset disease. The thesis firstly describes that the joint presence of M71V/Q141K results in highly impaired ABCG2 function. It proves that the investigated mutations are correlated with decreased protein expression, and this decrease is linked to clinical data such as elevated FUE and UUE values. These results establish a strong connection between ABCG2 dysfunction and renal-overload hyperuricemia in clinically diagnosed gout patients.

7. Summary

Gout is a common crystal induced disease with high personal and social burden. If left untreated, it is characterised by severe arthritis and comorbidities. ABCG2 urate transporter dysfunction is known to be strongly related to gout and lately it was also connected with renal-overload type hyperuricemia. However, despite being a key factor, our information is still limited on how ABCG2 genetic changes in gouty patients relate to protein expression and that to clinical parameters.

This thesis synthesises existing literature and our previous results concerning 78 gouty patients and 73 healthy controls from a Hungarian population. ABCG2 protein expression was measured on red blood cells by a flow cytometry-based method, while genetic background was determined by TaqMan-based qPCR. All urate and clinical parameters were determined among standardised circumstances.

The prevalence of ABCG2 mutations in gouty and control patients were 32.1% and 13.7%, respectively. Most common SNP was Q141K while one sample with R236X and with R383C was found. We firstly described a new M71V functional mutation that results in an active but structurally unstable protein. These ABCG2 mutations showed strong association with impaired protein expression while the latter was associated with higher disease severity and significantly elevated clinical indices of fractional urate excretion (FUE) and urinary urate excretion (UUE) compared to wild type gouty patients.

This thesis highlights the significance of the ABCG2 urate transporter in gout. It manages to firstly evaluate ABCG2 protein expression in a clinically defined gouty population while also proving its association between already known, and newly described ABCG2 genetic mutations and renal-overload hyperuricemia. The thesis also assesses relations between ABCG2 mutations, gout susceptibility and disease severity characterised by early onset disease with frequent flares and tophi formation.
The study was registered by the Scientific and Research Ethic Committee of the Medical Research Council, Hungary (41006-1/2013/EKU).

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9. Bibliography of the candidate's publications

9.1. Publications related to the thesis

1.

Pálinkás M, Szabó E, Kulin A, Mózner O, Rásonyi R, Juhász P, Nagy K, Várady G, Vörös D, Zámbó B, Sarkadi B, Poór G

Genetic polymorphisms and decreased protein expression of ABCG2 urate transporters are associated with susceptibility to gout, disease severity and renal-overload hyperuricemia

CLINICAL AND EXPERIMENTAL MEDICINE 2022 Paper: DOI: 10.1007/s10238-022-00848-7, 8 p. (2022)

Közlemény: 33061607 Szakcikk (Folyóiratcikk) | Tudományos

Scopus - Biochemistry, Genetics and Molecular Biology (miscellaneous) SJR indikátor: Q1

Scopus - Medicine (miscellaneous) SJR indikátor: Q1

IF: 5,057*

*Várható IF érték

2.

Járai Zoltán, Alföldi Sándor, Mátyus János, Ábrahám György, Balog Attila, Csiky Botond, Farsang Csaba, Kumánovics Gábor, **Pálinkás Márton**, Páll Dénes, Reusz György, Tislér András, Szekanecz Zoltán

A Magyar Hypertonia Társaság, a Magyar Nephrologiai Társaság és a Magyar Reumatológusok Egyesületének konszenzus-dokumentuma a hyperuricaemiás és a köszvényes betegek ellátásáról

MAGYAR REUMATOLÓGIA 61: 2 pp. 70-89. (2020)

Közlemény: 31350737 Jelentés (Folyóiratcikk) | Tudományos

3.

Pálinkás Márton, Balog Attila, Kumánovics Gábor, Szekanecz Zoltán

A kristályok bűvöletében - terápia

MAGYAR REUMATOLÓGIA 61: 4 pp. 246-251. (2020)

Közlemény: 31806040 Összefoglaló cikk (Folyóiratcikk) | Tudományos

4.

Kumánovics Gábor, Balog Attila, **Pálinkás Márton**, Szekanecz Zoltán

A kristályok bűvöletében - diagnosztika

MAGYAR REUMATOLÓGIA 61: 4 pp. 239-246. (2020)

Közlemény: 31806019 Összefoglaló cikk (Folyóiratcikk) | Tudományos

5.

Balog Attila, Kumánovics Gábor, **Pálinkás Márton**, Szekanecz Zoltán

A kristályok bűvöletében - patogenezis

MAGYAR REUMATOLÓGIA 61: 4 pp. 232-238. (2020)

Közlemény: 31806017 Összefoglaló cikk (Folyóiratcikk) | Tudományos

6.

Pálinkás M, Mester A, Poór Gy

A kettős energiájú CT alkalmazása a köszvény diagnosztikájában és a terápiamonitorozásban

ORVOSTOVÁBBKÉPZŐ SZEMLE 2020: Fájdalomcsillapítás Különszám pp. 2-6. (2020)

Közlemény: 33558160 Összefoglaló cikk (Folyóiratcikk) | Tudományos

7.

Zámbó B, Bartos Z, Mózner O, Szabó E, Várady G, Poór G, **Pálinkás M**, Andrikovics H, Hegedűs T, Homolya L, Sarkadi B

Clinically relevant mutations in the ABCG2 transporter uncovered by genetic analysis linked to erythrocyte membrane protein expression

SCIENTIFIC REPORTS 8: 1 Paper: 7487, 13 p. (2018)

Közlemény: 3370208 Szakcikk (Folyóiratcikk) | Tudományos

Scopus - Multidisciplinary SJR indikátor: D1

IF: 4,011

8.

Pálinkás M

Célok és eszközök az urátcsökkentő terápiában

ORVOSTOVÁBBKÉPZŐ SZEMLE 24: 12 pp. 47-53. (2017)

Közlemény: 33558182 Összefoglaló cikk (Folyóiratcikk) | Tudományos

9.

Pálinkás M, Poór Gy

A köszvény kardiovaszkuláris komorbiditása

ORVOSTOVÁBBKÉPZŐ SZEMLE 23: 3 pp. 77-80. (2016)

Közlemény: 33558238 | Összefoglaló cikk (Folyóiratcikk) | Tudományos

10.

Pálinkás M, Poór Gy

Újdonságok a köszvény klasszifikációjában, genetikájában és terápiájában

MAGYAR REUMATOLÓGIA 56: 2 pp. 69-75. (2015)

Közlemény: 2924337 | Összefoglaló cikk (Folyóiratcikk) | Tudományos

11.

Pálinkás M, Poór Gy

Kristályarthritisek.

In: Szekanecz, Zoltán; Nagy, György (szerk.) Reumatológia

Budapest, Magyarország: Medicina Könyvkiadó (2019) pp. 551-576.

Közlemény: 33558422 Könyvfejezet (Könyvrészlet) | Oktatási

9.2. Publications not related to the thesis

1.

Donáth Judit, Balla Bernadett, **Pálinkás Márton**, Rásonyi Rita, Vastag Gyula, Alonso Nerea, Prieto Beatriz Larraz, Vallet Mahéva, Ralston Stuart H, Poór Gyula

Pattern of SQSTM1 Gene Variants in a Hungarian Cohort of Paget's Disease of Bone

CALCIFIED TISSUE INTERNATIONAL 108: 2 pp. 159-164. (2021)

Közlemény: 31625608 Szakcikk (Folyóiratcikk) | Tudományos

Scopus - Orthopedics and Sports Medicine SJR indikátor: Q1

Scopus - Endocrinology SJR indikátor: Q2

Scopus - Endocrinology, Diabetes and Metabolism SJR indikátor: Q2

IF: 4,000

2.

Pálinkás M

Etoricoxib - új lehetőségek a fájdalomkezelés terén. A kezelés hatékonysága és biztonságossága: Kommentár

MOZGÁSSZERVI TOVÁBBKÉPZŐ SZEMLE: INTERDISZCIPLINÁRIS SZAKMAI LAP 3: 2 pp. 76-77. (2020)

Közlemény: 33558300 Ismertetés (Folyóiratcikk) | Tudományos

3.

Donáth Judit, **Pálinkás Márton**

Fájdalomcsillapítás: hogyan kezdjük el? A ketoprofen gél és a nimesulid granulátum helye a mindennapi praxisban

HÁZIORVOS TOVÁBBKÉPZŐ SZEMLE 19: 6 pp. 426-429. (2014)

Közlemény: 2876709 Összefoglaló cikk (Folyóiratcikk) | Tudományos

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