Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 498757, 9 pages
doi:10.1155/2011/498757

Research Article

Urinary Elimination of Coproporphyrins Is Dependent on *ABCC2* Polymorphisms and Represents a Potential Biomarker of MRP2 Activity in Humans

Isabelle Benz-de Bretagne,[1,2] Renaud Respaud,[3] Patrick Vourc'h,[1,4] Jean-Michel Halimi,[5,6] Agnès Caille,[7] Jean-Sébastien Hulot,[8] Christian R. Andres,[1,4] and Chantal Le Guellec[1,9]

[1] *CHRU de Tours, Laboratoire de Biochimie et Biologie Moléculaire, 37044 Tours, France*
[2] *Université Pierre et Marie Curie, École Doctorale de Physiologie et Physiopathologie, 75006 Paris, France*
[3] *CHRU de Tours, Unité de Pharmacie Clinique et Oncologique, 37044 Tours, France*
[4] *Université François Rabelais de Tours, UMR INSERM U930, CNRS ERL 3106, 37032 Tours, France*
[5] *CHRU de Tours, Service de Néphrologie-Immunologie Clinique, 37044 Tours, France*
[6] *Université François Rabelais de Tours, EA425, 37032 Tours, France*
[7] *Université François Rabelais de Tours, INSERM Center d'Investigation Clinique 202, 37044 Tours, France*
[8] *AP-HP, Hôspital Pitié-Salpêtrierè, Service de Pharmacologie, 75013 Paris, France*
[9] *Département de Pharmacologie, Université François Rabelais de Tours, 37032 Tours, France*

Correspondence should be addressed to Chantal Le Guellec, leguellec@med.univ-tours.fr

Received 12 October 2010; Revised 12 January 2011; Accepted 9 February 2011

Academic Editor: Yasushi Okazaki

MRP2 encoded by *ABCC2* gene is involved in the secretion of numerous drugs and endogenous substrates. Patients with Dubin-Johnson syndrome due to mutation in *ABCC2* gene have elevated urinary coproporphyrin ratio (UCP I/(I + III)). Here we investigated whether this ratio could serve as a biomarker of MRP2 function. Phenotype-genotype relationships were studied in 74 healthy subjects by measuring individual UCP I/(I + III) ratio obtained on 24-hour urine and by analyzing five common SNPs in *ABCC2* gene. The UCP I/(I + III) ratio varied from 14.7% to 46.0% in our population. Subjects with 3972TT genotype had a higher ratio ($P = .04$) than those carrying the C allele. This higher UCP I/(I + III) ratio was correlated with a higher level of isomer I excretion. This study provides a proof of concept that UCP I/(I + III) ratio can be used as a biomarker of MRP2 function in clinical studies as it provides quantitative information about the *in vivo* activity of MRP2 in a given patient.

1. Introduction

Excessive accumulation of drugs and consequent drug-induced toxicity may arise from a defect in the ability to secrete drugs into the bile or urine. Various membrane transporters, including those of the ATP-binding cassette (ABC) family, are involved in these processes in the kidneys and liver. One such transporter, multidrug resistance protein 2 (MRP2), encoded by the *ABCC2* (ABC subfamily C2) gene, plays a key role in the secretion of numerous drugs and drug metabolites [1]. Studies in Mrp2-deficient rodents have confirmed the relevance of this transporter in drug pharmacokinetics, with exposure levels reaching 140% to 615% of those in normal animals, depending on the drug considered [2–5].

MRP2 is also involved in the elimination of various endogenous compounds. Uroporphyrins and coproporphyrins, natural intermediates of heme biosynthesis, are the main porphyrins in excess in the plasma. Coproporphyrins exist in two isomeric forms (isomer I and isomer III), both physiologically excreted by the liver and the kidney. Although no direct lines of *in vitro* evidence indicated a role for MRP2 in coproporphyrins transport, animal studies recently confirmed the major role of Mrp2 in the elimination of these

compounds from the plasma [6]. The authors used a bile fistula rat model to study the biliary and urinary excretion of coproporphyrin isomers in wild-type and Mrp2-deficient rats receiving a continuous infusion of equimolar amounts of the coproporphyrin isomers. Mrp2-deficient rats excreted larger amounts of coproporphyrins in urine, with very low levels in bile compared to wild-type rats. The proportion of each isomer in urine also differed between the two groups of animals. Isomer III predominated in the urine of wild-type animals, whereas the proportion of isomer I was higher in the urine of Mrp2-deficient rats, accounting for 70% of the coproporphyrins present. These results confirmed that MRP2 is involved in the excretion of coproporphyrins and that the impairment of MRP2 activity leads to a switch in excretion pattern, from excretion in bile to excretion in urine.

Dubin-Johnson syndrome (DJS) is an autosomal recessive disorder in which the production or function of MRP2 protein is impaired [7, 8]. Missense, nonsense, or splice-site mutations in the *ABCC2* gene are responsible for this syndrome. It is characterized by conjugated hyperbilirubinemia and a deposition of a melanin-like pigment in hepatocytes, with liver function otherwise normal. DJS patients also present a modified pattern of coproporphyrin isomers excretion in urine, characterized by a high urinary coproporphyrin I-to-coproporphyrin I + III ratio (UCP I/(I + III) ratio) [9, 10]. Subjects carrying a homozygous mutation in the *ABCC2* gene have a UCP I/(I + III) ratio greater than 80%, whereas in normal subjects it is only about 30%. Subjects with heterozygous mutations have moderately high UCP I/(I + III) ratios (about 60%) [9]. Based on results from studies on the distribution of transporters in liver and kidney [11] and results of Moriondo et al. [6], Frank et al. [9], and Kaplowitz et al. [12], we propose a possible physiopathologic pathway for coproporphyrin elimination (Figure 1).

Apart from mutations, several common single nucleotide polymorphisms (SNPs) in the *ABCC2* gene have also been identified [13, 14]. The phenotypic consequences of these SNPs have been evaluated indirectly, by studying the pharmacokinetics of several MRP2 substrates. These studies showed an impact of particular SNPs (−24C/T, 1249G/A, 3563T/A, 3972C/T, and 4544G/A) on methotrexate [15], mycophenolic acid [16], irinotecan [17], pravastatin [18], and doxorubicin [19] elimination. Identifying patients with low levels of MRP2 activity before the administration of a drug that might act as a substrate for MRP2 could therefore potentially lower the risk of overexposure. However, no phenotypic marker of MRP2 activity has yet been identified. We hypothesized that the pattern of coproporphyrin elimination in urine would constitute a potential biomarker for MRP2 activity. We thus analyzed phenotype-genotype relationships, by studying the excretion of coproporphyrin isomers in urine and by genotyping several *ABCC2* SNPs in a group of healthy volunteers.

2. Methods

2.1. Subjects and Study Procedures. Eighty healthy human volunteers were recruited for the COVOL study (Clinicaltrial.gov number NCT00746044). Caucasian male and female subjects were eligible for participation, provided they met the following criteria: at least 18 years of age, no clinical abnormality (investigator's clinical judgment), no porphyria (according to the urine analysis results), and normal renal and liver functions. Subjects had no chronic treatment other than oral contraception in female subjects (comprising ethynylestradiol, 20 to 30 μg) and took no medication in the two weeks preceding the study. The COVOL protocol was approved by the ethics committee of Tours University Hospital, France. All subjects gave written informed consent for genetic testing and urine analysis. A blood sample was drawn from each subject for SNP analysis. For coproporphyrin determinations, urinary samples were obtained according to the following timetable: day 1, 5 mL of the morning micturition sample was dispensed into aliquots, and all subsequent urine over the next 24 hours was collected. We also collected 24-hour urine samples on two other occasions, within a maximum of two months. Sodium carbonate was immediately added to samples as an oxidizing agent, and samples were then kept frozen until analysis. Procedures for urine handling and storage were established before this study [20]. Urine volumes were precisely measured.

2.2. HPLC Analysis of Coproporphyrins and UCP I/(I + III) Ratio Determination. Urinary coproporphyrin levels were determined by HPLC (high-performance liquid chromatography), using a method described in more detail elsewhere [20]. Briefly, analyses were carried out with a chromatographic system composed of a pump and an Ultimate 3000 autosampler (Dionex, Sunnyvale, Calif, USA). The 474 fluorescence detector (Waters, Saint-Quentin-en-Yvelines, France) was set at 365 nm for excitation and 624 nm for emission. Chromatographic separation was achieved on a reverse-phase C18 Symmetry (Waters, Saint-Quentin-en-Yvelines, France) column (4.8 × 250 mm) with a precolumn. All chemicals were HPLC grade. The mobile phase was a gradient of acetonitrile (Carlo-Erba, Val de Reuil, France) and 0.015 M sodium acetate (Merck, Darmstadt, Germany) buffer, adjusted to pH 4 with glacial acetic acid (Carlo-Erba, Val de Reuil, France).

Urine samples (400 μL) were acidified by adding 20 μL 10 N hydrochloric acid (Carlo-Erba, Val de Reuil, France), vortexing for 20 seconds and centrifuging the mixture for 10 minutes at 18000 × g. We then injected 50 μL of the supernatant into the chromatography system. The limits of quantification were 7 nmol/L for isomer I and 10 nmol/L for isomer III. The UCP I/(I + III) ratio was determined with a precision of about 99%.

We quantified both coproporphyrin isomers in urine from standard curves obtained with commercially available calibrators (Recipe, Munich, Germany). The UCP I/(I + III) ratio was obtained by dividing the peak height of isomer I by the sum of peak heights of isomers I and III.

2.3. DNA Analysis. Genomic DNA was extracted from peripheral blood leukocytes according to the kit manufacturer's protocol (FlexiGene DNA kit, QIAGEN, Hilden, Germany). Five SNPs within the *ABCC2* gene were selected for genotyping on the basis of previous studies evaluating

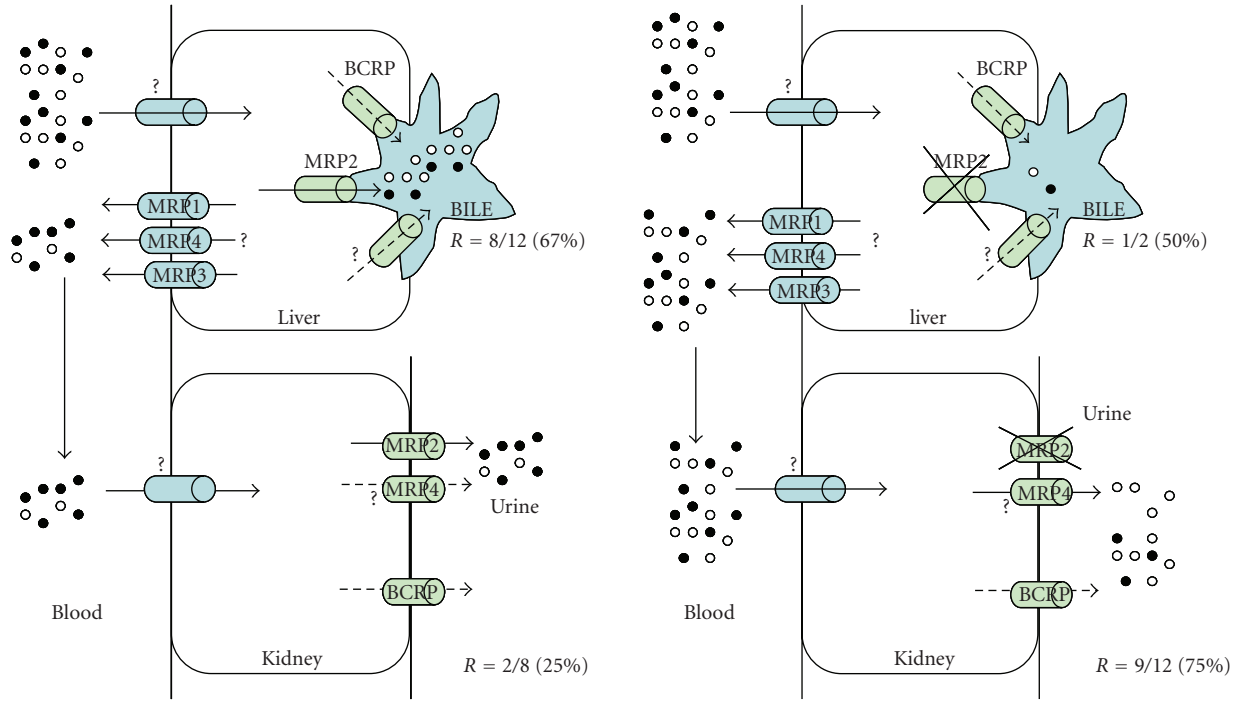

Figure 1: Proposed physiopathologic pathway for coproporphyrin elimination. This model was established on the basis of experimental data for both structure-affinity relationships for the interaction between coproporphyrin isomers and MRP2 [12] and of the measurement of coproporphyrin excretion in the bile and urine of $Mrp2^{-/-}$ or wild-type rats [6]. Calculations were based on an equimolar perfusion of coproporphyrin isomers in blood (for illustration, 10 molecules of each isomer are considered) and on the higher affinity of MRP2 for isomer I than for isomer III. The white squares represent molecules of isomer I and the black squares molecules of isomer III. Each part of the figure illustrates a particular situation: physiologic conditions (a); no MRP2 activity (b). R represents the UCP I/(I + III) ratio, calculated as the number of molecules of isomer I over the total number of molecules of isomer I and isomer III. In physiologic conditions (a), coproporphyrins enter the hepatocyte via an influx transporter, which may be OAT2, OATP1B1, OATP1B3, or another transporter. Coproporphyrins are then excreted predominantly in bile, via MRP2. As MRP2 has a higher affinity for isomer I than for isomer III due to a conformational advantage [12], the biliary coproporphyrin ratio is thus ~70% (67% in our theoretical example). A minor fraction may be secreted via BCRP and/or other transporters located in the canalicular membrane of hepatocytes. The fraction not excreted in bile returns to the blood via efflux transporters (possibly MRP3/*ABCC3,* which has a substrate specificity similar to or overlapping that of MRP2, MRP1/*ABCC1,* or MRP4/*ABCC4*, all located in the sinusoidal membrane; for review, see Borst et al. [26]). The remaining coproporphyrins (three times as much isomer III as isomer I) are excreted then in the urine via MRP2. The resulting UCP I/(I + III) ratio in normal subjects is thus ~30% (25% in our theoretical example). If MRP2 is entirely absent (b), as in DJS, only very small amounts of coproporphyrins are excreted in bile, probably via BCRP or other transporters. Most of the coproporphyrins are returned to the blood, presumably via MRP3, which is upregulated in $Mrp2^{-/-}$ mice [6, 27], or by MRP4. The kidney thus received both isomers, in equimolar proportions. Elimination in urine involves transporters other than MRP2 (possibly MRP4 or BCRP), with the same stereospecificity as MRP2 for the two isomers. According to this model, the urinary ratio should be about 80% (75% in our example), consistent with values observed in DJS patients.

their impact on MPR2 activity [13, 19, 21–23]. The five SNPs (−24C/T (rs717620), 1249G/A (rs2273697), 3563T/A (rs17216324), 3972C/T (rs3740066), and 4544G/A (rs8187710)) were genotyped by direct sequencing. PCR was carried out in a total volume of 50 μL containing 100 ng of total DNA, 10 pmol of each primer (Table 1) [24], 0.2 mmol/L dNTPs, 1.5 mmol/L $MgCl_2$, and 1.5 units of *Taq* polymerase (Invitrogen, Carlsbad, Calif, USA). The amplification protocol was as follows: initial denaturation at 94°C for 5 minutes, 35 cycles (1 minute at 94°C, 30 s at 60°C (rs17216324, rs2273697, and rs8187710), 56°C (rs717620), or 65°C (rs3740066), and 1 minute at 72°C), and a final extension at 72°C for 10 minutes. Then, PCR products were purified with a DNA gel extraction kit (Millipore, Billerica, Mass, USA) and sequenced with the Big Dye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Carlsbad, Calif, USA). Sequencing analysis was carried out on an Applied 3130XL automated DNA capillary sequencer (Applied Biosystems, Carlsbad, Calif, USA).

2.4. Statistical Analysis. The normality of the UCP I/(I + III) ratios's distribution was tested using the Shapiro-Wilk test. A Mann-Whitney test was performed to compare UCP I/(I + III) ratio between men and women. UCP I/(I + III) ratios were grouped into 4 categories based on distribution according to quartiles. The lower quartile (Q1) included the data of the 25% of patients with the lowest ratio; quartiles Q2 and Q3 extended 25% below and above the median ratio,

Table 1: Primers used for genotyping ABCC2 (MRP2) polymorphisms.

SNP	Reference	Primer sequence (5′ → 3′)		Fragment size, bp
ABCC2 5′UTR	rs717620	F	CCTTTACGGAGAACATCAGA	252
−24C/T		R	TTCTGGTTCTTGTTGGTGAC	
ABCC2 exon 10	rs2273697	F	GTGCCTTGGAGAAGCTGTGT	478
1249G/A		R	TTGCCCAAACTCCCATTAAG	
ABCC2 exon 25	rs17216324	F	GTAAGCTGTGCCCATCAAGG	383
3563T/A		R	CCTCCCACCGCTAATATCAA	
ABCC2 exon 28	rs3740066	F	GAGTCCTGGGTGGACTGTTC	291
3972C/T		R	CCAGCTGCTCTCCACTCTGT	
ABCC2 exon 32	rs8187710	F	AAATGCCTAGACTTGAGATGCTG	449
4544G/A		R	CGTGAAATTCAGGACAGTGG	

respectively; the upper quartile (Q4) included the 25% of patients with the highest ratio. Student's t test was used to compare the concentrations of the two isomers in patients with the highest ratio (Q4) versus others.

We used a Bland and Altman plot and its intraclass correlation coefficient (ICC) to compare the UCP I/(I + III) ratios obtained for the morning sample and the 24-hour urine samples. The same test was used to evaluate the stability of the UCP I/(I + III) ratio (obtained for the first, second, and third 24-hour urine samples) over time in a given subject (intraindividual variability on three separate occasions).

All SNPs were tested for Hardy-Weinberg equilibrium. A search for a relationship between UCP I/(I + III) ratio (obtained for the first 24-hour urine samples) and each SNP was first performed using a Kruskal-Wallis rank sum test and the post hoc Siegel-Castellan test. Then, a Mann-Whitney test was used to compare UCP I/(I + III) ratio as a function of each *ABCC2* polymorphism (wild-type allele carriers *versus* homozygous variant for each SNP). Statistical analysis was performed with *R* version 2.8.0, an open source program developed by the *R* Foundation for Statistical Computing (Vienna, Austria). We used THESIAS software (Paris, France) [25] to construct *ABCC2* haplotypes and to analyze associations between haplotypes and UCP I/(I + III) ratio.

3. Results

3.1. Distribution of UCP I/(I + III) Ratio in Healthy Volunteers. Eighty subjects were recruited (38 men and 42 women). Mean age was 25.2 (±6.5) years, mean weight was 63.1 (±9.9) kg, and mean height was 169.0 (±15.4) cm. Five subjects were excluded from the analysis because we were unable to obtain the totality of samples (urine or DNA) and one was excluded because he took a nonsteroidal anti-inflammatory drug (NSAID) during the study.

UCP I/(I + III) ratio varied from 14.7% to 46.0% in our group (as determined from the first 24-hour urine sample) with a median at 28.0% (Q1: 25.1%; Q4: 33.6%; Figure 2). The distribution of UCP I/(I + III) in our population was not Gaussian ($P = .21$). No difference in UCP I/(I + III) ratio was found between men (median (interquartile range)) (30.0 (7.8)%) and women (28.3 (9.1)%; $P = .24$). In parallel,

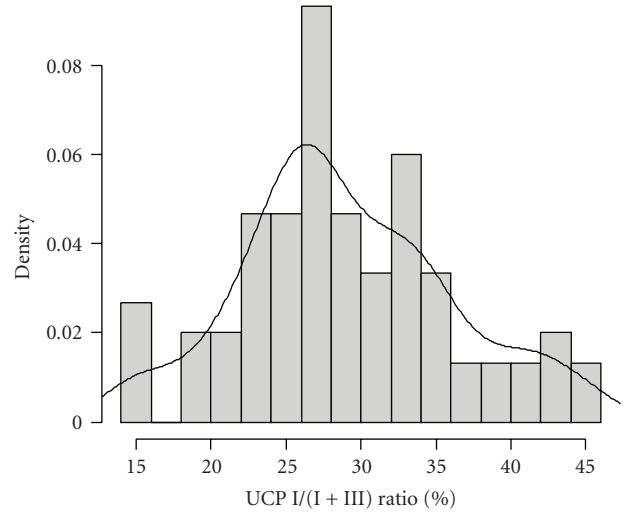

Figure 2: Distribution of UCP I/(I + III) ratio in our population.

we did not observe any correlation ($r^2 = 0.003$, $P = .66$) between the UCP I/(I + III) ratio and the age of the subjects. Subjects with a high UCP I/(I + III) ratio (greater than 33.6%) had higher isomer I concentrations in urine (mean ± sd; 25.6 ± 12.0 nmol/L) than did the other subjects (16.5 ± 8.6 nmol/L) ($P = .007$), whereas no difference in the concentration of isomer III was observed between these two groups (41.3 ± 24.5 nmol/L and 48.5 ± 29.7 nmol/L, resp.; $P = .35$).

3.2. Stability of UCP I/(I + III) Ratio over Time in Healthy Volunteers. Good concordance (ICC = 0.67 with a confidence interval of [0.42; 0.96]) between the UCP I/(I + III) ratios measured for the morning urine sample and those obtained for the 24-hour urine samples was observed in our population (Figure 3). The exclusion of the one subject with discordant UCP I/(I + III) ratios (black circle) increased the ICC to 0.90, with a confidence interval of [0.77; 0.97]. It is therefore possible to use morning urine samples in place of 24-h urine samples.

Comparisons of the UCP I/(I + III) ratios obtained for the same subject on three separate occasions (24-h urine samples) showed that the UCP I/(I + III) ratio was stable

Table 2: Relationships between UCP I/(I + III) ratio and each *ABCC2* polymorphism using the Mann-Whitney test.

Polymorphism	Genotype	Frequency no. (%)	Median [interquartile range] UCP I/(I + III) ratio (%)	*P* value*
ABCC2 −24 C/T	CC	46 (62.2)	26.9 [8.4]	.08
	CT	25 (33.8)		
	TT	3 (4.0)	33.8 [5.8]	
ABCC2 1249 G/A	GG	52 (70.2)	27.8 [8.4]	.39
	GA	21 (28.4)		
	AA	1 (1.4)	33.6 [na]	
ABCC2 3563 T/A	TT	64 (86.5)	28 [8.6]	—
	TA	10 (13.5)		
	AA	0	—	
ABCC2 3972 C/T	CC	29 (37.7)	26.7 [7.6]	.04
	CT	37 (49.3)		
	TT	8 (12.0)	33.1 [3.1]	
ABCC2 4544 G/A	GG	64 (86.5)	28 [8.6]	—
	GA	10 (13.5)		
	AA	0	—	

* Wild-type allele carriers versus homozygous variant.

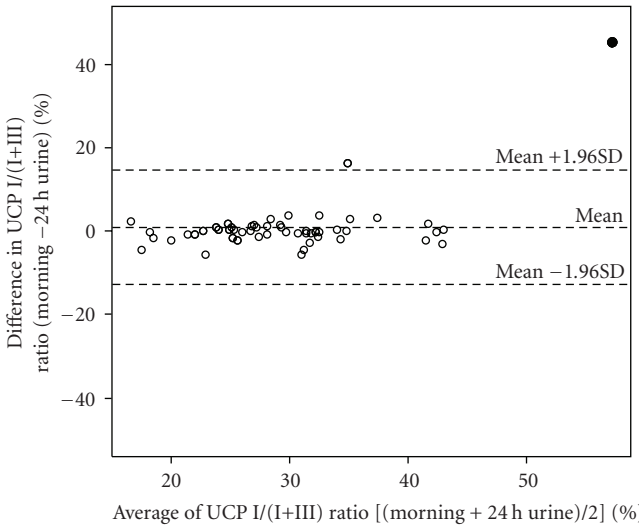

Figure 3: Mean UCP I/(I + III) ratio determined for morning and 24-hour urine samples (Bland and Altman plot). The black circle represents the subject with highly discordant UCP I/(I + III) ratio values between morning and 24-hour urine samples.

over time, with a median coefficient of variation (CV) of 7.8%, except in three subjects for whom CV values of 21 to 27% were obtained. The ICC values of these subjects were in the normal range, at 0.80 [0.64; 0.87]. Thus, UCP I/(I + III) ratio remains stable over time in a given subject.

3.3. Genotype-Phenotype Correlation in Healthy Subjects. The results of the genotypic analysis for the 74 volunteers are summarized in Table 2. The "minor" allele frequencies (MAFs) of the −24C/T, 1249G/A, 3563T/A, 3972C/T, and 4544G/A polymorphisms of *ABCC2* were 0.22, 0.15, 0.08, 0.34, and 0.08. All polymorphisms were in Hardy-Weinberg equilibrium. We found a linkage disequilibrium between −24C/T and 3972C/T ($r^2 = 0.47$, $D' = 1$) and complete linkage disequilibrium between 3563T/A, and 4544G/A ($r^2 = 1$, $D' = 1$). Haplotype analysis was then performed for four SNPs (−24C/T, 1249G/A, 3563T/A, and 3972C/T). Five haplotypes were observed: CGTC (f [frequency] = 0.42), CGTT ($f = 0.15$), CGAC ($f = 0.07$), CATC ($f = 0.16$), and TGTT ($f = 0.21$).

In a preliminary trend analysis, we observed a nonsignificant difference in UCP I/(I + III) ratio according to 3972C/T genotype ($P = .08$). The same was true for the −24C/T SNP ($P = .17$). When we grouped together CC and CT subjects, we found that subjects who were homozygous TT for the 3972C/T SNP had higher UCP I/(I + III) ratios ($P = .04$) than allele C carriers (Figure 4(a)). The same was true ($P = .08$) for −24TT subjects (Figure 4(b)). We found no relationship with any of the other three *ABCC2* SNPs studied. Similarly, no association was found between haplotypes and UCP I/(I + III) ratio.

4. Discussion

This is the first study exploring the role of MRP2 polymorphisms in coproporphyrin elimination in humans, with the ultimate aim of evaluating UCP I/(I + III) ratio as a biomarker of MRP2 function *in vivo*.

Measurements of the UCP I/(I + III) ratio in 74 healthy subjects effectively demonstrated wide interindividual variability, with a median (range) of 28.0 (14.7–46.0)%, consistent with values previously reported for the general population [9]. By contrast, UCP I/(I + III) ratio remained stable over three different measurements made in

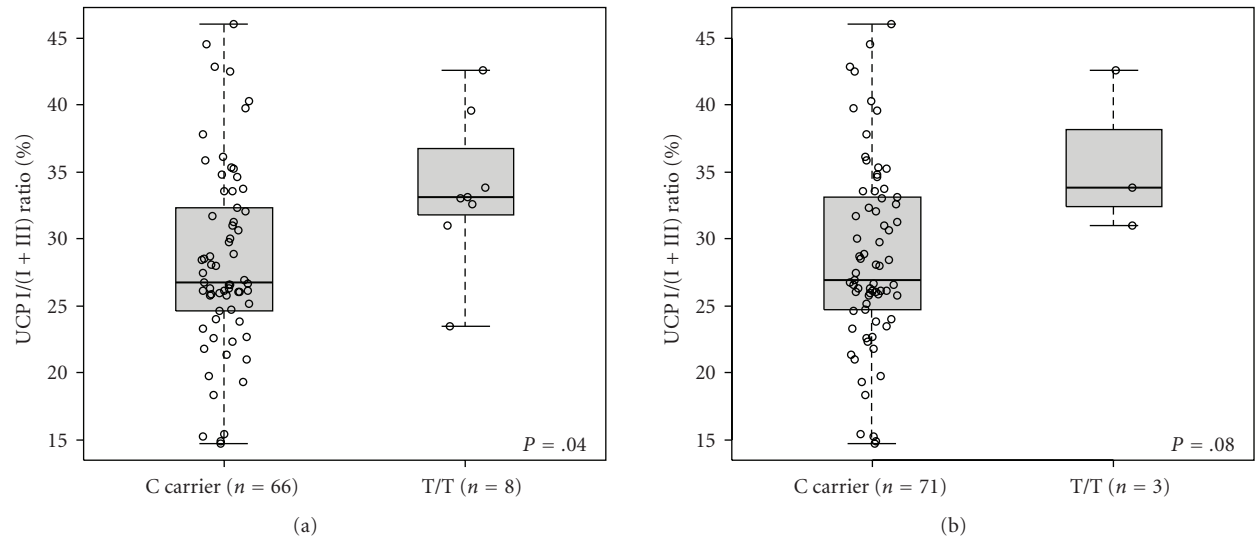

Figure 4: Relationship between UCP I/(I + III) ratio and *ABCC2* genotypes [(a) 3972C/T and (b) −24C/T]. Boxes represent the 25th and 75th percentiles. The median is indicated by a line, and the 10th and 90th percentiles are indicated by whiskers.

individual subjects taking no drug that might interfere with MRP2 at any time during the study period, indicating that this ratio is indeed an individual characteristic. Note that some female subjects took an oral contraception ($n = 20$) comprising ethinylestradiol (EE), known to decrease bile flow and to decrease expression and activity of Mrp2 in rats when administered subcutaneously at 5 mg/kg/day [28]. Consistent with the very low EE dosage used, we found no difference in UCP I/(I + III) ratio between women who took EE and those who did not (Mann-Whitney test, $P = .61$).

Genotype analysis was conducted with the five SNPs of the *ABCC2* gene previously shown to be involved in the pharmacokinetics or toxicity of MRP2 substrates. This genetic strategy was specifically selected to study the phenotypic consequences of these frequent common SNPs, and not of rare mutations leading to DJS (incidence = 0.05%). The minor allele frequencies (MAFs) for the *ABCC2* SNPs in our subjects were consistent with those reported in the HAPMAP project on a Caucasian population (residents of Utah descended from immigrants from northern and western Europe) [29] and none of these SNPs deviated from Hardy-Weinberg equilibrium. As 4544G/A was in total linkage disequilibrium with 3563T/A ($r^2 = 1, D' = 1$), it was not included in the haplotype analysis.

Our phenotype-genotype analysis revealed that homozygous 3972T and −24T subjects had higher UCP I/(I + III) ratios than wild-type allele carriers. We found no statistical relationship with the other polymorphisms of *ABCC2*.

These results are concordant with experimental results. The 3972C/T polymorphism leads to a decreased MRP2 protein expression associated with an impaired transport activity [30]. As the 3972C/T polymorphism is synonymous, its influence on MRP2 activity is probably mediated by the variation of another SNP in very strong linkage disequilibrium [16]. We effectively found a linkage disequilibrium between this SNP and the −24C/T polymorphism, although not complete ($r^2 = 0.47, D' = 1$), explaining why the trend for an association between −24C/T polymorphism and UCP I/(I + III) was not significant contrary to 3972C/T. Another hypothesis is that 3972C/T SNP may affect protein conformation and substrate specificity [31], modulate protein production by altering mRNA secondary structure and stability [32, 33], or lower translation efficacy [30]. The −24C/T polymorphism is located in the 5′-UTR, a region that appears essential for *ABCC2* transcription in liver cells [34]. Similarly, the −24T allele was associated with reduced *ABCC2* mRNA levels, [21] and reduced MRP2 protein expression albeit without significant difference in transport activity [30]. This SNP could affect *ABCC2* mRNA stability and/or translational activity [30].

Laechelt et al. [30] showed in cells transfected with −24T or 3972T *ABCC2* cDNA that 3 haplotypes, H9 (−24C/1249G/3972T), H10 (−24T/1249G/3972C), and H12 (−24T/1249G/3972T), were associated with altered MRP2 protein expression (33%, 73%, and 44%, resp., as compared to wild-type haplotypes) and altered efflux rates (significant for 3972T-containing haplotypes H9 and H12). This confirms that both polymorphisms influence the function of *ABCC2* transporter, the effect of 3972C/T being predominant. We did not find any relationship between haplotypes in our population and the UCP I/(I + III) ratio. Our results show a SNP-dependent coproporphyrin elimination and confirmatory studies in a larger population are required to analyze haplotypes and to increase the study power.

Several authors also assessed the functional consequences of *ABCC2* SNPs for the pharmacokinetics of drugs acting as substrates of MRP2. In their study on pediatric cancer patients, Rau et al. showed that children carrying the −24T allele had lower levels of MTX clearance than other children, this difference being most marked (tripling of the area under the curve AUC) in female patients [15]. Naesens et al. showed an increase of the mycophenolic acid

AUC (17% at day 42 after transplantation, $P = .008$) in renal allograft recipients carriers of the −24T allele and a similar relationship in patients carriers of the 3972T allele [16]. Recently, Laechelt et al. found that subjects bearing the 3972T allele with or without the −24T presented an increased AUC_{oral} of talinolol. This indicates that screening for *ABCC2* polymorphisms may help to better understand the interindividual differences in the pharmacokinetics of some drugs.

Beyond the genetic factors, the activity of MRP2 transporter can also be modulated by exogenous factors, and particularly drug interactions, by phenotypical modification of its activity [1]. Personal data, not yet published, show that the basal value of UCP I/(I + III) ratio in a given subject does indeed increase following the administration of an MRP2 inhibitor, suggesting that UCP I/(I + III) ratio reflects MRP2 activity at a given time in a given patient. In subjects of the present study taking no drug, comparable UCP I/(I + III) ratios were obtained for morning micturition and 24-hour urine. So, based on a simple urine sample, UCP I/(I + III) ratio is a practicable test for future prospective studies evaluating its predictive value for the pharmacokinetics of MRP2 substrates.

The precise mechanism controlling the secretion of coproporphyrin isomers into urine has yet to be fully described. Evidence for the involvement of MRP2 in this process is provided by the higher UCP I/(I + III) ratio observed in DJS patients and Mrp2-deficient rats and is supported by the results of our study showing that a moderate impairment of MRP2 function due to polymorphisms increases this ratio, although to a lesser extent than in subjects completely lacking the protein. Frank et al. suggested that the higher UCP I/(I + III) ratio in DJS patients resulted from the excretion of larger amounts of isomer I in urine [9], but these authors did not quantify each product separately. We showed, with 24-hour urine samples, that a high UCP I/(I + III) ratio (subjects from Q4) effectively resulted from an increase in isomer I excretion in urine. We are currently conducting transport studies on MDCKII (Madin Darby Canine Kidney II) cells to verify if one or both coproporphyrin isomers are substrates of MRP2. Increased excretion of isomer I in urine, associated with an inverted proportion of the two isomers in case of MRP2 defect (increase of the UCP I/(I + III) ratio), suggests that coproporphyrin elimination may involve at least a second transporter in the kidney. The proposed physiopathologic pathway in Figure 2, accounting for the inverted proportions of isomers in Dubin-Johnson patients (ratio ∼80%) compared to healthy subjects (ratio ∼30%), is consistent with the intermediate ratio (about 40%) observed in subjects with lower MRP2 activity, such as subjects with 24TT or 3972TT genotypes. In this case, only part of the coproporphyrins are excreted into the bile via MRP2, again with a higher proportion of isomer I (biliary ratio ∼70%) than isomer III, due to the higher affinity of MRP2 for isomer I. The remaining coproporphyrins are excreted in urine via MRP2 or other transporters (such as BCRP, MRP4). Knowing that there is less of isomer I than of isomer III which arrives at the kidney, no competition occurs and the UCP I/(I + III) ratio is about 35 to 45%.

In turn, UCP I/(I + III) ratio provides quantitative information about the *in vivo* activity of MRP2. This would account for the variation of UCP I/(I + III) ratio with *ABCC2* genotype, being high in patients with DJS mutations, moderately high in subjects with relevant polymorphisms, and normal in wild-type subjects.

In our opinion, this study provides a proof of concept that UCP I/(I + III) ratio can be used as a biomarker of MRP2 function in clinical studies. Indeed, it is stable over time in a given subject, provided that no environmental factor (e.g., drug interaction) modifies MRP2 function. It depends on genetic characteristics, and the administration of an MRP2 inhibitor increases the ratio (personal data). Thus, UCP I/(I + III) ratio may reflect the functionality of MRP2 in a given patient, at a given time, taking into account individual genotype and concurrent environmental factors. We now need to carry out a prospective study to demonstrate that the UCP I/(I + III) ratio measured at the time of drug intake is effectively correlated with the pharmacokinetics of the drug. We are currently carrying out such a study with methotrexate, a well-known substrate of MRP2.

5. Conclusion

MRP2 is involved in the elimination of several drugs. No biomarker of its activity is available so far. Previous studies have shown that the urinary coproporphyrin ratio UCP I/(I + III) is elevated in patients with Dubin-Johnson syndrome, as a consequence of mutations in the *ABCC2* gene. In healthy volunteers, the ratio was highly variable between subjects and tended to be elevated in subjects homozygous for two distinct SNPs, 3972C/T and −24C/T. This study provides a proof of concept that UCP I/(I + III) ratio can be used as a biomarker of MRP2 function in clinical studies as it provides quantitative information about the *in vivo* activity of MRP2 in a given patient. Based on these results, we propose the use of UCP I/(I + III) ratio to evaluate MRP2 activity in future pharmacokinetic studies.

Abbreviations

MRP1: Multidrug resistance-associated protein 1
MRP2: Multidrug resistance-associated protein 2
MRP3: Multidrug resistance-associated protein 3
MRP4: Multidrug resistance-associated protein 4
BCRP: Breast cancer resistance protein.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank all the volunteers who contributed to the study. They would also like to thank the staff of the Clinical Investigation Center of Tours University Hospital (research nurses, clinical research fellows, project manager, and biostatisticians) and the *Centre de Ressources Biologiques*

de Touraine (CRBT). Additional thanks go to Professor Philippe Lechat for his scientific contribution. This study was funded by grants from the Ligue *Contre le Cancer*, the *Entreprises du Médicament* (LEEM), and the CANCEN association (*Cancérologie Centre*).

References

[1] O. Fardel, E. Jigorel, M. Le Vee, and L. Payen, "Physiological, pharmacological and clinical features of the multidrug resistance protein 2," *Biomedicine and Pharmacotherapy*, vol. 59, no. 3, pp. 104–114, 2005.

[2] S. Ahmed, N. T. Vo, T. Thalhammer et al., "Involvement of Mrp2 (Abcc2) in biliary excretion of moxifloxacin and its metabolites in the isolated perfused rat liver," *Journal of Pharmacy and Pharmacology*, vol. 60, pp. 55–62, 2008.

[3] K. T. Kivisto, O. Grisk, U. Hofmann et al., "Disposition of oral and intravenous pravastatin in MRP2-deficient TR-rats," *Drug Metabolism and Disposition*, vol. 33, pp. 1593–1596, 2005.

[4] G. Luo, C. E. Garner, H. Xiong et al., "Effect of DPC 333 [(2R)-2-(3R)-3-amino-3-[4-(2-methylquinolin-4-ylmethoxy) phenyl]-2-oxopyrrolidin-1-yl-N-hydroxy-4-methylpentanamide], a human tumor necrosis factor α-converting enzyme inhibitor, on the disposition of methotrexate: a transporter-based drug-drug interaction case study," *Drug Metabolism and Disposition*, vol. 35, pp. 835–840, 2007.

[5] J. Wang, M. Figurski, L. M. Shaw, and G. J. Burckart, "The impact of P-glycoprotein and Mrp2 on mycophenolic acid levels in mice," *Transplant Immunology*, vol. 19, no. 3-4, pp. 192–196, 2008.

[6] V. Moriondo, S. Marchini, P. Di Gangi et al., "Role of multidrug-resistance protein 2 in coproporphyrin transport: results from experimental studies in bile fistula rat models," *Cellular and Molecular Biology*, vol. 55, no. 2, pp. 70–78, 2009.

[7] I. N. Dubin and F. B. Johnson, "Chronic idiopathic jaundice with unidentified pigment in liver cells; a new clinicopathologic entity with a report of 12 cases," *Medicine*, vol. 33, no. 3, pp. 155–197, 1954.

[8] D. Keppler, J. König, and M. Büchler, "The canalicular multidrug resistance protein, cMRP/MRP2, a novel conjugate export pump expressed in the apical membrane of hepatocytes," *Advances in Enzyme Regulation*, vol. 37, pp. 321–333, 1997.

[9] M. Frank, M. Doss, and D. G. De Carvalho, "Diagnostic and pathogenetic implications of urinary coproporphyrin excretion in the dublin-johnson syndrome," *Hepato-Gastroenterology*, vol. 37, no. 1, pp. 147–151, 1990.

[10] S. Toh, M. Wada, T. Uchiumi et al., "Genomic structure of the canalicular multispecific organic anion-transporter gene (MRP2/cMOAT) and mutations in the ATP-binding-cassette region in Dubin-Johnson syndrome," *The American Journal of Human Genetics*, vol. 64, pp. 739–746, 1999.

[11] K. M. Giacomini, S. M. Huang, and D. J. Tweedie, "Membrane transporters in drug development," *Nature Reviews Drug Discovery*, vol. 9, pp. 215–236, 2010.

[12] N. Kaplowitz, N. Javitt, and A. Kappas, "Coproporphyrin I and 3 excretion in bile and urine," *Journal of Clinical Investigation*, vol. 51, no. 11, pp. 2895–2899, 1972.

[13] S. Ito, I. Ieiri, M. Tanabe, A. Suzuki, S. Higuchi, and K. Otsubo, "Polymorphism of the ABC transporter genes, MDR1, MRP1 and MRP2/cMOAT, in healthy Japanese subjects," *Pharmacogenetics*, vol. 11, no. 2, pp. 175–184, 2001.

[14] H. Suzuki and Y. Sugiyama, "Single nucleotide polymorphisms in multidrug resistance associated protein 2 (MRP2/ABCC2): its impact on drug disposition," *Advanced Drug Delivery Reviews*, vol. 54, pp. 1311–1331, 2002.

[15] T. Rau, B. Erney, R. Göres, T. Eschenhagen, J. Beck, and T. Langer, "High-dose methotrexate in pediatric acute lymphoblastic leukemia: impact of ABCC2 polymorphisms on plasma concentrations," *Clinical Pharmacology and Therapeutics*, vol. 80, pp. 468–476, 2006.

[16] M. Naesens, D. R. J. Kuypers, K. Verbeke, and Y. Vanrenterghem, "Multidrug resistance protein 2 genetic polymorphisms influence mycophenolic acid exposure in renal allograft recipients," *Transplantation*, vol. 82, pp. 1074–1084, 2006.

[17] F. A. de Jong, T. J. Scott-Horton, D. L. Kroetz et al., "Irinotecan-induced diarrhea: functional significance of the polymorphic ABCC2 transporter protein," *Clinical Pharmacology and Therapeutics*, vol. 81, pp. 42–49, 2007.

[18] K. T. Kivistö and M. Niemi, "Influence of drug transporter polymorphisms on pravastatin pharmacokinetics in humans," *Pharmaceutical Research*, vol. 24, pp. 239–247, 2007.

[19] L. Wojnowski, B. Kulle, M. Schirmer et al., "NAD(P)H oxidase and multidrug resistance protein genetic polymorphisms are associated with doxorubicin-induced cardiotoxicity," *Circulation*, vol. 112, no. 24, pp. 3754–3762, 2005.

[20] R. Respaud, I. Benz-de Bretagne, H. Blasco, J. S. Hulot, P. Lechat, and C. Le Guellec, "Quantification of coproporphyrin isomers I and III in urine by HPLC and determination of their ratio for investigations of multidrug resistance protein 2 (MRP2) function in humans," *Journal of Chromatography B*, vol. 877, no. 30, pp. 3893–3898, 2009.

[21] S. Haenisch, U. Zimmermann, E. Dazert et al., "Influence of polymorphisms of ABCB1 and ABCC2 on mRNA and protein expression in normal and cancerous kidney cortex," *Pharmacogenomics Journal*, vol. 7, no. 1, pp. 56–65, 2007.

[22] M. Hirouchi, H. Suzuki, M. Itoda et al., "Characterization of the cellular localization, expression level, and function of SNP variants of MRP2/ABCC2," *Pharmaceutical Research*, vol. 21, no. 5, pp. 742–748, 2004.

[23] H. E. Meyer Zu Schwabedissen, G. Jedlitschky, M. Gratz et al., "Variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation," *Drug Metabolism and Disposition*, vol. 33, no. 7, pp. 896–904, 2005.

[24] H. Izzedine, J. S. Hulot, E. Villard et al., "Association between ABCC2 gene haplotypes and tenofovir-induced proximal tubulopathy," *Journal of Infectious Diseases*, vol. 194, no. 11, pp. 1481–1491, 2006.

[25] D. A. Tregouet and V. Garelle, "A new JAVA interface implementation of THESIAS: testing haplotype effects in association studies," *Bioinformatics*, vol. 23, no. 8, pp. 1038–1039, 2007.

[26] P. Borst, N. Zelcer, and K. Van De Wetering, "MRP2 and 3 in health and disease," *Cancer Letters*, vol. 234, no. 1, pp. 51–61, 2006.

[27] M. G. Donner and D. Keppler, "Up-regulation of basolateral multidrug resistance protein 3 (Mrp3) in cholestatic rat liver," *Hepatology*, vol. 34, no. 2, pp. 351–359, 2001.

[28] M. Trauner, M. Arrese, C. J. Soroka et al., "The rat canalicular conjugate export pump (Mrp2) is down-regulated in intrahepatic and obstructive cholestasis," *Gastroenterology*, vol. 113, no. 1, pp. 255–264, 1997.

[29] "The international hapmap project," *Nature*, vol. 426, pp. 789–796, 2003.

[30] S. Laechelt, E. Turrini, A. Ruehmkorf, W. Siegmund, I. Cascorbi, and S. Haenisch, "Impact of ABCC2 haplotypes on transcriptional and posttranscriptional gene regulation and function," *Pharmacogenomics Journal*, vol. 11, no. 1, pp. 25–34, 2011.

[31] C. Kimchi-Sarfaty, J. M. Oh, I. W. Kim et al., "A "silent" polymorphism in the MDR1 gene changes substrate specificity," *Science*, vol. 315, pp. 525–528, 2007.

[32] F. Capon, M. H. Allen, M. Ameen et al., "A synonymous SNP of the corneodesmosin gene leads to increased mRNA stability and demonstrates association with psoriasis across diverse ethnic groups," *Human Molecular Genetics*, vol. 13, no. 20, pp. 2361–2368, 2004.

[33] A. G. Nackley, S. A. Shabalina, I. E. Tchivileva et al., "Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure," *Science*, vol. 314, no. 5807, pp. 1930–1933, 2006.

[34] T. Tanaka, T. Uchiumi, E. Hlnoshlta et al., "The human multidrug resistance protein 2 gene: functional characterization of the 5'-flanking region and expression in hepatic cells," *Hepatology*, vol. 30, no. 6, pp. 1507–1512, 1999.