Comprehensive Pharmacogenomic Study Reveals an Important Role of UGT1A3 in Montelukast Pharmacokinetics

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To identify the genetic basis of interindividual variability in montelukast exposure, we determined its pharmacokinetics and sequenced 379 pharmacokinetic genes in 191 healthy volunteers. An intronic single nucleotide variation (SNV), strongly linked with UGT1A3*2, associated with reduced area under the plasma concentration–time curve (AUC0–∞) of montelukast (by 18% per copy of the minor allele; $P = 1.83 \times 10^{-10}$). UGT1A3*2 was associated with increased AUC0–∞ of montelukast acyl-glucuronide M1 and decreased AUC0–∞ of hydroxymetabolites M5R, M5S, and M6 ($P < 10^{-8}$). Furthermore, SNVs in SLCO1B1 and ABCC9 were associated with the AUC0–∞ of M1 and M5R, respectively. In addition, a candidate gene analysis suggested that CYP2C8 and ABCC9 SNVs also affect the AUC0–∞ of montelukast. The found UGT1A3 and ABCC9 variants associated with increased expression of the respective genes in human liver samples. Montelukast and its hydroxymetabolites were glucuronidated by UGT1A3 in vitro. These results indicate that UGT1A3 plays an important role in montelukast pharmacokinetics, especially in UGT1A3*2 carriers.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
- Interindividual variability in the pharmacokinetics of montelukast is high. It is not known how genetic variants in genes encoding drug-metabolizing enzymes, membrane transporters, and regulatory proteins contribute to this variability.

WHAT QUESTION DID THIS STUDY ADDRESS?
- This study investigated whether genetic variants in pharmacokinetic genes affect the pharmacokinetics of montelukast and its metabolites.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE
- The results indicate that UGT1A3 plays an important role in montelukast pharmacokinetics, especially in carriers of the UGT1A3*2 allele associated with increased UGT1A3 expression. Furthermore, the results suggest that also CYP2C8 and ABCC9 variants affect the exposure to montelukast.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE
- Genetic variants explain a significant proportion of interindividual variability in montelukast pharmacokinetics. This knowledge may aid in individualizing treatment with leukotriene receptor antagonists.

Montelukast is a leukotriene receptor antagonist, which is widely used in the treatment of asthma.1 After oral administration, montelukast is extensively metabolized and the majority of the metabolites are excreted into the bile.2 Previous studies have shown that the main enzyme involved in the oxidative metabolism of montelukast is cytochrome P450 (CYP) 2C8.3,4 Also, CYP2C9, CYP3A4, and uridine diphosphate-glucuronosyltransferase (UGT) 1A3 seem to contribute to the formation of montelukast metabolites.4–7 Additionally, montelukast has been suggested to be a substrate of organic anion transporting polypeptide (OATP) 1B1, 1B3, and 2B1 transporters.8–10

High interindividual variability exists in the pharmacokinetics of montelukast. We hypothesized that variation in genes encoding drug-metabolizing enzymes and membrane transporters, as well as proteins that affect their expression or biochemistry, contributes to this variability. Therefore, the aim of this study was to investigate the possible effects of genetic variability in these pharmacokinetic genes on montelukast pharmacokinetics. To this end, we determined the pharmacokinetics of montelukast after a...
10-mg dose in 191 healthy volunteers and fully sequenced 379 pharmacokinetic genes using massively parallel sequencing.

RESULTS
Montelukast pharmacogenomics
In the present study, substantial interindividual variability was observed in the pharmacokinetic variables of montelukast and its acyl-glucuronide (M1) and hydroxymetabolites (M5R, M5S, and M6) (Supplementary Table S1). The areas under the plasma concentration–time curve from 0 h to infinity (AUC_{0-\infty}) of montelukast, M1, M5R, M5S, and M6 varied 8.7-fold, 13-fold, 30-fold, 22-fold, and 23-fold between individual subjects, respectively.

A total of 105,145 single nucleotide variations (SNVs) were found in the 379 analyzed pharmacokinetic genes (Supplementary Table S2), of which 46,064 had a minor allele frequency (MAF) of at least 0.05. In a stepwise linear regression analysis fixed for demographic covariates, nine common variants (MAF \( \geq 0.05 \)) in three genes were independently associated with montelukast or its metabolite pharmacokinetics at a Bonferroni-corrected significance level of 1.09 \( \times 10^{-6} \) (Table 1). The AUC_{0-\infty} of montelukast and its metabolites showed the strongest associations with variants in the UGT1A gene (Figure 1, Table 1). For montelukast AUC_{0-\infty}, the strongest association was observed with rs7604115, located in the first intron of UGT1A3. The AUC_{0-\infty} of montelukast was 18% smaller per copy of the variant allele \( (P = 1.83 \times 10^{-10}) \). After adjusting for this variant, no other variant remained statistically significantly associated with montelukast AUC_{0-\infty}. The investigated genetic variants had no significant effect on the peak plasma concentration (C\(_{\text{max}}\)) or the elimination half-life \( (t_{1/2}) \) of montelukast.

In agreement with the effects of UGT1A variants on parent montelukast, the AUC_{0-\infty} of montelukast acyl-glucuronide (M1) was 25% larger per copy of the variant allele \( (P = 6.02 \times 10^{-7}) \) (Table 1). R3806592 is in a strong linkage disequilibrium with the UGT1A3 rs7604115 SNV associated with the AUC_{0-\infty} of parent montelukast \( (r^2 = 0.95, P = 3.65 \times 10^{-11}) \). UGT1A variants were also significantly associated with the C\(_{\text{max}}\) of M1 and the C\(_{\text{max}}\)/montelukast AUC_{0-\infty} ratio. Furthermore, the solute carrier organic anion transporter gene 1B1 (SLCO1B1) SNVs rs73063122 and rs4149056 were significantly associated with the AUC_{0-\infty} and C\(_{\text{max}}\) of M1, respectively. These two SNVs are in a strong linkage disequilibrium with each other \( (r^2 = 0.60, P = 5.73 \times 10^{-2}) \), suggesting that both of these associations are due to the rs4149056 missense SNV known to markedly impair the activity of OATP1B1.\(^{11}\)

The effects of UGT1A variants on the AUC_{0-\infty} of the hydroxylated M5R, M5S, and M6 metabolites of montelukast were larger than what was observed for montelukast (Table 1). The AUC_{0-\infty} of M5R, M5S, and M6 were 46% \( (P = 1.26 \times 10^{-30}) \), 33% \( (P = 8.14 \times 10^{-14}) \), and 39% \( (P = 2.89 \times 10^{-2}) \) smaller per copy of the rs7604115 variant allele, respectively. UGT1A variants were also significantly associated with the C\(_{\text{max}}\) and the metabolite/montelukast AUC_{0-\infty} ratios of M5R, M5S, and M6 and the t\(_{1/2}\) of M5R and M5S. The AUC_{0-\infty} of M5R was also associated with the rs704212 SNV in the ATP binding cassette subfamily C member 9 (ABCC9) transporter gene.

UGT1A linkage disequilibrium and haplotype analyses
The UGT1A gene region (±20 kb) was found to consist of nine linkage disequilibrium (LD) blocks (Figure 1). The SNVs associated with montelukast or its metabolite pharmacokinetics were located in blocks 3 and 4. Within these blocks, 28 haplotypes were inferred (Supplementary Figure S1). The SNVs showing the strongest associations with montelukast pharmacokinetics were strongly linked to the missense variants rs3821242 and rs6431625 \( (r^2 \geq 0.69) \), which together define the UGT1A3*2 haplotype. Based on the missense variants rs3821242, rs6431625, and rs45449995, the inferred haplotypes were grouped to subtypes of UGT1A3\(^*1\) (wildtype), \(^*2\) (rs3821242 and rs6431625; MAF 0.39), \(^*3\) (rs3821242; MAF 0.060), and \(^*6\) (rs3821242, rs6431625, and rs45449995; MAF 0.018). The effects of the UGT1A3*2 haplotype on montelukast and its metabolite pharmacokinetics were similar to the effects of the individual, intronic UGT1A SNVs (Tables 1 and 2, Figure 2).

Functional validation
Next, we investigated whether montelukast or its hydroxymetabolites are substrates of UGT1A enzymes in vitro. As the most strongly associated variants are localized around the first exon of UGT1A3, we focused on this enzyme, together with UGT1A1 and UGT1A9, which are also known to catalyze the glucuronidation of carboxylic acids.\(^{12}\) Montelukast, M5R, M5S, and M6 were all metabolized by UGT1A3, but not significantly by UGT1A1 or UGT1A9 (Supplementary Figure S2).

To further elucidate the mechanisms of associations between UGT1A, ABCC9, and SLCO1B1 SNVs and montelukast or its metabolite pharmacokinetics, we then investigated the effects of the SNVs on the respective gene expression in human liver samples (Table 3). UGT1A3 expression showed a strong association with the SNVs associated with montelukast pharmacokinetics. The strongest association was observed with rs4663969, present in both UGT1A3\(^*2\) and \(^*3\) haplotypes. UGT1A3 gene expression was 24% higher per copy of the variant allele \( (P = 2.27 \times 10^{-4}) \). Of the UGT1A3\(^*2\), \(^*3\), and \(^*6\) haplotypes, only UGT1A3\(^*2\) was significantly associated with increased UGT1A3 gene expression. The UGT1A3 expression was 24% higher per copy of the UGT1A3\(^*2\) haplotype \( (P = 2.08 \times 10^{-4}) \) (Figure 3). ABCC9 expression was 25% higher \( (P = 3.41 \times 10^{-6}) \) per copy of the rs704212 variant allele. SLCO1B1 expression was 13% lower \( (P = 0.0146) \) per copy of the rs4149056 variant allele. Of SLCO1B1 haplotypes, \(^*15\) (rs2306283 and rs4149056) was associated with a 15% lower SLCO1B1 expression per copy of the haplotype \( (P = 0.00733) \).

Candidate gene analysis
We next carried out a candidate gene analysis for montelukast AUC_{0-\infty}, focusing on common (MAF \( \geq 0.05 \)) missense variants in genes suggested to be involved in montelukast pharmacokinetics (CYP2C8, CYP2C9, CYP3A4, SLCO1B1, SLCO1B3,
### Table 1. Results of the stepwise forward linear regression analysis of the effects of 46,064 SNVs in 379 genes on montelukast and its metabolite pharmacokinetics

| Pharmacokinetic variable | dbSNP ID   | Gene     | Location | Nucleotide change | MAF | Effect \(a\) | Average | 90% CI    | \(P\) value |
|--------------------------|------------|----------|----------|-------------------|-----|--------------|---------|----------|------------|
| Montelukast              |            |          |          |                   |     |              |         |          |            |
| \(AUC_{0\infty}\)        | rs7604115  | UGT1A3   | intron 1/4| c.868-17564C>T    | 0.40| -17.7%       | -21.6%, | -13.7%   | \(1.83 \times 10^{-10}\) |
| \(C_{\text{max}}\)       | —          |          |          |                   |     |              |         |          |            |
| \(t_{1/2}\)              | —          |          |          |                   |     |              |         |          |            |
| M1                       |            |          |          |                   |     |              |         |          |            |
| \(AUC_{0\infty}\)        | 1. rs7604115 | UGT1A3   | intron 11/14 | c.1497 + 2246A>C | 0.32| 26.4%        | 18.5%,  | 34.8%    | \(9.20 \times 10^{-9}\) |
|                          | 2. rs704212 | ABCC9    | intron 12/37| c.1802 + 2622G>A | 0.14| -27.6%       | -34.6%, | -19.8%   | \(4.83 \times 10^{-7}\) |
| \(C_{\text{max}}\)       | rs7604115  | UGT1A3   | intron 1/4 | c.868-17564C>T    | 0.40| -33.9%       | -38.6%, | -28.8%   | \(7.13 \times 10^{-17}\) |
| \(t_{1/2}\)              | rs7556676  | UGT1A3   | intron 1/4 | c.868-17430A>G    | 0.46| -24.8%       | -28.2%, | -21.2%   | \(2.81 \times 10^{-18}\) |
| M5R                      |            |          |          |                   |     |              |         |          |            |
| \(AUC_{0\infty}\)        | 1. rs7604115 | UGT1A3   | intron 1/4 | c.868-17564C>T    | 0.40| -33.2%       | -38.5%, | -27.5%   | \(8.14 \times 10^{-14}\) |
|                          | 2. rs704212 | ABCC9    | intron 12/37| c.1802 + 2622G>A | 0.14| -26.2%       | -31.2%, | -20.8%   | \(1.82 \times 10^{-17}\) |
| \(C_{\text{max}}\)       | rs7604115  | UGT1A3   | intron 1/4 | c.868-17564C>T    | 0.40| -33.9%       | -38.6%, | -28.8%   | \(2.17 \times 10^{-17}\) |
| \(t_{1/2}\)              | rs2361501  | UGT1A3   | intron 1/4 | c.867 + 51A>T     | 0.47| -20.1%       | -24.7%, | -15.3%   | \(1.71 \times 10^{-9}\) |
| M5S                      |            |          |          |                   |     |              |         |          |            |
| \(AUC_{0\infty}\)        | rs7604115  | UGT1A3   | intron 1/4 | c.868-17564C>T    | 0.40| -39.4%       | -43.2%, | -35.4%   | \(2.89 \times 10^{-27}\) |
| \(C_{\text{max}}\)       | rs7604115  | UGT1A3   | intron 1/4 | c.868-17564C>T    | 0.40| -34.0%       | -37.8%, | -29.9%   | \(2.22 \times 10^{-23}\) |
| \(t_{1/2}\)              | rs463969   | UGT1A3   | intron 1/4 | c.867 + 16674C>A  | 0.46| -25.4%       | -28.1%, | -22.7%   | \(2.77 \times 10^{-20}\) |

\(AUC_{0\infty}\) area under the plasma concentration-time curve from 0 h to infinity; CI, confidence interval; \(C_{\text{max}}\), peak plasma concentration; dbSNP, National Center for Biotechnology Information Short Genetic Variations database; MAF, minor allele frequency; SNV, single nucleotide variation; \(t_{1/2}\), elimination half-life.

\(a\)Per copy of the minor allele.

\(SLCO2B1, UGT1A3\), as well as the \(ABCC9\) rs704212 SNV (Supplementary Table S3). In a stepwise linear regression analysis, \(UGT1A3*2\) was associated with a 17% \((P = 2.99 \times 10^{-10})\), \(ABCC9\) rs704212 with a 14% \((P = 2.19 \times 10^{-4})\), and \(CYP2C8*3\) (rs10509681 and rs11572080) with an 11% \((P = 0.00659)\) reduced, and \(CYP2C8*4\) (rs1058930) with a 13% \((P = 0.0184)\) increased \(AUC_{0\infty}\) of montelukast per copy of each minor allele (Table 4; adjusted \(R^2 = 0.41\)).

### DISCUSSION

In this study we used targeted massively parallel sequencing of 379 pharmacokinetic genes to characterize the genetic basis of interindividual variability in montelukast pharmacokinetics. The \(UGT1A3*2\) haplotype and variants located around the first exon of \(UGT1A3\) were strongly associated with the systemic exposure to parent montelukast and its metabolites. We further demonstrated that montelukast and its hydroxymetabolites are substrates
of UGT1A3 in vitro and that the variants associated with the pharmacokinetic variables also significantly affect UGT1A3 gene expression in human liver. In addition, our results indicate involvement of ABCC9 and SLCO1B1 in the pharmacokinetics of montelukast metabolites. Moreover, a candidate gene approach suggested that, in addition to UGT1A3 variants, also CYP2C8 and ABCC9 variants affect parent montelukast exposure.

The UGT1A gene encodes the UGT1A family enzymes, which catalyze the formation of hydrophilic glucuronide metabolites. Individual UGT1A genes have unique first exons but share exons 2–5. Strong linkage disequilibrium exists throughout the whole UGT1A gene (Figure 1c). The intronic UGT1A SNVs showing the strongest associations with montelukast or its metabolite pharmacokinetics are strongly linked to the UGT1A3 missense
variants rs3821242 (c.31T>C, p.Trp11Arg) and rs6431625 (c.140T>C, p.Val47Ala) that together define the haplotype UGT1A3*2. The UGT1A3*2 haplotype and the individual intronic SNVs similarly reduced the exposure to montelukast and its hydroxymetabolites, and increased the exposure to montelukast acyl-glucuronide, indicating enhanced glucuronidation. Consistently, UGT1A3*2 has previously been shown to increase the metabolism of the UGT1A3 substrates atorvastatin, telmisartan, and febuxostat in humans.14–16

The UGT1A3*2 haplotype and the UGT1A SNVs associated with montelukast pharmacokinetics significantly increased UGT1A3 mRNA expression in human liver samples. Similarly, a previous study also showed that UGT1A3 mRNA and protein expression are significantly increased in UGT1A3*2 carriers.14 In addition to UGT1A3*2, the UGT1A3*6 haplotype has been associated with increased UGT1A3 expression.14 In our study, the number of UGT1A3*6 carriers was relatively small, and only a tendency towards increased UGT1A3 expression could be observed (Figure 3).

The causal variant of UGT1A3*2 affecting UGT1A3 expression has remained unknown.14 We identified several intronic SNVs that are strongly linked to UGT1A3*2 (Supplementary Material).
Figure S1). Of these, rs3806597 (c.-204A>G) is located on a proposed farnesoid X receptor (FXR) binding site upstream of UGT1A3, but the variant allele has not affected FXR-mediated induction of UGT1A3 by the bile acid chenodeoxycholic acid.17 None of the other strongly linked variants appear to be located in a transcription factor binding site upstream of UGT1A3.18 In addition to being associated with expression, the UGT1A3*2 missense variants might also alter the enzymatic activity of UGT1A3. However, in vitro studies with these variants have shown conflicting results, with reduced, increased, and unchanged activity.19–21 Altogether, although the causal variant cannot be identified, the increased UGT1A3 expression in association with UGT1A3*2 provides a mechanistic explanation for our pharmacokinetic results.

In accordance with a recently published in vitro study,7 our results demonstrate that montelukast is efficiently glucuronidated by UGT1A3. We also showed that montelukast hydroxymetabolites M5R, M5S, and M6 are efficiently glucuronidated by UGT1A3 in vitro, which could explain why the concentrations of the hydroxymetabolites were affected more by the UGT1A3 variants than those

**Figure 2** Geometric mean (90% CI) plasma concentrations of montelukast, its acyl-glucuronide (M1), and hydroxymetabolites (M5R, M5S, M6) after a single 10-mg oral dose of montelukast in 191 healthy volunteers with different UGT1A3 genotypes. Open circles indicate noncarriers of UGT1A3*2 (n = 72), solid circles subjects heterozygous for the UGT1A3*2 (n = 90), and solid triangles subjects homozygous for the UGT1A3*2 haplotype (n = 29). The insets depict the same data on a semilogarithmic scale. Plasma concentrations of montelukast were adjusted for BSA and those of M1 and M5S for lean body weight, as according to the linear regression models of their AUC0-24 values.
of parent montelukast. Taken together, our results indicate that glucuronidation plays an important role in the metabolism of both parent montelukast and its hydroxymetabolites. However, due to low plasma concentrations, the hydroxymetabolites are unlikely to contribute to the pharmacological effects of montelukast.

In addition to UGT1A3 variants, SNVs in the SLC01B1 gene were associated with significantly higher AUC and C\textsubscript{max} of montelukast acyl-glucuronide, M1. SLC01B1 encodes OATP1B1, an influx transporter mediating the hepatic uptake of its substrates from sinusoidal blood.\textsuperscript{11} Of the associated SNVs,

### Table 3 Results of the linear regression analysis of the effects of UGT1A SNVs and haplotypes on UGT1A3 mRNA expression, ABCC9 rs704212 SNV on ABCC9 mRNA expression, and SLC01B1 SNVs and haplotypes on SLC01B1 mRNA expression in liver samples from patients undergoing laparoscopic gastric bypass operation

| Gene   | n   | dpSNP ID/Haplotypes | MAF (n = 185-197) | Effect* | 90% CI | P value |
|--------|-----|---------------------|------------------|---------|--------|---------|
| UGT1A3 | 136 | rs7604115 0.44       | 22.3% 11.3%, 34.5%| 5.65 x 10^{-4} |
|        | 136 | rs3806592 0.43       | 22.9% 11.7%, 35.2%| 4.78 x 10^{-4} |
|        | 136 | rs7556676 0.47       | 23.2% 12.4%, 35.0%| 2.57 x 10^{-4} |
|        | 136 | rs4663969 0.47       | 23.6% 12.7%, 35.6%| 2.27 x 10^{-4} |
|        | 136 | rs1875263 0.44       | 22.9% 11.7%, 35.2%| 4.78 x 10^{-4} |
|        | 136 | UGT1A3*2 0.41        | 23.8% 12.8%, 35.8%| 2.08 x 10^{-4} |
|        | 136 | UGT1A3*3 0.058       | -1.2% -18.7%, 20.0%| 0.918   |
|        | 136 | UGT1A3*6 0.10        | 29.9% -13.3%, 94.7%| 0.286   |
| ABCC9  | 187 | rs704212 0.11        | 25.2% 13.1%, 38.6%| 3.41 x 10^{-4} |
| SLC01B1| 124 | rs4149056 0.24       | -13.5% -21.4%, -4.7%| 0.0146  |
|        | 124 | rs2306283 0.47       | -7.5% -14.7%, 0.4%| 0.117   |
|        | 123 | SLC01B1*1B 0.18      | 10.4% 0.8%, 20.9%| 0.0740  |
|        | 123 | SLC01B1*5 0.019      | 11.2% -18.3%, 51.3%| 0.570   |
|        | 123 | SLC01B1*15 0.22      | -15.2% -23.2%, -6.3%| 0.00733 |

MAF, minor allele frequency.

*Per copy of the minor allele.

Figure 3 Boxplots of the effects of UGT1A3*2, *3, and *6 haplotypes on UGT1A3 mRNA expression in human liver samples. The horizontal lines inside the boxes represent the median, the box edges show the lower and upper quartiles, and the whiskers show the 10th and 90th percentiles. Individual data points are given as circles for men and as triangles for women.
Our finding that the OATP1B1 function-impairing an important determinant of parent montelukast pharmacokinetics, OATP1B1, 1B3, and 2B1, and that OATP-CYP2C8 interplay is of the haplotype, which contains rs2306283 without rs4149056, showed a no identified transporter function. Notably, another SLCO1B1 appeared to have no effect. Nevertheless, the SLCO1B1 p.Asn130Asp) missense variant was associated with increased > OATP1B1. In a previous study, the rs2306283 (c.388A>T) substitution in ABCC9 showed a BSA effect per 10% increase; genetic variant effect per copy of the minor allele. BSA, body surface area; AUC0-165, area under the plasma concentration-time curve from 0 h to infinity; CI, confidence interval; MAF, minor allele frequency.

Table 4 Results of the candidate gene analysis on montelukast AUC0-∞

| Variable | MAF     | Effecta | 90% CI       | P value | Adjusted R² for each step |
|----------|---------|---------|--------------|---------|--------------------------|
| BSA      | —       | −11.5%  | −14.0%, −9.0% | 2.10 × 10⁻¹¹ | 0.17                     |
| UGT1A3*2 | 0.39    | −16.8%  | −20.5%, −12.9% | 2.99 × 10⁻¹⁰ | 0.32                     |
| ABCC9 rs704212 | 0.14  | −13.7%  | −19.1%, −7.9%  | 2.19 × 10⁻⁴ | 0.37                     |
| CYP2C8*3 | 0.11    | −11.3%  | −17.5%, −4.7%  | 0.00659  | 0.40                     |
| CYP2C8*4 | 0.07    | 12.7%   | 3.7%, 22.5%    | 0.0184   | 0.41                     |

BSA, body surface area; AUC0-∞, area under the plasma concentration-time curve from 0 h to infinity; CI, confidence interval; MAF, minor allele frequency.

The rs4149056 missense variant (c.521T>G, p.Val174Ala, SLCO1B1*5 or *15) markedly impairs the function of OATP1B1, as demonstrated both in vitro and in vivo in humans. Many glucuronide conjugates are OATP1B1 substrates and our results suggest that also M1 is a substrate of OATP1B1. In a previous study, the rs2306283 (c.388A>T, p.Asn130Asp) missense variant was associated with increased SLCO1B1 expression in human liver samples (n = 143), whereas rs4149056 had no effect. In our study, however, rs4149056 was associated with decreased SLCO1B1 expression, but rs2306283 appeared to have no effect. Nevertheless, the SLCO1B1*1B haplotype, which contains rs2306283 without rs4149056, showed a tendency for a 10% increased expression of SLCO1B1 per copy of the haplotype.

A recent study suggested that montelukast is a substrate of OATP1B1, 1B3, and 2B1, and that OATP-CYP2C8 interplay is an important determinant of parent montelukast pharmacokinetics. Our finding that the OATP1B1 function-impairing rs4149056 SNV did not significantly affect the pharmacokinetics of parent montelukast, even in the candidate gene analysis with no correction for multiple testing, indicates that OATP1B1 is not of major importance for the hepatic elimination of montelukast in vivo in humans. Moreover, we fully sequenced the SLCO1B3 and SLCO2B1 genes and found no association between montelukast AUC and variants in these genes, indicating that these genes are also not important determinants of interindividual variability in montelukast pharmacokinetics.

Interestingly, the ABCC9 rs704212 SNV was associated with reduced plasma concentrations of montelukast hydroxymetabolite M5R, and with increased mRNA expression of ABCC9 in human liver samples. Based on these results, the SNV was selected for the candidate gene analysis, where it was found to be associated also with reduced AUC of montelukast. Therefore, the effects of genetic variants on the steady-state pharmacokinetic parameters measured after a single dose. Therefore, the effects of genetic variants on the steady-state plasma concentrations of montelukast should be similar to the effects on the AUC0-∞ of montelukast observed in our study after a single dose. Because montelukast dose-dependently improves chronic asthma, the reduced plasma concentrations of montelukast due to the UGT1A3*2 haplotype might impair its efficacy. Even though the effect of the UGT1A3*2 haplotype on montelukast pharmacokinetics is modest, the haplotype is common (Supplementary Figure S3). Therefore, it may be an important factor explaining the variability in montelukast response at the population level. Body surface area and the UGT1A3*2 allele together explained 32% of interindividual variability.
variability in montelukast exposure. When the ABCC9 and CYP2C8 alleles were added to the model, this percentage increased to 41%. Together with pharmacodynamic markers, this knowledge might aid in individualizing treatment with leukotriene receptor antagonists.

In conclusion, genetic variability in UGT1A3 significantly affects montelukast pharmacokinetics. This indicates that glucuronidation via UGT1A3 has a larger role in the metabolism of montelukast than previously thought, especially in subjects carrying the UGT1A3*2 haplotype. These results also further confirm that the UGT1A3*2 haplotype enhances the glucuronidation of UGT1A3 substrates. Moreover, the candidate gene analysis suggested that also CYP2C8*3, CYP2C8*4, and ABCC9 rs704212 affect the pharmacokinetics of montelukast.

METHODS
Study participants
In all, 201 healthy unrelated Finnish Caucasian volunteers participated in the pharmacokinetic study after giving written informed consent. Their health was confirmed by medical history, clinical examination, and laboratory tests. Participants were not on any continuous medication nor were tobacco smokers. The study was approved by the Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa and the Finnish Medicines Agency Fimea. Ten participants discontinued the study before montelukast administration and thus the pharmacokinetic data were obtained from 191 participants. Of these, 92 were women and 99 men. Their mean ± SD age was 24 ± 4 years, height 174 ± 9 cm, weight 70 ± 12 kg, and body mass index (BMI) 22.8 ± 2.5 kg/m².

A whole-blood DNA sample and a liver biopsy were obtained from 201 patients undergoing laparoscopic gastric bypass operation at the Kuopio University Hospital, as part of the Kuopio Obesity Surgery Study. Good quality RNA expression data and genotypes were obtained from 188 patients. These patients consisted of 129 women and 59 men (mean ± SD: age 48 ± 9 years and BMI 44 ± 6 kg/m²). Seventy-six patients had type 2 diabetes, 34 had nonalcoholic fatty liver, 32 had nonalcoholic steatohepatitis, and 58 used lipid-lowering medication. The degree of liver steatosis was graded from 0 to 3 and that of lobular inflammation from 0 to 2. Written informed consent was obtained from all patients and the study protocol was approved by the Ethics Committee of the Northern Savo Hospital District.

Montelukast pharmacokinetics
After fasting overnight, the healthy volunteers ingested a single 10-mg dose of montelukast (Singulair tablet, Merck Sharp & Dohme, Haarlem, The Netherlands) with 150 ml of water at 8 AM. Standardized meals were served at 4, 7, and 10 h after montelukast ingestion. Timed blood samples (4–9 ml each) were collected into light-protected ethylenediaminetetraacetic acid (EDTA) tubes prior to and up to 24 h after montelukast administration. Tubes were immediately placed on ice and plasma was separated within 30 min. Samples were stored at –70°C until analysis.

The concentrations of plasma montelukast, montelukast acylglucuronide (M1), montelukast 1,2-diol (M6), 21R-hydroxy montelukast (M5R), and 21S-hydroxy montelukast (M5S) were measured using a Nexera X2 liquid chromatography instrument (Shimadzu, Kyoto, Japan) interfaced with a 5500 Qtrap tandem mass spectrometer (AB Sciex, Toronto, ON). Prior to quantification, the plasma sample was purified from proteins and phospholipids using a Phree phospholipid removal plate (Phenomenex, Torrance, CA) according to the manufacturer’s instructions. In short, the plasma sample was mixed with acetonitrile containing 1% formic acid and internal standards (1:4 v/v) and drawn through the Phree cartridges. The chromatographic separation was achieved on a reversed-phase Kinetex C18 analytical column (100 × 2.1 mm internal diameter, 2.6 μm particle size; Phenomenex) using 2 mM ammonium acetate (A) (pH 4.0) adjusted with 98% formic acid and acetonitrile (B) as a mobile phase. The injection volume was 3 μl and the column temperature was held at 30°C. The gradient profile was set as follows: a linear increase from 35% B to 62% B over 2.2 min, held at 62% B for 2 min, a gradient from 62% B to 95% B over 0.6 min, and maintained at 95% B for 1.4 min followed by a reversion to the initial conditions. The mass spectrometer was operated in a positive electrospray ionization mode (ESI+) employing scheduled multiple reaction monitoring (MRM) for the optimal dwell time for each analyte. A corresponding deuterated reference compound served as internal standard for each analyte, except for M6, which utilized montelukast-d6. The target mass-to-charge ratios (m/z) for montelukast, M1, M5R/M5S, and M6 were 586→422, 762→422, 602→147, and 602→438, and the limits of quantification (ng/ml) were 1.0, 0.2, 0.1, and 0.1, respectively. The day-to-day coefficient of variation (CV) was below 10% at relevant concentrations for all analytes. The AUC0–∞, Cmax, and t1/2 values were calculated for montelukast, M1, M5R, M5S, and M6 with standard noncompartmental methods using Phoenix WinNonlin, v. 6.3 (Certara, Princeton, NJ).

DNA sequencing and genotyping
Genomic DNA was extracted using the Maxwell 16 LEV Blood DNA Kit on a Maxwell 16 Research automated nucleic acid extraction system (Promega, Madison, WI; pharmacokinetic study) or DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany; liver samples). DNA concentration and absorbance 260/280 ratio (A260/A280) were determined with the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Targeted massively parallel sequencing of the 379 pharmacokinetic genes ±20 kb (Supplementary Table S2; genome build GRCh37) was performed in all pharmacokinetic study participants (n = 201). For library preparation, 3 μg of genomic DNA was processed according to the NEBNext DNA Sample Prep protocol (New England Biolabs, Ipswich, MA). Target enrichment capture was performed using the NimbleGen SeqCap EZ Choice capture protocol (Roche Sequencing, Pleasanton, CA). Sequencing was done on the Illumina HiSeq2000 platform with 100 bp paired-end reads (Illumina, San Diego, CA). Quality control, short read alignment, and variant calling and annotation were carried out using an in-house-developed pipeline, as described previously. The sequencing and bioinformatics pipelines were carried out at the Technology Centre at the Institute for Molecular Medicine Finland (Helsinki, Finland). Mean coverage depth was 37.2×. Coverage depth ≥10×, Hardy–Weinberg equilibrium P < 3.15 × 10⁻⁷ (Bonferroni correction), and proportion missing <0.05 were employed as quality thresholds for including genotype data in statistical analysis.

The pharmacokinetic study participants and liver samples were genotyped for the UGT1A rs7604115, rs8306592, rs7556676, rs4663969, rs1875263, rs3821242, rs6431625, and rs45449995, ABCC9 rs704212, and SLCO1B1 rs4149056 and rs2306283 SNVs with TaqMan genotyping assays on a QuantStudio 12K Flex Real-Time PCR System according to the manufacturer’s protocol (Thermo Fisher Scientific). Call identity with sequencing data was 99–100%. In case of discordant results, genotypes obtained by sequencing were used in the statistical analysis.

Reverse transcription quantitative real-time PCR
RNA from liver samples was extracted using the mirNeasy Mini Kit (Qiagen, Chatsworth, CA) and stored at −80°C. RNA was reverse-transcribed using the SuperScript Vilo cDNA Synthesis Kit, according to the manufacturer’s instructions (Thermo Fisher Scientific). The cDNA samples were preamplified (14 cycles) with a custom TaqMan pre amp pool containing the assays for UGT1A1, ABCC9, SLCO1B1 and reference genes before quantitative real-time PCR (qPCR), according to the manufacturer’s instructions (Thermo Fisher Scientific).

The qPCR was carried out using OpenArray technology on the QuantStudio 12K Flex Real-Time PCR System. The custom OpenArray plate
In vitro studies with UGT1A1, 1A3, and 1A9 recombinant enzymes
The incubation mixtures (triplicate samples) contained 100 nM phosphate buffer, pH 7.4, 5 mM MgCl₂, 0.3 mg/ml of suprasomal protein (Conring, Woburn, MA), 2 mM UDP-glucuronic acid (UDPGA, trimonium salt), and 0.1 μM montelukast, M5R, M5S, or M6, in a total volume of 500 μl. Control incubations (duplicate samples) were performed with control supersomes, which do not contain active UGT enzyme. The reactions were initiated by the addition of the substrate and incubated in a shaking water bath at 37°C. Reactions were stopped by moving 50 μl samples to 50 μl acetonitrile containing 1% formic acid and internal standards at timepoints 0, 6, 12, 20, 40, and 60 min. After centrifugation, the supernatants were analyzed using SCIEX API 2000 tandem mass spectrometer (AB Sciex), as described previously. Depletion (percentage) of the substrates at each timepoint was calculated for UGT1A1, 1A3, 1A9, and control samples.

Statistical analysis
The data were analyzed with the statistical programs JMP Genomics 7.0 (SAS Institute, Cary, NC) and IBM SPSS 22.0 for Windows (Armonk, NY). The pharmacokinetic variables were logarithmically transformed before analysis. Possible effects of demographic covariates on pharmacokinetic variables and gene expression were investigated using stepwise linear regression analysis with P-value thresholds of 0.05 for entry and 0.10 for removal. Sex, body weight, lean body weight,45 and body surface area,46,47 hepatosteatosis, and use of lipid-lowering medication for gene expression analysis were fixed for significant demographic covariates. Additive coding was employed for genetic variants. The effects of gene and genetic variants on glucuronidation of montelukast were investigated using stepwise linear regression analysis (SAS Institute, Cary, NC) and IBM SPSS 22.0 for Windows (Armonk, NY). The data were analyzed with the statistical programs JMP Genomics 7.0 (SAS Institute, Cary, NC) and IBM SPSS 22.0 for Windows (Armonk, NY). Additive coding was employed for genetic variants. The effects of gene and genetic variants on glucuronidation of montelukast were investigated using stepwise linear regression analysis (SAS Institute, Cary, NC) and IBM SPSS 22.0 for Windows (Armonk, NY)

Additional Supporting Information may be found in the online version of this article.

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CONFLICT OF INTEREST
The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS
P.H. and M.Ni. wrote the article; P.H., A.T., J.T.B., and M.Ni. designed the research; P.H., A.T., M.Ne., T.T., M.P-H., V.K., V.T.M., J.P., J.T.B., and M.Ni. performed the research; P.H. and M.Ni. analyzed the data.

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