Identification of Glycochenodeoxycholate 3-O-Glucuronide and Glycodeoxycholate 3-O-Glucuronide as Highly Sensitive and Specific OATP1B1 Biomarkers

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The aim of this study was to investigate the sensitivity and specificity of endogenous glycochenodeoxycholate and glycodeoxycholate 3-O-glucuronides (GCDCA-3G and GDCA-3G) as substrates for organic anion transporting polypeptide 1B1 (OATP1B1) in humans. We measured fasting levels of plasma GCDCA-3G and GDCA-3G using liquid chromatography-tandem mass spectrometry in 356 healthy volunteers. The mean plasma levels of both compounds were ~ 50% lower in women than in men ($P = 2.25 \times 10^{-18}$ and $P = 4.73 \times 10^{-9}$). In a microarray-based genome-wide association study, the *SLCO1B1* rs4149056 (c.521T>C, p.Val174Ala) variation showed the strongest association with the plasma GCDCA-3G ($P = 3.09 \times 10^{-30}$) and GDCA-3G ($P = 1.60 \times 10^{-17}$) concentrations. The mean plasma concentration of GCDCA-3G was 9.2-fold ($P = 8.77 \times 10^{-31}$) and that of GDCA-3G was 6.4-fold ($P = 2.45 \times 10^{-13}$) higher in individuals with the *SLCO1B1* c.521C/C genotype than in those with the c.521T/T genotype. No other variants showed independent genome-wide significant associations with GCDCA-3G or GDCA-3G. GCDCA-3G was highly efficacious in detecting the *SLCO1B1* c.521C/C genotype with an area under the receiver operating characteristic curve of 0.996 ($P < 0.0001$). The sensitivity (98–99%) and specificity (100%) peaked at a cutoff value of 180 ng/mL for men and 90 ng/mL for women. In a haplotype-based analysis, *SLCO1B1*5* and **15* were associated with reduced, and *SLCO1B1*1B, *14,* and **35* with increased OATP1B1 function. *In vitro*, both GCDCA-3G and GDCA-3G showed at least 6 times higher uptake by OATP1B1 than OATP1B3 or OATP2B1. These data indicate that the hepatic uptake of GCDCA-3G and GDCA-3G is predominantly mediated by OATP1B1. GCDCA-3G, in particular, is a highly sensitive and specific OATP1B1 biomarker in humans.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Glycochenodeoxycholate 3-O-glucuronide (GCDCA-3G) and glycodeoxycholate 3-O-glucuronide (GDCA-3G) are circulating OATP substrates and promising candidates as endogenous biomarkers for organic anion transporting polypeptide (OATP)–mediated drug–drug interaction (DDI) risk assessment. The plasma concentrations of GCDCA-3G and GDCA-3G have been shown to increase in response to a nonspecific OATP inhibitor and to correlate with the exposure to OATP1B probe drugs.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ Which of the hepatic influx transporters are responsible for GCDCA-3G and GDCA-3G uptake? Are GCDCA-3G and GDCA-3G sensitive endogenous biomarkers for OATB1B1 phenotyping *in vivo* in humans?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ GCDCA-3G is a highly sensitive and specific endogenous biomarker for identifying impaired OATP1B1 activity. GDCA-3G is somewhat less sensitive than GCDCA-3G, but also shows relatively high specificity. In healthy volunteers, the plasma GCDCA-3G and GDCA-3G levels are higher in men than in women.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ GCDCA-3G could be a useful biomarker for assessing OATP1B1-mediated DDI risk and evaluating the mechanisms of complex DDIs. Importantly, it presents as the most sensitive OATP1B1 phenotyping biomarker to date.

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Pharmacokinetic drug–drug interactions (DDIs) mediated by drug-metabolizing enzymes and drug transporters are an important cause of adverse effects and treatment failures.[1] Such disadvantageous drug reactions commonly occur when a perpetrator drug inhibits the metabolism or the transport of a victim drug.[2] Consequently, prior to human dosing, new drug candidates are routinely evaluated *in vitro* as inhibitors of various drug-metabolizing enzymes and transporters. If the evaluated risks exceed regulatory guidance thresholds, it is recommended to carry out a thorough assessment of the propensity of an investigational drug to cause DDIs *in vivo* in humans.[3,4]

Limitations in preclinical transporter assays and *in vitro*–to–*in vivo* extrapolations may lead to false positive predictions and potentially unnecessary clinical DDI studies.[5] In this regard, endogenous transporter substrates have the potential to aid in assessing the risk of transporter-mediated DDIs during early clinical drug development. Hence, there has been a growing interest in validating endogenous biomarkers for specific drug transporters, in order to substitute or supplement formal clinical DDI trials.[6] Recently, significant progress has been made, in particular, in the assessment of endogenous biomarkers for the hepatic organic anion transporting polypeptide 1B (OATP1B) transporters.[7–12] For example, biomarkers such as coproporphyrin I, hexadecanedioate, tetradecanedioate, glycochenodeoxycholate 3-sulfate (GCDCA-S), glycodeoxycholate 3-sulfate (GDCA-S), glycodeoxycholate 3-O-glucuronide (GDCA-3G), glycochenodeoxycholate 3-O-glucuronide (GCDCA-3G), and direct bilirubin have shown increased exposures after administration of the nonselective OATP1B inhibitor rifampin (international nonproprietary name, rifampicin).[7–10]

In humans, the OATP1B subfamily consists of two closely related transporters, OATP1B1 and OATP1B3. They are both exclusively expressed on the sinusoidal membrane of hepatocytes and play a crucial role in the hepatic uptake of a variety of drugs.[13–18] OATP1B1 and OATP1B3 are known to mediate clinically significant DDIs. In addition, a common single-nucleotide variation (SNV), c.521T>C (rs4149056, p.Val174Ala), in the *SLCO1B1* gene impairs OATP1B1 function, resulting in markedly elevated plasma levels of many drugs, such as statins.[18–27]

Besides plasma drug concentrations, the reduced function *SLCO1B1* c.521T>C SNV may also raise the levels of circulating endogenous substrates, offering an additional tool in biomarker research. This was successfully demonstrated in a metabolomics and genome-wide association study, which identified potential OATP1B1 biomarkers, including hexadecanedioate and tetradecanedioate and many partly unidentified biomarker candidates.[28] Thereafter, a few studies have investigated the effects of *SLCO1B1* genotype on endogenous biomarkers in healthy volunteers, but with limited statistical power due to small sample sizes.[29,30]

Up until recently, the only bile acid glucuronide known to present as an OATP substrate was the 24-O-glucuronide of chenodeoxycholic acid.[8] However, GCDCA-3G and GDCA-3G have also been identified as candidate biomarkers for OATP-mediated DDIs.[10] Compared with nonglucuronidated amidated (glycine or taurine conjugated) bile acids, and even chenodeoxycholic acid 24-O-glucuronide, both GCDCA-3G and GDCA-3G exhibit robust dose-dependent responses to the OATP1B inhibitor rifampin, and their plasma levels correlate with the pharmacokinetics of OATP1B substrate drugs.[10] These data suggest that both glucuronides could serve as endogenous biomarkers for OATP1B transporters, but further assessments are warranted to elucidate their specificity and sensitivity regarding OATP1B1 and OATP1B3. Therefore, the aim of this study was to investigate the specificities and sensitivities of GCDCA-3G and GDCA-3G as OATP1B1 substrates in humans using a comprehensive genomic analysis in healthy volunteers. In addition, both glucuronides were assessed as OATP substrates *in vitro*.

METHODS

Study participants and samples

A total of 356 healthy unrelated Finnish White subjects (183 women, 173 men) participated in the study after giving a written informed consent. The mean ± standard deviation age of the participants was 24.1 ± 4.1 years, weight 69.7 ± 12.1 kg, and body mass index 22.9 ± 2.7. Blood samples for measuring endogenous compounds were collected as part of a previously published[25] and a yet unpublished single dose pharmacokinetic study (EudraCT numbers 2011-004645-40 and 2015-000540-41). Following an overnight fast at 7–8 AM and before the study drug administration, a 10 mL blood sample was collected from each participant into a light-protected ethylenediaminetetraacetic acid-containing tube. Because the samples were collected before the study drug administration, it is not possible that the administered drugs could have affected the concentrations of GCDCA-3G or GDCA-3G. The tube was placed on ice immediately after sampling, and plasma was separated within 30 minutes. The plasma samples were stored at −80°C until analysis. The study protocols were approved by the coordinating ethics committee of the Hospital District of Helsinki and Uusimaa (Helsinki, Finland).

Chemicals

Glycochenodeoxycholate (GCDCA), glycodeoxycholate (GDCA), stable labeled GCDCA D7, GDCA D5, GCDCA-3G D4, and nonlabeled GCDCA-3G and GCDCA-3G were purchased from Toronto Research Chemicals (Toronto, Canada). Stable labeled GDCA-3G C13N15 was prepared at Pfizer (Supplemental Methods). Other chemicals and organic solvents were of commercially available analytical grade.

Quantification of plasma GCDCA-3G and GDCA-3G

Plasma samples were thawed at room temperature and prepared by protein precipitation on a 96-well Impact filter plate (Phenomenex, Torrance, CA) using a Freedom EVO automated liquid handling system (Tecan Group, Männedorf, Swizerland). In short, 100 µL aliquot of plasma and 400 µL of acetonitrile, which contained stable isotope-labeled internal standards (25 ng/mL each), were mixed on the filter plate. The sample mixture was allowed to incubate for 15 minutes before the supernatant was separated. The sample extract was dried using a GeneVac centrifugal evaporator (Thermo Fisher Scientific, Waltham, MA), followed by reconstitution in 50 µL of 50% methanol. An aliquot of 1–2 µL was delivered into a Sciex 6500 QTRAP + liquid chromatography-tandem mass spectrometry system (Sciex, Toronto, Canada). The simultaneous quantification of plasma GCDCA-3G, GDCA-3G, GCDCA, and GDCA were performed as previously described.[31] The lower limits of quantification were 0.25 ng/mL, and the interday coefficient of variations were below 10% at relevant concentrations for all analytes.

Genotyping

Genomic DNA was extracted from ethylenediaminetetraacetic acid anticoagulated blood samples using the Maxwell 16 LEV Blood DNA

Kit on a Maxwell 16 Research automated nucleic acid extraction system (Promega, Madison, WI). The participants were genotyped using either the Illumina Infinium Core Exome (n = 183) or Global Screening Array (n = 173) microchips at the Technology Center at the Institute for Molecular Medicine Finland (Helsinki, Finland). Only the SNVs, which were available on both chips and had minor allele frequencies over 0.01, were included in the analyses. Success rate of 97% and Hardy-Weinberg equilibrium $P < 10^{-5}$ were employed as quality thresholds for including genotype data in statistical analysis. In addition, the participants were genotyped for the *SLCO1B1* c.388A>G (rs2306283, p.N130D), c.463C>A (rs11045819, p.P155T), c.521T>C, and c.1929A>C (rs34671512, p.L643F) SNVs with TaqMan genotyping assays on a QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). The *SLCO1B1* haplotypes **1A* (c.388A-c.463C-c.521T-c.1929A), **1B* (c.388G-c.463C-c.521T-c.1929A), **5* (c.388A-c.463C-c.521C-c.1929A), **14* (c.388G-c.463A-c.521T-c.1929A), **15* (c.388G-c.463C-c.521C-c.1929A), and **35* (c.388G-c.463C-c.521T-c.1929C) were computed with PHASE v2.1.1.[32–36]

Statistical analysis

The data were analyzed with the statistical programs JMP Genomics 8.0 (SAS Institute, Inc., Cary, NC) and IBM SPSS 25 for Windows (Armonk, NY). The concentrations of GCDCA-3G, GDCA-3G, GCDCA, and GDCA as well as the glucuronide/parent compound ratios were logarithmically transformed before analysis. Possible effects of demographic covariates (sex and logarithmically transformed bodyweight and body mass index) were investigated using a forward stepwise linear regression analysis with P value thresholds of 0.05 for entry and 0.10 for removal as the stepping method criteria. Genome-wide association analyses were carried out using linear regression analysis with additive coding and the significant demographic covariates set as fixed factors. A P value of below 5×10^{-8} was considered genome-wide significant. For genome-wide significant SNVs and for haplotypes of the genome-wide significant genes, analysis of variance adjusting for significant demographic covariates was carried out with pairwise comparisons with the Fisher's Least Significant Difference method. A P value of below 0.05 was considered statistically significant. Possible correlations of fasting plasma GCDCA-3G and GDCA-3G concentrations with 3R,5S-fluvastatin area under the concentration-time curve (AUC) were investigated using partial correlation analysis controlling for body surface area and cytochrome P450 2C9 (CYP2C9) genotype in 183 of the participants with previously published pharmacokinetic data on fluvastatin.[25] Receiver operating characteristic (ROC) analysis was carried out with GraphPad Prism version 8.2.1 for Windows (GraphPad Software, San Diego, CA).

In vitro studies

Both GCDCA-3G and GDCA-3G were screened *in vitro* as substrates of various human liver solute carriers individually transfected into Human Embryonic Kidney (HEK) 293 cells as previously described.[37] In this instance, however, additional uptake studies were conducted with OATP1B1*5 and OATP1B1*15 similarly transfected into HEK293 cells (provided by Yuichi Sugiyama, Tokyo University, Japan), and OATP1B1*14 transfected into HEK293 cells (acquired from Absorption Systems, Exton, PA). OATP1B1*1A expressed in HEK293 cells was prepared in-house as described in Supplemental Methods. Rosuvastatin (1 μM) served as a positive control probe for OATP1B1*1A, OATP1B1*14, OATP1B1*5, OATP1B1*15, OATP1B3, and OATP2B1, fluvastatin (1 μM) for OATP1B1*1A, OATP1B1*14, OATP1B1*5, OATP1B1*15, taurocholate (0.2 μM) for sodium-taurocholate co-transporting polypeptide (NTCP), cyclic guanosine monophosphate (0.5 μM) for organic anion transporter 2 (OAT2), and metformin (20 μM) for organic cation transporter 1 (OCT1). Briefly, HEK293 cells (wild type and stably transfected with NTCP, OATP1B1 forms, OATP1B3, OATP2B1, OAT2, and OCT1) were seeded at a density of $0.5–1.2 \times 10^5$ cells/well on BioCoat 48 or 96-well poly-D-lysine coated plates (Corning Inc., Corning NY), grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% sodium pyruvate for 48 hours at 37°C, 90% relative humidity, and 5% carbon dioxide. OATP1B1 forms, OATP1B3, OATP2B1, and NTCP-HEK293 cells were supplemented with nonessential amino acids and GlutaMAX (Thermo Fisher Scientific). OCT1-HEK293 and OAT2-HEK293 cells were supplemented with 1% gentamycin, 1% sodium pyruvate, and 50 μg/mL hygromycin B. To obtain an uptake ratio, both bile acid glucuronides (1 μM) were incubated for 2 minutes with wild-type and transporter-transfected HEK293 cells. For the OATP forms, uptake ratios were determined up to 5 minutes of incubation and at three different concentrations of glucuronide substrate (1, 5, and 10 μM), but only 2-minute data at 1 μM are reported herein. The performance of the different transporter-transfected cells was validated using the probe substrates (incubated over 2 to 4 minutes) described above.[37] At the end of the incubation, cellular uptake was determined by washing the cells four times with ice-cold transport buffer followed by lysis with 0.2 mL of methanol containing internal standard. Quantification of GCDCA-3G and GDCA-3G in cells was as described in Supplemental Methods. Sample processing and bioanalysis of rosuvastatin, taurocholic acid, metformin, fluvastatin, and cyclic guanosine monophosphate has already been described.[37]

Both GCDCA-3G and GDCA-3G (~ 0.01 to 100 μM) were also studied as *in vitro* inhibitors of OATP1B1*1A, OATP1B3, and OATP2B1 (expressed in HEK293 cells) using rosuvastatin (0.5 μM) as substrate. Where possible, the concentration of glucuronide rendering 50% inhibition of rosuvastatin uptake (IC_{50}) was determined. In addition, GCDCA-3G and GDCA-3G were evaluated as substrates of adenosine triphosphate (ATP)-binding cassette (ABC) efflux transporters expressed in vesicles; human bile salt export pump (BSEP), and multidrug resistance-associated proteins 2, 3, and 4 (MRP2, MRP3, MRP4) as described in Supplemental Methods.

RESULTS

Genome-wide association study for plasma GCDCA-3G and GDCA-3G

The mean fasting plasma concentrations of GCDCA-3G and GDCA-3G were ~ 50% lower ($P = 2.25 \times 10^{-18}$ and $P = 4.73 \times 10^{-9}$) and those of GCDCA and GDCA were ~ 20% lower ($P = 0.0165$ and $P = 0.0328$) in women than in men. Accordingly, the GCDCA-3G/GCDCA and GDCA-3G/GDCA concentration ratios were 42% and 32% lower in women than in men ($P = 7.27 \times 10^{-10}$ and $P = 1.50 \times 10^{-6}$). In a genome-wide association analysis adjusted for sex, the *SLCO1B1* rs4149056 (c.521T>C, p.Val174Ala) SNV showed the strongest associations with the plasma concentrations of both GCDCA-3G ($P = 3.09 \times 10^{-30}$) and GDCA-3G ($P = 1.60 \times 10^{-17}$) (**Figure 1**). After adjusting for the c.521T> C SNV, no other SNVs remained associated with GCDCA-3G and GDCA-3G concentrations at the genome-wide significance level. In subjects homozygous for the *SLCO1B1* c.521T>C SNV, the mean plasma concentrations of GCDCA-3G and GDCA-3G were 9.2-fold and 6.4-fold of those in individuals with the T/T genotype ($P = 8.87 \times 10^{-31}$ and $P = 2.45 \times 10^{-13}$) (**Table 1**, **Table S1**, **Figure 2**). In T/C heterozygotes, the mean plasma concentrations of GCDCA-3G and GDCA-3G were 1.7-fold and 1.9-fold of those in individuals with the T/T genotype ($P = 1.21 \times 10^{-13}$ and $P = 1.35 \times 10^{-9}$). The *SLCO1B1* c.521T>C SNV was not significantly associated with the

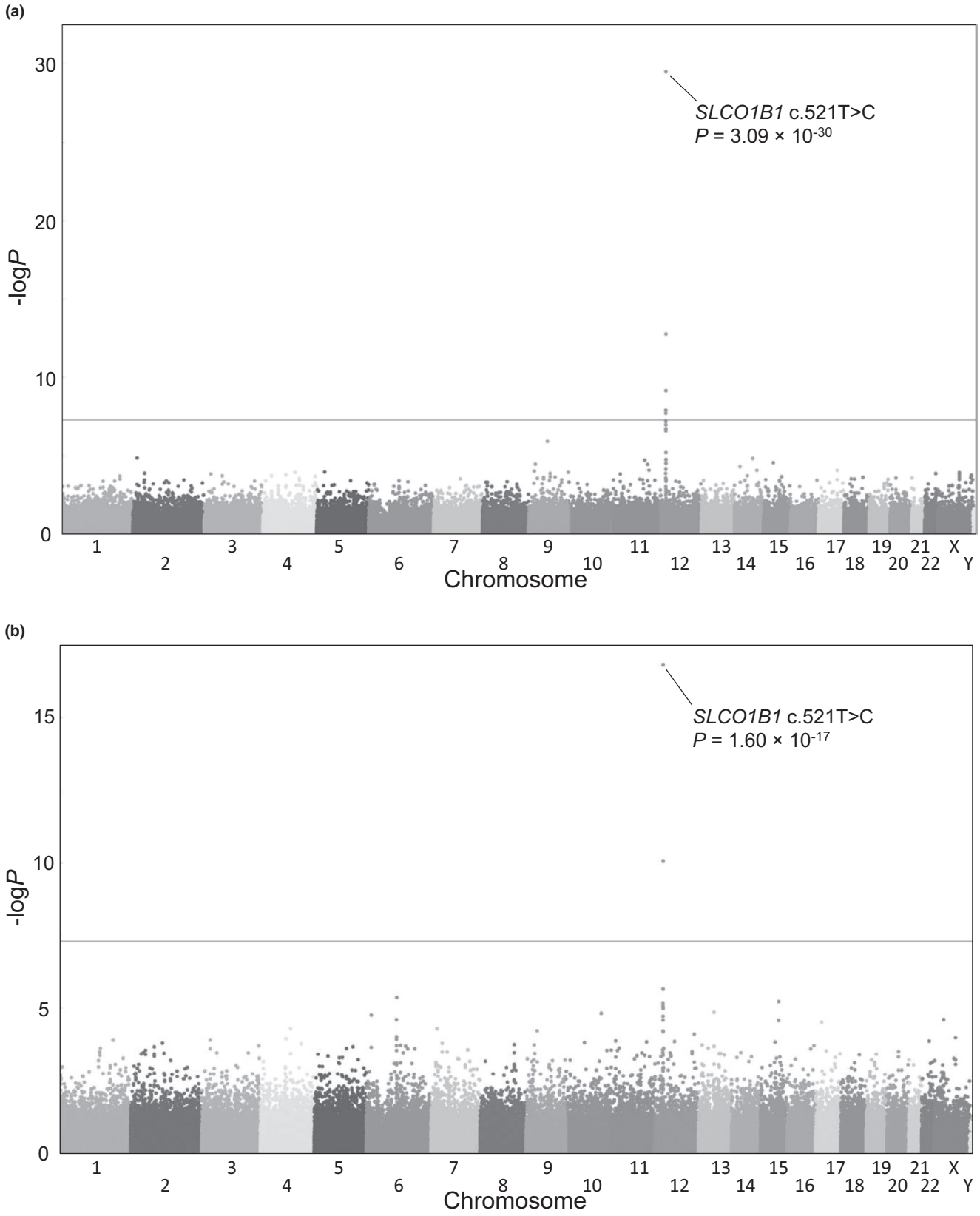

Figure 1 Manhattan plots of the fasting plasma concentrations of (**a**) GCDCA-3G and (**b**) GDCA-3G. Horizontal lines indicate the genome-wide significance level of 5×10^{-8}.

Table 1 Effects of the *SLCO1B1* c.521T>C single nucleotide variation on the plasma concentrations of GCDCA-3G, GDCA-3G, GCDCA, and GDCA

Variable	T/T genotype (control; *n* = 230)	T/C genotype (*n* = 113)			C/C genotype (*n* = 13)		
	Geometric mean (95% CI)	Geometric mean (95% CI)	Ratio to control (95% CI)	*P*	Geometric mean (95% CI)	Ratio to control (95% CI)	*P*
GCDCA-3G (ng/mL)	25.8 (23.8, 28.0)	44.6 (39.8, 50.0)	1.73 (1.50, 1.99)	1.21×10^{-13}	238 (171, 333)	9.23 (6.55, 13.0)	8.77×10^{-31}
GDCA-3G (ng/mL)	56.9 (51.0, 63.7)	106 (90.0, 124)	1.85 (1.53, 2.25)	1.35×10^{-9}	367 (229, 585)	6.43 (3.98, 10.4)	2.45×10^{-13}
GCDCA (ng/mL)	419 (378, 465)	378 (326, 438)	0.900 (0.751, 1.08)	0.252	567 (367, 876)	1.35 (0.865, 2.11)	0.185
GDCA (ng/mL)	122 (107, 138)	124 (104, 149)	1.02 (0.822, 1.27)	0.839	141 (83.3, 237)	1.16 (0.675, 1.98)	0.598
GCDCA-3G/GCDCA ratio	0.0615 (0.0563, 0.0673)	0.118 (0.104, 0.134)	1.92 (1.64, 2.24)	3.55×10^{-15}	0.420 (0.289, 0.611)	6.83 (4.65, 10.0)	2.51×10^{-20}
GDCA-3G/GDCA ratio	0.468 (0.431, 0.509)	0.848 (0.753, 0.955)	1.81 (1.57, 2.10)	1.49×10^{-14}	2.61 (1.84, 3.70)	5.57 (3.89, 7.98)	7.00×10^{-19}

Data are adjusted for sex. CI, confidence interval; GCDCA, glycochenodeoxycholate; GCDCA-3G, glycochenodeoxycholate 3-O-glucuronide; GDCA, glycodeoxycholate; GDCA-3G, glycodeoxycholate 3-O-glucuronide.

plasma concentrations of parent GCDCA or GDCA, but their concentrations tended to be slightly increased in C/C homozygotes. In line with these results, the plasma GCDCA-3G to GCDCA and GDCA-3G to GDCA concentration ratios were markedly increased in C/C homozygotes compared with T/T homozygotes, but the increases were slightly smaller than those of the respective glucuronide concentrations (**Table 1**, **Table S1**). Moreover, GCDCA-3G ($r = 0.218$, $P = 0.003$), but not GDCA-3G ($r = 0.108$, $P = 0.149$) concentration showed a positive correlation with 3R,5S-fluvastatin AUC.

Receiver operating characteristic (ROC) analysis

To quantify the sensitivity and specificity of GCDCA-3G and GDCA-3G to detect reduced OATP1B1 activity, we calculated the areas under the ROC curves (AUROCs) and determined the cutoff values for the optimal discrimination of the C/C genotype from the T/C and T/T genotypes (**Figure S1**). The AUROC of GCDCA-3G was larger than 0.99 for both men and women ($P \leq 0.0002$), and the cutoff values with the highest true positive rate together with the lowest false positive rate were 180 and 90 ng/mL, respectively. With these cutoff values, the sensitivity was 100% and specificity higher than 98%. For GDCA-3G, the AUROCs for both sexes were larger than 0.85 ($P \leq 0.0071$) and the optimal cutoff values (430 and 280 ng/mL) resulted in 75–80% sensitivity and 97% specificity. GCDCA-3G and GDCA-3G separated C/C homozygotes from the T/C heterozygotes nearly as well as from the T/C and T/T genotypes, but the separation between T/C heterozygotes and T/T homozygotes was not as clear (**Figure S1**).

Haplotype analysis

In order to investigate how *SLCO1B1* haplotypes affect OATP1B1 function, we next investigated their associations with the plasma concentrations of GCDCA-3G and GDCA-3G. In this analysis, individuals homozygous or compound heterozygous for the *SLCO1B1*5* or **15* alleles had markedly increased concentrations of both of these biomarkers (**Table 2**, **Table S2**, **Figure 3**). Moreover, individuals compound heterozygous for **1B*, **14*, and **35* showed significantly reduced concentrations of GCDCA-3G, but the associations of these alleles with GDCA-3G were less clear.

In vitro studies

Screening of human liver solute carriers (SLCs) showed that GCDCA-3G and GDCA-3G were strongly taken up by OATP1B1*1A *in vitro*, as indicated by 86.0-fold and 77.8-fold higher cellular uptake at 1 μM in the presence of OATP1B1*1A-transfected cells vs. mock-transfected HEK293 cells (**Table 3**). Both GCDCA-3G and GDCA-3G were also transported by OATP1B3 and OATP2B1, although with markedly lower uptake ratios compared with OATP1B1*1A. Of note, NTCP, OAT2, and OCT1 showed no uptake of either GCDCA-3G or GDCA-3G. Both glucuronides were also shown to inhibit the OATP-dependent uptake of rosuvastatin into HEK293 cells, with OATP1B1*1A presenting lower IC_{50} values (≤ 28 μM vs. 34 to >100 μM) compared with OATP1B3 and OATP2B1 (**Figure S2**). It was also shown that the OATP1B1*14 variant protein presented significantly increased,

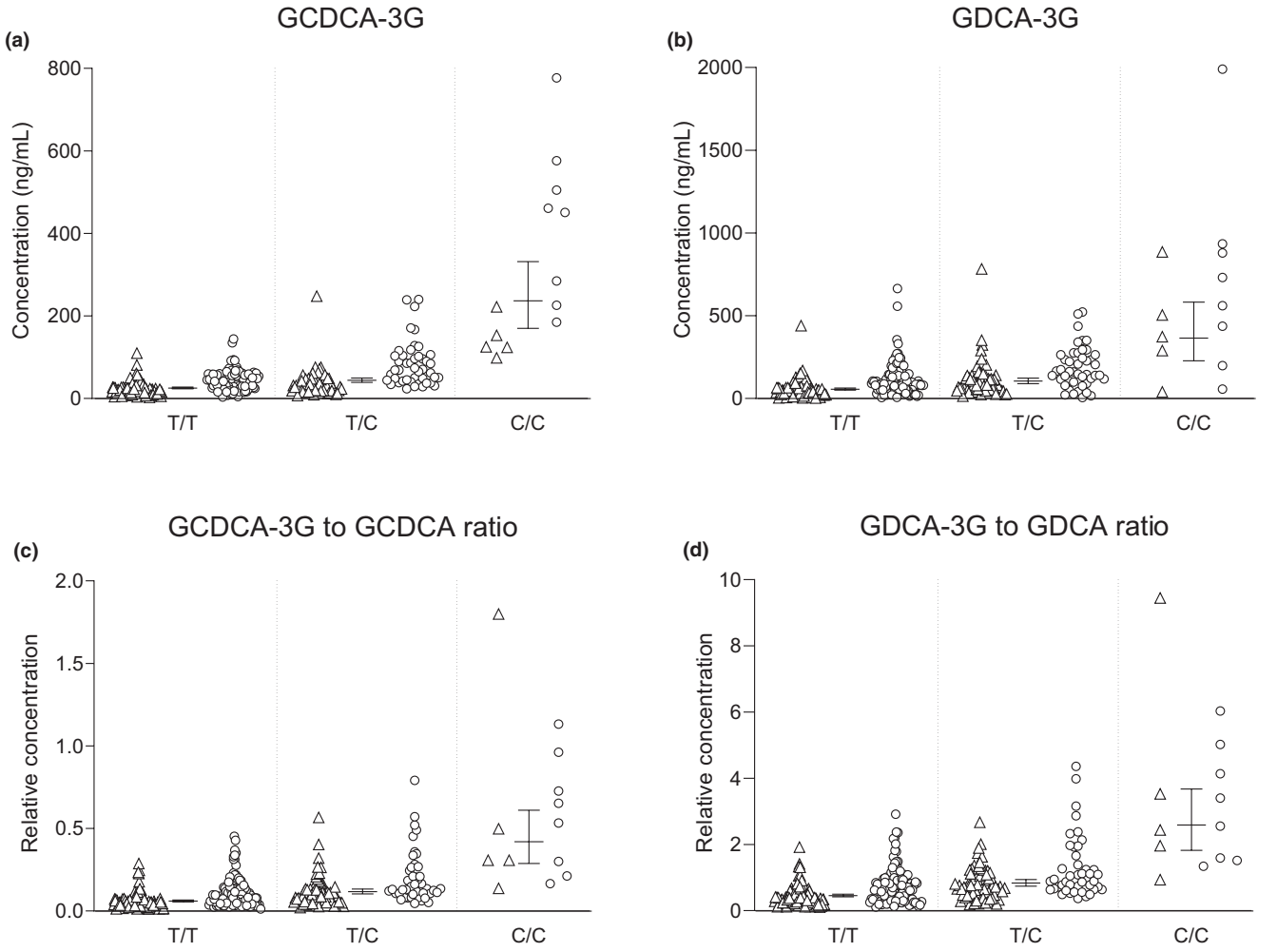

Figure 2 Effects of *SLCO1B1* c.521T>C single nucleotide variation on the plasma concentrations of (**a**) GCDCA-3G and (**b**) GDCA-3G, (**c**) the ratio of GCDCA-3G to GCDCA, and (**d**) the ratio of GDCA-3G to GDCA. Horizontal lines indicate geometric estimated marginal mean values and whiskers indicate 95% confidence intervals. Triangles, women; circles, men.

and the OATP1B1*5 and *15 variants significantly reduced, uptake of GCDCA-3G and GDCA-3G (**Table 4**). When incubated with membrane vesicles, GCDCA-3G and GDCA-3G (0.2 μM) were found to be good substrates (uptake ratios between 10 and 30) of the MRP2 and MRP3 efflux transporters (**Table S3**). Both glucuronides also presented as MRP4 substrates but with vesicle uptake ratios ~ 4.0. In addition, when compared with taurocholic acid, GCDCA-3G was a relatively weak substrate of BSEP with a vesicle uptake ratio of 2.4.

DISCUSSION

In this study, we identified endogenous GCDCA-3G and GDCA-3G as highly sensitive and specific OATP1B1 substrates. In a genome-wide analysis, the *SLCO1B1* c.521T>C reduced-function SNV showed the strongest associations with both of these candidate circulating biomarkers, whereas no other SNVs were independently associated with their concentrations at the genome-wide significance level. In particular, GCDCA-3G showed very high sensitivity and specificity, being able to almost completely differentiate individuals homozygous for the variant allele from heterozygotes and noncarriers. Moreover, the plasma levels of GCDCA-3G correlated positively with the AUC of the known OATP1B1 substrate drug 3R,5S-fluvastatin. In a haplotype-based analysis, *SLCO1B1*5* and **15* were associated with increased, and *SLCO1B1*1B*, **14*, and **35* with reduced plasma levels of GCDCA-3G and GDCA-3G. *In vitro* screening with transfected HEK293 cells supported the specificity of the compounds for OATP1B1*1A with several-fold higher uptake vs. OATP1B3 or OATP2B1. GCDCA-3G also presented higher affinity as an inhibitor of OATP1B1*1A (IC_{50} = 16 μM) when compared with OATP1B3 and OATP2B1 (IC_{50} > 100 μM).

The functional effects of *SLCO1B1* c.521T>C are well characterized both *in vitro* and *in vivo*. The variant impairs the trafficking of OATP1B1 protein onto the plasma membrane and leads to nearly abolished OATP1B1 activity *in vitro*.[18,25,38] Individuals homozygous for the variant allele have shown markedly elevated plasma concentrations of several OATP1B1 substrate drugs, consistent with reduced hepatic uptake.[18,19,21–25,39,40] Our results are therefore compatible with reduced hepatic uptake of GCDCA-3G and GDCA-3G in individuals homozygous for the *SLCO1B1*

Table 2 Effects of *SLCO1B1* haplotypes on the plasma concentrations of GCDCA-3G and GDCA-3G in 356 healthy volunteers

Genotype (*n*)	Geometric mean ng/mL (95% CI)	Ratio to **1A/*1A* (95% CI)	*P*
GCDCA-3G			
**14/*14* (1)	6.42 (2.01, 20.6)	0.229 (0.071, 0.738)	
**14/*35* (4)	12.9 (7.22, 23.1)	0.461 (0.255, 0.831)	0.0102
**1B/*35* (7)	16.7 (10.7, 25.8)	0.595 (0.378, 0.935)	0.0245
**1B/*14* (6)	16.8 (10.5, 27.0)	0.600 (0.369, 0.977)	0.0399
**1B/*1B* (3)	18.8 (9.58, 36.8)	0.671 (0.339, 1.33)	0.250
**1A/*35* (12)	22.5 (16.1, 31.4)	0.803 (0.565, 1.14)	0.221
**1A/*14* (34)	25.1 (20.5, 30.6)	0.895 (0.713, 1.12)	0.336
**14/*15* (11)	25.6 (18.0, 36.3)	0.912 (0.632, 1.32)	0.624
**1A/*1A* (116)	28.0 (25.1, 31.2)	1.00	
**1A/*1B* (47)	28.2 (23.8, 33.4)	1.01 (0.822, 1.23)	0.959
**1B/*15* (17)	36.4 (27.4, 48.2)	1.30 (0.960, 1.76)	0.0894
**15/*35* (6)	38.9 (24.2, 62.5)	1.39 (0.853, 2.26)	0.185
**5/*14* (3)	39.8 (20.4, 77.9)	1.42 (0.721, 2.81)	0.309
**5/*35* (1)	46.7 (14.6, 149)	1.67 (0.519, 5.36)	
**1A/*15* (69)	50.3 (43.7, 57.9)	1.80 (1.50, 2.15)	3.06×10^{-10}
**1A/*5* (6)	65.5 (40.7, 105)	2.34 (1.44, 3.81)	6.76×10^{-4}
**5/*15* (2)	166 (72.9, 376)	5.91 (2.58, 13.5)	3.13×10^{-5}
**15/*15* (11)	255 (180, 362)	9.10 (6.31, 13.1)	2.53×10^{-27}
GDCA-3G			
**14/*14* (1)	12.5 (2.37, 64.3)	0.191 (0.0363, 1.00)	
**1B/*35* (7)	25.0 (13.5, 46.5)	0.385 (0.203, 0.731)	0.00366
**1A/*35* (12)	32.0 (19.9, 51.6)	0.495 (0.300, 0.815)	0.00586
**1B/*14* (6)	42.5 (21.7, 83.3)	0.655 (0.329, 1.31)	0.229
**1A/*14* (34)	52.5 (39.6, 70.0)	0.810 (0.587, 1.12)	0.200
**5/*35* (1)	57.3 (11.0, 299)	0.885 (0.169, 4.64)	
**14/*35* (4)	58.7 (25.8, 133)	0.906 (0.392, 2.09)	0.817
**1A/*1B* (47)	59.6 (46.9, 75.9)	0.920 (0.692, 1.22)	0.566
**1A/*1A* (116)	64.9 (55.6, 75.6)	1.00	
**14/*15* (11)	65.4 (39.8, 108)	1.01 (0.599, 1.70)	0.974
**1B/*1B* (3)	96.9 (37.3, 252)	1.49 (0.568, 3.93)	0.414
**5/*14* (3)	100 (38.7, 259)	1.55 (0.589, 4.05)	0.375
**1A/*15* (69)	101 (82.9, 123)	1.56 (1.21, 2.01)	5.72×10^{-4}
**15/*35* (6)	115 (58.9, 226)	1.78 (0.892, 3.55)	0.102
**1B/*15* (17)	133 (89.4, 199)	2.06 (1.34, 3.16)	0.00103
**1A/*5* (6)	216 (110, 423)	3.33 (1.67, 6.64)	6.95×10^{-4}
**15/*15* (11)	345 (210, 568)	5.33 (3.17, 8.96)	7.95×10^{-10}
**5/*15* (2)	516 (161, 1650)	7.96 (2.46, 25.8)	5.83×10^{-4}

Data are adjusted for sex. CI, Confidence interval; GCDCA-3G, glycochenodeoxycholate 3-O-glucuronide; GDCA-3G, glycodeoxycholate 3-O-glucuronide.

c.521T>C SNV. Interestingly, a previous metabolomic and genome-wide association study found an unidentified endogenous compound to be very strongly associated with the *SLCO1B1* c.521T>C SNV.[28] However, the compound structure remained partly uncharacterized but was likely to be a glucuronide conjugate of GCDCA. Our results suggest that the compound is the 3-O-glucuronide of GCDCA.

In the present study, the fasting plasma concentrations of GCDCA-3G were nine times as high in individuals with the homozygous *SLCO1B1* c.521C/C genotype as in those with the reference T/T genotype. The concentrations of GDCA-3G showed larger between-subject variability and the effect of *SLCO1B1* genotype was smaller, with a six-fold difference between C/C and T/T homozygotes. Similarly, in a previous study, GCDCA-3G was more

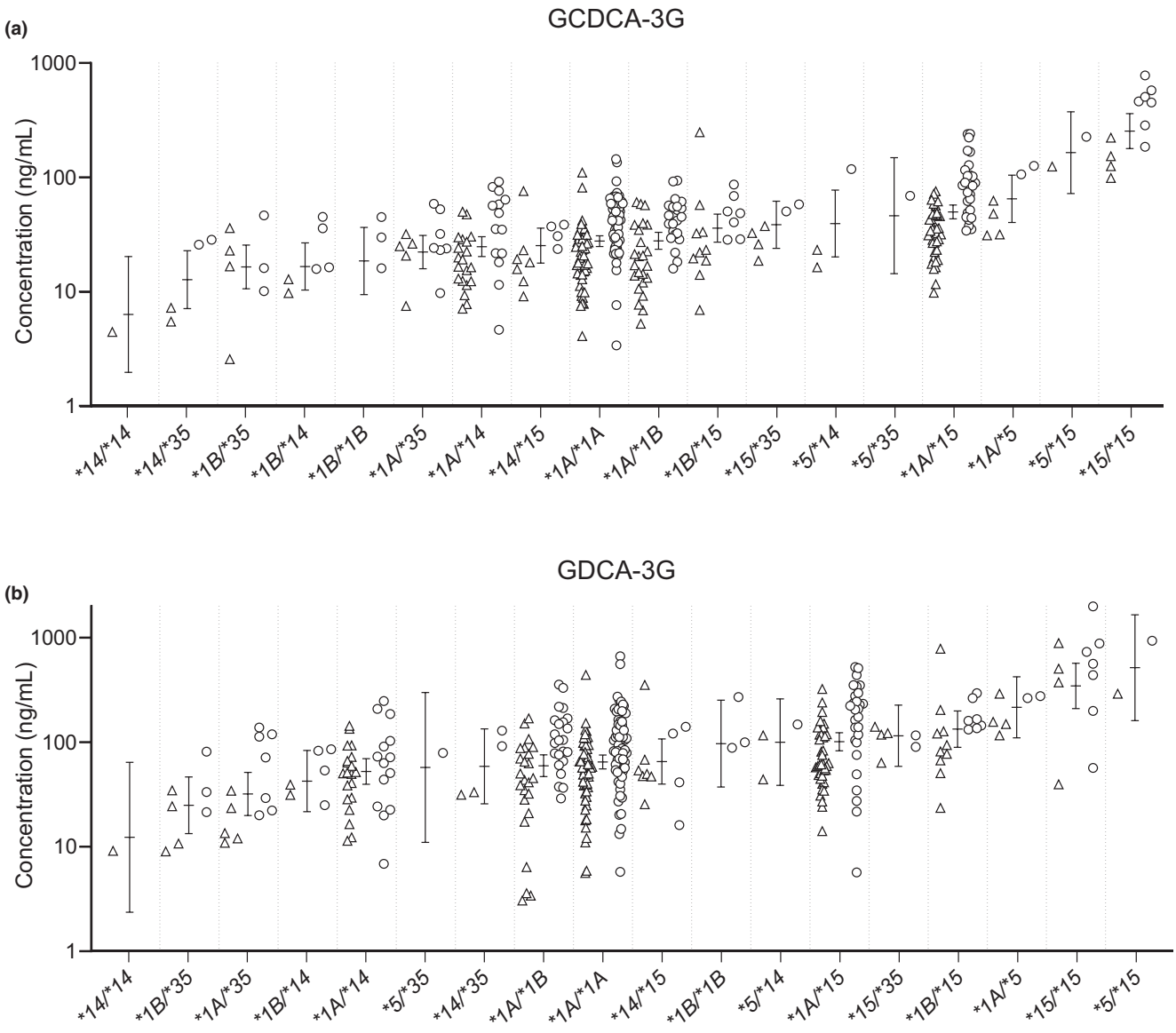

Figure 3 Effects of *SLCO1B1* haplotypes on the plasma concentrations of (**a**) GCDCA-3G and (**b**) GDCA-3G. Horizontal lines indicate geometric estimated marginal mean values and whiskers indicate 95% confidence intervals. Triangles, women; circles, men.

sensitive to OATP1B inhibition by rifampin than GDCA-3G and there was larger between-subject variability in the concentrations of GDCA-3G.[10] Our finding that the *SLCO1B1* genotype has a marked effect on the plasma levels of both candidate biomarkers strongly supports the hypothesis that OATP1B1 is mainly responsible for their hepatic uptake. Assuming that the differences

Table 3 Uptake of 1 μM GCDCA-3G and GDCA-3G by various human solute carriers

	Uptake ratio (vs. wild-type HEK293 cells)					
Substrate	OATP1B1*1A	OATP1B3	OATP2B1	NTCP	OAT2	OCT1
GCDCA-3G	86.0 ± 5.5 (1.7)	13.9 ± 0.4 (0.2)	2.8 ± 0.5 (< 0.1)	1.13 ± 0.27 (<0.1)	0.72 ± 0.20 (< 0.1)	0.56 ± 0.03 (< 0.1)
GDCA-3G	77.8 ± 6.9 (1.5)	5.5 ± 0.4 (< 0.1)	2.0 ± 0.5 (< 0.1)	0.34 ± 0.03 (< 0.1)	1.32 ± 0.19 (< 0.1)	0.93 ± 0.10 (< 0.1)
Probe	51.3 ± 1.4	69.8 ± 1.9	71.1 ± 9.6	102.4 ± 7.9	23.6 ± 0.58	15.4 ± 1.06

Uptake ratio was determined over two minutes for both bile acid glucuronides and two to four minutes for the probe compounds. Data are arithmetic means ± SD (ratio to probe substrate) of $n = 3$ different determinations in one experiment. Values in parentheses represent the uptake ratio of ratios for each glucuronide vs. the chosen probe. Rosuvastatin (1 μM) served as a probe for OATP1B1*1A, OATP1B3, and OATP2B1; taurocholate (0.2 μM) for NTCP; cyclic guanosine monophosphate (0.5 μM) for OAT2; and metformin (20 μM) for OCT1. GCDCA-3G, glycochenodeoxycholate 3-O-glucuronide; GDCA-3G, glycodeoxycholate 3-O-glucuronide; NTCP, sodium-taurocholate co-transporting polypeptide; OAT2, organic anion transporter 2; OATP, organic anion transporting polypeptide; OCT1, organic cation transporter 1.

Table 4 Effects of OATP1B1 variants on the uptake of GCDCA-3G, GDCA-3G, rosuvastatin, and fluvastatin

		Uptake ratio (vs. wild-type HEK293 cells)			
Experiment	Substrate (1 μM)	OATP1B1*5	OATP1B1*15	OATP1B1*14	OATP1B1*1A
1	GCDCA-3G	2.2 ± 0.5 (17%)	2.3 ± 0.3 (18%)	13 ± 0.5 (100%)	nd
	Rosuvastatin[a]	2.4 ± 0.4 (16%)	2.3 ± 0.2 (15%)	15 ± 0.2 (100%)	nd
	Fluvastatin	1.8 ± 0.2 (56%)	1.5 ± 0.1 (47%)	3.2 ± 0.1 (100%)	nd
2	GDCA-3G	8.2 ± 0.5 (13%)	7.0 ± 0.8 (11%)	63 ± 5.2 (100%)	nd
	Rosuvastatin	5.9 ± 0.5 (15%)	5.0 ± 0.2 (13%)	40 ± 3.3 (100%)	nd
	Fluvastatin	1.9 ± 0.1 (61%)	1.5 ± 0.1 (48%)	3.1 ± 0.1 (100%)	nd
3	GCDCA-3G	nd	nd	157 ± 6.4	86 ± 5.4
	GDCA-3G	nd	nd	142 ± 5.0	78 ± 6.9
	Rosuvastatin	nd	nd	85 ± 5.3	51 ± 1.4
	Fluvastatin	nd	nd	4.4 ± 0.1	2.7 ± 0.1

Data are arithmetic means ± SD (percentage of OATP1B1*14 in the same experiment). Uptake was determined over 2 minutes (n = 3 determinations in each experiment). OATP, organic anion transporting polypeptide; GCDCA-3G, glycochenodeoxycholate 3-O-glucuronide; GDCA-3G, glycodeoxycholate 3-O-glucuronide; nd, not determined.
[a]Substrate concentration was 0.5 μM.

in GCDCA-3G and GDCA-3G plasma concentrations between the genotypes are due to differences in their clearances, one could estimate that the fraction of OATP1B1-mediated clearance among individuals with the *SLCO1B1* c.521T/T genotype is at least 90% for GCDCA-3G and 85% for GDCA-3G. This is well in line with our *in vitro* screening data showing several-fold higher uptake of both compounds by OATP1B1*1A compared with the two other hepatic influx transporters OATP1B3 and OATP2B1.

Our *in vitro* experiments showed that the *SLCO1B1* c.521T>C SNV, that is, the **5* and **15* haplotypes, similarly reduced the uptake of GCDCA-3G and GDCA-3G. This indicates that the differences in the sensitivity for the effects of the SNV observed *in vivo* between the compounds cannot be explained by substrate-dependent effect of the genetic variant. It is therefore likely that other hepatic transporters or elimination pathways, such as renal excretion, could be more important determinants of GDCA-3G clearance. No qualitative differences between the compounds were seen in our experiments with other hepatic influx and efflux transporters. Further studies are therefore needed to clarify the mechanisms underlying the difference in the sensitivity of GCDCA-3G and GDCA-3G resulting from reduced OATP1B1 activity.

In our screening experiments, both GCDCA-3G and GDCA-3G seemed to be good substrates of MRP2, MRP3, and MRP4. It is therefore possible that drug interactions or genetic variability affecting their activities could affect the plasma concentrations of GCDCA-3G and GDCA-3G. However, considering the large effect of *SLCO1B1* genotype on GCDCA-3G and GDCA-3G concentrations, the roles of these MRP transporters in the clearance of GCDCA-3G and GDCA-3G should be relatively small. In addition to GCDCA-3G and GDCA-3G, bilirubin glucuronides (MRP2 and MRP3) and CP-I (MRP2) are known substrates of MRP transporters, but little is known about the possible role of these transporters in the kinetics of other endogenous OATP1B biomarkers.[6]

In spite of a relatively high between-subject variation in fasting plasma concentrations, GCDCA-3G completely differentiated the *SLCO1B1* c.521C/C homozygous men from the noncarriers and only two out of 45 T/C heterozygotes overlapped with the C/C group. Among women, the C/C genotype was differentiated except one out of 110 noncarrier subjects and one out of 68 heterozygotes. Accordingly, GCDCA-3G showed a very high efficacy for detecting OATP1B1 deficiency, i.e., the *SLCO1B1* c.521C/C genotype with an area under the ROC curve of nearly 1. The specificity and sensitivity to distinguish individuals with the C/C genotype from those with the T/C or T/T genotype peaked at a GCDCA-3G concentration cutoff value of 180 ng/mL for men and 90 ng/mL for women, with 98–99% specificity and 100% sensitivity. GDCA-3G, exhibited higher variability and did not show as high efficacy in detecting OATP1B1 deficiency. Taken together, these data indicate that the fasting plasma GCDCA-3G concentration is a highly sensitive and specific biomarker for OATP1B1 deficiency.

We then employed these endogenous substrates to clarify the effects of *SLCO1B1* haplotypes on OATP1B1 activity in humans. Consistent with the *in vitro* data, individuals who carried two reduced function alleles, either **5* or **15*, showed the highest and those carrying **14* showed lowered plasma concentrations of both substrates. In addition to **14*, also the **1B* and **35* alleles seemed to reduce the plasma concentrations of these biomarkers. Of note, GCDCA-3G concentration in the individual homozygous for **14* was 1/40 of the mean value in **15/*15* homozygotes. In a previous study, the c.388A>G SNV and the c.388G-allele containing **1B*, **14*, and **35* haplotypes were associated with increased OATP1B1 protein expression.[34] Similarly to our study, the effect of **1B* was smaller than those of **14* and **35*. Both *SLCO1B1*14* and **35* have been associated with increased clearance of OATP1B1 substrate drugs in humans.[33,34]

Endogenous biomarkers provide a potentially effective tool to investigate DDI risks during early clinical drug development. To serve as a tool in decision making, sensitivity and specificity of the endogenous biomarker for a given pharmacokinetic process are of high importance.[5] GCDCA-3G could therefore be a

useful biomarker for OATP1B1 activity in DDI risk estimation.[8,27] Moreover, it could be useful in elucidating the mechanisms of DDIs, similar to what was recently demonstrated with other OATP biomarkers such as coproporphyrin I, chenodeoxycholic acid 24-O-glucuronide, GCDCA-S, and GDCA-S.[8,10,31,41]

Unlike coproporphyrin I, chenodeoxycholic acid 24-O-glucuronide, and GCDCA-S, which present as substrates of both OATP1B1 and OATP1B3 *in vitro*, GCDCA-3G is more selective for OATP1B1.[7,8] As discussed, it is hypothesized that the fraction transported by OATP1B1 (vs. other OATP forms and liver SLCs) is higher for GCDCA-3G vs. GDCA-3G, GDCA-S, GCDCA-S, coproporphyrin I, and chenodeoxycholic acid 24-O-glucuronide. Such a distinction is important because it has been reported that the magnitude of inhibitory OATP DDIs (e.g., perpetrator-to-placebo area under the concentration-time curve ratio (AUCR) for statins, repaglinide and coproporphyrin I) is modulated by *SLCO1B1* genotype.[30,42,43] If this phenomenon is widespread with new chemical entities entering the clinic, beyond extensively described perpetrators such as rifampicin and cyclosporine, then GCDCA-3G could be deployed as a useful phenotyping tool and used in conjunction with statin probe drugs and other OATP biomarkers such as coproporphyrin I, GCDCA-S, and GDCA-S. As described by others, it is possible to quantitate multiple OATP1B biomarkers in plasma and generate AUCR signatures at increasing doses of perpetrator drug.[10,31] Such "biomarker multiplexing" is useful when assessing OATP1B DDI risk in healthy volunteers and diseased individuals, when considering the impact of *SLCO1B1* genotype on DDI magnitude, and evaluating DDI with new chemical entities presenting different OATP1B1 vs. OATP1B3 inhibition signatures *in vitro*.

In this study, the plasma levels of GCDCA-3G and GDCA-3G were ~ 50% lower in women than in men. In theory, these differences could be explained by differences in overall bile acid synthesis rate, glycine conjugation of CDCA and DCA, or 3-glucuronidation of GCDCA and GDCA. Previous studies have shown that overall the plasma concentrations of bile acids are lower in women than in men.[44,45] Moreover, the concentration of the bile acid synthesis intermediate C4 and its ratio to cholesterol have been higher in men than in women, indicating a higher rate of bile acid synthesis in men.[46] In our study, the plasma levels of parent GCDCA and GDCA were ~ 20% lower in women than in men, suggesting that the sex differences in GCDCA-3G and GDCA-3G concentrations could be partly due to differences in bile acid synthesis rate. In a previous study, the fasting plasma concentrations of parent CDCA and DCA and their glycine conjugates were similarly lower in women than in men, indicating that sex has no effect on the glycine conjugation of CDCA and DCA.[45] On the other hand, the differences in glucuronide/parent compound concentration ratios suggest that the 3-glucuronidation of GCDCA and GDCA is more efficient in men than in women. Interestingly, a previous study showed that the 6-glucuronidation of the secondary bile acid hyodeoxycholate is similarly more efficient in men than in women.[47]

Our study had two main limitations. First, the supportive *in vitro* experiments characterizing GCDCA-3G and GDCA-3G as substrates of influx and efflux transporters, inhibitors of hepatic OATP transporters, and the impact of *SLCO1B1* variant alleles on GCDCA-3G and GDCA-3G uptake were carried out as single, screening experiments with technical replicates only. Second, this study did not investigate which of the uridine diphosphate (UDP)-glucuronosyltransferase isoforms (UGTs) are responsible for the 3-glucuronidation of GCDCA and GDCA. If the isoforms are genetically polymorphic, such as UGT1A1, 1A3, or 2B17, the possible effects of genetically varying glucuronidation should be taken into account when interpreting results with GCDCA-3G and GDCA-3G.[48] Further studies are required to verify the preliminary *in vitro* findings and to determine which UGT enzymes are involved in GCDCA-3G and GDCA-3G formation.

In conclusion, this study identified GCDCA-3G and GDCA-3G as highly sensitive and specific substrates of OATP1B1 in healthy volunteers. In particular, plasma GCDCA-3G was able to detect genetically determined OATP1B1 deficiency with high efficacy and is therefore a promising biomarker to support the investigation of OATP1B1 function, DDIs, and OATP1B1 phenotype-*SLCO1B1* genotype associations.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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CONFLICT OF INTEREST

All authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

M.Ne., A.D.R., and M.Ni. wrote the manuscript. D.R. and M.Ni. designed the research. M.Ne., P.H., A.T., B.R., M.W., S.L., S.M., M.V., M.A.C., C.C., R.R., and M.Ni. performed the research. M.Ne., B.R., M.W., S.L., D.R., and M.Ni. analyzed the data.

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