Breast cancer resistance protein polymorphism ABCG2 c.421C>A (rs2231142) moderates the effect of valproate on lamotrigine trough concentrations in adults with epilepsy

Ivana Šušak Sporiš[1], Nada Božina[2], Iva Klarica Domjanović[3], Davor Sporiš[1], Silvio Bašić[1], Ivana Marković[1], Mila Lovrić[4], Lana Ganoci[4], and Vladimir Trkulja[2]

[1]Dubrava Clinical Hospital
[2]University of Zagreb School of Medicine
[3]Croatian Agency for Medical Products and Medical Devices
[4]University Hospital Centre Zagreb Department of Laboratory Diagnostics

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Abstract

To test the hypothesis that the effect of valproate on dose-adjusted lamotrigine troughs was moderated by the ABCG2 c.421C>A (rs2231142) polymorphism, we conducted two studies in adults with epilepsy. Since Study 2 replicated the findings of Study 1, we analysed combined data [total N=471; 140 exposed to valproate (treated), 331 not exposed (controls)]. With adjustment for cotreatments and comorbidities, age, sex, body weight and polymorphisms (and linked polymorphisms) suggested to affect exposure to lamotrigine (UGT1A4*3 c.142T>G, rs2011425; UGT2B7 -161C>T, rs7668258; ABCB1 1236C>T, rs1128503) (by entropy balancing), primary analysis indicated: in variant carriers, geometric means ratio (GMR) [treated (n=21) vs. controls (n=72)] was around 60% higher than in wild-type subjects [treated (n=119) vs. controls (n=259)] – ratio of GMRs 1.61 (95%CI 1.23-2.11) and 1.63 (95%CrI 1.26-2.10), in the frequentist and Bayesian analysis, respectively. Similar differences in valproate effects between ABCG2 c.421C>A variant carriers and wild-type subjects were found in secondary analysis (adjustment by exact matching) when exposure to valproate was defined as valproate troughs up to 364 ?mol/L or [?]364 ?mol/L (vs. no exposure to valproate). Susceptibility of the estimates to (hypothetical) unmeasured confounding was low. Data suggests that polymorphism rs2231142 moderates the effect of valproate on exposure to lamotrigine.

Introduction

Lamotrigine, an antiepileptic drug (AED), shows considerable interindividual variability in systemic exposure[1-4]and is subject to therapeutic drug monitoring (TDM).[3]Exposure variability stems primarily from factors affecting its clearance.[1,2,4,5] Elimination of lamotrigine is almost exclusively by hepatic uridine-diphosphate-glucuronidases (UGTs), predominantly UGT1A4 with a contribution of UGT2B7 and possibly UGT1A3 and/or UGT1A,[2,3] and 10% of the dose is recovered as unchanged lamotrigine in urine.[5] Lamotrigine is a substrate for two efflux transporters, P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2).[2]

Lamotrigine clearance increases in pregnancy, and slightly with higher body weight; it is reduced in people with moderate-severe liver failure, and slightly decreases with older age and advanced renal failure.[1,4,5] The major source of clearance alterations, however, are drug-drug interactions.[4-6]Several antiretroviral drugs, classical AEDs and estrogen/gestagen combinations greatly increase lamotrigine glucuronidation[1,6]. On the other hand, valproate inhibits lamotrigine glucuronidation in human liver microsomes and human recombinant UGT2B7[7] *in vitro* , reduces lamotrigine clearance by 50-60% and increases exposure by around

2-fold.[1,6] Finally, lamotrigine induces its own glucuronidation (results in a mild increase in clearance), but this is completed within two weeks from the start of treatment.[8]

Both *UGT1A4* and *UGT2B7* genes are highly polymorphic.[9] As recently reviewed,[2]only some of the single nucleotide polymorphisms (SNPs)/haplotypes in these genes have been evaluated regarding lamotrigine pharmacokinetics in studies largely varying in size, sampled populations, design, methodology and control of confounding, with commonly inconsistent outcomes. The most consistent evidence points out two SNPs as relevant for lamotrigine clearance - *UGT1A4*3 142T>G*(rs2011425), which is in complete linkage disequilibrium (LD) with two promoter SNPs (rs3732219 and res3732218) also suggested to affect lamotrigine pharmacokinetics[2]; and *UGT2B7 -161C>T* (rs7668258), which is in complete LD with*UGT2B7 802C>T* (rs7439366)[10], another SNP reported associated with lamotrigine exposure.[2] Regarding efflux transporter polymorphisms, it has been suggested, although not unequivocally, that *ABCB1*SNPs *1236C>T* (rs1128503),*2677G>T/A* (rs2032582) and*3435C>T* (rs1045642) either individually or as haplotypes, since in a strong LD,[11] might impact systemic levels of lamotrigine.[2] It is also suggested that the loss of function *ABCG2 c.421C>T* (rs2231142) SNP contributes to pharmacokinetic variability of lamotrigine.[2]

In an earlier exploratory study[12] in a sample of Central-Eastern European patients with epilepsy, we suggested that valproate might have interacted with the *ABCG2 c.421C>T* (rs2231142) genotype in the effect on dose-adjusted lamotrigine troughs. However, the study was moderate in size (N=205, 74 patients co-treated with valproate) and data were analysed in rather complex regression models.[12] In the present report we: i) re-analyse the study data[12](addressed here as Study 1) in a way more straightforwardly focused specifically to test moderation of the valproate effect by the*ABCG2 c.421C>T* (rs2231142) genotype; ii) provide data from a new and identically designed study (Study 2) undertaken to re-evaluate the Study 1 results; and iii) analyse the combined (Study 1 and Study 2) data.

Patients and Methods

Outline

Study 1[12] and Study 2 (Study 2 followed after completion of Study 1) each included consecutive adults and adolescents (age [?]16 years) with epilepsy on lamotrigine monotherapy or lamotrigine + valproate co-treatment with no other AEDs, who underwent TDM as a part of their standard management. Both treatments were gradually titrated as*per* approved labels. After at least 21 days of treatment (to attain steady state and to bridge the period of the initial UGT induction by lamotrigine[8]), patients provided blood samples for determination of morning (07:00-09:00 hours) lamotrigine/valproate troughs. They were included: i) if consented to be genotyped for the pharmacogenes of interest, i.e., *ABCG2 c.421C>T* (rs2231142) SNP and SNPs that have been suggested to affect exposure to lamotrigine - *UGT1A4*3 c.142T>G* (rs2011425) (in complete LD with rs3732219 and rs3732218)[2], *UGT2B7 -161C>T*(rs7668258) [in complete LD with *UGT2B7 802C>T*(rs7439366)[10]] and *ABCB1 1236C>T* (rs1128503) [in strong LD with *ABCB1 2677G>T/A* (rs2032582) and *ABCB1 3435C>T* (rs1045642)];[11] ii) were non-smokers and, based on routine assessments, had preserved renal and liver function and no indication of chronic heart failure, unregulated diabetes mellitus, hyper- or hypothyroidism, history of or an on-going malignant disease, or of any acute illness, and were free of treatments known to affect lamotrigine or valproate, and/or activity of ABCG2, P-glycoprotein, UGT2B7 or UGT1A4 within the previous month. Pregnant women and HIV-positive patients were not included.

For the present report, we conceived a *primary* and a*secondary* analysis with the following main elements: i) exposure to valproate is treatment; ii) dose-adjusted lamotrigine trough concentration (per 100 mg) is the outcome; iii) *ABCG2 c.421C>T* (rs2231142) genotype is a (presumed) moderator of the effect of treatment on the outcome. For the *primary* analysis, patients were considered "treated" i.e., exposed to valproate if their valproate troughs were >lower limit of quantification (LLOQ) (20.8 μmol/L) (treatment= valproate >LLOQ), and were considered as "controls" if on lamotrigine monotherapy or if valproate troughs were below the limit of quantification (BLOQ) (control = valproate 0/BLOQ). For the *secondary* analysis, patients were "controls" if valproate 0/BLOQ, and "treated" if i) valproate >LLOQ but <364 μmol/L (median of

the measured values which approximated the recommended lower limit of targeted valproate troughs[13]), designated as "Low valproate"; or if ii) valproate [?]364 µmol/L, which was designated as "Target/high valproate". The (presumed) moderator was categorized as *ABCG2 c.421C>A* wild-type homozygosity (wt), or as variant allele carriage [since variant homozygotes were (expectedly) only sporadic]. The analysis was focused on the hypothesis of no moderation of the valproate effect by the *ABCG2 c.421C>A*genotype (i.e., the valproate-lamotrigine relationship is the same in*ABCG2 c.421C>A* wt subjects and in variant carriers) (see Appendix A, 1. Conceived analysis and hypothesis tests, for details). Although data were cross-sectional, two basic conditions were met that enabled consideration of causal effects (valproate – lamotrigine) and effect moderation: the cause (treatment, exposure to valproate) preceded the outcome (lamotrigine troughs), and there was no reverse causation (i.e., effect of outcome on treatment) - the effect of valproate on lamotrigine has been well established, as has been a lack of the effect of lamotrigine on valproate concentrations.[14-16] Therefore, when valproate and lamotrigine troughs are determined concomitantly, at steady state, after completion of the initial lamotrigine self-induction, it is reasonable to consider that the latter is consequent to the former, but not*vice-versa* (see Appendix A, 2. Lamotrigine does not affect valproate concentrations, for details). The same reasoning applies to the lack of effect of the outcome on other UGT enzymes or transporters.[3,6] Finally, the condition that the "other" causative i.e., the (presumably) moderating factor –*ABCG2 c.421C>A* genotype - preceded the outcome was also met.

We combined inclusion/exclusion criteria and statistical adjustment to achieve conditional exchangeability between treated and control subjects (see Appendix A, 3. Measures to achieve conditional exchangeability, for details), and generated estimates were submitted to analysis of sensitivity to unmeasured confounding.

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[17] It was approved by the Institutional Ethics Committee (approval class: 8.1.-19/12-2, registration number: 02/21 AG).

Bioanalytical methods and genotyping

Plasma lamotrigine was measured using a validated high-performance liquid chromatography with a diode-array detector (Shimadzu, Japan), as described previously,[18] while serum valproate was measured by an immunoassay (PETINIA) on a Dimesion Expand analyzer (Siemens; calibrator and control samples by Siemens, Germany). Both analytes are included in external quality control schemes (DGKL RfB and UK NEQAS).

Genomic DNA was extracted from three milliliters of whole blood using the FlexiGene DNA Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Genotyping of *MDR1/ABCB1 1236C>T* , *ABCG2 421C>A* and *UGT2B7 –161C>T* was performed using TaqMan Drug Metabolism Genotyping assays ID C_7586662_10, ID C_15854163_70, ID C_27827970_40, respectively, while genotyping of *UGT1A4 142 T>G* was performed using Custom TaqMan SNP Genotyping assay (Applied Biosystems, Foster City, CA, USA) by real-time polymerase chain reaction (PCR) genotyping method on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Genotyping of *UGT1A4 142T>G* was confirmed by a PCR-RFLP method[19] on the Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA).[18]

Methods of statistical adjustment

To achieve a balance between treated (exposed to valproate) and controls (not exposed) on measured covariates in the primary analysis, we used entropy balancing[20] implemented in package*WeightIt* [21] in R[22], with average treatment effect (ATE) as the estimand. Entropy balancing is a form of distance matching: the procedure assigns weights under given enforced restrictions on distance between treated and controls (that is, the distance between moments of covariates), taking into account the estimand.[23] In the secondary analysis, for this purpose we employed exact matching on categorical covariates combined with optimal full matching (estimand ATE) with age and body weight (bw) as continuous covariates[24,25] implemented in package*MatchIT* [26] in R (see Appendix A, 4. Methods of statistical adjustment, for details). To estimate the main valproate effects, balancing/matching were undertaken in the entire sample; to test the

valproate**ABCG2 c.421C>A* genotype interaction, treated and controls were balanced separately at each level of the genotype.[27] Owing to the fact that exact matching is applicable only to binary treatments, in the secondary analysis Low valproate-treated and Target/high valproate-treated subjects were separately matched to control subjects (valproate 0/BLOQ).

Data analysis

All analyses were done on ln-transformed dose-adjusted lamotrigine troughs using frequentist and Bayesian methods: i) first, we used raw data in the primary analysis setting (valproate >LLOQ vs. valproate 0/BLOQ) to estimate the main valproate effect and valproate**ABCG2 c.421C>A* SNP interaction, i.e., valproate effects in *ABCG2 c.421C>A* wt subjects and in variant carriers, separately in Study 1 and Study 2, and in a combined Study 1 + Study 2 data using one-stage fixed-effect individual patient data meta-analysis (meta-regression) approach. Since Study 1 and Study 2 results were closely similar (homogenous) and there was no relevant "study effect", all further analyses were conducted on the*combined dataset* ; ii) next, we generated adjusted estimates (after covariate entropy balancing with additional adjustment for age and body weight, regardless of the achieved balance between treated and controls subjects) of the main valproate effect and of the valproate*SNP interaction in the primary analysis setting; iii) we then used combined data in the secondary analysis to estimate the main valproate effect (Low valproate vs. valproate 0/BLOQ and Target/high valproate vs. valproate 0/BLOQ) and valproate*SNP interaction, i.e., valproate effects at each of the two *ABCG2 c.421C>A* genotype levels, using raw data, and data after matching between treated and controls (with further adjustment for age and body weight, regardless of the balance achieved by matching).

The main and the interaction effects were estimated in separate models, and were expressed as geometric means ratios (GMRs) (treatment vs. control) and as ratio of GMRs[27]: GMR for treatment vs. control in *ABCG2 c.421C>A* variant carriers / GMR for treatment vs. control in *ABCG2 c.421C>A* wt subjects. Details on data analysis are provided in Appendix A, 5. Frequentist and Bayesian estimation. We used SAS 9.4 for Windows (SAS Inc., Cary, NC) and package *rstanarm* [28] in R. We used CubeX[29] to evaluate Hardy-Weinberg equilibrium and linkage disequilibrium.

Sensitivity to unmeasured confounding

The starting point was an assumption that whatever difference in the size of the effect of valproate on lamotrigine troughs was observed between *ABCG2 c.421C>A* variant carriers and wt subjects – it was due to bias arising from some unmeasured confounding factor or a set of factors (i.e., biasing set) that "interfered" with the valproate effect in variant carriers but not in wt subjects. We first calculated E-value as an indicator of severity of uncontrolled confounding (i.e., size of the hypothetical confounding effect) that would be needed to at least partly explain away the observed heterogeneity of the valproate effect (difference in effects between variant carriers vs. wt subjects)[30] (package*E-value* [31] in R) (see Appendix A, 6. Sensitivity of ratio of GMRs to unmeasured confounding, for details). We further hypothesized: i) that the overall prevalence of this (assumed) biasing set was 25%; ii) that in *ABCG2 c.421C>A* wt subjects, prevalence of the biasing set was balanced between treated and controls, and hence had no effect on lamotrigine troughs, but that in*ABCG2 c.421C>A* variant carriers it was markedly more prevalent in valproate-treated than in control subjects in a 3:1 ratio, and that it exerted a moderate or a strong effect, illustrated by GMRs of 1.25 and 1.50, respectively, i.e, that it generated a ratio of GMRs of 1.25 or 1.50, respectively. We then adjusted the observed ratios of GMRs for this hypothesized imbalance and confounding effect[32] (package *episensr* [33]in R) (see Appendix A, 6. Sensitivity of ratio of GMRs to unmeasured confounding, for details).

Results

Patients: Study 1, Study 2 and combined data

Study 1 included 205 patients, 131 (63.9%) on lamotrigine monotherapy and 74 co-treated with valproate (Table 1). Three of the latter subjects (250 mg/day valproate) had valproate troughs below the lower limit of quantification (BLOQ), hence 134 were considered to have valproate troughs 0/BLOQ and 71 had quantifiable concentrations (valproate >LLOQ) (Table 1). Study 2 included 266 subjects, 197 (74.1%)

on lamotrigine monotherapy and 69 were co-treated with valproate (all with valproate troughs >LLOQ) (Table 1). Hence, combined data pertain to 331 subjects with valproate troughs 0/BLOQ and 140 subjects with valproate >LLOQ (Table 1). With respect to *ABCG2 c.421C>A* (rs2231142) polymorphism, 80% of the patients in each study were wild type (wt) homozygous and 20% were heterozygous, while variant homozygotes were sporadic (5 patients overall) (Table 1). Similarly, 75-80% of the patients were *UGT1A4*3 c.142T>G* (rs2011425) wt homozygous, while the rest were heterozygous (only 4 subjects were variant homozygotes) (Table 1). With respect to *UGT2B7 -161C>T* (rs7668258) and *ABCB1 1236C>T*(rs1128503) polymorphisms, 45-50% of the patients across the studies were heterozygous, followed by wt homozygous subjects (Table 1). There were no departures from the Hardy-Weinberg equilibrium for any SNP, and no indication of LD between the *ABCG2* and *UGT2B7* loci (long arm chromosome 4) (D'=0.239 r^2=0.0068, Chi^2=3.2) (combined data).

Median dose-adjusted lamotrigine troughs (μmol/L/100 mg) were clearly higher in patients with valproate >LLOQ ("treated") than in patients with valproate 0/BLOQ ("controls") (174 vs. 68, 159 vs. 68 and 166 vs. 68, in Study 1, Study 2 and combined, respectively) (Table 1).

ABCG2 c.421C>A moderates the effect of valproate on lamotrigine troughs: primary analysis

Considering raw data, dose-adjusted lamotrigine troughs were in each study around 2.5 times higher in subjects with valproate >LLOQ (treated) than in those with valproate 0/BLOQ (controls) (Figure 1A). In Study 1, lamotrigine was around 2.3 times higher in treated (n=61) than in controls (n=106) if they were all*ABCG2 c.421C>A* wt homozygotes, and was around 4.25 times higher in treated (n=10) than in controls (n=38) if they were*ABCG2 c.421C>A* variant carriers (Figure 1A). Ratio of geometric means ratios (GMRs) for treated vs. control subjects if variant carriers vs. if wt homozygotes was 1.85 (Figure 1B), with >99% probability that it was >1.0 and >95% probability that it was >1.25 indicating a marked quantitative interaction between the exposure to valproate and*ABCG2 c.421C>A* genotype (Figure 1B). Study 2 replicated these findings: among *ABCG2 c.421C>A* wt subjects, lamotrigine troughs in treated (n=58) were around 2.3 time higher than in controls (n=153) (Figure 1A), while among *ABCG2 c.421C>A* variant carriers the difference was around 3.2 times (11 treated vs. 44 controls) (Figure 1A): 95%CI/CrI around the ratio of GMRs was again entirely >1.0 with a high probability that the ratio was >1.25 (Figure 1B). In the combined data (one-stage fixed effect individual patient data meta-analysis), lamotrigine troughs were 2.29 times higher in treated (n=119) than in controls (n=259) among *ABCG2 c.421C>A* wt subjects, and were around 3.7 times higher in treated (n=21) than in controls (n=72) among *ABCG2 c.421C>A* variant carriers (Figure 1A). Probability that the ratio of GMRs was >1.0 was 100%, and probability that it was >1.25 was [?]96.7% (Figure 1B).

Considering the combined data, entropy balancing enabled a perfect covariate balance between treated (valproate >LLOQ) and control (valproate 0/BLOQ) subjects overall (Table 2), and also when done separately in *ABCG2 c.421C>A* wt subjects and variant carriers (Table 3) (see Appendix B for the assigned weights). With further adjustment for age and body weight, the difference between treated and control subjects regarding dose-adjusted lamotrigine troughs was clearly higher among *ABCG2 c.421C>A* variant carriers than among wt subjects (Figure 2), with 100% probability that ratio of GMRs was >1.0, and close to 100% probability that it was >1.25 (Figure 2).

ABCG2 c.421C>A moderates the effect of valproate on lamotrigine troughs: secondary analysis

Considering raw combined data, there were overall 70 patients with valproate troughs [?]364 μmol/L (Target/high) ("treated"), 60 of whom were *ABCG2 c.421C>A* wt homozygotes and 10 were variant carriers (Table 4); there were 70 patients with valproate troughs >LLOQ but <364 μmol/L (Low) ("treated"), 59 of whom were wt homozygotes and 11 were variant carriers (Table 4); and of 331 patients with valproate 0/BLOQ ("controls"), 259 were wt subjects and 72 were variant carriers (Table 4). In patients with Low valproate (treated), lamotrigine was around 1.9 times higher than in patients with valproate 0/BLOQ (controls) among*ABCG2 c.421C>A* wt homozygotes, and around 2.7 times higher among variant carriers (Figure 3). In patients with Target/high valproate (treated), lamotrigine was around 2.7 times higher than in controls

among *ABCG2 c.421C>A* wt homozygotes and around 5.0 times higher among variant carriers (Figure 3). In both cases, 95%CI/CrI around ratios of GMRs were clearly >1.0 (Figure 3). Eventually, among *ABCG2 c.421C>A* wt subjects, 60/60 patients with Target high valproate (treated) and 59/59 with Low valproate (treated) could be matched to 251 and 254/259 controls, respectively (Table 4). Among *ABCG2 c.421C>A* variant carriers, 10/10 with Target/high valproate and 10/11 with Low valproate could be matched to 54 and 30/72 controls, respectively (Table 4). In all matched sets, minor imbalances between treated and controls remained regarding age and body weight (Table 4). With further adjustment for these suboptimally matched covariates: i) Lamotrigine was around 1.8 higher in Low valproate-treated vs. controls among *ABCG2 c.421C>A*wt subjects, and it was around 2.4 times higher among variant carriers, with 95%CI/CrI around ratio of GMRs >1.0 (Figure 3); ii) Lamotrigine was around 2.7 times higher in Target/high valproate-treated vs. controls among *ABCG2 c.421C>A* wt subjects, and it was around 5.2 times higher among variant carriers, with 95%CI/CrI around ratio of GMRs >1.0 (Figure 3).

Sensitivity to unmeasured confounding

We hypothesised that the observed differences in valproate effects in*ABCG2 c.421C>A* variant carriers and wt subjects (ratios of GMRs >1.0) were due to some unmeasured confounding factor (or a set of confounders) that caused considerably greater effects of valproate in variant carriers than in wt controls, and that, hence, the ratio of GMRs was high. First, we calculated values of this assumed confounding effect that would be needed to at least partly explain away the observed moderating effect, i.e., to "push" the observed point-estimate ratios of GMRs to the value of 1.25 (Table 5). The lowest such (hypothetical) effect was 1.53, needed to "push" the ratio of GMRs of 1.42 to 1.25 (Table 5). The largest such (hypothetical) effect was 2.39, needed to "push" the ratio of GMRs of 1.89 to 1.25 (Table 5). Next, we hypothesized that there existed a considerable imbalance (of 3:1) in prevalence of a (set of) confounding factor(s) in valproate-exposed vs. control subjects when they were*ABCG2 c.421C>A* variant carriers, and that it had a moderate (ratio of GMRs=1.25) or a marked (ratio of GMRs=1.50) biasing effect, and we corrected the observed ratios of GMRs (from adjusted primary and secondary analyses of combined data) for this bias (Figure 4): even with such a large (hypothetical) imbalance (51% vs. 17% or 60% vs. 20%) in prevalence of the biasing set, and with its marked effect, the lowest bias-corrected ratio of GMRs was 1.26 (Figure 4), i.e., still higher than the conventional upper limit of "equivalence".

Discussion

Present data strongly suggest that the *ABCG2 c.421C>A* genotype moderates the effect of valproate on exposure to lamotrigine: the effect is more pronounced in *ABCG2 c.421C>A* variant carriers than in wt subjects. This is supported by: i) consistent findings in Study 1 and Study 2; ii) consistent estimates based on raw and adjusted data, regardless of the mode of adjustment; iii) consistent findings in frequentist and Bayesian models; iv) consistent findings regardless of the definition of exposure to valproate (valproate >LLOQ; valproate >LLOQ and <364 μmol/L; or valproate [?]364 μmol/L vs. valproate 0/BLOQ); v) low susceptibility of the estimates to unmeasured confounding. In this regard, three points need to be discussed: first, despite data replication and measures undertaken to control confounding, how probable it is that the observed is an artefact, i.e., a result of bias?; second, if the observed is true, is it practically relevant?; finally, what is the biological background of the observed effect moderation? They should be addressed having in mind study limitations: i) drug exposures are represented by TDM troughs and not by, e.g., total exposures over dosing intervals. However, for both drugs, troughs are considered reliable indicators of exposure; ii) present estimates were obtained in Caucasian patients of Central-Eastern European descent (Slavic) and might not hold in other populations; iii) although the total number of subjects across the two studies was reasonably high, some patients subsets were relatively modest in size.

With respect to residual confounding/bias, and having in mind factors that we accounted for (by inclusion/exclusion criteria and statistical adjustments), the most feasible "candidate sources" are transporter and enzyme polymorphisms that remained unaddressed – primarily those in the *ABCG2* , *ABCG1* and *UGT1A4* and *UGT2B7*genes. However, it seems highly unlikely that they could completely or in a larger part explain the observed differences in valproate effects conditional on *ABCG2 c.421C>A* rs2231142 geno-

type. For this purpose, they would need to act synchronously in the same direction, their prevalence across the valproate-by-*ABCG2c.421C>A* genotype subsets would need to be markedly imbalanced, and their cumulative effect would need to be considerable – i.e., the "scenario" would need to be considerably more "unfavourable" than the one addressed in the present analysis of sensitivity to unmeasured confounding. Considering the *current knowledge* on the topic, this seems highly improbable. Of the*ABCG2* SNPs, apart from the investigated rs2231142 with global minor allele prevalence of 12%[34], reduced transporter function has been reported associated with three further SNPs (rs34783571, rs192169062 and rs34264773), for three SNPs no effect on function is reported and for the rest functional consequences are unknown.[34] The estimated global cumulative minor allele prevalence of all "reduced function" SNPs is 0.68%, and for combined "unknown" and "reduced" it is 1.3%[34] - it is unreasonable to expect more than a few subjects in the current sample with minor alleles on any of these loci, and, hence, to assume that these (presumed) SNPs contributed to the observed effects. Situation is more complex regarding genes encoding the two main lamotrigine-metabolizing enzymes: both *UGT1A4* and *UGT2B7*are highly polymorphic. However, based on combined *in vitro* , pharmacokinetic and clinical data, polymorphisms that we presently genotyped - *UGT2B7 -161C>T* (rs7668258) and*UGT1A4*3 142T>G* (rs2011425) – are the only two with reasonably convincing evidence of relevance for lamotrigine clearance, although not all individual study results have been consistent.[2] Moreover, they are each in LD with other polymorphisms. *UGT2B7 -161C>T* (rs7668258) is in a complete LD with numerous other *UGT2B7* promoter polymorphisms forming two major haplotypes[10] and with a number of other SNPs, and participates in several haplotypes[9] -*UGT2B7*1a, *1j, *1k, *2b, *2c, *2d, *2f* . Similarly,*UGT1A4*3 c.142T>G* (rs2011425) is in a complete LD with several promoter SNPs, e.g., -*219C>T* and-*163G>A* (rs3732219 and rs3732218) to form the*UGT1A4*3a* haplotype, but also with -*419* and -*463* , and with several other SNPs (form haplotypes *5 and *7a).[9,35-37] Also, at least in Caucasians, rs2011425 is in a complete LD with *UGT1A4*2 c.70C>A*(rs6755571, Pro24Tre)[38,39] which *in vitro* is associated with a reduced enzyme activity[35,40], but reports about its association with lamotrigine troughs have been contradictory (e.g., in Scandinavian subjects[41,42]). Hence, by identification of heterozygous or variant homozygous*UGT2B7 -161C>T* or *UGT1A4*3 c.142T>G* genotype, one identifies subjects with "broader" genetic makeups that differ from those in their respective wild-type (wt) homozygous controls. Although most of the elements of these "broader makeups" are not identified, they are (at least partly) controlled for by identification of the *UGT2B7 -161C>T* or *UGT1A4*3 c.142T>G*polymorphisms. In this context, it is important to note that, according to the current (incomplete) knowledge, practically all *UGT1A4*polymorphisms with a prevalence of around 10-15% ("common") are in LD with *UGT1A4*3 c.142T>G* ,[9,36]whereas cumulative prevalence of all other SNPs is around 5-10%. Similarly, the most common (known) *UGT2B7* haplotypes/haplotype pairs include *UGT2B7 -161C>T,* [9,43]while cumulative prevalence of haplotype pairs not including this SNP may be approximated at around 15%.[43] In the present sensitivity analysis, we hypothesized overall prevalence of a "biasing set" of 25% - which is considerably higher than the estimated prevalence of any of these two potential sources of bias (and particularly markedly higher than probability of their joint occurrence). Hence, it appears highly unlikely that the observed effect moderation could be due to the *UGT* polymorphisms that remained undetermined.

Among numerous *ABCG1* gene polymorphisms,[44] we determined (and adjusted for) *ABCB1 1236T>C*(rs1128503). However, it has been repeatedly shown in a strong LD with two further polymorphisms [*2677T>G/A* (rs2032582),*3435T>C* (rs104564)].[11] In a sample of patients originating from the same general population as the patients in the present study, we also observed an almost complete LD between these three *ABCB1* SNPs.[45] Therefore, by controlling for the rs1128503 genotype, one largely controls for the other two SNPs. In Caucasians, these are the three most prevalent*ABCB1* SNPs and have been extensively evaluated with respect to bioavailability of a range of drugs, but with extremely variable outcomes disabling any consensus.[44] Regarding lamotrigine, several studies tested involvement of individual SNPs or of the haplotype[2], but the most recent larger study in Scandinavian patients[42] found no signal that would relate *1236T>C* or *3435T>C* to dose-adjusted lamotrigine troughs. Cumulative prevalence of other six coding *ABCB1* SNPs in Caucasians is around 10%,[44] and their potential involvement in the present observations is highly unlikely. Finally, a recent comprehensive systematic review[46] identified a number of studies evaluating SNPs in other ABC transporters in relation to pharmacokinetics and response to a variety of drugs – just

to find mostly weak or none and unreproducible associations, suggesting that the impact of these SNPs on drug pharmacokinetics is generally minor (if any).[46] This appears applicable to lamotrigine, as well. Based on the current knowledge,[2] it is also reasonable to conclude that polymorphisms in the SCL superfamily transporters are highly unlikely to be relevant for exposure to lamotrigine.

Present estimates suggest around 65-70% higher effect of valproate on lamotrigine troughs in *ABCG2 c.421C>A* variant carriers than in wt subjects. As elaborated, it appears reasonable to assign this difference (or the largest part of it) indeed to the valproate-SNP interaction rather than to bias. Practically, it means that the recommended reduction in lamotrigine dosage if co-administered with valproate – guided by the expected two-fold increase in exposure to lamotrigine – might be too modest in this limited subset of patients carrying the *ABCG2 c.421C>A* variant allele.

Considering the biological distribution of the ABCG2 transporter,[34] location at which its reduced efflux activity (consequent to the *ABCG2 c.421C>A*mutation[47]) might be relevant for systemic bioavailability of oral lamotrigine is primarily the gastrointestinal system. A modest contribution might come also from the involvement of ABCG2 in the urinary excretion of drugs.[48] However, since valproate (unlike lamotrigine) is not an ABCG2 substrate,[49] mechanisms underlying the observed difference between valproate effects on lamotrigine troughs in*ABCG2 c.421C>A* variant allele carriers and wt subjects are not straightforwardly conceivable. Nevertheless, at least one potential (hypothetical) explanation appears plausible. The reduced ABCG2 activity consequent to the *ABCG2 c.421C>A* SNP is due to a reduced number of transporters since the variant protein is more extensively degraded.[34] Interestingly, this has been explicitly demonstrated in the human placenta.[34]On the other hand, perfusion of the human placental tissue with valproate [concentrations ranging from 290 to 1150 μmol/L (correspond to "Low"-to-"Target/high" valproate as defined in the present analysis)] reduces the ABCG2 expression (reduced transcripts) by 2-3 fold.[50] Similarly, placental ABCG2 expression is reduced in mice exposed to valproate during pregnancy.[51] Assuming that the phenomenon is generalizable to other tissues that physiologically express ABCG2, it appears that the increase in lamotrigine exposure induced by valproate might not be due solely to inhibited lamotrigine glucuronidation, but also to reduced ABCG2 efflux activity in the gastrointestinal tract. In this context, a greater difference in lamotrigine troughs between valproate-exposed (treated) and non-exposed (control) patients if they are *ABCG2 c.421C>A* variant carriers than if they are*ABCG2 c.421C>A* wt homozygotes (i.e., moderation of the valproate effect by this SNP) might be reasonably explained by a multiplicative (and not only additive) effects of valproate (reduced transporter transcription/expression) and of the *ABCG2 c.421C>A* SNP (increased transporter degradation), since they both "work in the same direction" (reduced ABCG2 efflux activity), but by different mechanisms (as illustrated in Figure 5). This possibility is supported by the fact that the moderating effect of the *ABCG2 c.421C>A* SNP appeared more pronounced when exposure to valproate was higher: ratio of GMRs [ratio (GMR for valproate-exposed vs. non-exposed variant carriers / GMR for valproate-exposed vs. non-exposed wt subjects)] tended to be numerically larger when exposure to valproate was "Target/high" (1.80 frequentist, 1.89 Bayes), than when it was "Low" (1.48 frequentist, 1.58 Bayes) as shown in Figure 5.

In conclusion, present analysis of two separate studies and of combined data strongly suggests that the effect of valproate on exposure to lamotrigine is moderated by the *ABCG2 c.421C>A* SNP: the valproate-induced increase in lamotrigine levels is more pronounced in variant allele carriers than in wt subjects to the extent that is likely clinically relevant.

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Conflict of Interest Statement

The authors declare no competing interests.

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Table 1 . Subject characteristics in Study 1, Study 2 and in the combined dataset: for all patients, and by level of valproate troughs categorized as "valproate (VAL) 0/BLOQ" [patients on lamotrigine (LAM) monotreatment and those co-treated with valproate, but with troughs below the lower limit of quantification (BLOQ, 28.0 μmol/L)] and as "valproate >lower limit of quantification (LLOQ)". Data are count (percent), median (range), mean±SD (range). For the three valproate co-treated patients with troughs BLOQ, individual valproate doses are listed.

	Study 1	Study 1	Study 1	Study 1	Study 1	Study 2	Study 2	Study 2	Study 2	Study 2	Combined	Co
	All		VAL 0/BLOQ		VAL>LLOQ	All		VAL 0/BLOQ		VAL>LLOQ	All	
N	205		134		71	266		197		69	471	
LAM + VAL	74 (36.1)		3		71	69 (25.9)		0		69	143 (30.4)	
LAM only	131 (63.9)		131		0	197 (74.1)		197		0	328 (69.6)	
VAL dose (g/day)	0 (0-2.0)		0.25, 0.25, 0.25		1.0 (0.25-2.0)	0 (0.0-2.0)		—		1.0 (0.25-2.0)	0 (0-2.0)	

	Study 1	Study 1	Study 1	Study 1	Study 1	Study 2	Study 2	Study 2	Study 2	Study 2	Combined	Co
LAM dose (mg/day)	125 (12.5-400)		100 (12.5-400)		150 (25-300)	200 (50-550)		200 (50-500)		200 (50-550)	175 (12.5-550)	
VAL trough 0/BLOQ	134 (65.4)		—		–	197 (74.1)		197		0	331 (70.2)	
VAL >BLOQ	71 (34.6)		—		–	69 (25.9)		0		69	70 (14.9)	
Age (years)	37±14 (16-77)		37±13 (16-74)		36±14 (16-77)	41±15 (19-70)		43±15 (19-70)		35±13 (19-70)	39±15 (16-77)	
Men	69 (33.7)		41 (30.6)		28 (39.4)	119 (44.7)		75 (38.1)		44 (63.8)	188 (39.9)	
Body weight (kg)	71±15 (27-140)		72±16 (46-140)		69±15 (27-100)	78±18 (46-143)		77±18 (46-143)		79±17 (47-117)	75±17 (27-143)	
ABCG2 c.421 C>A												
CC	167 (81.5)		106 (79.1)		61 (85.9)	211 (79.3)		153 (77.7)		58 (84.1)	378 (80.2)	
CA	36 (17.1)		26 (19.4)		10 (14.1)	52 (19.6)		41 (20.8)		11 (15.9)	88 (18.7)	
AA	2 (1.0)		2 (1.5)		0	3 (1.1)		3 (1.5)		0	5 (1.1)	
ABCB1 1236 C>T												
CC	75 (36.6)		50 (37.3)		25 (35.2)	84 (31.6)		59 (30.0)		25 (36.2)	159 (33.8)	
CT	93 (45.4)		62 (46.3)		31 (43.7)	126 (47.4)		94 (47.7)		32 (46.4)	219 (46.5)	
TT	37 (18.0)		22 (16.4)		15 (21.1)	56 (21.1)		44 (22.3)		12 (17.4)	93 (19.7)	
UGT2B7 - 161 C>T												
CC	51 (24.9)		29 (21.6)		22 (31.0)	68 (25.5)		46 (23.3)		22 (31.9)	119 (25.3)	
CT	98 (47.8)		65 (48.5)		33 (46.5)	139 (52.3)		104 (52.8)		35 (50.7)	237 (50.3)	
TT	56 (27.3)		40 (29.9)		16 (22.5)	59 (22.2)		47 (23.9)		12 (17.4)	115 (24.4)	
*UGT1A4*3 c.142 T>G*												
TT	156 (76.1)		102 (76.1)		54 (76.1)	209 (78.6)		153 (77.7)		56 (81.2)	365 (77.5)	

	Study 1	Study 1	Study 1	Study 1	Study 1	Study 2	Study 2	Study 2	Study 2	Study 2	Combined	Co
TG	47 (22.9)		31 (23.1)		16 (22.5)	55 (20.7)		42 (21.3)		13 (18.8)	102 (21.7)	
GG	2 (1.0)		1 (0.8)		1 (1.4)	2 (0.7)		2 (1.0)		0	4 (0.8)	
LAM (µmol/L)	10.5 (0.5-102)		6.7 (0.5-39.7)		25.9 (2.3-102)	14.3 (2.1-74.0)		11.0 (2.1-60.1)		34.1 (5.5-74.0)	12.8 (0.5-102)	
LAM / 100 mg	92 (6.5-464)		68 (6.5-318)		174 (31-464)	79.5 (13.5-300)		68 (13.5-201)		159 (59-300)	84.0 (6.5-464)	

Table 2 . Subject characteristics and dose-adjusted lamotrigine (LAM) troughs in combined data before and after covariate entropy balancing between patients with valproate (VAL) troughs >lower limit of quantification (LLOQ) and those with valproate troughs 0/below the limit of quantification (BLOQ). Standardized differences (d) <0.1 indicate irrelevant differences. Data are (weighted) count (%), mean±SD and geometric mean (% coefficient of variation) for dose-adjusted lamotrigine troughs.

	Before weighting	Before weighting	Before weighting	Before weighting	Before weighting		After weighting	After weighting	After weighting	After weighting	Afte weig
	VAL>LLOQ		VAL 0/BLOQ	d	d		VAL>LLOQ		VAL 0/BLOQ		
N	140		331	—	—		140		331		
Weighting co-variates											
Men	72 (51.4)		116 (35.1)	0.335	0.335		55.9 (39.8)		132.1 (39.8)		
Age (years)	35±13		41±15	-0.392	-0.392		39±15		39±15		
Body weight (kg)	74±16		75±17	-0.067	-0.067		75±16		75±17		
ABCG2 c.421C>A											
CC (wild type)	119 (85.0)		259 (78.2)	0.175	0.175		112.4 (80.3)		265.7 (80.3)		
CA or AA (variant carriers)	21 (15.0)		72 (21.8)	-0.175	-0.175		27.6 (19.7)		65.3 (19.7)		
ABCB1 1236 C>T											

	Before weighting	Before weighting	Before weighting	Before weighting	Before weighting	After weighting	After weighting	After weighting	After weighting	Afte weig
CC	50 (35.7)	109 (32.9)	0.059	0.059		47.3 (33.8)	111.7 (33.8)			
CT	63 (45.0)	156 (47.1)	-0.043	-0.043		65.1 (46.5)	153.9 (46.5)			
TT	27 (19.3)	66 (19.9)	-0.016	-0.016		27.6 (19.7)	65.3 (19.7)			
UGT2B7 - 161 C>T										
CC	44 (31.4)	75 (22.7)	0.198	0.198		35.4 (25.3)	83.6 (25.3)			
CT	68 (48.6)	169 (51.1)	-0.050	-0.050		70.4 (50.3)	166.5 (50.3)			
TT	28 (20.0)	87 (26.3)	-0.149	-0.149		34.2 (24.4)	80.8 (24.4)			
*UGT1A4*3 c.142 T>G*										
TT (widl type)	110 (78.6)	255 (77.0)	0.037	0.037		108.5 (77.5)	256.6 (77.5)			
TG or GG (variant carriers)	30 (21.4)	76 (23.0)	-0.037	-0.037		31.5 (22.5)	74.5 (22.5)			
Outcome										
LAM troughs (µmol/L/100 mg)	159 (46)	63 (59)	1.858	1.858		159 (48)	64 (59)			

Table 3 . Subject characteristics and dose-adjusted lamotrigine (LAM) troughs in combined data before and after covariate entropy balancing between patients with valproate (VAL) troughs >lower limit of quantification (LLOQ) and those with valproate troughs 0/below the limit of quantification (BLOQ) performed separately in *ABCG2 c.421C>A* wild type subjects and variant allele carriers (to enable test of valproate*polymorphism interaction). Standardized differences (d) <0.1 indicate irrelevant differences. Data are (weighted) count (%), mean±SD and geometric mean (% coefficient of variation) for dose-adjusted lamotrigine troughs.

	Before weighting	Before weighting	Before weighting	Before weighting	Before weighting	After weighting	After weighting	After weighting	After weighting	A w
	VAL>LLOQ		VAL 0/BLOQ		d	VAL>LLOQ		VAL 0/BLOQ		d

	Before weighting	Before weighting	Before weighting	Before weighting	Before weighting	After weighting	After weighting	After weighting	After weighting	A w
ABCG2 c.421C>A wild type										
N	119		259		—	119		259		–
Weighting co-variates										
Men	62 (52.1)		93 (35.9)		0.331	48.8 (41.0)		106.2 (41.0)		0
Age (years)	35±13		41±15		-0.372	39±15		39±15		0
Body weight (kg)	74±16		75±18		-0.091	75±16		75±17		0
ABCB1 1236 C>T										
CC (wild type)	40 (33.6)		78 (30.1)		0.075	37.1 (31.2)		80.9 (31.2)		0
CT or TT (variant carriers)	79 (66.4)		181 (69.9)		-0.075	81.9 (68.8)		178.1 (68.8)		0
UGT2B7 -161 C>T										
CC (wild type)	40 (33.6)		63 (24.3)		0.206	32.4 (27.2)		70.6 (27.2)		0
CT or TT (variant carriers)	79 (66.4)		196 (75.7)		-0.206	86.6 (72.8)		188.4 (72.8)		0
*UGT1A4*3 c.142 T>G*										
TT (wild type)	93 (78.2)		207 (79.9)		-0.044	94.4 (79.4)		205.6 (79.4)		0
TG or GG variant carriers)	26 (21.8)		53 (20.1)		0.044	24.6 (20.6)		53.4 (20.6)		0
Outcome										

	Before weighting	Before weighting	Before weighting	Before weighting	Before weighting	After weighting	After weighting	After weighting	After weighting	A w
LAM troughs (µmol/L/100 mg)	155 (45)		68 (57)		1.719	153 (47)		68 (57)		1
ABCG2 c.421C>A variant carriers										
N	21		72		—	21		72		–
Weighting covariates										
Men	10 (47.6)		23 (31.9)		0.324	7.5 (35.5)		25.5 (35.5)		0
Age (years)	34±14		41±14		-0.476	40±15		40±14		0
Body weight (kg)	76±17		75±18		0.068	76±17		76±18		0
ABCB1 1236 C>T										
CC (wild type)	10 (47.6)		31 (43.1)		0.092	9.3 (44.1)		31.7 (44.1)		0
CT or TT (variant carriers)	11 (52.4)		41 (56.9)		-0.092	11.7 (55.9)		40.3 (55.9)		0
UGT2B7 -161 C>T										
CC (wild type)	4 (19.1)		12 (16.7)		0.062	3.6 (17.2)		12.4 (17.2)		0
CT or TT (variant carriers)	17 (80.9)		60 (83.3)		-0.062	17.4 (82.8)		59.6 (82.8)		0
*UGT1A4*3 c.142 T>G*										

	Before weighting	Before weighting	Before weighting	Before weighting	Before weighting	After weighting	After weighting	After weighting	After weighting	A
TT (wild type)	17 (81.0)		48 (66.7)		0.329	14.7 (69.9)		50.3 (69.9)		0
TG or GG variant carriers)	4 (19.0)		24 (33.3)		-0.329	6.3 (30.1)		21.7 (30.1)		0
Outcome										
LAM troughs (µmol/L/100 mg)	185 (49)		50 (60)		2.564	178 (48)		50 (60)		2

Table 4 . Patient characteristics when reorganized by the level of valproate troughs [[?]364 µmol/L (Target/high), above lower limit of quantification but < 364 µmol/L (Low) or 0/BLOQ (not treated or below the limit of quantification) (control)]: before and after matching.

	Before matching	Before matching	Before matching	Before matching	Before matching
All patients	Target/high		Low		0/BLOQ
N	70		70		331
Men	33 (47.1)		39 (55.7)		116 (35.1)
Age (years)	34±13		36±13		41±15
Body weight (kg)	72±17		76±16		75±17
ABCG2 wild type	60 (85.7)		59 (84.3)		259 (78.2)
ABCG2 variant allele	10 (14.3)		11 (15.7)		67 (20.2)
ABCB1 wild type	20 (28.6)		30 (42.9)		109 (32.9)
ABCB1 variant allele	40 (71.4)		40 (57.1)		222 (67.1)
UGT2B7 wild type	26 (37.1)		18 (25.7)		75 (22.7)
UGT2B7 variant allele	44 (62.9)		52 (74.3)		256 (77.3)
*UGT1A4*3* wild type	55 (789.6)		55 (78.6)		255 (77.0)
*UGT1A4*3* variant allele	15 (21.4)		15 (21.4)		76 (23.0)
ABCG2 c.421C>A wt					
N	60		59		259
Men	30 (50.0)		32 (54.2)		93 (35.9)
Age (years)	34±13		37±14		41±15
Body weight (kg)	73±18		75±15		75±17
ABCB1 wild type	16 (26.7)		24 (40.7)		78 (30.1)
ABCB1 variant allele	44 (73.3)		35 (59.3)		181 (69.9)
UGT2B7 wild type	24 (40.0)		16 (27.1)		63 (24.3)
UGT2B7 variant allele	36 (60.0)		43 (72.9)		196 (75.7)
*UGT1A4*3* wild type	47 (78.3)		46 (78.0)		207 (79.9)
*UGT1A4*3* variant allele	13 (21.7)		13 (22.0)		52 (20.1)
ABCG2 c.421C>A variant					
N	10		11		72
Men	3 (30.0)		7 (63.6)		23 (31.9)
Age (years)	36±18		33±9		41±14
Body weight (kg)	71±13		82±19		75±18
ABCB1 wild type	4 (40.0)		6 (54.5)		31 (43.1)

	Before matching	Before matching	Before matching	Before matching	Before matching
ABCB1 variant allele	6 (60.0)		5 (45.5)		41 (56.9)
UGT2B7 wild type	2 (20.0)		2 (18.2)		12 (16.7)
UGT2B7 variant allele	8 (80.0)		9 (81.8)		60 (83.3)
*UGT1A4*3* wild type	8 (80.0)		9 (81.8)		48 (66.7)
*UGT1A4*3* variant allele	2 (20.0)		2 (18.2)		24 (33.3)

Table 5 . Sensitivity to unmeasured confounding of moderation of the valproate effect on dose-adjusted lamotrigine troughs by *ABCG2 c.421C>A* polymorphism. In the primary analysis, moderating effect is illustrated by a ratio of two geometric means ratios (GMRs): numerator is GMR for valproate >lower limit of quantification (LLOQ) vs. valproate 0/below the limit of quantification (BLOQ) in *ABCG2 c.421C>A* variant carriers and denominator is GMR for valproate >LLOQ vs. valproate 0/BLOQ in *ABCG2 c.421C>A* wild type subjects. In the secondary analysis, moderating effect is illustrated by two ratios of GMRs: i) in the first one, numerator is GMR for Low valproate (valproate >LLOQ but <364 μmol/L) vs. valproate 0/BLOQ in*ABCG2 c.421C>A* variant carriers and denominator is GMR for Low valproate vs. valproate 0/BLOQ in *ABCG2 c.421C>A* wild type subjects; ii) in the second one, numerator is GMR for Target/high valproate ([?]364 μmol/L) vs. valproate 0/BLOQ in *ABCG2 c.421C>A* variant carriers and denominator is GMR for Target/high valproate vs. valproate 0/BLOQ in*ABCG2 c.421C>A* wild type subjects. Shown are observed point-estimates of ratios of GMRs and corresponding confounding effect (i.e., a "confounding" ratio of GMRs) that would be needed to reduce the observed ratio to 1.25 (i.e., to largely "explain-away" the observed moderating effect of the *ABCG2 c.421C>A*polymorphism).

	Observed ratio of GMRs	Needed confounding effect
Primary analysis raw data		
Frequentist	1.61	1.90
Bayes	1.63	1.93
Primary adjusted analysis		
Frequentist	1.64	1.95
Bayes	1.61	1.90
Secondary analysis raw data (Low valproate)		
Frequentist	1.42	1.53
Bayes	1.45	1.59
Secondary analysis raw data (Target/high valproate)		
Frequentist	1.88	2.37
Bayes	1.68	2.02
Secondary analysis adjusted (Low valproate)		
Frequentist	1.46	1.61
Bayes	1.58	1.84

	Observed ratio of GMRs	Needed confounding effect
Secondary analysis adjusted (Target/high valproate)		
Frequentist	1.80	2.24
Bayes	1.89	2.39

Figure 1 . Raw dose-adjusted lamotrigine trough concentrations organized in line with the concept of primary analysis where subjects exposed to valproate (treated) are defined as those with valproate troughs >lower limit of quantification (valproate >LLOQ) and controls are defined as subjects not co-treated with valproate or with valproate troughs below the limit of quantification (BLOQ) (valproate 0/BLOQ). **A** . Shown is number of subjects (n) and geometric mean (GM) dose-adjusted lamotrigine troughs with coefficient of variation (%CV) for each study and for the combined data, overall and separately for *ABCG2 c.421C>A* wild type (wt) subjects and variant allele carriers. Shown are also valproate effects as geometric means ratios (GMR) with confidence/credible intervals (separately for each study and for combined data; the latter was based on one stage fixed-effect individual patient data meta-analysis/meta-regression approach): overall or main effects (estimated in simple models with "treatment" as the only effect; "study" was included in the meta-analysis approach), and then treatment effects in wild type subjects and variant carriers (models with treatment*polymorphism interaction). **B** . Moderation of the valproate effect on lamotrigine troughs by *ABCG2 c.421C>A* polymorphism. For each study and for combined data, we generated 40000 samples of the sampling distributions (frequentist) and of the posterior distributions (Bayesian) of the differences in valproate effects between *ABCG2 c.421C>A* variant carriers and wt subjects, i.e., of the ratios of GMRs [GMR (valproate >LLOQ vs. valproate 0/BLOQ in variant carriers) / GMR (valproate >LLOQ vs. valproate 0/BLOQ in wt subjects)]. Depicted are ratios (95%CI/CrI) with probabilities that they are >1.0 and >1.25 (values denoted by vertical grey lines).

Figure 2 . Adjusted (after entropy balancing with further adjustment for age and body weight) primary analysis of the effect of valproate [valproate >lower limit of quantification (LLOQ) vs. valproate 0/below the lower limit of quantification (BLOQ)] and of effect moderation by the *ABCG2 c.421C>A*polymorphism in the combined data. **A.** Shown are valproate effects as geometric means ratios (GMR), overall (models with valproate, age and body weight as fixed effects), and in *ABCG2 c.421C>A* wild type (wt) subjects and variant carriers (models with valproate, genotype, valproate*genotype interaction, age and body weight as fixed effects). **B** . Moderation of the valproate effect on lamotrigine troughs by the *ABCG2 c.421C>A* polymorphism. We generated 40000 samples of the sampling distribution (frequentist) and of the posterior distribution (Bayesian) of the ratios of GMRs [GMR (valproate >LLOQ vs. valproate 0/BLOQ in variant carriers) / GMR (valproate >LLOQ vs. valproate 0/BLOQ in wt subjects)]. Depicted are ratios (95%CI/CrI) with probabilities that they are >1.0 and >1.25 (values denoted by vertical grey lines).

Figure 3 . Dose-adjusted lamotrigine trough concentrations organized in line with the concept of secondary analysis where patients exposed to valproate (treated) were defined as those with valproate troughs >lower limit of quantification but <364µmol/L (valproate "Low") or as those with valproate troughs [?]364 µmol/L (valproate "Target/high"), and controls were subjects with valproate 0/below the lower limit of quantification (BLOQ). Depicted are number of subjects (n) and geometric mean (GM) dose-adjusted lamotrigine troughs with coefficient of variation (%CV) in the combined set for "Low", "Target/high" and "valproate 0/BLOQ" subjects, overall and for *ABCG2 c.421C>A*wild type (wt) patients and variant carriers. First, shown are raw data and then data after matching between treated ("Low" or "Target/high") and control subjects (valproate 0/BLOQ). Shown are also valproate effects as geometric means ratios (GMR) with confidence/credible intervals, overall and in *ABCG2 c.421C>A* wt subjects and variant carriers. Overall or main effects were generated in models with "treatment" as the only fixed effect (matched data always included additional

adjustment for age and body weight). With raw data, effects in wt patients and variant carriers were derived from an interaction term between a 3-level treatment (valproate "Low", "Target/high" or 0/BLOQ) and *ABCG2 c.421C>A* genotype. With matched data, two models with interactions (treatment*genotype) were fitted, one with "Low" vs. valproate 0/BLOQ, and the other one with "Target/high" vs. valproate 0/BLOQ. For each interaction term, provided are ratios of GMRs (i.e., differences in valproate effects between variant carriers and wt subjects), frequentist with a P-value for interaction, and Bayesian with a probability that ratios were >1.0.

Figure 4 . Sensitivity to unmeasured confounding of moderation of the valproate effect on dose-adjusted lamotrigine troughs by*ABCG2 c.421C>A* polymorphism: bias-corrected ratios of geometric means ratios (GMRs) observed in the adjusted primary and secondary analysis of the combined data. **A.** Sensitivity analysis of the ratio of GMRs in the primary adjusted analysis (depicted in Figure 2). Observed frequentist and Bayes ratios of GMRs (depicted as "GMR ratio to correct") corrected for a moderate (ratio of GMRs 1.25) or strong (ratio of GMRs 1.50) biasing effect generated by (hypothetical) "biasing set" that is markedly more prevalent in valproate-exposed (51%) vs. control subjects (17%) if *ABCG2 c.421C>A* variant carriers (and well balanced if wild type subjects, 25% vs. 25%). **B** . Sensitivity analysis of the ratio of GMRs in the secondary adjusted analysis of "Low valproate" vs. valproate 0/below the lower limit of quantification (BLOQ) (depicted in Figure 3). Observed ratios are corrected for a moderate or strong biasing effect generated by a biasing set more prevalent in valproate exposed (60%) than in control subjects (20%) if variant carriers (and well balanced if wild type subjects, 25% vs. 25%). **C** . Sensitivity analysis of the ratio of GMRs in the secondary adjusted analysis of "Target/high valproate" vs. valproate 0/BLOQ (depicted in Figure 3). Observed ratios are corrected for a moderate or strong biasing effect generated by a biasing set more prevalent in valproate exposed (51%) than in control subjects (17%) if variant carriers (and well balanced if wild type subjects, 25% vs. 25%). A bias-corrected ratio of GMRs of 1.25 is depicted (in gray and dashed line) to indicate a conventional limit of "equivalence".

Figure 5 . Possible mechanism of moderation of the valproate effect on exposure to lamotrigine by *ABCG2 c.421C>A*polymorphism (suggested by the present data). Inhibition of the main lamotrigine-metabolizing UGT enzymes resulting in reduced lamotrigine clearance is a well-known major mechanism by which valproate increases exposure to lamotrigine. Experimental data also suggest that valproate might reduce transcription, and consequently, the number of ABCG2 transporters. In people who are *ABCG2 c.421C>A* wild type homozygotes, with preserved ("normal") ABCG2 protein degradation, this effect would only mildly reduce the overall ABCG2 efflux activity, with a mild contribution (in addition to UGT inhibition) to the increase in lamotrigine levels. However, in *ABCG2 c.421C>A*variant carriers, this effect would be "combined" with enhanced ABCG2 protein degradation (consequent to the mutation), and the ABCG2 numbers/efflux activity would be markedly reduced resulting in a greater increase in exposure to lamotrigine i.e., a greater effect of valproate in variant carriers than in wild type subjects.

List of appendices

Appendix A

1. Conceived analysis and hypothesis tests
2. Lamotrigine does not affect valproate concentrations
3. Measures to achieve conditional exchangeability
4. Methods of statistical adjustment
5. Frequentist and Bayesian estimation
6. Sensitivity of ratio of GMRs to unmeasured confounding

Appendix B

Weights assigned by entropy balancing