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Source / Izvornik: **European Journal of Clinical Pharmacology, 2023, 79, 1117 - 1129**

Journal article, Accepted version
Rad u časopisu, Završna verzija rukopisa prihvaćena za objavljivanje (postprint)

https://doi.org/10.1007/s00228-023-03526-z

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:062920

Bearing variant alleles at uridine glucuronosyltransferase polymorphisms *UGT2B7 -161C>T* (rs7668258) or *UGT1A4*3 c.142T>G* (rs2011425) has no relevant consequences for lamotrigine troughs in adults with epilepsy

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Running title: Lamotrigine and *UTG2B7/UGT1A4* polymorphisms

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Abstract

Purpose. To estimate whether epilepsy patients with variant *UGT2B7 -161C>T* (rs7668258) or *UGT1A4*3 c.142T>G* (rs2011425) alleles differ from their wild-type (wt) peers in exposure to lamotrigine.

Methods. Consecutive adults on lamotrigine monotherapy or lamotrigine+valproate co-treatment undergoing routine therapeutic drug monitoring, otherwise generally healthy and free of interacting drugs, were genotyped for *UGT2B7 -161C>T* and *UGT1A4*3 c.142T>G.* Heterozygous, variant homozygous, or combined heterozygous/variant homozygous subjects were compared to their wt controls for dose-adjusted lamotrigine troughs with adjustment for age, sex, body weight, rs7668258/rs2011425, polymorphisms of efflux transporter proteins *ABCG2 c.421C>A* (rs2231142) and *ABCB1 1236C>T* (rs1128503), and level of exposure to valproate using covariate entropy balancing.

Results. Of the 471 included patients, 328 (69.6%) were on monotherapy and 143 were co-treated with valproate. Dose-adjusted lamotrigine troughs in *UGT2B7 -161C>T* heterozygous (CT, n=237) or variant homozygous (TT, n=115) subjects were closely similar to those in their wt controls (CC, n=119): geometric means ratios (GMRs) (frequentist and Bayes) 1.00 (95%CI 0.86-1.16) and 1.00 (95%CrI 0.83-1.22) for CT vs. CC; and 0.97 (0.81-1.17) and 0.97 (0.80-1.20) for TT vs. CC subjects. Lamotrigine troughs were also closely similar in *UGT1A4*3 c.142T>G* variant carriers [n=106: 102 TG + 4 GG subjects) and wt controls (TT, n=365): GMR= 0.95 (0.81-1.12) frequentist, 0.96 (0.80-1.16) Bayes. GMRs for variant carriers vs. wt controls were around unity also at different levels of exposure to valproate.

Conclusion. Dose-adjusted lamotrigine troughs in epilepsy patients with variant *UGT2B7 -161C>T* or *UGT1A4*3 c.142T>G* alleles are equivalent to those in their respective wt peers.

Keywords: lamotrigine, uridine glucuronosyltransferases (UGTs), polymorphism, bioavailability

Disclosures and Declarations

Authorship. All authors meet the ICMJE criteria for authorship.

Competing interests. The authors declare no financial or non-financial competing interests that would be relevant to the present article.

Funding. This study received no funding.

Ethics approval. This study was approved by the Ethics Committee at the Zagreb University Hospital Center (approval class: 8.1.-19/12–2, registration number: 02/21/AG).

Consent to participate. All participants provided a signed informed consent for participation in the study, i.e., donation of a blood sample for genotyping of pharmacogenes for research purposes.

Consent to publish. All participants provided a signed informed consent for participation in the study, including a consent to publish (anonymized) research results in scholarly journals.

Author contributions. All authors were fully involved in manuscript development and assume responsibility for the direction and content. N.B.,VT., IŠS., I.K.D., conceived the study. M.L., L.G.,L.Š. and N.B. performed and reviewed the bioanalytical analyses. V.T. performed data analysis and drafted the manuscript. N.B., V.T.,I.Š.S., I.K.D.,M.L., Z.Č.R. Ž.P.G. participated in the preparation of the manuscript. All authors reviewed the manuscript and provided their approval for submission.

Acknowledgments. We thank Zrinka Mirković and Maja Mezak Herceg for their technical support.

Data availability. Data can be obtained upon a reasonable request from the corresponding author.

Introduction

Lamotrigine is a commonly used broad-spectrum antiepileptic drug (AED) known for a considerable inter-subject variability in systemic exposure due to variable total body clearance [1-6], resulting in a rather wide range of recommended trough concentration in therapeutic drug monitoring (TDM) [3,5]. It is cleared almost exclusively by hepatic uridine diphosphate glucuronosyltransferases (UGTs), predominantly UGT1A4 with a contribution of UGT2B7 (possible contribution of UGT1A3 and/or UGT1A2 has also been suggested) [2,3], while ~10% is excreted unchanged *via* kidneys [1,6]. Consequently, UGT inducers (several antiretrovirals, classical AEDs and estrogens/gestagens) reduce exposure to lamotrigine up to 40-50%, while valproate (commonly used with lamotrigine) inhibits UGTs [7], reduces clearance by 50-60% and increases exposure to lamotrigine by approximately 2-fold [1,8]. This is reflected in dosing recommendations in co-treated (inducers, valproate) patients [1]. Other "classical" factors also contribute somewhat to variability in lamotrigine clearance [1,2, 4, 6]: i) it is reduced in moderate-severe liver failure and moderately decreases with older age and advanced renal failure; ii) it increases in pregnancy and slightly with increasing body weight; iii) over the initial 2-3 weeks of treatment, lamotrigine mildly induces its own glucuronidation [1,6,7]. Accounting for UGT inducer or valproate use, age and body weight reduces the inter-subject coefficient of variation (%CV) of lamotrigine clearance from 90% to around 45-50% - a still high inter-individual variability [9]. Lamotrigine is a substrate for efflux transporter proteins P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2). Limited and equivocal data suggest [2,10] that single nucleotide polymorphisms (SNPs) *ABCB1 1236C>T* (rs1128503), *2677G>T/A* (rs2032582) and *3435C>T* (rs1045642) [in a strong linkage disequilibrium (LD) [10]], and *ABCG2 c.421C>T* (rs2231142) might affect systemic lamotrigine exposure. However, the main pharmacogenetic "targets" in attempts to understand the variability of lamotrigine clearance are *UGT1A4* and *UGT2B7* polymorphisms [2]. Both genes are highly polymorphic [11]. The most consistent findings pertain to *UGT1A4*3 c.142T>G* (Leu48Val, rs2011425): i) *in vitro*, the 48Val variant displays increased glucuronidation (tamoxifen as a probe) [12]; ii) *in vivo*, several studies indicated associations between the variant allele/variant homozygosity (GG) and lower exposure and less clinical effect of lamotrigine [2]. Some studies, however, failed to demonstrate such an association (Japanese [13] or Danish patients [14]). Of the *UGT2B7* SNPs, most of the (rather limited) *in vivo* human data pertain to *UGT2B7 -161C>T* (rs7668258) and *UGT2B7 802C>T* (rs7439366) [2]. In human liver tissue, *UGT2B7 -161C>T* is associated with reduced enzyme content and overall reduced glucuronidation capacity [15]. The two SNPs are in a

complete LD [16,17], and a few smaller studies suggested a mildly reduced lamotrigine clearance in heterozygous/variant homozygous subjects [2]. A recent larger study in Danish patients suggested around 9% higher dose-adjusted lamotrigine troughs in the *UGT2B7 802C>T* variant than in wild-type homozygotes [14], while a study in Mexican patients suggested no relevant association between either of the SNPs and lamotrigine troughs [18]. The apparent inconsistencies could be due to a variety of factors, e.g., ethnic specificities, study designs, sample size, control of confounding and assessed outcomes. Moreover, considering the large number of SNPs in each of the two genes, attempts to evaluate relevance of any single one of them for bioavailability of lamotrigine might seem meaningless if one does not account ("control") for all of the others. Obviously, such an effort would require studies including tens of thousands of subjects that are unlikely to ever happen. However, both rs7668258 and rs2011425 are in complete LD with many other SNPs in the respective genes. *UGT2B7 -161C>T* (rs7668258) is in a complete LD with numerous other *UGT2B7* promoter polymorphisms forming two major haplotypes [16] and with a number of other SNPs, and participates in several haplotypes [11] - *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) [11,12,19, 20]. Also, at least in Caucasians, rs2011425 is in a complete LD with *UGT1A4*2 c.70C>A* (rs6755571, Pro24Tre) [21,22] which *in vitro* is associated with a reduced enzyme activity [12, 23], but reports about its association with lamotrigine troughs have been ambiguous (e.g., in Scandinavian subjects [14,24]). 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 that in their respective wild-type (wt) homozygous controls. Elements of these makeups may or may not be related to lamotrigine exposure, and it might not be possible to untangle their individual contributions. Consequently, by contrasting subjects heterozygous or variant homozygous at *UGT2B7 -161C>T* or *UGT1A4*3 c.142T>G* to their wt peers, one may not be able to estimate the effects of these specific polymorphisms, but could still estimate the effects of the respective "broader makeups" represented by these genotypes. In this context, we aimed to estimate the effect of *UGT1A4*3 c.142T>G* and of *UGT2B7 -161C>T* heterozygous/variant homozygous genotypes (i.e., related "broader makeups") on (dose-adjusted) lamotrigine troughs in adult and adolescent epilepsy patients of Central-Eastern European descent.

Patients and Methods

Study outline

Otherwise generally healthy patients on lamotrigine or on combined lamotrigine + valproate therapy undergoing routine TDM after at least 3 weeks of (co-)treatment were genotyped for *UGT2B7 -161C>T* (rs7668258) and *UGT1A4*3 c.142T>G* (rs2011425), and also for two efflux transporter SNPs - *ABCG2 c.421C>A* (rs2231142) (classified as wt or variant carriers, since only 1.0% of patients were variant homozygous), and *ABCB1 1236C>T* (rs1128503). Patients were also classified with respect to exposure to valproate as (i) valproate trough=0 (patients on lamotrigine monotreatment) or below the lower limit of quantification (BLOQ) (20.8 μmol/L), (ii) low valproate, i.e., 0/BLOQ < valproate trough <364 μmol/L (median of the quantified values, and approximate lower limit of recommended valproate troughs [5]), and (iii) target/high valproate (≥364 μmol/L). The study concept was as follows: i) heterozygous or variant homozygous subjects are considered to differ from the respective wt controls not only regarding the determined genotype, but regarding a "broader makeup" consisting of linked polymorphisms; ii) these "broader makeups" have no other means of affecting exposure to lamotrigine but by affecting the (respective) UGT enzyme activity; iii) however, whether or not enzyme activity is affected is of no interest – the outcome of interest are lamotrigine troughs, and "enzyme activity" is considered an unobserved true exposure represented by an instrumental variable, i.e., the *UGT2B7 -161C>T* or *UGT1A4*3 c.142 C>T* genotype. To estimate the effects of *UGT2B7 -161C>T* (i.e., the associated broader makeup), in the entire sample (main effects) we emulated a randomized experiment in which "treated" were heterozygous (CT) and variant homozygous subjects (TT), whereas wt subjects were controls. To estimate the main effects of *UGT1A4*3 c.142T>G* (i.e., the associated broader makeup) we emulated a trial in which "treatment" was variant allele carriage (TG or GG; since there were <1% variant homozygotes) and wt patients were controls. Finally, we emulated two trials to test potential moderation of the polymorphism effects by exposure to valproate, i.e., the genotype*valproate interaction: "treated" were variant carriers (CT/TT in the case of rs7668258, or TG/GG in the case of rs2011425) and controls were their wt peers, and differences were estimated at valproate 0/BLOQ and at valproate >0/BLOQ. Although cross-sectional, we deemed data as appropriate for the purpose: i) the presumed cause (genotype/associated broader makeup) preceded the outcome (lamotrigine troughs); ii) it was plausible to assume no reverse causation, i.e., no effect of the outcome on "treatment" - samples were taken after the initial lamotrigine self-induction had been completed [25]; iii) it was plausible to assume also no effect of outcome on other

possible causes, i.e., confounders/outcome ancestors. This is primarily of interest in the sense of no effect of lamotrigine on valproate levels. Since valproate is partly eliminated by UGTs (26), it has been suggested that valproate-lamotrigine interaction could be bi-directional (27), considering the initial UGT induction by lamotrigine. However, present samples were taken after this process had been completed, and individual and population pharmacokinetic studies have refuted the (hypothetical) effects of lamotrigine on valproate clearance [28,29]. The same reasoning applies to the lack of effect of the outcome on other UGT enzymes or transporters.

We used inclusion/exclusion criteria and covariate entropy balancing to control for the effects of confounders/outcome ancestors (Table 1) (details in Supplementary Information - Methods to achieve conditional exchangeability [Fig S1, Fig S2]). Since it was reasonable to expect residual confounding (Table 1), the estimated effects were subjected to analysis of sensitivity to unmeasured confounding.

The study was conducted in line with the Declaration of Helsinki (the 2008 version) and was approved by the Institutional Ethics Committee.

Patients

Consecutive epilepsy patients on lamotrigine (immediate-release tablets) or on combined lamotrigine + valproate (extended-release tablets) regimen with gradual dose titration as *per* approved labels, scheduled for routine TDM after at least 21 days of (co-)treatment provided blood samples for determination of morning (07:00-09:00 hours) lamotrigine/valproate troughs. From initiation of the monotherapy or from initiation of the combined treatment (addition of valproate to lamotrigine, or, less commonly, lamotrigine to pre-existing valproate), patients were seen in two-week intervals, and at a pre-TDM interview to assess (by self-report) tolerability, treatment compliance and possible violation of the inclusion/exclusion criteria. They were included in the study if: i) willing to donate blood samples and provided signed informed consent for genotyping of pharmacogenes; ii) aged ≥16 years; iii) non-smokers or ex-smokers; iv) not using other AEDs or other drugs known to affect lamotrigine or valproate, and/or activity of UGTs, P-glycoprotein or ABCG2 within the previous month; v) had preserved cardiac, renal and liver function, based on routine assessment. Patients suffering unregulated diabetes mellitus, hypo- or hyperthyroidism, those with a history of or an ongoing malignant disease or any acute illness, pregnant women and patients with HIV/AIDS were not included.

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 [30], while serum valproate was measured by an immunoassay (PETINIA) on a Dimension 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*3 c.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*3 c.142T>G* was confirmed by a PCR-RFLP method on the Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) [31].

Weighting and data analysis

To achieve a balance between "treated" and "controls" on measured covariates, we used entropy balancing [32] implemented in package *WeightIt* [33] in R [34] 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 [35]. To estimate the main effects, balancing was undertaken in the entire sample; to test the genotype*valproate interaction, "treated" and "controls" were balanced separately at each level of exposure to valproate. We used generalized frequentist (robust variance estimator) and Bayesian weighted models to analyze (ln-transformed) dose-adjusted lamotrigine troughs with geometric means ratios (GMRs) as effect measures. In Bayesian analysis, we defined moderate-strength skeptical normal prior for the polymorphism effect [normal (0.0, 0.355)] compatible with the a priori hypothesis of no treatment effect. In models testing the interaction, we additionally defined a moderate-strength normal prior for the effect of valproate [normal (0.693, 0.40)] in line with the expected twice higher, on average, exposure to lamotrigine with valproate co-treatment. We used SAS 9.4 for

Windows (SAS Inc., Cary, NC) and R package *rstanarm* [36]. We used CubeX [37] to evaluate Hardy-Weinberg equilibrium and linkage disequilibrium.

Sensitivity to unmeasured confounding/bias

We considered that bias arising from unmeasured confounders was primarily due to (hypothetical) effects of *UGT2B7* and *UGT1A4* SNPs that were not accounted for, i.e., the "remaining" genetic makeups besides those consisting of the evaluated SNPs and their linked polymorphisms. We assumed that this hypothetical bias might have "pushed" the observed GMRs to >1.0 or to <1.0 with a "moderate" (i.e., 1.25 or 0.80, respectively) or a "strong" effect (i.e., 1.43 or 0.70, respectively): GMRs 1.25/0.80 correspond to standard upper and lower limits of equivalent exposure, while GMRs 1.43/0.70 are their "extended" values applicable to compounds showing high variability, i.e., inter-subject %CV of 50% (corresponds to the "inherent" variability in lamotrigine clearance, after adjusting for age, body weight and concomitant use of UGT inducers or valproate) [9]. According to the present (incomplete) knowledge, practically all *UGT1A4* polymorphisms with a prevalence of around 10-15% ("common"), are in LD with *UGT1A4*3 c.142T>G* [11, 19], whereas cumulative prevalence of all other SNPs is around 5-10%. Similarly, the most common (known) *UGT2B7* haplotypes/haplotype pairs include *UGT2B7 -161C>T* [11, 17], while cumulative prevalence of haplotype pairs not including this SNP may be approximated at around 15% [17]. We (conservatively) assumed that the prevalence of these genetic constellations that we did not account for in the present sample was 25% for *UGT1A4* and 25% for *UGT2B7* (regardless of whether they were considered as "competing instrument" or as "outcome ancestor"). Since their occurrence is independent, the probability of their joint occurrence is 6.25%, hence we stayed with a more unfavorable scenario with prevalence of 25%. Finally, we assumed that this total prevalence resulted from a marked imbalance between "treated" and "control" subjects of 2:1 and 4:1. Hence, we corrected the observed estimates of the "treatment" effect for unobserved confounding effect [38] of GMR 1.25 and 1.43 (and their reciprocal values), assuming 2:1 and 4:1 imbalance of a biasing set of covariates between "treated" and "controls" assuming its total prevalence of 25% (R package *episensr* [39]) (see also Supplementary Information – Sensitivity of GMR to unmeasured confounding).

Results

Patients

We included 471 patients, 143 (30.4%) co-treated with valproate and 328 on lamotrigine monotherapy (Table 2). Three co-treated patients had valproate troughs BLOQ, hence 331 (70.2%) patients had valproate 0/BLOQ, while "low" and "target/high" valproate were seen in 70 patients each (Table 2). Regarding *UGT2B7 -161C>T*, 50% of the patients were heterozygotes, while wt and variant homozygotes were comparably prevalent (Table 2). Only 4 (0.8%) patients were *UGT1A4*3 c.142T>G* variant homozygous, and wt subjects prevailed (77.5%) (Table 2). Variant homozygotes were also sporadic regarding *ABCG2 c.421C>A* (Table 2). Patient subsets based on *UGT2B7 -161C>T* and on *UGT1A4*3 c.142T>C* genotypes numerically differed with respect to a number of characteristics, however dose-adjusted lamotrigine troughs apparently only mildly differed across the respective subsets (Table 2). 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).

Balanced/weighted data

In the overall sample, all treated (*UGT2B7 -161* CT or TT, or *UGT1A4*3 c.142* TG/GG genotype) and respective wt control patients (CC and TT genotypes, respectively) were well balanced (Supplementary Information - Table S1 summarizes information on weights) on all covariates (d=0.000) and their dose-adjusted lamotrigine troughs were closely similar (Table 3). All comparisons (main effects) yielded GMRs close to 1.0 with CI/CrI within the conventional range of equivalent exposure (Fig 1A). For both polymorphisms, variant allele carriers (CT/TT or TG/GG) were well balanced on all covariates vs. their respective wt controls at valproate 0/BLOQ and at valproate >0/BLOQ (Table 4). Dose-adjusted lamotrigine troughs were (expectedly) considerably higher with valproate >0/BLOQ than with valproate 0/BLOQ (Table 4), and for both polymorphisms, variant carriers and wt controls had closely similar values at both valproate levels (Table 4). All GMRs (variant carriers vs. wt controls) were close to 1.0 (Fig 2A) while some CIs/CrIs were wide (exceeded the conventional limits of equivalence) (Fig 2A) due to high inter-subject variability and a limited number of subjects in some of the valproate-by-polymorphism subsets. Overlapping distributions of GMRs (variant carriers vs. wt controls) estimated at the two levels of exposure to valproate (Fig 2B) illustrate lack of polymorphism*valproate interaction.

Sensitivity to unmeasured confounding

Based on previous reports, variant *UGT2B7 -161C>T* allele should be expected associated with higher exposure to lamotrigine. We hence assumed that the observed GMRs of 1.00 (CT vs. CC) and 0.97 (TT vs. CC) were due to the effect of confounding bias that "pushed" the "true" GMR towards ≤1.0 (Fig 2A): however, even assuming a considerable imbalance in the prevalence of the "biasing" covariates and their moderate (0.80) the or strong (0.70) effect, the bias-corrected estimates did not suggest any relevant effect of this polymorphism on dose-adjusted lamotrigine troughs (Fig 2A). On the other hand, considering previous reports, variant *UGT1A4*3 c.142T>G* allele should be expected associated with lower exposure to lamotrigine. We hence assumed that the observed GMR of 0.95 (TG/GG vs. TT) was due to the effect of confounders that "increased" the "true" GMR towards ≥1.0 (Fig 2B): however, even under a huge assumed imbalance in prevalence of the biasing covariates (60% vs. 15%) and with a marked biasing effect (1.43) "corrected" GMR estimate (GMR==0.804) still did not cross the limit of what is generally considered "a practically relevant difference" (i.e., outside the limits of "equivalent exposure") (Fig 2B).

Discussion

Polymorphisms in genes encoding UGT1A4 and UTG2B7 – considered the main enzymes in lamotrigine metabolism – have been commonly evaluated in attempts to elucidate sources of inter-individual variability in lamotrigine clearance. The largest body of evidence pertains to *UGT2B7 -161C>T* (rs7668258) and *UGT1A4*3 c.142T>G* (rs2011425), both of which are *in vitro* associated with altered enzyme activity [12, 15]. *In vivo* data, however, are equivocal: some studies reported associations between heterozygosity (CT) / variant homozygosity (TT) at *UGT2B7 -161C>T* with mildly increased lamotrigine levels, and some reported associations between the variant allele at *UGT1A4*3 c.142T>G* (TG/GG) and reduced lamotrigine concentrations – but several studies reported no association of either polymorphism with exposure to lamotrigine (reviewed in [2], exemplified in e.g., [13, 14, 18, 24]). As in any complex setting investigated using observational data, these somewhat inconsistent reports might be due to any one or more of several reasons, e.g., ethnicity-related specifics, sample size, outcome measures, bioanalytical methods and control of confounding. The present analysis included adult Caucasian epilepsy patients of Central-Eastern European descent and used dose-adjusted lamotrigine troughs obtained through routine TDM as an outcome. We *a priori* accepted the fact that it was impossible to assess specific relationships between either of the two SNPs and the outcome due to their complete LD with many other

polymorphisms within the respective genes i.e., that genotypes at the two loci were parts of broader "genetic makeups" whose actual "composition" remained unknown (we did not determine genotypes at other respective polymorphisms and, currently, not all linkages among numerous SNPs in *UGT2B7* and *UGT1A4* genes might be known). Finally, we *a priori* acknowledged that many polymorphisms were likely *not linked* to two genotyped polymorphisms, and could have been (reasonable) sources of bias. Otherwise, we accounted for a range of classical and (pharmaco)genetic factors known or suspected to affect exposure to lamotrigine by combining inclusion/exclusion criteria and "statistical" adjustment. For the latter, we used a method (covariate entropy balancing) that is model-independent and more appropriate for a given setting than a "standard" regression analysis. For example, in a *UGT1A4*3 c.142T>G* TG/GG vs. wt control comparison, considered covariates formed a total of 108 strata (3 x 3 x 3 x 2 x 2), with a further need for adjustment for age and body weight. For a regression model to yield a reasonably accurate "adjusted" estimate of a difference, i.e., one that is not dependent on model extrapolations that might be considerably astray, each stratum would need to contain at least a few "treated" and a few "controls" – which in the present case would not be possible, since there were 106 TG/GG patients – and in each stratum values of age and body weight between "treated" and "controls" would need to at least partly overlap.

Under these circumstances, all observed GMRs (main effects) - for *UGT2B7 -161C>T* CT or TT vs. wt controls (CC) and for *UGT1A4*3 c.142T>G* TG/GG vs. wt controls (TT) – were closely around 1.0 with CIs/CrIs within the classical limits of equivalent exposure. Even GMRs (point-estimates) corrected for a hypothetical considerable biasing effect of unmeasured confounders with (unrealistically) high imbalance between "treated" and "controls" did not signal any practically relevant effect. We assigned this (hypothetical) biasing effect primarily to unmeasured variables pertaining to other potential SNPs in the *UGT2B7* and *UGT1A4* genes that so far have not been suggested related to exposure to lamotrigine, nor shown linked to the two genotyped SNPs, although it could be viewed as a result of any number of biasing factors. However, based on the current knowledge, those factors that could be identified have likely not contributed to this hypothetical bias. For example, we adjusted for the loss-of-function SNP in the *ABCG2* gene (*ABCG2 c.421C>A*, rs2231142) that apparently moderately affects lamotrigine troughs [40], and for which global minor allele prevalence has been estimated at around 12% [41]. Reduced transporter function has been reported associated with three further *ABCG2* SNPs (rs34783571, rs192169062 and rs34264773), for three SNPs no effect on function has been reported and for the rest, functional

consequences are unknown [41]. The cumulative estimated prevalence of combined other (besides rs2231142) "loss-of-function" and "unknown effect on function" SNPs is around 1.0% [41]. This suggests that it would be reasonable to expect at most 5 patients in the current sample bearing any of these "other" SNPs – hence, it is highly unlikely that these (undetermined) SNPs have biased the present results. Similar reasoning is applicable to SNPs in the *ABCB1* gene, as well. We adjusted for the *ABCB1 1236T>C* (rs1128503) polymorphism which is in a strong LD [10] with two further common coding SNPs - *2677T>G/A* (rs2032582) and *3435T>C* (rs104564)]. In a sample of renal transplant patients from the same general population as in the present study, we recently also observed almost complete LD among these three SNPs [42]. Hence, by controlling for the rs1128503 genotype, one largely controls for the other two SNPs. In Caucasians, these three SNPs are the most prevalent ones, and are the most commonly evaluated among numerous *ABCB1* SNPs with respect to bioavailability of a range of drugs, but with extremely variable outcomes disabling any consensus [43]. In respect to lamotrigine, several studies tested involvement of individual SNPs or of the haplotype [with T/G/T having higher lamotrigine concentration than C/G(A)/C] in lamotrigine pharmacokinetics [2], but the most recent larger study in Scandinavian patients [44] 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% [43], suggesting that in the "worst case scenario" at most 50 patients in the current sample might have harbored any of those SNPs. Even if one were to assume that each of them "worked in the same direction" regarding exposure to lamotrigine, and that there was an unrealistically huge imbalance in their simultaneous prevalence between "treated" and "controls", and their considerable effect, these "other" SNPs could not have relevantly biased the present estimates. Finally, a recent comprehensive systematic review [45] 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 the none and unreproducible associations, suggesting that the impact of these SNPs on drug pharmacokinetics is generally minor (if any) [45], and this appears applicable to lamotrigine, as well. Based on the current knowledge (reviewed in [2]), it is also reasonable to conclude that polymorphisms in the SCL superfamily transporters are highly unlikely to be relevant for exposure to lamotrigine. Therefore, the hypothetical strong bias used in the present analysis to "correct" the observed estimates might have had different sources, albeit it seems reasonable to assign it to *UGT2B7* and/or *UGT1A4* SNPs that have not been addressed and are not linked (or are not known to be linked) to the two typed polymorphisms.

In addition to the main effects, the present analysis demonstrates closely similar dose-adjusted troughs between variant carriers (*UGT2B -161* CT/TT or *UGT1A4*3* TG/GG subjects) and their wt peers at each of the two levels of exposure to valproate, i.e., lack of an interaction between genotype and valproate. In this analysis, genotypes used for adjustment, and exposure to valproate were dichotomized since, despite the total number of 471 patients, number of subjects in some of the strata formed by multiple 3-level and multiple 2-level factors was very low. Values in CT/TT or TG/GG patients were equivalent to those in CC or TT patients (respectively) at each of the two levels of exposure to valproate, or, point-estimates were within the narrow range between 0.90 and 1.11, with CIs/CrIs slightly exceeding the conventional limits of equivalence. In this respect, it should be noted that even with a GMR of 1.0, with 50% CV (this corresponds to %CV in lamotrigine clearance after adjustment for age, body weight, use of UGT inducers and/or valproate) a sample of 96 vs. 44 or of 30 vs. 110 subjects achieves only around 60% power to "place" the 90%CIs/CrIs within the range 0.80-1.25.

Comparing results across observational studies that differ in sampling populations and methodology is not straightforward – it seems more reasonable to assess each individual study for its own merit. We believe that in the present analysis we generated reasonably unbiased estimates to support a view that heterozygosity or variant homozygosity at *UGT2B7 -161C>T* (rs7668258) or at *UGT1A4*3 c.142T>G* (rs2011425) – each representing a "broader genetic makeup" that differs from that represented by the wt genotype – have no relevant consequences for dose-adjusted lamotrigine troughs in adult epilepsy patients. Present estimates were obtained in Caucasian patients of Central-Eastern European descent (Slavic) and might not hold in other populations, e.g., those in which the typed polymorphisms are potentially linked to different other SNPs, or in which prevalence of functionally relevant non-linked SNPs is considerably different.

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Table 1 Confounders [may affect both the outcome (dose-adjusted lamotrigine troughs) and exposure - activity of UGT enzyme whose polymorphism (*UGT2B7 -161 C>T* or *UGT1A4*3 142 T>G*) is used as an instrumental variable] and outcome ancestors (may affect the outcome) considered in an attempt to achieve conditional exchangeability between "treated" and "controls" (see Supplementary Information 1: Methods to achieve conditional exchangeability, with directed acyclic graphs [Fig S1, Fig S2]).

Confounders/outcome ancestors	Controlled by
Fully controlled	
UGT2B7 -161 C>T or *UGT1A4*3 142 T>G*	Entropy balancing
Age, sex, body weight	Entropy balancing
Exposure to valproate	Entropy balancing
ABCG2 c.421 C>A genotype	Entropy balancing
ABCB1 1236 C>T genotype	Entropy balancing
Lamotrigine dose	Dose-adjusted lamotrigine trough as the outcome
Drugs that may affect lamotrigine by any mechanism (except for valproate)	Inclusion-exclusion criteria
Comorbidities that can affect lamotrigine by any mechanism	Inclusion-exclusion criteria
Drugs and comorbidities that may affect valproate by any mechanism	Inclusion-exclusion criteria; entropy balancing for valproate troughs
Polymorphisms in genes encoding UGTs and other enzymes that may affect exposure to valproate	Entropy balancing for valproate troughs. Around 50% of valproate clearance is by glucuronidation by, presumably, a number of UGT enzymes, around 40% by beta-oxidation and around 10-20% by cytochrome P-450 enzymes.
Partly controlled	
UGT2B7 /UGT1A4 enzyme activity (regardless of the "role")	Exclusion of drugs and comorbidities, and entropy balancing with respect to the *-161C>T* or *c.142T>G* SNPs and valproate exposure only partly "controlled" the respective enzyme(s) activity since other *UGT2B* and *UGT1A4* polymorphisms remained undetermined (unmeasured)
P-glycoprotein and/or ABCG2 activity	Exclusion of drugs and comorbidities, and entropy balancing with respect to *ABCB1 1236 C>T* and *ABCG2 c.421C>A* SNPs and valproate exposure only partly "controlled" the respective transporter activity since other *ABCB1* and *ABCG2* polymorphisms remained undetermined (unmeasured)
Uncontrolled – unknown/unmeasured	
Factors currently unknown to affect lamotrigine exposure, e.g., polymorphisms in genes encoding transporter proteins other than P-glycoprotein and ABCG2	---

Table 2 Subject characteristics (raw data) overall and by *UGT2B7 -161C>T* and *UGT1A4*3 c.142T>G* polymorphisms (with standardized differences [d] for balancing variables and the outcome). Data are count (%), median (range), mean±SD (range), geometric (geo) mean (%CV) for ln(lamotrigine [LAM]/dose)

		By *UGT2B7 -161C>T* (rs7668258)				By *UGT1A4*3 c.142T>G* (rs2011425)		
	All	CC (wild type)	CT	TT	Max d	TT (wild type)	TG or GG	d
N	471	119	237	115	---	365	106	---
Lamotrigine + valproate	143 (30.4)	47 (39.5)	68 (28.7)	28 (24.4)	---	112 (30.7)	31 (21.2)	---
Lamotrigine only	328 (69.6)	72 (60.5)	169 (71.3)	87 (75.6)	---	253 (69.3)	75 (70.8)	---
Lamotrigine dose (mg/day)	175 (12.5-550)	175 (25-500)	200 (25-550)	150 (12.5-500)	---	150 (12.5-500)	200 (25-500)	---
Valproate dose (g/day)	0 (0-2.0)	0 (0-2.0)	0 (0-2.0)	0 (0-2.0)	---	0 (0-2.0)	0 (0-2.0)	---
Age (years)	39±15 (16-77)	40±15 (16-72)	38±15 (16-77)	40±13 (19-70)	0.210	39±15 (16-77)	39±14 (16-70)	-0.049
Men	188 (39.9)	45 (37.8)	98 (41.4)	45 (39.1)	0.034	153 (41.9)	35 (33.0)	0.089
Body weight (kg)	75±17 (27-143)	74±16 (27-110)	76±17 (35-130)	75±18 (47-143)	0.122	76±17 (27-140)	71±16 (35-143)	0.273
UGT2B7 -161 C>T rs7668258								
CC	119 (25.3)	---	---	---	---	90 (24.7)	29 (27.4)	-0.027
CT	237 (50.3)	---	---	---	---	194 (53.1)	43 (40.6)	0.126
TT	115 (24.4)	---	---	---	---	81 (22.2)	34 (32.1)	-0.099
*UGT1A4*3 142 T>G* rs2011425								
TT	365 (77.5)	90 (75.6)	194 (81.8)	81 (70.4)	0.114	---	---	---
TG	102 (21.7)	29 (24.4)	40 (16.9)	33 (28.7)	(TT vs.	---	---	---
GG	4 (0.8)	0	3 (1.3)	1 (0.9)	TG/GG)	---	---	---
ABCG2 c.421 C>A rs 2231142								
CC	378 (80.2)	103 (86.6)	186 (78.5)	89 (77.4)	0.092	300 (82.2)	78 (73.6)	0.086
CA	88 (18.7)	15 (12.6)	48 (20.2)	25 (21.7)	(CC vs.	64 (18.5)	24 (22.6)	(CC vs.
AA	5 (1.1)	1 (0.8)	3 (1.3)	1 (0.9)	CA/AA)	1 (0.3)	4 (3.8)	CA/AA)
ABCB1 1236 C>T rs1128503								
CC	159 (33.8)	32 (26.9)	81 (34.2)	46 (40.0)	0.131	129 (35.3)	30 (28.3)	0.070
CT	219 (46.5)	66 (55.5)	103 (43.5)	50 (43.5)	0.120	159 (43.6)	60 (56.6)	-0.130
TT	93 (19.7)	21 (17.6)	53 (22.4)	19 (16.5)	0.058	77 (21.1)	16 (15.1)	0.060
Valproate trough (μmol/L)	0 (0-813)	0 (0-662)	0 (0-724)	0 (0-813)	---	0 (0-813)	0 (0-691)	---
0 (NT/BLOQ)[1]	331 (70.2)	75 (63.0)	169 (71.3)	87 (75.7)	0.126	255 (69.8)	76 (71.7)	-0.018
Low (0< to 364 μmol/L)	70 (14.9)	18 (15.1)	33 (13.9)	19 (16.5)	0.026	55 (15.1)	15 (14.1)	0.009
Target/high (≥ 364 μmol/L)	70 (14.9)	26 (21.9)	35 (14.8)	9 (7.8)	0.140	55 (15.1)	15 (14.1)	0.009
LAM (μmol/L)	12.8 (0.5-102)	16.8 (0.5-69)	12.6 (1.3-102)	9.9 (1.5-102)	---	12.6 (0-102)	13.6 (1.3-47.7)	---
LAM/dose (μmol/L/100 mg)	84.0 (6.5-464)	89.7 (10.0-314)	84.0 (6.5-464)	82.0 (10.4-340)	---	85.3 (10-464)	80 (6.5-247)	---
Geo mean [Ln(LAM/dose)]	83 (75)	92 (74)	83 (74)	76 (75)	0.295	85 (75)	79 (73)	0.105

[1]BLOQ – below the lower limit of quantification (20.8 μmol/L); NT – not co-treated; 3 co-treated patients had valproate BLOQ

Table 3 Subject characteristics by *UGT2B7 -161C>T* and *UGT1A4*3 c.142T>G* genotypes after balancing/weighting. "Treated" are *UGT2B7 -161C>T* heterozygous or variant homozygous patients and *UGT1A4*3 c.142T>G* variant allele carriers (TG/GG) (only 4 patients were variant homozygous), and controls are their respective wild type (wt) subjects. Data are weighted counts (percent), mean±SD or geometric mean (%CV) for lamotrigine (LAM) dose-adjusted troughs (on ln-transformed data). Shown are also standardized differences (d) for balancing variables (maximum d for any pairwise comparison) and for the outcome.

	UGT2B7 -161C>T				*UGT1A4*3 c.142T>G*		
	Treated: CT	Treated: TT	Control: CC	Max d	Treated: TG/GG	Control: TT	d
N	237	115	119		106	365	
Balancing covariates							
Women	142.4 (60.1)	69.1 (60.1)	71.5 (60.1)	0.000	63.7 (60.1)	219.3 (60.1)	0.000
Men	94.6 (39.9)	45.9 (39.9)	47.5 (39.9)	0.000	42.3 (39.9)	145.7 (39.9)	0.000
Age (years)	39±15	39±13	39±15	0.000	39±15	39±15	0.000
Body weight (kg)	75±17	75±18	75±17	0.000	75±18	75±17	0.000
Valproate trough (μmol/L)							
0 (NT/BLOQ)[1]	166.5 (70.2)	80.8 (70.2)	83.6 (70.2)	0.000	74.4 (70.2)	256.4 (70.2)	0.000
Low (0 < and < 364)	35.2 (14.9)	17.1 (14.9)	17.7 (14.9)	0.000	15.8 (14.9)	54.3 (14.9)	0.000
Target/high (≥ 364)	35.2 (14.9)	17.1 (14.9)	17.7 (14.9)	0.000	15.8 (14.9)	54.3 (14.9)	0.000
ABCG2 c. 421 CC	190.2 (80.3)	92.3 (80.3)	95.5 (80.3)	0.000	85.1 (80.3)	292.9 (80.3)	0.000
ABCG2 c. 421 CA/AA	46.8 (19.7)	22.7 (19.7)	23.5 (19.7)	0.000	20.9 (19.7)	72.1 (19.7)	0.000
ABCB1 1236 CC	80.0 (33.8)	38.8 (33.8)	40.2 (33.8)	0.000	35.8 (33.8)	123.2 (33.8)	0.000
ABCB1 1236 CT	110.2 (46.5)	53.5 (46.5)	55.3 (46.5)	0.000	49.3 (46.5)	169.7 (46.5)	0.000
ABCB1 1236 TT	46.8 (19.7)	22.7 (19.7)	23.5 (19.7)	0.000	20.9 (19.7)	72.1 (19.7)	0.000
UGT2B7 -161 CC	---	---	---		26.8 (25.3)	92.2 (25.3)	0.000
UGT2B7 -161 CT	---	---	---		53.3 (50.3)	183.7 (50.3)	0.000
UGT2B7 -161 TT	---	---	---		25.9 (24.4)	89.1 (24.4)	0.000
*UGT1A4*3 142 TT*	183.7 (77.5)	89.1 (77.5)	92.2 (77.5)	0.000	---	---	
*UGT1A4*3 142 TG/GG*	53.3 (22.5)	25.9 (22.5)	26.8 (22.5)	0.000	---	---	
Outcome							
LAM (μmol/L/100 mg)	84 (74)	82 (79)	84 (73)	0.046	81 (76)	85 (77)	-0.075

BLOQ – below the lower limit of quantification (20.8 μmol/L); NT- not co-treated

Table 4 Subject characteristics by *UGT2B7 -161C>T* and *UGT1A4*3 c.142T>G* genotypes before and after covariate entropy balancing, separately at different levels of exposure to valproate [valproate 0 or below the limit of quantification (BLOQ); valproate >0/BLOQ] for evaluation of the effect of variant carriage (CT/TT or TG/GG vs. respective wild type) at different exposure to valproate. Data are (weighted) counts (percent), mean±SD or geometric mean (%CV) for lamotrigine (LAM) dose-adjusted troughs (on ln-transformed data) (outcome). Shown are also standardized differences (d) for balancing variables and for the outcome.

	Before entropy balancing			After entropy balancing		
UGT2B7 -161C>T	Treated: CT/TT	Control: CC	d	Treated: CT/TT	Control: CC	d
Valproate 0/BLOQ						
N	256	75	---	256	75	---
Men	90 (35.2)	26 (34.7)	0.010	89.7 (35.0)	26.3 (35.0)	0.000
Age (years)	40±15	43±16	-0.160	41±15	41±15	0.000
Body weight (kg)	76±18	74±17	0.086	75±17	75±17	0.000
ABCG2 c.421 CA/AA	60 (23.4)	12 (16.0)	0.188	55.7 (21.7)	16.3 (21.7)	0.000
ABCB1 1236 CT/TT	169 (66.0)	53 (70.7)	-0.100	171.7 (67.1)	50.3 (67.1)	0.000
*UG1A4*3 c.142* TG/GG	57 (22.3)	19 (25.3)	-0.072	58.8 (23.0)	17.2 (23.0)	0.000
LAM (μmol/L/100 mg)	64(61)	65 (55)	-0.038	64 (60)	63 (55)	0.032
Valproate >0/BLOQ						
N	96	44	---	96	44	---
Men	53 (55.2)	19 (43.2)	0.242	49.4 (51.4)	22.6 (51.4)	0.000
Age (years)	35±13	36±13	-0.076	35±14	35±13	0.000
Body weight (kg)	75±17	73±16	0.112	74±17	74±14	0.000
Ln(valproate) (μmol/L)	5.78±0.49	5.82±0.39	-0.312	5.83±0.47	5.83±0.42	0.000
ABCG2 c.421 CA/AA	17 (17.7)	4 (9.1)	0.255	14.4 (15.0)	6.6 (15.0)	0.000
ABCB1 1236 CT/TT	56 (58.2)	34 (77.3)	-0.414	61.7 (64.3)	28.3 (64.3)	0.000
*UG1A4*3 c.142* TG/GG	20 (20.8)	10 (22.7)	-0.046	20.6 (21.4)	9.4 (21.4)	0.000
LAM (μmol/L/100 mg)	155 (48)	170 (40)	-0.223	157 (48)	161 (39)	-0.059
*UGT1A4*3 c.142 T>G*	Treated:TG/GG	Control: TT	d	Treated:TG/GG	Control: TT	d
Valproate 0/BLOQ						
N	76	255	---	76	255	---
Men	20 (26.3)	96 (37.7)	-0.245	26.6 (35.0)	89.4 (35.0)	0.000
Age (years)	40±14	41±15	-0.031	41±15	41±15	0.000
Body weight (kg)	71±16	76±18	-0.307	75±19	75±18	0.000
ABCG2 c.421 CA/AA	24 (41.6)	48 (18.8)	0.297	16.5 (21.7)	55.5 (21.7)	0.000
ABCB1 1236 CT/TT	52 (68.2)	170 (66.7)	0.037	51.0 (67.1)	171.0 (67.1)	0.000
UG2B7 -161 CT/TT	57 (75.0)	199 (78.0)	-0.072	58.8 (77.3)	197.2 (77.3)	0.000
LAM (μmol/L/100 mg)	62 (61)	64 (59)	-0.058	64 (61)	64 (59)	0.000
Valproate >0/BLOQ						
N	30	110	---	30	110	---
Men	15 (50)	57 (51.8)	-0.036	15.4 (51.4)	56.6 (51.4)	0.000
Age (years)	34±13	36±13	-0.128	35±14	35±13	0.000
Body weight (kg)	72±17	75±16	-0.181	74±16	74±16	0.000
Ln(valproate) (μmol/L)	5.81±0.53	5.83±0.45	-0.035	5.83±0.51	5.83±0.44	0.000
ABCG2 c.421 CA/AA	4 (13.3)	17 (15.5)	-0.060	4.5 (15.0)	16.5 (15.0)	0.000
ABCB1 1236 CT/TT	24 (80.0)	66 (60.0)	0.447	19.3 (64.3)	70.7 (64.3)	0.000
UG2B7 -161 CT/TT	20 (66.7)	76 (69.1)	-0.051	20.6 (68.6)	75.4 (68.6)	0.000
LAM (μmol/L/100 mg)	147 (43)	163 (46)	-0.242	147 (40)	164 (47)	-0.265

Fig 1 A Differences [as geometric means ratios (GMR) with 95% and 90% confidence/credible intervals] in dose-adjusted lamotrigine troughs between patients heterozygous/variant homozygous at *UGT2B7 -161C>T* or at *UGT1A4*3 c.142T>G* and their respective wild-type (wt) controls: overall ("main effects") and at different levels of valproate exposure [valproate trough 0 or below the limit of quantification (BLOQ) and valproate trough >0/BLOQ]. Vertical gray lines indicate GMRs 0.90 and 1.11, a range within which typically GMR point-estimates fall under equivalent exposure; vertical black lines indicate GMRs 0.80 and 1.25, a conventional acceptance range for the 90% CIs around point estimates for a claim of equivalent exposure. **B** Frequentist sampling distributions (left) and Bayesian posterior distributions (right) (we simulated 40000 distributions for each) of GMRs for variant allele carriers (i.e., *UGT2B7 -161 CT/TT* or *UGT1A4 c.142 TG/GG*) vs. respective wt controls estimated at valproate 0/BLOQ and at valproate >0/BLOQ. Vertical dashed lines indicate GMR point estimates. The general overlap of estimated effect distributions illustrates their close similarity at both levels of exposure to valproate for both polymorphisms.

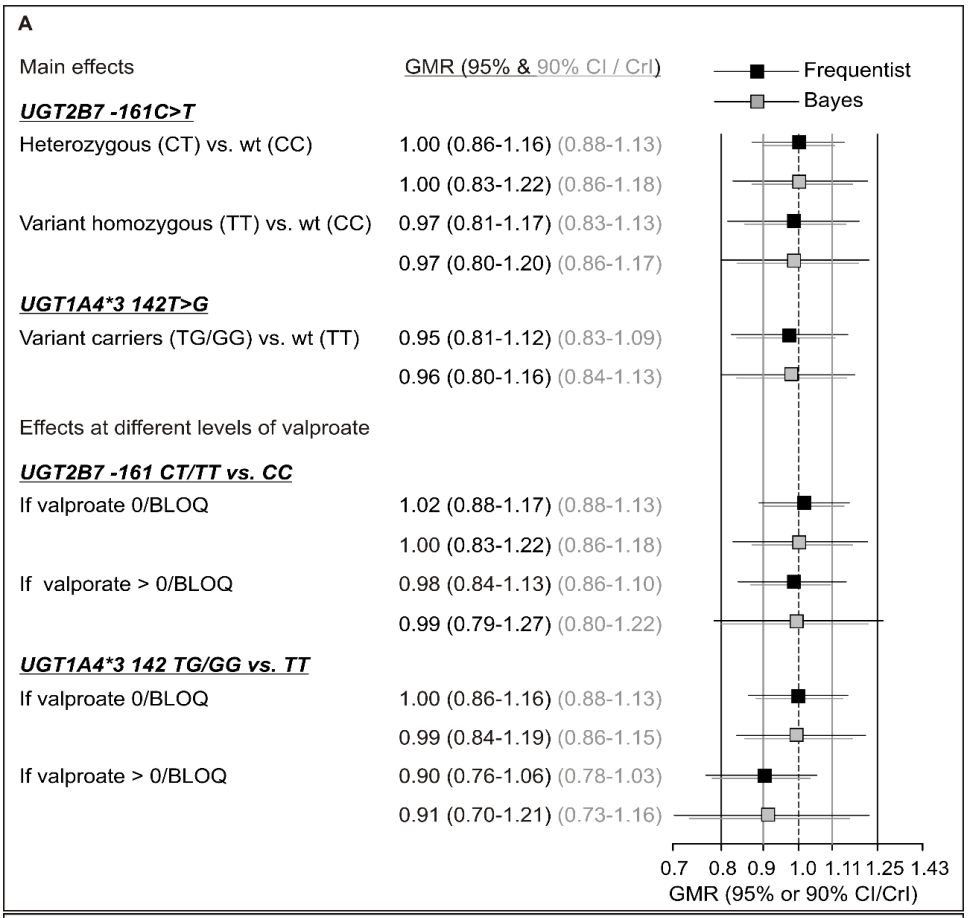
Fig 2 Sensitivity analysis – shown are observed (main) effects (point-estimate geometric means ratios, GMRs) corrected for bias due to unmeasured confounding. We assumed that a set of unmeasured covariates ("biasing set") had an effect on dose-adjusted lamotrigine troughs and that it could have either increased them or reduced them. We further assumed that the total prevalence of such a set in the current sample was 25%, but with imbalance between "treated" (in the case of *UGT2B7 -161C>T* polymorphism, treated are either CT or TT subjects; in the case of *UGT1A4*3 c.142T>G*, treated are TG/GG subjects) and "control" subjects (CC and TT, respectively) of 2:1 or 4:1 (see Sensitivity to unmeasured confounding for details). **A** In the case of *UGT2B7* polymorphism, previous reports suggested that CT or TT genotypes were associated with higher lamotrigine levels. Hence, it is assumed that the observed GMRs for CT vs. CC subjects (GMR=1.00) and for TT vs. CC subjects (GMR=0.97) are due to a biasing effect of unmeasured confounders that "pushed" GMR to <1.0, and was moderate (GMR=0.80) or strong (0.70). **B** In the case of *UGT1A4* polymorphism, previous reports suggested that variant allele was associated with lower lamotrigine troughs. Hence, it is assumed that the observed GMR for TG/GG vs. GG subjects (GMR=0.95) is due to a biasing effect that "pushed" GMR towards 1.0 (i.e., towards >1.0) and was moderate (GMR=1.25) or strong (1.43).

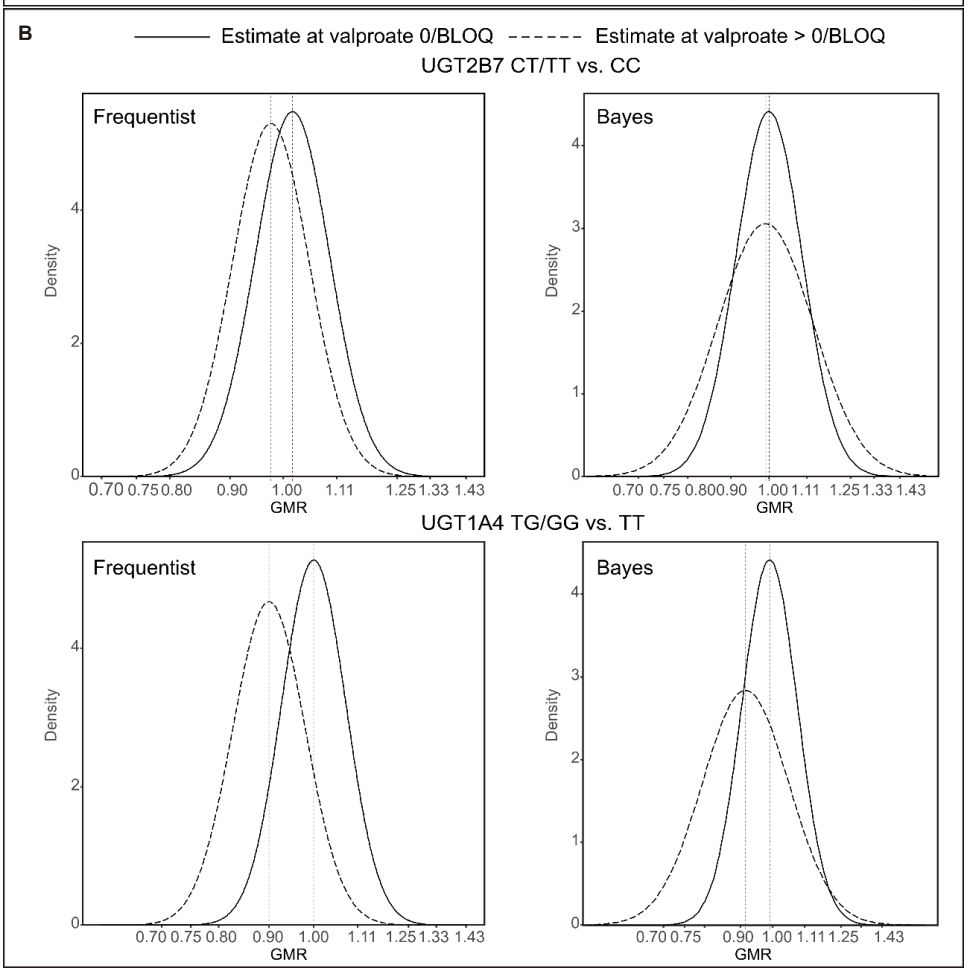
Supplementary Information - Content

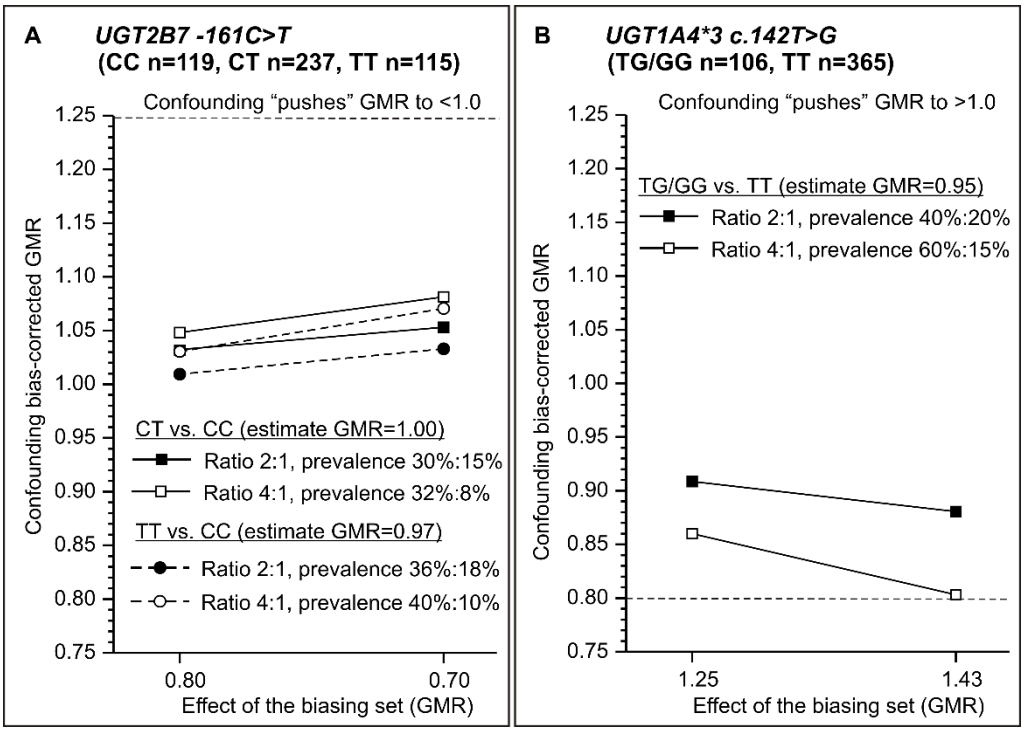
Methods to achieve conditional exchangeability – Fig S1, Fig S2

Sensitivity of GMR to unmeasured confounding

Weights assigned to "treated" and "control" subjects – Table S1


A
Main effects
GMR (95% & 90% CI / CrI)
Frequentist
Bayes
UGT2B7 -161C>T
Heterozygous (CT) vs. wt (CC)
1.00 (0.86-1.16) (0.88-1.13)
1.00 (0.83-1.22) (0.86-1.18)
Variant homozygous (TT) vs. wt (CC)
0.97 (0.81-1.17) (0.83-1.13)
0.97 (0.80-1.20) (0.86-1.17)
UGT1A4*3 142T>G
Variant carriers (TG/GG) vs. wt (TT)
0.95 (0.81-1.12) (0.83-1.09)
0.96 (0.80-1.16) (0.84-1.13)
Effects at different levels of valproate
UGT2B7 -161 CT/TT vs. CC
If valproate 0/BLOQ
1.02 (0.88-1.17) (0.88-1.13)
1.00 (0.83-1.22) (0.86-1.18)
If valporate > 0/BLOQ
0.98 (0.84-1.13) (0.86-1.10)
0.99 (0.79-1.27) (0.80-1.22)
UGT1A4*3 142 TG/GG vs. TT
If valproate 0/BLOQ
1.00 (0.86-1.16) (0.88-1.13)
0.99 (0.84-1.19) (0.86-1.15)
If valproate > 0/BLOQ
0.90 (0.76-1.06) (0.78-1.03)
0.91 (0.70-1.21) (0.73-1.16)
0.7 0.8 0.9 1.0 1.11 1.25 1.43
GMR (95% or 90% CI/CrI)


B
Estimate at valproate 0/BLOQ
Estimate at valproate > 0/BLOQ
UGT2B7 CT/TT vs. CC
Frequentist
Bayes
Density
GMR
0.70 0.75 0.80 0.90 1.00 1.11 1.25 1.33 1.43
UGT1A4 TG/GG vs. TT
Frequentist
Bayes
Density
GMR
0.70 0.75 0.80 0.90 1.00 1.11 1.25 1.33 1.43


A
UGT2B7 -161C>T
(CC n=119, CT n=237, TT n=115)
Confounding "pushes" GMR to <1.0
Confounding bias-corrected GMR
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
CT vs. CC (estimate GMR=1.00)
Ratio 2:1, prevalence 30%:15%
Ratio 4:1, prevalence 32%:8%
TT vs. CC (estimate GMR=0.97)
Ratio 2:1, prevalence 36%:18%
Ratio 4:1, prevalence 40%:10%
0.80
0.70
Effect of the biasing set (GMR)
B
UGT1A4*3 c.142T>G
(TG/GG n=106, TT n=365)
Confounding "pushes" GMR to >1.0
Confounding bias-corrected GMR
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
TG/GG vs. TT (estimate GMR=0.95)
Ratio 2:1, prevalence 40%:20%
Ratio 4:1, prevalence 60%:15%
1.25
1.43
Effect of the biasing set (GMR)

Supplemental Information: Online Resource 1

European Journal of Clinical Pharmacology

Bearing variant alleles at polymorphic uridine glucuronosyltransferase loci *UGT2B7 -161C>T* (rs7668258) or *UGT1A4*3 c.142T>G* (rs2011425) has no relevant consequences for lamotrigine troughs in adults with epilepsy

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Contents

Methods to achieve conditional exchangeability

We adopted the concept of causality as set forth by Pearl [1-4] and as implemented in R package *daggity* [5] – we generated a directed acyclic graph (DAG) in order to adequately identify roles of individual variables, define those that need to be controlled for in order to "exclude" and not introduce confounding/bias (as much as reasonably possible) (i.e., to "close" and not to "open" "backdoor paths" between the presumed cause and the outcome of interest) [6], and to identify sources of confounding that are practically almost impossible to control (considering, for example, a large number of potentially relevant polymorphisms). The essential elements of the emulated trials conceived to evaluate the effects of interest are shown in Figure S1 and most of the substantiative knowledge needed to "build" the graph is elaborated in the main text (additional elaboration added where appropriate): 1. The (potential) causal path (i.e., the one investigated) starts with a black circle (Fig S1) indicating genotype at the evaluated polymorphism – *UGT2B7 -161C>T* [CC (wild-type), CT or TT] or *UGT1A4*3 c.142T>G* [TT(wild-type) or TG/GG taken together since <1% patients were variant homozygous] – with a thick full black arrow projecting to the respective (UGT2B7 or UGT1A4) enzyme activity (depicted as a dark gray-black outlined circle), and a further thick black arrow projecting to the outcome(lamotrigine trough) depicted by a black circle. Typically, such a path indicates a causal effect of the "starting point" (exposure, treatment) on the outcome. The setting is specific in that the actual "exposure" or "treatment" is UGT2B7 or UGT1A4 enzyme activity important for lamotrigine clearance and known, at least *in vitro*, to be affected by the respective polymorphisms. However, as it cannot be measured directly *in vivo*, it remains unmeasured (hence – gray). The starting point, i.e., the genotype, on the other hand, can be determined and is used as a "proxy" of exposure. It has no other ways of affecting the outcome but by the effect on enzyme activity, has no effect on any other variable in the constellation of various elements, and thus qualifies as an instrumental variable. Still, it is not an "ideal" instrument. Since the two polymorphisms are each in complete linkage equilibrium (LD) with a variety of other polymorphisms in the respective genes, by identifying a specific genotype at these polymorphisms, one identifies subjects with particular broader genetic makeups that include all the linked polymorphisms (which, however, remain undetermined). Another shortcoming of this instrument is that it is not "exhaustive" – both *UGT2B7* and *UGT1A4* genes each harbor several tens of polymorphisms that are not linked (or are not known to be linked) to the genotyped loci and that might, although this is currently not known for a fact, also reflect on the respective enzyme activity: hence, they are depicted as "competing instrument" which, however, remains unmeasured (depicted as a dark gray circle); 2. Other elements in the DAG are depicted by blue circles indicating outcome ancestors (may affect the outcome) and their parents, or by red circles indicating "classical confounders" (may affect both the outcome and the exposure) and their parents; 3. Full arrows in the DAG indicate causality: thick black arrows indicate the assessed causal path, while thinner gray arrows depict the effect of parent variables on their descendants, i.e., outcome ancestors or "classical confounders". Black dashed arrows indicate "biasing paths" (with the respect to the investigated causal path), i.e., (known or very likely) effects of outcome ancestors on the outcome, and the effects of "classical confounders" on both the outcome and the exposure (UGT2B7 or UGT1A4 enzyme activity). These biasing paths might be direct, or mediated through one or more "downstream" variables (mediators);

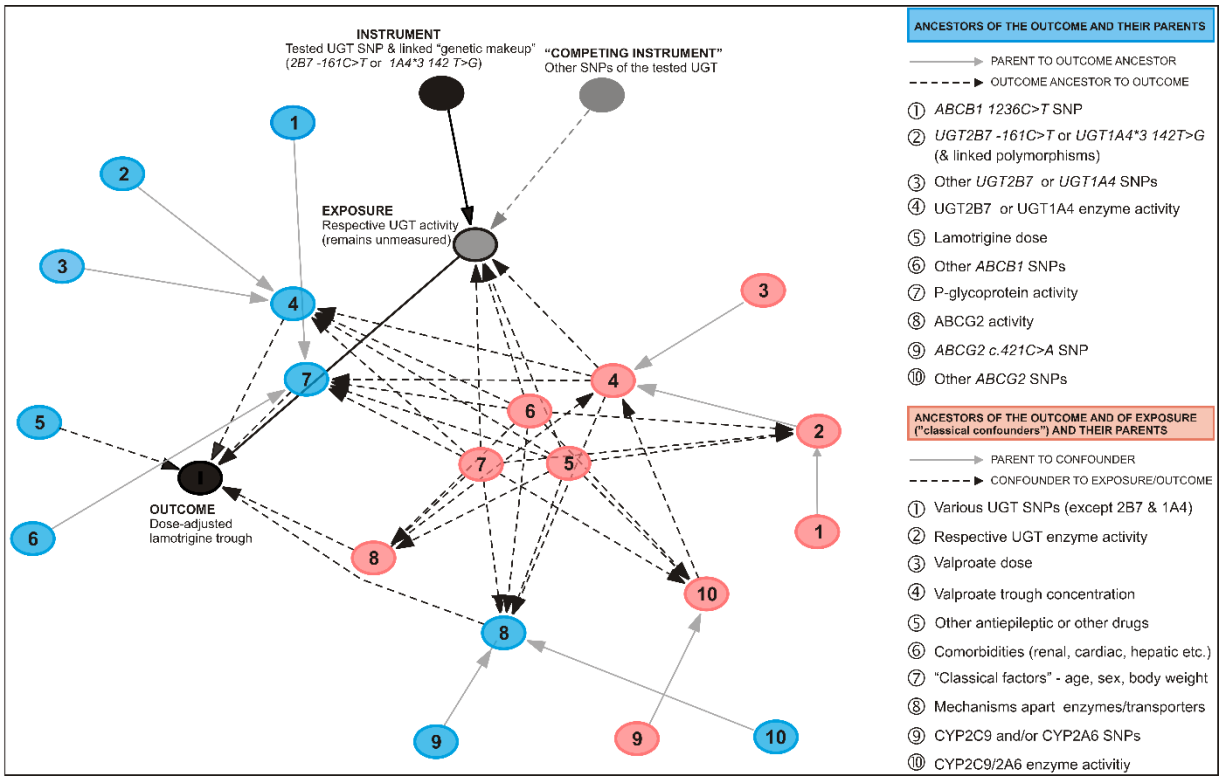

Fig S1 Directed acyclic graph (DAG, generated using package *daggity*) representing variables of interest in the investigated setting, and their roles. Black & dark gray-black outlined circles connected with thick black arrows depict the relationship of interest, i.e., the presumed (potential) causal path that is investigated: the outcome of interest is dose-adjusted lamotrigine trough; the actual exposure ("treatment") is the activity of the UGT2B7 or UGT1A4 enzyme(s) which, however, remains unmeasured but it is "represented" by an instrument: genotype at the respective polymorphism [*UGT2B7 -161C>T* (CC, CT or TT); or *UGT1A4*3 c.142T>G* (TT or TT/TG taken jointly)] and polymorphisms linked to it, which also are not measured (but are acknowledged based on the existing knowledge). The concept implies that the detected genotype represents a broader genetic makeup (includes polymorphisms in LD) that differs in wild-type (wt), heterozygous, and variant homozygous subjects with consequently different enzyme activity (i.e., results in "treated" vs. "control" activity). A completely dark gray circle denoted as a "competing instrument" indicates that the used instrument is not "ideal": it is known that both *UGT2B7* and *UGT1A4* genes harbor many other polymorphisms which are not (or are not known to be) linked with the typed polymorphisms and these (although, this is also not known for a fact, but is assumed) might also reflect on the respective enzyme activity ("exposure"). All black dashed arrows depict "direct" or "mediated" effects on only the outcome (arising from outcome ancestors – blue circles) or on both the outcome and the "exposure" (arising from confounders – red circles) that bias the investigated causal path. Most or some of the outcome ancestors and confounders have "parents", i.e., variables by which they are defined (or given birth) – causal effects of parents on their "children" (descendants) are denoted as gray full arrows. Note: in some cases, confounders affect the outcome through their "downstream" effects on the outcome ancestors (i.e., "red circles work 'through' the effects on blue circles").

4. It should be noted that the lack of arrows emerging from the outcome and ending in "exposure" or in any outcome ancestor or confounder (or their parent) illustrates that the condition of *no reverse causation* is met: (i) lamotrigine does not affect genotype (*UGT* or *ABCB1* and *ABCG2* genes); (ii) since blood samples were taken after at least 21 days of treatment, the initial induction of UGT1A4 and UGT2B7 enzymes (or other UGT enzymes) by lamotrigine has been completed, hence UGT1A4 or UGT2B7 activity (whether considered as "exposure" or as "outcome ancestor") or activity of any other UGT enzyme is not affected by lamotrigine; (iii) consequently, valproate levels (a confounder) are not affected by lamotrigine; (iv) lamotrigine does not affect the activity of ABCB1 or ABCG2 transporters; and (v) clearly, lamotrigine has no "reverse" effect on classical confounders/outcome ancestors like age, body weight, comorbidities or comedication. 5. Blue circles in Fig S1 – outcome ancestors: (i) we considered lamotrigine dose as a particularly relevant outcome ancestor; (ii) depending on which one was considered "exposure", UGT2B7 or UGT1A4 enzyme activity was considered an outcome ancestor, with several obvious and potential "parents" – polymorphisms *UGT2B7 -161C>T* or *UGT1A4*3 c.142T>G* (depending on which one was considered "instrument") and "other" (undetermined) polymorphism in the respective genes (depending on which were considered "competing instrument"), and antiepileptic (AED) or other drugs, comorbidities, exposure to valproate and "classical" factors such as age, sex, body weight – that might affect the enzyme activity (and are, otherwise, considered as confounders – see below); (iii) activity of the ABCG2 transporter (to which lamotrigine is a substrate) with several obvious and potential parents – polymorphism *ABCG2 c.421C>A*, other *ABCG2* polymorphisms, exposure to valproate (although valproate is not an ABCG2 substrate [7], there is evidence from human placental tissue that valproate might alter expression of the ABCG2 protein [8]), various AEDs and non-AED drugs that might reflect on ABCG2 activity, and comorbidities and classical factors such as age, body weight and sex, that might potentially reflect on ABCG2 activity (and are, otherwise, considered as confounders – see below); (iv) activity of the ABCB1 transporter (P-glycoprotein), with several obvious and potential parents – polymorphism *ABCB1 1236C>T* and other *ABCB1* polymorphisms, and other factors (as in the case of ABCG2 or UGT activity) that are otherwise considered as confounders (as in the case of ABCG2, valproate is not an ABCB1 substrate, but might alter expression of ABCB1 [7, 8]). 6. Red circles in Fig S2 – ("classical") confounders: (i) exposure to valproate. It is well known that valproate inhibits lamotrigine-metabolizing UGTs, i.e., UGT1A4 and UGT2B7, hence, depending on which is considered "exposure" and which is considered "outcome ancestor" valproate affects both the exposure and the outcome. It can further affect outcomes by affecting ABCB1 and/or ABCG2 activity (as mentioned). There are many obvious and potential "parents" to exposure to valproate: valproate dose; polymorphisms in genes encoding other UGTs (apart form UGT1A4 and UGT2B7) that affect activity of the respective enzymes involved in valproate clearance [9]; closely related "parents" are drugs and comorbidities that might reflect on activity of these UGTs (directly or through gene expression); activity of CYP2C9 and 2A6 (key for valproate clearance [10]) affected by polymorphisms and/or by other AED and non-AED drugs and/or comorbidities or "general" subject characteristics as age, sex, body weight and including unknown (assumed) mechanisms apart from the effects on enzymes and transporters; (ii) other AEDs or non-AED drugs, that might affect "exposure" (UGT2B7 or UGT1A4 activity) directly or by affecting exposure to valproate by affecting relevant UGT and/or CYP activity, or via any other mechanism apart from enzymes and transporters, and may affect the

outcome via effects on any of the outcome ancestors (UGT1A4 or UGT2B7 activity, ABCB1 activity, ABCG2 activity, or via a further mediator, which is exposure to valproate); (iii) comorbidities and/or "classical" factors like age, body weight and sex – that might affect both the "exposure" and the outcome via the same possible routes as depicted for comedication (apart from valproate).

Figure S2 shows the same DAG as Figure S1, but with different messages summarizing the results of the attempts to control confounding and, potentially, specifically estimate the tested causal path denoted by the black circles (instrument and outcome) connected by thick black arrows "via" exposure (dark gray-black outlined circle). All white circles indicate (potential) sources of confounding bias that were controlled for by different means. *Daggity* enables one to identify the "minimum adjustment set", i.e., the smallest possible set of confounders that need to be conditioned upon to close all backdoor paths, i.e., prevent spurious associations between the instrument ("exposure") and the outcome – i.e., it may not be needed to condition upon all of them individually to achieve the goal: for example, valproate dose is a direct and the most important parent to valproate trough – an important confounder – but, if one conditions on valproate trough, one does not need to condition on its parent valproate dose. *Daggity* "recognizes" confounders (and their parents or descendants) by their "classical" definition which includes links to both exposure and the outcome. It does not "recognize" variables that affect only the outcome (i.e., outcome ancestors and their parents) – but these also need to be controlled to evaluate the causal path of interest [6]. In Figure S2, white black-outlined circles depict confounders/outcome ancestors that we directly conditioned upon by different means: i) inclusion/exclusions criteria – relevant comorbidity and comedication (AED or non-AED) apart from valproate; ii) definition of the outcome as "dose-adjusted" lamotrigine trough controls for the effect of the outcome ancestor "lamotrigine dose"; iii) statistical adjustment (entropy balancing) – *ABCB1 1236C>T* polymorphism (and polymorphisms that are in a strong or complete LD), thus "removing" their contribution to P-glycoprotein activity; *UGT2B7 -161C>T* or *UGT1A4*3 c.142T>G* polymorphism when considered as a covariate (and polymorphisms that are in a strong or complete LD with it), thus "removing" their impact on their descendant "enzyme activity"; *ABCG2 c.421C>A* polymorphism (and polymorphisms in a strong or complete LD), thus removing its contribution to ABCG2 activity; valproate trough concentrations and classical factors age, body weight and sex, thus removing their impact (direct or mediated) on exposure and the outcome. White-red outlined circles in Figure 2S depict confounder parents that were controlled "indirectly", i.e., by directly controlling their children: by conditioning on valproate trough, blocked is the influence of all of its ancestors, i.e., valproate dose, CYP2C9/2A6 polymorphisms reflecting on enzyme activity, polymorphisms/activity of UGTs (other than 2B7 and 1A4) and others that are known to- or might affect valproate clearance. Light gray circles in Figure S2 denote elements that were not controlled for and that remain potential sources of confounding bias: i) "competing instrument"– an (apparent) effect of the assessed instrument on the outcome, or a lack of an effect might be due to the biasing effect of the "competing instrument" on exposure; ii) *UGT2B7* or *UGT1A4* polymorphisms other than the genotyped ones (and their linked polymorphisms) when considered as outcome ancestors might have affected the activity of the respective enzyme; similarly, *ABCG2* and *ABCB1* polymorphisms other than genotyped ones (and their linked polymorphisms) might have affected the activity of the respective transporters. Note, however, that

UGT2B7/UGT1A4 activity, P-glycoprotein activity and ABCG2 activity are denoted as light gray-black outlined circles: their activity was partially "controlled for" by controlling other factors, but not in full since their mentioned parents were not controlled for. Finally, there is always a possibility of some unmeasured confounding arising from factors that have not been known so far to impact lamotrigine concentrations. Open biasing paths from "competing instrument" to exposure, and those coming into only "partly controlled" outcome ancestors from their parents are depicted as full gray arrows. The dashed black arrows indicate biasing paths emerging from "only partly controlled" outcome ancestors towards the outcome. Other black dashed lines are blocked and indicate blocked backdoor paths.

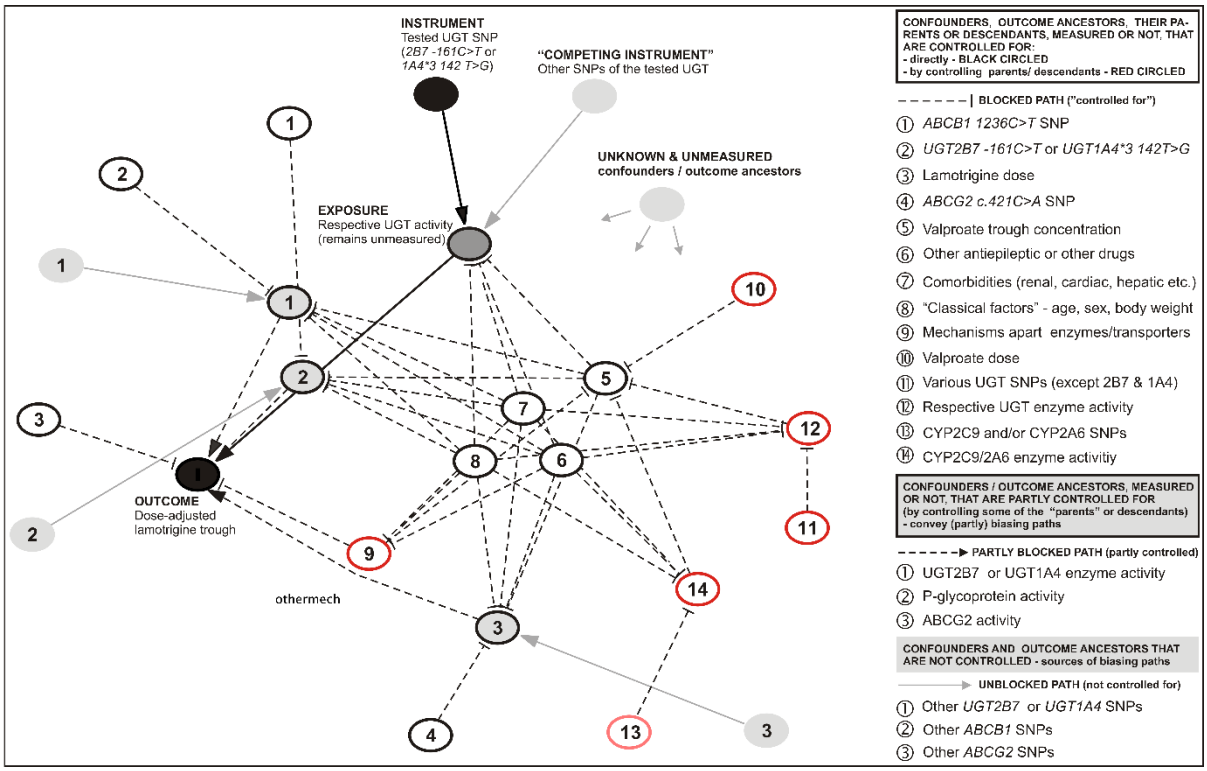

Figure S2. DAG from Figure S1 with denoted ancestors/confounders and their parents and/or descendants that were fully controlled for (white circles – black outline if controlled directly, red outline if controlled by controlling descendants), that were partly controlled for (light gray-black outlined circles), or were not controlled for (light gray circles). The full gray arrow from "competing instrument" to "exposure" indicates its (presumed) effect on the exposure. Other full gray arrows indicate effects "coming in" to partly controlled outcome ancestors from (some of) their parents. Dashed black arrows indicate biasing effects of outcome ancestors on the outcome. Other, blocked, dashed lines indicate blocked backdoor paths.

Therefore, the whole concept *a priori* acknowledged existence of otherwise known or reasonably suspected confounders/outcome ancestors that remained uncontrolled– this emphasized the need to evaluate susceptibility of the generated estimates to unmeasured confounding.

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Sensitivity of GMR to unmeasured confounding

The method used to address sensitivity of the observed estimates to unmeasured confounding, i.e., to generate bias-corrected estimates was developed for relative risks (RR), while geometric means ratio (GMR) was the outcome measure used in the present study. Still, we deemed the method appropriate for the purpose due to certain common features of RR and GMR: (i) both are exponents of difference in means of ln-transformed quantities (risk or a right-tailed continuous variable like drug concentration); (ii) both ln(risk) and ln(right-tailed continuous variable) have a normal distribution and their interpretation is similar as they provide information about a relative difference between treatment and control. If for a treatment vs. control RR >1.0, e.g., 1.5, it means relatively by 50% higher risk with treatment, just as is the case with GMR: if 1.5, it means relatively by 50% higher value of the measured quantity with treatment.

Weights assigned to "treated" and "control" subjects

The entropy balancing algorithm defines constraints on the moments of covariates in a balancing set and then searches for weights to be assigned to each "treated" and "control" subject (or subjects in other subsets if more than two) so that the defined constraint is met [1]. For example, if a "balancing set"

contains several continuous and several categorical covariates, the procedure searches for weights that will, in the end, result in identical means and proportions across the balanced subsets (i.e., standardized mean differences will be 0), or in closely similar means/proportions so that the standardized mean differences will be <0.1 (a limit typically used to identify adequate balance). At the same time, the mean of the weights in each subject subset will be 1.0 with a moderate standard deviation, weight ranges will overlap and there will be no extreme weights that would require trimming [to avoid overt dependence on a few highly (under)weighted individuals]. For this to be possible, raw data across the subject subsets should show a reasonable level of overlap in the values of all covariates. When such an overlap is modest, some assigned weights will be rather high (or, reciprocally, low), standardized differences will differ from 0 or might exceed the limit of 0.1. However, there are also situations where raw data overlap is so poor that entropy balancing is not possible. In the present study, for all "emulated trials", entropy balancing was successful with all standardized differences being 0 and with assigned weights within reasonably narrow ranges (Table S1).

Table S1 Summary of weights assigned by entropy balancing in all "emulated trials" in the present study.

	Mean	Min - Max	RSD
Overall *UGT2B7 -161 C>T*			
CC	1.00	0.496-2.243	0.327
CT	1.00	0.673-1.427	0.161
TT	1.00	0.565-2.256	0.331
Overall *UGT1A4 c.142T>G*			
TT	1.00	0.753-1.455	0.118
TG/GG	1.00	0.311-2.463	0.432
UGT2B7 -161C>T at valproate 0/BLOQ			
CC	1.00	0.508-1.927	0.265
CT/TT	1.00	0.827-1.120	0.066
UGT2B7 -161C>T at valproate >0/BLOQ			
CC	1.00	0.539-2.391	0.453
CT/TT	1.00	0.598-1.386	0.181
UGT1A4 c.142T>G at valproate 0/BLOQ			
TT	1.00	0.761-1.320	0.106
TG/GG	1.00	0.485-3.180	0.377
UGT1A4 c.142T>G at valproate >0/BLOQ			
TT	1.00	0.713-1.295	0.115
TG/GG	1.00	0.452-2.498	0.514

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