

Single nucleotide polymorphisms in the intergenic region between metformin transporter OCT2 and OCT3 coding genes are associated with short-term response to metformin monotherapy in type 2 diabetes mellitus patients

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Abstract

Objective(s): High variability in clinical response to metformin is often observed in type 2 diabetes (T2D) patients, and it highlights the need for identification of genetic components affecting the efficiency of metformin therapy. Aim of this observational study is to evaluate the role of tagSNPs (tagging single nucleotide polymorphisms) from genomic regions coding for six metformin transporter genes with respect to the short-term efficiency.

Design: 102 tagSNPs in 6 genes coding for metformin transporters were genotyped in the group of 102 T2D patients treated with metformin for 3 months.

Methods: Most significant hits were analyzed in the group of 131 T2D patients from Slovakia. Pharmacokinetic study in 25 healthy nondiabetic volunteers was conducted to investigate the effects of identified polymorphisms.

Results: In the discovery group of 102 patients, minor alleles of rs3119309, rs7757336 and rs2481030 were significantly nominally associated with metformin inefficiency ($P = 1.9 \times 10^{-6}$ to 8.1×10^{-6}). Effects of rs2481030 and rs7757336 did not replicate in the group of 131 T2DM patients from Slovakia alone, whereas rs7757336 was significantly associated with a reduced metformin response in combined group. In pharmacokinetic study, group of individuals harboring risk alleles of rs7757336 and rs2481030 displayed significantly reduced AUC_{∞} of metformin in plasma.

Conclusions: For the first time, we have identified an association between the lack of metformin response and SNPs rs3119309 and rs7757336 located in the 5' flanking region of the genes coding for Organic cation transporter 2 and rs2481030 located in the 5' flanking region of Organic cation transporter 3 that was supported by the results of a pharmacokinetic study on 25 healthy volunteers.

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Introduction

Metformin (1,1-dimethylbiguanide) is prescribed to at least 120 million people worldwide to treat hyperglycemia (1). It is recommended as the first drug of choice for type 2 diabetes (T2D) according to the guidelines of the ADA (American Diabetes Association) and EASD (European Association for the Study of Diabetes), unless not tolerated or contraindicated (2, 3). Metformin provides approximately 1–1.5% improvement of HbA_{1c} and decreases insulin resistance (4, 5). A feature of the metformin therapy is the high variability in glycemic response ranging from good clinical response up to showing no benefit, and high prevalence of common side effects (5–30%) (6, 7, 8). Up to about a third of the patients using metformin monotherapy were unable to achieve the HbA_{1c} target of 7% (53 mmol/mol) as a treatment goal (9).

Metformin is not metabolized in the human body (10); its passive diffusion through the cell membranes is very limited (11). Its distribution, transportation to the target tissues and subsequent excretion from the organism are ensured by organic cation transporters (OCTs), multidrug and toxin extrusion antiporters (MATEs) and plasma membrane monoamine transporter (PMAT) to which metformin is a substrate. PMAT (*SLC29A4*) is expressed in human small intestine tissues and is thought to be involved in the gastrointestinal absorption of metformin (12). Organic cation transporter 1, OCT1 (*SLC22A1*), is expressed mainly in the liver and is a major mediator for the metformin uptake into the blood stream from enterocytes and the hepatic uptake of metformin (13). Organic cation transporter 3, OCT3 (*SLC22A3*), has a diverse expression pattern and is thought to mediate metformin uptake in the gastrointestinal tract, muscles, heart and liver; thus, it possibly may balance decreased function of OCT1 and PMAT (13, 14, 15). Organic cation transporter 2, OCT2 (*SLC22A2*), is expressed mainly in the kidney, where it transports metformin into the proximal tubule cells (16). Finally, multidrug and toxin extrusion antiporters 1 and 2, MATE1 and MATE2K (*SLC47A1*, *SLC47A2*) are expressed in brush border of the renal proximal tubule and excrete metformin into the urine (17, 18).

Genome-wide complex trait analysis (GCTA) results showed that contribution of heritability in glycemic response to metformin could be 20–34% and suggested that mainly a spectrum of many genetic variants with small or moderate effect is involved (19). Results of the first genome-wide association study (GWAs) on the long-term efficiency of metformin support this hypothesis as

the polymorphism rs11212617, located near the ataxia-telangiectasia-mutated gene (*ATM*) and significantly associated with metformin response, could explain only approximately 2.5% of its variability (20). Several case-control pharmacogenetic studies have investigated the associations between metformin efficiency and pharmacokinetic parameters with SNPs in *OCT1*, *OCTN1*, *OCT2*, *OCT3*, *MATE1* and *MATE2K* coding genes (21, 22, 23, 24). Current findings indicate that research on metformin efficiency requires complex methods involving the identification of genetic variability in metformin transporters and pharmacokinetic parameters obtained in different mediums (like plasma, erythrocytes and urine) simultaneously.

In this study, we aimed to evaluate the clinical consequences of systematically selected tagSNPs from genomic regions coding for six metformin transporter genes with respect to the relatively short-term efficiency, where the metformin transporter effects may be the most prominent.

Research design and methods

Study group

Study was based on a prospective OPTIMED cohort that was launched in 2010 in the framework of the Latvian National Research Programme BIOMEDICINE. 28 endocrinologists from health care centers and hospitals were involved in the recruitment of patients with T2D to maintain up to a 3-year long follow-up study. All participants were included in the government-funded Genome Database of Latvian Population (LGDB) (27). Inclusion criteria were the following: patients with ICD-10 E11 diagnosis (fasting blood glucose test result ≥ 7 mmol/L and/or OGT test result ≥ 11 mmol/L), drug naïve, over 18 years old, signed informed consent and lack of pregnancy in women. Baseline data on other diagnoses, history of gestational diabetes, anthropometric measurements (height, weight, waist circumference and blood pressure), intolerance of antidiabetic drugs and biochemical analysis were gathered. Overall, 313 prospective study participants (OM) were recruited in the study on January 2015. 102 patients who have received metformin monotherapy for 3 months and had corresponding HbA_{1c} measurements (95.5 \pm 9.0 days after beginning of the therapy) were selected for this study. Time before baseline HbA_{1c} measurement and start of the therapy was <30 days and was not significantly associated

with ΔHbA_{1c} values. OPTIMED project protocol was approved by the Central Medical Ethics Committee of Latvia (Protocol No. 01-29.1/22).

In Slovakia, the study was conducted in a university hospital setting. T2D was diagnosed in patients according to the criteria of the American Diabetes Association. The Louis Pasteur University Hospital Review Board gave ethical approval for this study. All participating subjects gave a written consent to be included in the study. 148 patients of Caucasian origin were recruited from three out-patient clinics. Patients with malignancies, another endocrine disorders, chronic kidney disease stage 3–5, severe liver disease and systemic inflammatory disease were excluded. Only drug-naïve patients with HbA_{1c} in the range of 6.5–11% (48–97 mmol/mol) were included. Baseline HbA_{1c} measurement was performed within 1 week before the treatment initiation and second measurement was taken after 6 months of metformin monotherapy. 131 patients were further included in the study as they had HbA_{1c} measurements in the corresponding period (101.5 ± 20.6 days).

Twenty-five volunteers from Latvia in the pharmacokinetic study group ranged between the ages of 22 and 37 years and have been evaluated to be healthy by a medical doctor. All participants have signed informed consent, and the study was approved by the Committee of Ethics (Nr.3000610-18L). Study group and determination of metformin in blood are described in [Supplementary methods](#) (see section on [supplementary data](#) given at the end of this article).

SNP selection and genotyping

We developed a genotyping panel using HaploView 4.2 (27 genome release) for SNP selection in CEU+TSI population. Due to the relatively small expected sample size, only variations exceeding minor allele frequency (MAF) of 0.05 in Caucasians were included. Altogether 192 SNPs and tagSNPs in regions covering the 52 genes that are previously reported as influencing the efficiency and tolerability of peroral antidiabetic therapy were selected. For a particular study, 108 tagSNPs located in OCT1 (*SLC22A1*), OCT2 (*SLC22A2*), OCT3 (*SLC22A3*), MATE1 (*SLC47A1*), MATE2K (*SLC47A2*) and PMAT (*SLC29A4*) regions were used. DNA samples for genotyping analysis were provided by LGDB. Genotyping was performed in all patients using GoldenGate Genotyping Assay with VeraCode technology (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. One positive control

sample in each assay plate was used; 19 duplicate samples were randomly distributed among the plates. Concordance rate of the randomly distributed duplicate samples was 99.7%. The GeneCall threshold was set to 0.25, and samples with call rates lower than 0.9 were excluded. The cluster images of signal intensity were reviewed manually for all SNPs. 18 from 192 SNPs were excluded from the panel after quality control due to failed genotyping or weak genotype separation due to low fluorescence. Primary data analyses were performed using Illumina GenomeStudio software as previously reported (26).

In the patient group from Slovakia, OCT2 rs7757336 and OCT2-OCT3 rs2481030 polymorphisms were analyzed by high-resolution melting analysis after real-time PCR in the presence of LCGreen Plus dye and an unlabeled probe on Eco Real-Time PCR System (Illumina). Genotypes were identified using Eco Software 4.1. Genotyping success rate for all the examined variants was 100%, and duplicate genotyping concordance was 100% (15 samples for each SNP. Where available, 5 samples for each genotype were used).

Genetic testing of rs2481030 and rs7757336 in pharmacokinetic group was carried out using the Applied Biosystems TaqMan SNP (Applied Biosystems). Genotyping assay was performed with a modified protocol using 4.75 mL TaqMan Genotyping Mix, 0.25 mL SNP genotyping assay and 5 mL Millipore H_2O on a 7500 Real-Time PCR system (Applied Biosystems). AutoCaller 1.1 (Applied Biosystems) software was used to assign genotype calls for all samples simultaneously (genotype call rate – 100%, concordance rate – 100%).

Statistical analysis

To assess the genotyping quality, statistical analysis was performed with the PLINK v1.07 software (<http://pngu.mgh.harvard.edu/purcell/plink/>) (27). Two SNPs from genotyping panel were excluded due to deviation from the Hardy–Weinberg equilibrium $P < 0.05$ in the controls of discovery group. Altogether 102 tagSNPs with a genotyping rate of 97.2% in 102 individuals were used in further statistical analysis. Statistical power was calculated using Quanto software (Natara Software, Naperville, IL, USA). Our sample size provided 80% power (at $P = 0.05$) to detect an odds ratios (ORs) from 1.55 to 2.9 depending on MAF of SNPs.

Tidwell-Box linearity test, standardized residual values, standard errors of mean (S.E.M.) of independent variables and Pearson's r were obtained using SPSS 13.0

(Standard version, Chicago, IL, USA) to analyze the quality of data and confirm the use of samples in logistic and linear regressions performed. The Durbin–Watson test, Kolmogorov–Smirnov test and Shapiro–Wilk test, VIF and tolerance and standardized residuals were obtained using SPSS 13.0 to analyze the quality of data retrieved from pharmacokinetic study.

Logistic regression of responders vs non-responders (reduction vs no reduction or increase in HbA_{1c}) was performed using PLINK 1.07 open software assuming an additive mode of inheritance to estimate the association of SNPs with non-responsiveness after 3 months of metformin monotherapy in the discovery and replication groups and using a number of cofactors (age, sex, BMI, HbA_{1c}, days of treatment, dose of metformin and creatinine clearance) to adjust the analysis for other non-genetic factors. Linear regression was performed using PLINK 1.07 open software to estimate the association of SNPs with absolute changes in the obtained pharmacokinetic parameters and to correct for covariates (creatinine clearance, age, sex and weight).

HaploReg2 was used for exploring annotations of the noncoding genome at variants on the haplotype blocks and LD information from the 1000 Genomes Project (<http://www.broadinstitute.org/mammals/haploreg/haploreg.php>). IMPUTE version 2 was used for a genotype imputation and haplotype phasing with combined data from 1000 Genomes Pilot project and HapMap 3 (28) (https://mathgen.stats.ox.ac.uk/impute/impute_v2.html). The 1000 Genomes project browser (<http://browser.1000genomes.org/index.html>), which contains the database of phase3 autosomal variants, was used to predict the linkage disequilibrium among SNPs of interest.

Results

Genetic association study in discovery and replication groups

Characteristics of T2D patients included in the study are given in Table 1. Baseline HbA_{1c} level was similar in both groups; however, the discovery group showed significantly better treatment results in a shorter period in comparison with the replication group (Δ HbA_{1c}, 0.9% vs 0.6%). BMI and dose of metformin showed significant differences between both the groups. Δ HbA_{1c} values were not normally distributed. Thus, Tidwell–Box linearity test (P value >0.05), Cook's distance values <1, standardized residual values <2.58 and under maximum leverage value confirmed the use of logistic regression to ascertain the effects of age, creatinine clearance, baseline BMI and HbA_{1c}, sex, length of therapy, dose of metformin and 102 SNPs of the 6 metformin transporters (OCT1, OCT2, OCT3, MATE1, MATE2-K and PMAT) on the likelihood that participants have non-responder phenotype (no changes vs increased HbA_{1c} level) after 3 months of metformin monotherapy as described previously (29).

Data for the associations of all SNPs in the discovery group are shown in Supplementary Table 1. In total, 26 SNPs were nominally (P <0.05) associated, whereas only 3 SNPs remained significantly associated after the correction for multiple testing. Table 2 shows 3 SNPs that were significantly associated with non-responder phenotype after Bonferroni correction, rs2481030, rs7757336 and rs3119309. The 1000 Genomes project browser (<http://browser.1000genomes.org/index.html>) predicted a high linkage disequilibrium (D' =1.0) among rs7757336, rs3119309 and rs2481030 in the Caucasians

Table 1 Characteristics of T2D patient groups from Latvia and Slovakia.

Characteristics	Discovery group (n=102)	Replication group (n=131)	P values
Male, n (%)	33 (32.4)	67 (51.1)	0.006
Female, n (%)	69 (67.6)	64 (48.9)	0.006
Mean age \pm s.d., years	59.7 \pm 10.6	57.4 \pm 10.7	0.104
Mean BMI \pm s.d., kg/m ² , baseline	33.8\pm4.8	31.4\pm4.5	<0.0001
Creatinine clearance \pm s.d., mL/min	120.1\pm43.7	105.4\pm37.1	0.006
Dose of metformin \pm s.d., mg/per day	1525.0\pm533.5	1053.1\pm486.4	<0.0001
Non-responders to the treatment (decrease of HbA _{1c} , balance) n, (%)	18 (17.6)	19 (14.5)	0.644
Days between HbA _{1c} measurements \pm s.d.	95.5\pm9.0	101.5\pm20.6	0.006
HbA _{1c} \pm s.d., %, mmol/mol, baseline	7.4 \pm 1.5 (57 \pm 16.4)	7.6 \pm 1.0 (60 \pm 10.9)	0.225
HbA _{1c} \pm s.d., %, mmol/mol, after treatment, %	6.5 \pm 0.6 (48 \pm 6.6)	7.0 \pm 0.7 (53 \pm 7.7)	<0.0001
Decrease of HbA _{1c} \pm s.d., after treatment, %	0.9\pm1.3	0.6\pm0.9	0.039

P values <0.05 are marked with bold. P values were assessed using t-test and comparison of proportions.
s.d., standard deviation; BMI, body mass index.

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Table 2 SNPs associated with efficiency of metformin monotherapy in patients from Latvia and Slovakia.

SNP		Genotype number		OR (95% CI) [†]	P [‡]	P [‡]
		Non-responders	Responders			
rs7757336	Discovery group	2/9/7	0/14/70	50.360 (5.998–422.900)	3.06*e⁻⁴	0.002
	Replication group	0/7/12	1/22/89	1.323 (0.4089–4.282)	0.640	0.826
	Combined analysis	2/16/19	1/36/159	4.029 (1.956–8.301)	1.578*e⁻⁴	3.137*e⁻⁴
rs3119309	Discovery group	1/8/9	0/9/75	26.580 (4.631–152.500)	2.34*e⁻⁴	0.001
	Replication group	NA	NA	NA	NA	NA
	Combined analysis	NA	NA	NA	NA	NA
rs2481030	Discovery group	10/7/1	8/38/38	13.700 (3.435–54.670)	2.09*e⁻⁴	0.002
	Replication group	2/9/8	10/50/52	1.279 (0.563–2.907)	0.557	0.342
	Combined analysis	12/16/9	18/88/90	2.933 (1.674–5.137)	1.686*e⁻⁴	0.052

Discovery group, Latvia, $n=102$ patients, Replication group, Slovakia, $n=131$ [†]OR and P values estimated from logistic regression adjusted for age, sex, baseline BMI, number of treatment days, dose of metformin mg/per day, creatinine clearance (Cockcroft-Gault equation).

[‡] P value estimated from logistic regression adjusted for age, sex, baseline BMI, dose of metformin mg/per day, creatinine clearance (Cockcroft-Gault equation), number of treatment days and baseline HbA_{1c}. P values significant after Bonferroni correction are marked bold.

(CEU) with variable r^2 due to differences between MAFs ($r^2=0.182$ – 0.657).

The logistic regression model of combined minor alleles in the discovery group (count of minor alleles of rs2481030 and rs7757336) was statistically significant (χ^2 (7)=67.729, $P<0.001$). The model explained 80.0% (Nagelkerke R^2) of the variance in response to metformin, and carriers of minor alleles were 12.8 times more likely to exhibit non-responder phenotypes than participants with reference alleles. Longer time of the therapy, higher baseline HbA_{1c} and women gender were associated with a lower likelihood of exhibiting non-responder phenotype ($P<0.05$). Wide confidence intervals of models can be explained by small sample size and not as a consequence of the high standard errors as S.E.M. values for all logistic regression models ≤ 1.01 and Pearson's $r<0.8$.

SNP imputation of OCT1/OCT2/OCT3 locus was performed, but none of the imputed SNPs displayed stronger association compared with rs2481030, rs7757336 and rs3119309.

Two SNPs, rs7757336 and rs2481030, were selected for replication from a group of 131 Slovakian T2D patients (replication group). In total, 148 Slovakian patients were included in a 6-month duration study. Of them, we had data on HbA_{1c} from a 3-month visit in 131 patients, and these data were analyzed. Minor allele frequencies in replication group showed decreased MAF of SNPs rs7757336 (0.118, 0.132) and rs2481030 (0.317, 0.397) in comparison with the discovery group and HapMap data (0.173, 0.398 respectively).

We found no significant associations of SNPs rs7757336 and rs2481030 (Omnibus test P value >0.05) with non-responder phenotype in group from Slovakia

after 3 months of metformin monotherapy in a model with covariates sex, age, BMI, length of therapy, dose (mg/per day), baseline HbA_{1c} and creatinine clearance.

On analysis of a combined group (233 participants), logistic regression model is statistically significant (χ^2 (7)=76.070, $P<0.001$). The model included a number of minor alleles of rs2481030 or rs7757336 and age, sex, baseline BMI and HbA_{1c}, number of treatment days, dose of metformin mg/per day and creatinine clearance as covariates. Carriers of minor alleles were 2.00 times more likely to exhibit non-responder phenotypes than participants with reference alleles ($P=0.002$). Higher creatinine clearance, higher baseline HbA_{1c}, age and women gender were associated with a lower likelihood of exhibiting non-responder phenotype ($P<0.05$).

Pharmacokinetic study

Association of rs2481030 and rs7757336 with metformin pharmacokinetics was analyzed in a group of 25 participants. Phenotypic and biochemical data of those subjects are displayed in Table 3. Nine heterozygotes (AG) and 4 homozygotes (GG) of rs2481030 risk allele were identified. Eight heterozygotes (AC) of rs7757336 risk allele were identified, all being carriers of minor alleles of rs2481030 (AG and GG genotypes). Due to the small group size, all risk alleles were counted for each participant and used as covariates for linear regression analysis. For clarity of presentation, 12 participants with 0 risk alleles were assigned to the 'reference' group, whereas 13 participants with at least 1 risk allele were assigned to the 'risk group'. There are no significant differences in the sex, age, weight and creatinine clearance between the groups.

Table 3 Characteristics and pharmacokinetics parameters of 25 healthy participants in relation to combined alleles of rs2481030 and rs7757336 associated with metformin inefficiency after a single-dose oral administration of 500 mg metformin. Data are shown as mean value \pm s.d.

Characteristics	Study sample	Comparison of groups		
		Reference (n=12)	Risk group (n=13)	P values
Male n, %	9 (36.00)	3 (25.00)	6 (46.15)	0.473 [‡]
Weight, kg, s.d.	72.68 \pm 13.32	71.58 \pm 16.59	73.69 \pm 10.01	0.701 [‡]
Age, years, s.d.	26.44 \pm 3.99	26.17 \pm 4.73	26.69 \pm 3.35	0.752 [‡]
Creatinine clearance, mL/min/BSA, \pm s.d.	121.06 \pm 13.72	118.54 \pm 16.34	123.38 \pm 10.93	0.390 [‡]
AUC ₀₋₂₄ , μ g*h/mL, \pm s.d.	5.42 \pm 1.62	6.30 \pm 1.51	4.62 \pm 1.29	0.009
C _{max} , μ g/mL, plasma, \pm s.d.	0.72 \pm 0.24	0.84 \pm 0.25	0.60 \pm 0.18	0.022
T _{max} , plasma, h, \pm s.d.	2.32 \pm 1.25	2.42 \pm 1.51	2.23 \pm 1.01	NS
C _{max} , μ g/mL, erythrocytes, \pm s.d.	0.15 \pm 0.06	0.16 \pm 0.07	0.14 \pm 0.05	NS
Estimated half-life, h, \pm s.d.	4.44 \pm 0.62	4.41 \pm 0.56	4.46 \pm 0.70	NS
CL/F, L/h, \pm s.d.	51.40 \pm 16.79	42.47 \pm 9.50	59.64 \pm 18.11	0.010
V/F, L, \pm s.d.	333.56 \pm 146.18	268.04 \pm 59.66	394.03 \pm 176.70	NS
Metformin excreted in the urine, % of dose \pm s.d.	39.09 \pm 12.94	42.19 \pm 16.42	36.23 \pm 8.31	NS

[‡]P value derived from t-test. All other P values derived from linear regression with covariates: number of negative alleles, age, sex, weight, creatinine clearance (corrected for body surface area (BSA)). NS: linear regression model is not significant (Omnibus test).

The concentration of metformin in plasma was higher in the individuals from the reference group compared with those in the risk group in 4 sampling points (Fig. 1).

Quality measures for the regression analysis including combined number of risk alleles of rs7757336 and rs2481030 polymorphisms and covariates – weight, age, sex and creatinine clearance (calculated from 24-h urine samples and corrected for body surface area) – were as follows: $R^2=44.2\%$, result of the Durbin-Watson test =1.83, VIF was 1.06–1.56 and tolerance 0.64–0.95 and standardized residuals of linear regression with values $<\pm 2.17$ were normally distributed ($P=0.2$) (Table 3).

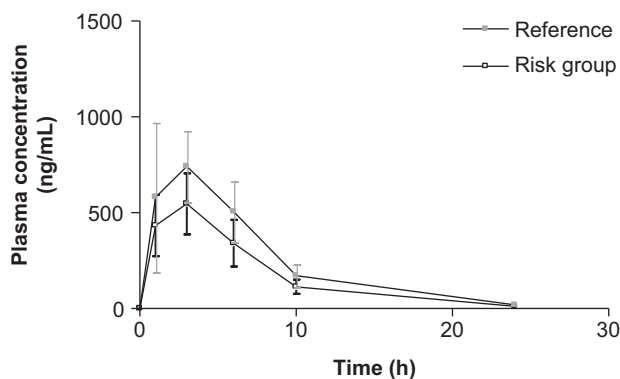


Figure 1

Concentration of metformin in plasma of 25 healthy individuals grouped based on the presence of the risk alleles after a single-dose oral administration of 500 mg metformin.

AUC_∞ of metformin plasma was significantly lower ($P=0.009$) in the risk group ($4.62 \pm 1.29 \mu\text{g/h/mL}$) vs reference group ($6.30 \pm 1.51 \mu\text{g/h/mL}$) in linear regression analysis. Model including number of risk alleles, age, sex, weight and creatinine clearance statistically significantly predicted metformin AUC_∞ in plasma (sig. *F* change (5, 19)=3.005, $P=0.036$). Weight ($P=0.037$) and number of minor alleles ($P=0.009$), but not age ($P=0.775$), sex ($P=0.581$) and creatinine clearance ($P=0.728$) were statistically significant variables to the prediction. C_{max} in plasma was significantly increased in reference group (0.84 ± 0.25 vs $0.60 \pm 0.18 \mu\text{g/mL}$, $P=0.022$), and apparent clearance (CL/F) was significantly higher in risk group ($59.64 \pm 18.11 \text{ L/h}$ vs $42.47 \pm 9.50 \text{ L/h}$, $P=0.01$). There were no significant differences between groups with respect to T_{max} in plasma, C_{max} in erythrocytes, estimated half-life and apparent volume of distribution, as well as dose of metformin excreted in the urine 24 h after drug administration. T_{max} in erythrocytes was not analyzed due to low variability in study sample.

Discussion

In this study, we show that minor alleles of SNPs rs3119309, rs7757336 and rs2481030 located in the intergenic region between OCT2 and OCT3 coding genes (*SLC22A2* and *SLC22A3*) are significantly associated with metformin inefficiency in the 233 newly diagnosed and well-described T2D patients. Testing of pharmacokinetic effects of these genetic markers was performed in 25 nondiabetic volunteers from Latvia.

Logistic analysis was performed using robust segregation of outcomes in the responder group showing decrease in HbA_{1c} vs non-responder group displaying no change or even increasing levels of HbA_{1c} 3 months after the beginning of metformin monotherapy. The main reason to choose this type of outcome is due to our intention to perform robust analysis that is better suited for the investigation in the small, well-phenotyped group (29). A number of studies investigating the efficiency of metformin including the only GWAS performed so far (20) have used the Δ HbA_{1c} or achievement of treatment goal of <6.5 or 7% HbA_{1c} (48–53 mmol/mol) as an outcome. However, patients with high baseline HbA_{1c} level will usually have larger Δ HbA_{1c} in comparison with other participants due to the natural limits. Nevertheless, they often will be included in the non-response group if treatment goals are set as criteria even if absolute decrease of HbA_{1c} is substantial. This may explain the significant deviation of Δ HbA_{1c} from the normal distribution in our research group. For the same reason, use of treatment goals can be misleading in the patients with extreme baseline HbA_{1c} values. Patients with high baseline HbA_{1c} will have larger HbA_{1c} decrease than patients with HbA_{1c} close to normal levels but more often will be classified as non-responders (30, 31).

As previously mentioned, GWAS identified an association with rs11212617 near *ATM*, whereas no association was found in any loci of known metformin transporter genes (20). Our results showing the association of SNPs in metformin transporter genes in contrast to this GWAS may be explained by relatively short time period used to estimate the efficiency of metformin in our study. Factors influencing the pharmacokinetics (e.g. organic cation transporters) will show their effects on efficiency mainly at the beginning of the treatment, especially in the case of metformin where gradual accumulation of drug has been observed in the organism over the time of treatment (21). Short time period is also less dependent on factors like diet, physical activities and adherence to the drug in comparison with the long-term treatment when metformin secondary failure due to decreasing body functions interferes. In addition, one should take into account the fact that initial metformin therapy is subject to change after the first 3 months of the therapy in cases where it failed to display the decrease of HbA_{1c}. A study investigating metformin therapy outcomes assessed initial nonadherence to be 17% in participants starting metformin therapy. One-third attempted another pharmacotherapy within 6 months, and side effects were found to be one of the

major limiting factors (32, 33, 34). Thus, the patients whose treatment inefficiency is caused by the defect in metformin transport may become eliminated from the studies involving longer observation times.

Our failure to replicate the association of identified SNPs in the replication group of 131 T2D participants from Slovakia may be explained by the differences in the phenotypical and genotypical characteristics of the groups. Among the phenotypical differences, significantly lower BMI, lower dose of metformin used and subsequently a lower reduction in HbA_{1c} were observed in the replication group in comparison with those in the discovery group. SNPs rs7757336 and rs2481030, age, sex, BMI, HbA_{1c}, metformin dose and creatinine clearance were not associated with non-responder phenotype, indicating a strong influence of other genetic or environmental factors like diet, physical activities and adherence to the metformin. MAF of the investigated SNPs in the replication group was lower compared with that found in the discovery group, and linkage disequilibrium differed ($r^2=0.255$ vs 0.194) indicating significant genetic differences between populations. However, it should be noted that the effect direction in the Slovakian group was similar to that observed in the Latvian group (OR 1.32, although non-significant). The results from combined analysis probably correspond better with the real-life effect size of the polymorphism with response to metformin, which could have been inflated in the original Latvian study as a result of statistical phenomenon called the 'winner's curse'. The combined analysis was made across the wider spectrum of metformin dosage, which was also taken into account as one of the covariates.

In line with the results of the pharmacogenetic study, in the pharmacokinetic study, the minor alleles of rs775336 and rs2481030 were also consistently associated with lower concentration of metformin and AUC_∞ of the plasma in a small independent group of 25 healthy nondiabetic volunteers. Due to the limited number of participants, it is necessary to confirm these findings in studies with a larger number of subjects. However, if these findings hold true, this will support the influence of OCTs in pharmacokinetics of metformin and importance of short-term study design in cases where factors influencing bioavailability are investigated. No significant differences were found in observed C_{max} and T_{max} in erythrocytes between controls and carriers of risk alleles indicating that observed changes of metformin pharmacokinetics cannot be explained by exposure time to metformin. When other pharmacokinetic measures were analyzed, we found that oral clearance (CL/F) is

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higher in the subjects with at least one inefficient allele, whereas apparent volume of distribution (V/F), half-life and total dose of metformin excreted in the first 24 h after administration were not different. It should be noted however that calculation of renal clearance and apparent volume of distribution were done assuming that there is no difference in the bioavailability of 500 mg of metformin taken orally. This assumption may not be true as a number of factors including the genetic variation in transporter genes may alter the bioavailability. The calculated CL/F and V/F values are strongly related to AUC_{∞} measurement, and their difference among groups should be interpreted with caution.

Variation in the bioavailability and volume of distribution is considered a major source of variation in the pharmacokinetics of metformin as it does not undergo metabolism in the human body and slow absorption is thought to be the rate-limiting factor in metformin disposition (10). It is not clear what proportion of metformin is sequestered in the enterocytes due to the effects of inward net flow and OCT related mechanism vs its paracellular route in human small intestine. If latter is true, the uptake and excretion remain the major factors influencing metformin plasma levels. Lower metformin level in plasma may indicate lower metformin uptake in the gastrointestinal tract by altered OCT3 transporter or can be explained by OCT2 variant that eliminates the drug more quickly.

Polymorphisms investigated in this study are noncoding and could be in linkage disequilibrium with causal SNPs within coding/regulatory regions of *SLC22A2*, *SLC22A3* or even *SLC22A1* resulting in altered transport activity or expression level in target tissues. In large GWAS, minor allele A of SNP rs3127573 (MAF 0.13) located near *SLC22A2* ($r^2=0.96$ with SNP rs3119309 investigated in our study) was identified to be associated with higher serum creatinine and lower estimated GFR (35). However, it is not clear how variants in OCT2 or its regulatory regions could be a basis for increased creatinine levels and decreased metformin levels simultaneously.

On the other hand, these can be explained by altered transport activity of creatinine and metformin transporter OCT3 (*SLC22A3*), which is expressed in the liver, small intestine and muscle tissue. Indeed, in the experiments with OCT2 and OCT3 expressing HEK293 cells (36), increased creatinine uptake in comparison with cells transfected with the null vector was identified, whereas such uptake was not observed in case of OCT1. Polymorphisms rs2292334, rs2048327, rs1810126 and rs3088442 were associated with reduced OCT3 mRNA levels, whereas a common variant of rs555754 was

associated with a greater transcription rate and higher expression levels of OCT3 in the liver (37). While none of the above-mentioned SNPs altering the OCT3 transporter efficiency were in strong linkage disequilibrium with SNPs investigated in our study, rs2481030 and rs2481031 (LD with $r^2=0.99$) (http://www.broadinstitute.org/mammals/haploreg/haploreg_v2.php). Possibly, rs2481030 could be a marker of reduced uptake OCT3 variant or lower transporter expression level and subsequently characterized by slower metformin absorption from gastrointestinal tract and decreased uptake in the liver, muscle, heart and adipose tissues.

Major limitations of our study are the relatively small sample size, possible effects of co-medications and adherence. Variable co-medications in antidiabetic therapy are very common, however, the stratification based on this variable or analysis of patients without concomitant diseases would result in significant selection bias and insignificant sample size. Particular functional variants, for example, OCT1 risk alleles of metformin intolerance were not included in this study (38) mainly because of low allele frequency that would result in very low statistical power due to small sample size. Our findings should be evaluated in larger groups of participants. Further investigation should be conducted to confirm whether rs7757336, rs3119309 and rs2481030 are valid markers of metformin inefficiency and if they can be useful in the prediction of treatment response to metformin in the T2D patients.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-16-0347>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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