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ORIGINAL RESEARCH

Intronic Variants in OCT1 are Associated with All-Cause and Cardiovascular Mortality in Metformin Users with Type 2 Diabetes

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Purpose: Organic cation transporters (Octs) use cations like endogenous compounds, toxins, and drugs, such as metformin, as substrates. Therefore, these proteins determine the pharmacokinetics and -dynamics of metformin and thus its efficacy. Of note, metformin is today the most commonly used pharmaceutical in the treatment of type 2 diabetes (T2DM) with nevertheless a great variability in clinical response, which attributes to genetic variances. The aim of this study was to determine the influence of intronic OCT1 SNPs on prevalence of all-cause and cardiovascular death.

Patients and Methods: Genotypes of 27 intronic SNPs in OCT1 were investigated in the LURIC study, a prospective cohort of 3316 participants scheduled for coronary angiography. We investigated whether these variants were associated with all-cause and cardiovascular death in 73 individuals with T2DM under metformin therapy, in individuals without diabetes, individuals with T2DM and individuals with T2DM without metformin therapy.

Results: In a multivariate Cox regression analysis adjusted for classical cardiovascular risk factors, 4 intronic OCT1 SNPs were significantly associated with all-cause and cardiovascular mortality in individuals with T2DM on metformin therapy.

Conclusion: According to their OCT1 genotype, some individuals with T2DM on metformin therapy might be prone to an increased risk of cardiovascular death.

Keywords: organic cation transporter 1, SNP, T2DM, cardiovascular death, metformin

Introduction

According to WHO data collected in 2014, cardiovascular diseases (CVDs) are globally the primary cause of death. Data obtained in the year 2016 showed that at least 17.9 million of people died from CVDs representing 31% of all deaths worldwide.[1] In Europe, more than half of all deaths are caused by CVDs.

Individuals with type 2 diabetes mellitus (T2DM) show significantly increased cardiovascular morbidity and all-cause mortality compared to subjects without diabetes.[2,3] In T2DM, coronary artery disease (CAD) and stroke increase 2.4-fold and the risk of heart failure increases 2.8-fold due to diabetic vascular disease.[4]

Metformin is the recommended first-line therapy and hence the most frequently prescribed drug in T2DM treatment. A recent review by Griffin and colleagues targeted the influence of metformin therapy on cardiovascular diseases and showed that all outcomes, except the risk of stroke, were improved by the use of metformin; however, none of these associations achieved statistical significance.[5]

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Metformin reduces glucose absorption in the gastrointestinal tract and suppresses hepatic gluconeogenesis by inhibiting the mitochondrial respiratory chain complex[6,7] I: leading to a decrease in gluconeogenetic enzymes,[8] II: via AMPK to an increased fatty acid uptake and beta-oxidation[9] and III: inhibition of mitochondrial glycerophosphate dehydrogenase.[10] AMPK activation can also occur independent of AMP, complex I and mitochondria.[11] In the gastrointestinal tract metformin changes the microbiota composition.[11] As seen in mice, metformin increases the number of Lactobacillus species, which in turn leads to a normalization of sodium–glucose cotransporter-1 (SGLT1) expression in the host which is changed in T2DM. Via the induction of GLP-1 secretion, SGLT1 decreases glucose production.[12–14]

In addition to its effects on glycemic control, it exerts favourable effects on surrogate parameters like body mass index (BMI) and waist circumference and reduces micro- and macrovascular complications.[15] In the last 6 years, increasing evidence points to the effectiveness of metformin in the treatment of cancer.[16] Metformin not only proved to be useful in the treatment of T2DM but also of pre-diabetes, type 1 diabetes mellitus, polycystic ovary syndrome and gestational diabetes. Positive effects of metformin were also seen in congestive heart failure, chronic liver and kidney disease, multiple sclerosis, or non-alcoholic fatty liver disease (reviewed in[17]). This drug not only might be used in the future for treatment of further disorders but also in anti-aging therapy. Current research to examine the potential of metformin on slowing the progress of age-related and age-dependent diseases in elderly individuals is ongoing.[18]

Metformin needs membrane transporters to penetrate organs and cross cell membranes due to its low hydrophobicity. Since metformin is not metabolized in the body, transport proteins regulating its gastrointestinal and hepatic uptake and renal elimination are particularly important in determining metformin pharmacokinetics and -dynamics. Organic cation transporter family members (Oct) are involved in the transport of small organic cations, including drugs, endogenous compounds or toxic substances with distinct molecular structures.[19] Metformin is a substrate of the gastrointestinal and liver expressed Oct1, which is mainly responsible for the uptake.[20,21] Of note, there is a reproducible heritability of glycemic response to metformin, up to 34%, based on genome-wide complex trait analyses. This suggests an important influence of genetic variants on the variance in glycemic response to metformin.[22] The effects of coding SNPs in OCT1 on uptake, and thus efficacy of metformin, have extensively been investigated by a variety of studies.[23–25] Recent work done in the field has begun to look more closely onto intronic SNPs in this region, since, on the one hand, genome-wide association studies (GWAS) targeting type 1 and type 2 diabetes showed top hits located in intronic regions of various genes[26,27] and, on the other hand, metformin pharmacokinetics and -dynamics have been linked to genetic variants in transcription factors.[28] Thus, coding as well as non-coding SNPs in *OCT 1* play an important role in inter-patient difference of metformin efficacy.[29–34] The investigated SNPs might therefore be important for metformin use in a variety of diseases.

The aim of this study is to determine the influence of intronic SNPs in one of the Oct transporter genes (*OCT1*), on critical outcomes in a large European cardiovascular risk cohort, such as the prevalence of all-cause and cardiovascular death where endpoint data were available. We investigate, whether this effect is metformin-dependent and thus relevant to metformin-users only or might be a more generalized effect.

Patients and Methods

Participants, Study Description, and Definition of Comorbidities

Data were obtained from the Ludwigshafen Risk and Cardiovascular Health (LURIC) study, a prospective cohort study designed to evaluate the determinants of cardiovascular health.[35–37] 3316 Caucasian subjects, aged 62.6 ± 10 years, referred for coronary angiography between July 1997 and January 2000, were recruited at a coronary care tertiary referral center (Herzzentrum Ludwigshafen, Germany). Participants with acute illness (except for acute coronary syndrome), non-cardiac chronic disease, or malignant neoplasms within the past 5 years were excluded. Written informed consent from each participant and the study approval by the institutional review board at the Ärztekammer Rheinland-Pfalz were obtained. The study was conducted in accordance with the Declaration of Helsinki. More detailed information about subjects in the study, examinations, recruitment and comorbidities have been previously described.[35,38]

Of note, during the recruitment phase of the LURIC study, metformin was not the first-line therapy option in type 2 diabetes therapy, due to the occurrence of several cases of lactic acidosis and consecutive safety concerns, leading to only 73 metformin users in this study. In the follow-up period (median 9.9 years), 894 (27%) of the study participants died.

During follow-up no patient was lost. Local registries were used to gain information on mortality. Classification of death due to cardiovascular or non-cardiovascular causes was done by the use of death certificates. Classification of causes of death was done by two physicians who reviewed death certificates and hospital records without knowledge of study participants' baseline characteristics. Cardiovascular deaths included sudden cardiac death (SCD), fatal myocardial infarction, deaths due to heart failure, death after intervention to treat CAD, stroke, and other deaths due to heart disease. SCD was defined as a sudden unexpected death either within 1 h of onset of symptoms or within 24 h of having been observed alive and without symptoms.

The presence of a visible luminal narrowing (>20% stenosis) in at least 1 of 15 coronary segments in coronary imaging was used to define coronary artery disease (CAD) according to the classification of the American Heart Association.[35] Hypertension was defined as a systolic and/or diastolic blood pressure ≥140 and/or ≥90 mmHg or a significant history of hypertension. The glomerular filtration rate was estimated by using the 2012 CKD-EPI eGFRcreatcys equation.[39] Pre-diabetes and diabetes were determined according to the American Diabetes Association (ADA) criteria.[40] Impaired fasting glycemia (IFG) was determined by plasma glucose concentrations between 5.6 and 6.9 mM, and fasting type 2 diabetes mellitus was determined by plasma glucose concentrations ≥7.0 mM or HbA1c levels ≥6.5%. Based on a 2 h post-oral glucose tolerance test (oGTT), impaired glucose tolerance (IGT) was diagnosed by plasma glucose concentrations between 7.8 and 11.0 mM, and 2 h post-load type 2 diabetes mellitus by plasma glucose concentrations ≥11.1 mM. Individuals who required antidiabetic medication (ie oral antidiabetic and/or insulin use for control of glycemia) were also defined as diabetic[41]. Number of subjects per group is shown in Figure 1. Individuals included in this analysis either belonged to the group of individuals without diabetes (no diabetes: ND) or to the group of individuals with type 2 diabetes mellitus (T2DM), both groups together are referred to as all individuals (all). The latter was divided into metformin users (metformin users with type 2 diabetes: MUT) and non-metformin users (non-metformin users with type 2 diabetes: NMUT). Number of individuals in each group is described in the flow chart (Figure 1).

SNP Selection and Analysis of Functional Consequences

Based on a minor allele frequency (MAF) >0.01 in a central European population, we selected 34 non-coding, intronic SNPs in the transporter gene OCT1 (gene SLC22A1 and 4 kb upstream of the first translational start site). Linkage disequilibrium (LD) analysis was performed with the LDlink tool of the NIH National cancer institute (RRID: SCR_011403, https://analysistools.nci.nih.gov/LDlink/) and was additionally checked in HaploReg v4.1 (RRID: SCR_006796).[42,43] The investigated SNPs were not in LD with any coding SNPs in *OCT1*.

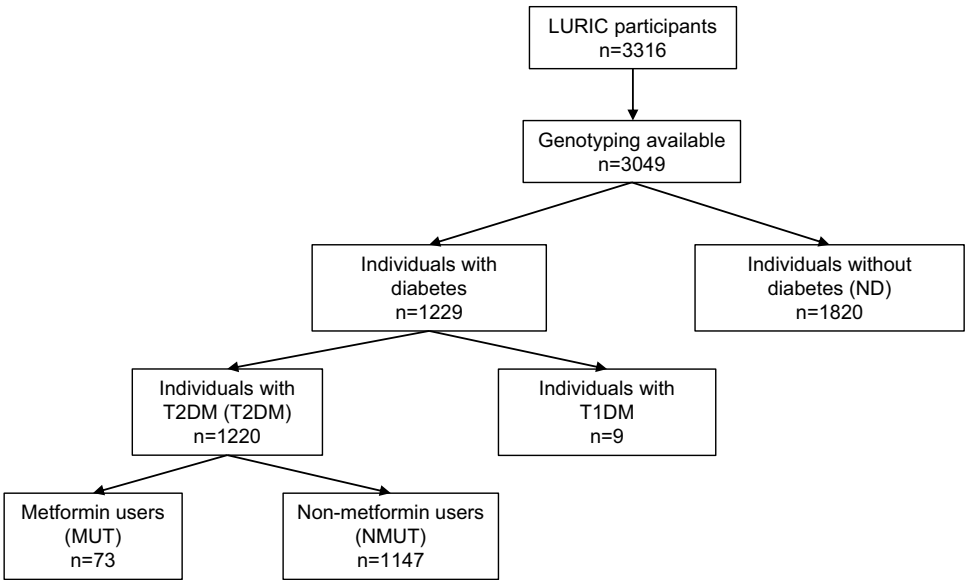

Figure 1 Flow chart of subjects per investigated group.
Abbreviations: T2DM, type 2 diabetes mellitus; T1DM, type 1 diabetes mellitus.

OCT1 genotyping data were available from 3061 individuals (92.3% of the entire cohort) due to technical reasons. OCT1 SNPs were imputed in pre-existing genotyping data and an in silico analysis was performed.

Analysis of cis or trans regulation of gene expression was determined by database search (GTEx Portal V8 (RRID: SCR_001618) or HaploReg v4.1 (RRID: SCR_006796).[42,43] Changes in transcription factor binding sites and sites of epigenetic modification were determined via HaploReg v4.1.

The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The data used for the analyses described in this manuscript were obtained from the GTEx Portal on 02/05/2020 and/or dbGaP accession number phs000424.vN.pN on 02/05/2020.

Statistical Analysis

Associations between the non-coding SNPs in OCT1 and changes in all-cause and cardiovascular mortality were analyzed using multivariate Cox regression. Hazard ratios (HR) with 95% CIs for the mortality categories all-cause death and cardiovascular death were calculated using Cox proportional hazards regression models, which enabled adjustment for potential confounding parameters. In these analyses, an additive model was calculated, the unadjusted model describes the crude association, the adjusted model was adjusted for sex, BMI, systolic blood pressure, hypertension, lipid parameters, C-reactive protein (CRP), sodium levels, cortisol, cystatin c, NT-pro-BNP, arterial fibrillation, left ventricular hypertrophy, smoking and coronary artery disease stages, respectively.

To determine the impact of glycemic control on the SNP effects on CVD, we adjusted in a third model additionally for HbA1c and HOMA-IR. HOMA-IR was calculated as follows: (fasting glucose [mmol/L] × fasting insulin [U/L])/22.5. Changes in HR are given per minor allele present and are referred to the homozygous major allele of each SNP, respectively.

Associations between investigated SNPs and mortality categories were determined for all LURIC participants. Subclassification of individuals with type 2 diabetes in metformin users, non-metformin users and comparison with subjects without diabetes should indicate whether the effect is metformin dependent or not.

Statistical significance was defined as $p<0.05$. Statistical analyses were done using the statistical software package STATA, StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC.

Results

Baseline Characteristics of the Study Cohort

The baseline characteristics of the study cohort are given in Table 1.

Data on Mortality of the Investigated Cohort and Subgroups

Data on all-cause and cardiovascular death of all subjects and the investigated subgroups are given in Table 2. Data on all-cause and cardiovascular death per genotype in all subjects and metformin users with T2DM are given in Table 3.

Minor Allele Frequency of the SNPs

The minor allele frequency of the intronic OCT1 SNPs rs461473, rs609468, rs622591, rs3777392, rs9295125/rs3818678 and rs456598 were 0.118, 0.229, 0.228, 0.114, 0.412 and 0.127 respectively in our cohort.

Association of OCT1 SNPs with All-Cause and Cardiovascular Death in Individuals with Type 2 Diabetes with and Without Metformin Therapy and Non-Diabetic Individuals

Only in metformin users with T2DM, SNP rs461473 was significantly associated with all-cause as well as cardiovascular death even after adjustment (see the 'Patients and Methods' section). Each copy of the minor A allele was significantly associated with an increase in HR for all-cause and cardiovascular death (see Table 4 for details). No association with either all-cause or cardiovascular death was seen in non-metformin users with T2DM and non-diabetic subjects (see Tables 5 and 6 for details).

SNPs rs609468 and rs622591 were borderline associated with all-cause death and only after adjustment significantly associated with cardiovascular death in metformin users with T2DM. Each copy of the minor T allele was associated with an increased HR, respectively. No associations with either all-cause or cardiovascular death were seen in non-metformin users with T2DM and non-diabetic subjects (see Tables 5 and 6 for details).

Table 1 Baseline Characteristics of the Study Participants

	All (n=3049)	ND (n=1820)	T2DM (n=1220)	p-values[a]	MUT (n=73)	NMUT (n=1147)	p-values[b]
Age (years)	62.7 (10.6)	60.9 (11.2)	65.5 (9.0)	<0.001	65.1 (7.2)	65.5 (9.1)	0.718
Male sex (%)	70.0	70.0	70.1	0.973	72.6	69.9	0.621
Body mass index (kg/m^2)	27.5 (4.0)	26.9 (3.8)	28.3 (4.2)	<0.001	29.2 (4.6)	28.2 (4.1)	0.041
Hypertension (%)	72.6	66.7	81.2	<0.001	83.6	81.1	0.302
Smoking (%)	19.4	21.3	16.7	0.076	19.2	16.6	0.080
Systolic blood pressure (mm Hg)	141 (23)	138 (22)	145 (24)	<0.001	153 (23)	144 (23)	0.002
LDL cholesterol (mmol/L)	3.02 (0.89)	3.07 (0.90)	2.95 (0.88)	<0.001	2.70 (0.88)	2.97 (0.88)	0.013
HDL cholesterol (mmol/L)	1.00 (0.28)	10.40 (0.29)	0.94 (0.25)	<0.001	0.91 (0.21)	0.94 (0.25)	0.243
Triglycerides (mmol/L)	1.94 (1.39)	1.81 (1.39)	2.13 (1.37)	<0.001	2.28 (1.25)	2.44 (1.37)	0.318
Total cholesterol (mmol/L)	5.40 (1.14)	5.5 (1.12)	5.3 (1.18)	0.010	5.02 (1.05)	5.4 (1.18)	0.017
HbA1c	6.3 (1.24)	5.7 (0.44)	7.2 (1.48)	<0.001	8.0 (1.40)	7.1 (1.46)	<0.001
NT-pro-BNP (ng/L)	912 (2069)	735 (1753)	1177 (2450)	<0.001	852 (1437)	1198 (2499)	0.246
Cystatin c (mg/L)	1.01 (1.99)	0.96 (0.38)	1.06 (0.43)	<0.001	0.95 (0.31)	1.07 (0.44)	0.020
C-reactive protein (mg/L)	10.07 (19.93)	8.44 (17.09)	12.5 (23.38)	<0.001	9.13 (16.15)	12.76 (23.75)	0.207
Sodium (mmol/l),	141 (2)	141 (2)	140 (3)	<0.001	140 (2)	140 (3)	0.151
Cortisol (nmol/L)	22 (8)	21 (8)	22 (7)	0.003	22 (7)	22 (7)	0.920
Atrial fibrillation (%)	12.11	10.95	13.87	0.016	11.27	14.03	0.514
Left-ventricular hypertrophy (%)	8.17	6.98	10.00	0.004	13.70	9.76	0.268
STA-CAD (%)				<0.001[c]			
Class1	47.23	45.16	50.08		50.68	50.04	
Class2	19.55	18.08	21.72		24.66	21.53	
Class3	12.23	11.43	13.52		13.70	13.51	

Notes: Data are given as mean (SD) if not otherwise stated. [a]p-value stating differences between non-diabetics and individuals with T2DM. [b]p-value stating differences between individuals with T2DM with and without metformin therapy. [c]Only overall test available.

Abbreviations: All, all individuals; ND, non-diabetic individuals; T2DM, individuals with type 2 diabetes mellitus; MUT, metformin users with T2DM; NMUT, non-metformin users with T2DM; SD, standard deviation; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HbA1c, haemoglobin A1c; NT-pro-BNP, N-terminal prohormone of brain natriuretic peptide; STA-CAD, coronary artery disease stage.

Table 2 Mortality Data of the Investigated Subgroups

	All		ND		MUT		NMUT		p-value[a]
	n	% Male	n	% Male	n	% Male	n	% Male	
ACD	891	73.4	399	73.4	24	83.3	463	73.0	0.201
CVD	555	72.8	226	73.9	18	77.8	307	71.7	0.683
Non CVD	336	74.4	173	72.8	6	100	156	75.6	0.189
Survival	2158	68.63	1421	69.0	49	67.4	684	67.8	

Note: [a]The given p-value states the difference of individuals with T2DM with and without metformin therapy.

Abbreviations: All, all individuals; ND, non-diabetic individuals; MUT, metformin users with T2DM; NMUT, non-metformin users with T2DM; T2DM, type 2 diabetes mellitus; ACD, all-cause death; CVD, cardiovascular death; Non CVD, death other than cardiovascular death.

SNP rs3777392 was significantly associated with cardiovascular death in the unadjusted model, the association was only seen by trend after adjustment in metformin users with T2DM. None of the mentioned associations were seen in non-metformin users with T2DM or non-diabetics (see Tables 4–6 for details).

SNPs rs9295125, rs3818678 and rs456598 showed an association with all-cause death in metformin users with T2DM only in the unadjusted model, which disappeared after adjustment (see Table 4). The minor alleles of SNPs rs9295125 and rs3818678 showed a by trend association with all-cause death in the unadjusted model. Each copy of the minor allele was associated with a decrease in HR for all-cause death. In individuals with T2DM who were non-metformin users, both SNPs were by trend associated with all-cause and cardiovascular death in the unadjusted model, which was significant only after adjustment (see Table 5 for details). In this patient subgroup, the minor

Table 3 Mortality Data per Genotype in All Individuals and Individuals with T2DM Using Metformin

SNPs	Alleles	n	All	n=3049		n	MUT	n=73	
			ACD	CVD	% of ACD/n		ACD	CVD	% of ACD/n
rs461473	GG	2385	691	431	62/18	60	18	14	78/23
G/A	GA/AA	664	200	124	62/19	13	6	4	67/31
rs609468	CC	1811	521	331	64/18	48	12	9	75/19
C/T	CT/TT	1238	370	224	61/18	25	12	9	75/36
rs622591	CC	1813	521	331	64/18	48	12	9	75/19
C/T	CT/TT	1236	370	224	61/18	25	12	9	75/36
rs3777392	CC	2403	702	435	62/18	57	17	12	71/21
C/T	CT/TT	646	189	120	63/19	16	7	6	80/38
rs9295125 G/T	GG	1049	295	188	64/18	26	11	8	73/31
rs3818678 G/C	GT/TT GC/CC	2000	596	367	62/18	47	13	10	77/21
rs456598	GG	2326	686	419	61/18	60	17	13	76/27
G/A	GA/AA	723	205	136	66/19	13	8	6	75/46

Abbreviations: All, all individuals; SNPs, single-nucleotide polymorphisms; MUT, metformin users with T2DM; T2DM, type 2 diabetes mellitus; ACD, all-cause death; CVD, cardiovascular death.

Table 4 Association of Intronic OCT1 SNPs with All-Cause and Cardiovascular Death in Metformin Users with T2DM (MUT)

rs Number		All-Cause Death		Cardiovascular Death	
Major/Minor		Unadjusted	Adjusted	Unadjusted	Adjusted
Allele	n	73	73	73	73
rs461473	p-value	0.006	0.006	0.021	0.018
G/A	HR	3.31	11.46	3.16	28.86
	CI	1.41–7.78	1.99–66.06	1.19–8.39	1.76–473.31
rs609468	p-value	0.053	0.057	0.118	0.031
C/T	HR	1.65	2.27	0.16	4.43
	CI	0.99–2.75	0.98–5.25	0.88–2.98	1.15–17.11
rs622591	p-value	0.053	0.057	0.118	0.031
C/T	HR	1.65	2.27	0.16	4.43
	CI	0.99–2.75	0.98–5.25	0.88–2.98	1.15–17.11
rs3777392	p-value	0.147	0.698	0.047	0.066
C/T	HR	1.65	1.32	2.08	8.67
	CI	0.84–3.25	0.32–5.37	1.01–4.28	0.86–87.05
rs9295125	p-value	0.082	0.290	0.204	0.183
G/T	HR	0.57	0.62	0.63	0.47
rs3818678 G/C	CI	0.31–1.07	0.25–1.51	0.31–1.29	0.16–1.43
rs456598	p-value	0.095	0.308	0.157	0.867
G/A	HR	2.12	2.30	2.17	0.84
	CI	0.88–5.13	0.46–11.41	0.74–6.35	0.12–5.99

Note: The statistically tested allele is the minor allele.
Abbreviations: HR, hazard ratio; CI, 95% confidence interval.

allele was associated with an increase in HR. In non-diabetic subjects, both SNPs showed an association with all-cause death only in the adjusted model. As in non-metformin users with T2DM, the minor allele of both SNPs was associated with an increased HR for all-cause death (see Table 5 for details). An overview of the influence of each minor allele of the investigated SNPs on the hazard ratio of cardiovascular risk is given in Figure 2.

Table 5 Association of Intronic OCT1 SNPs with All-Cause and Cardiovascular Death in Non-Metformin Users with T2DM (NMUT)

rs Number		All-Cause Death		Cardiovascular Death	
Major/Minor		Unadjusted	Adjusted	Unadjusted	Adjusted
Allele	n	1147	1147	1147	1147
rs461473 G/A	p-value	0.866	0.973	0.467	0.635
	HR	0.98	1.00	0.91	0.94
	CI	0.80–1.21	0.82–1.24	0.70–1.18	0.72–1.22
rs609468 C/T	p-value	0.898	0.793	0.440	0.246
	HR	1.01	0.98	0.93	0.89
	CI	0.87–1.18	0.84–1.15	0.76–1.12	0.73–1.08
rs622591 C/T	p-value	0.869	0.805	0.459	0.252
	HR	1.01	0.98	0.93	0.89
	CI	0.87–1.18	0.84–1.15	0.77–1.13	0.73–1.09
rs3777392 C/T	p-value	0.796	0.922	0.457	0.673
	HR	0.97	1.01	0.91	0.95
	CI	0.80–1.18	0.83–1.23	0.71–1.17	0.73–1.22
rs9295125 G/T rs3818678 G/C	p-value	0.094	0.039	0.068	0.038
	HR	1.12	1.15	1.16	1.19
	CI	0.98–1.28	1.01–1.32	0.99–1.36	1.01–1.41
rs456598 G/A	p-value	0.637	0.496	0.843	0.780
	HR	0.95	0.93	0.98	0.97
	CI	0.79–1.16	0.76–1.14	0.77–1.24	0.76–1.23

Note: The statistically tested allele is the minor allele.
Abbreviations: HR, hazard ratio; CI, 95% confidence interval.

Impact of Glycemic Control on the Influence of Intronic OCT1 SNPs on All-Cause and Cardiovascular Death

To determine whether glycemic control may affect the described influence of intronic OCT1 SNPs on all-cause and cardiovascular death in metformin users with T2DM we included HbA1c and HOMA-IR in our analysis. Rs461473, rs609468 and rs622591 were still significantly associated with an increased risk of all-cause death per minor allele. An association with an increased risk of cardiovascular death per minor allele was still seen for rs609468 and rs622591 (both p=0.012) and rs461473 (p=0.061), rs1777392 (p=0.070) and rs9295125/rs3818678 (p=0.077). For details see Table 7.

Prediction of Functional Consequences

Functional consequences of SNPs were determined by database search in HaploReg v4.1 and GETx Portal V8.

QTL Results

Rs461473, rs609468, rs622591, rs9295125, rs3818678 and rs456598 correlate with SLC22A1 expression. Rs9295125 correlates with expression of RP3-393E18.2, a large intergenic non-coding RNA locus, rs456598 with SOD2 expression and the presence of metabolites of the tryptophan and acylcarnitine metabolism.

Epigenomic Information

According to the chromatin 25-states model using 12 imputed marks, rs461473 creates a poised promoter in fat tissue, a primary H3K27ac possible enhancer in rectal mucosa and pancreatic islets and an active transcription start site in liver. It creates an active enhancer in the right atrium, duodenum mucosa, liver pancreas and an active promoter in the liver. In the liver it inactivates an enhancer and a promoter. Rs609468 creates an active enhancer in aorta, liver and an active promoter in the spleen. Rs622591 creates an active enhancer in the liver. Rs9295125 creates an active enhancer in fat, aorta and liver and rs3818678 in aorta, stomach smooth muscle, placenta and liver. Rs465698 generates an active enhancer in the right ventricle and lung and an active promoter in liver and spleen, whereas an inactive enhancer in the right ventricle is generated.

Table 6 Association of Intronic OCT1 SNPs with All-Cause and Cardiovascular Death in Non-Diabetic Patients (ND)

rs Number		All-Cause Death		Cardiovascular Death	
Major/Minor		Unadjusted	Adjusted	Unadjusted	Adjusted
Allele	n	1820	1820	1820	1820
rs461473 G/A	p-value	0.991	0.813	0.539	0.534
	HR	1.00	1.03	1.09	1.09
	CI	0.82–1.22	0.83–1.26	0.84–1.40	0.83–1.42
rs609468 C/T	p-value	0.458	0.715	0.861	0.935
	HR	0.94	0.97	0.98	1.01
	CI	0.79–1.11	0.82–1.15	0.79–1.22	0.81–1.26
rs622591 C/T	p-value	0.379	0.612	0.794	0.908
	HR	0.93	0.96	0.97	1.00
	CI	0.78–1.10	0.81–1.14	0.78–1.21	0.80–1.25
rs3777392 C/T	p-value	0.180	0.376	0.660	0.975
	HR	0.85	0.90	0.94	1.00
	CI	0.68–1.08	0.71–1.14	0.70–1.26	0.75–1.35
rs9295125 G/T rs3818678 G/C	p-value	0.118	0.074	0.876	0.837
	HR	1.12	1.14	1.01	1.02
	CI	0.97–1.28	0.99–1.31	0.84–1.22	0.85–1.23
rs456598 G/A	p-value	0.303	0.595	0.669	0.413
	HR	0.89	0.94	1.06	1.13
	CI	0.71–1.11	0.75–1.18	0.80–1.41	0.85–1.50

Note: The statistically tested allele is the minor allele.
Abbreviations: HR, hazard ratio; CI, 95% confidence interval.

Changes in Transcription Factor Binding Motifs

Rs461473 and rs609468 do not lead to changes in transcription factor binding sites. All other SNPs change various transcription factor binding sites: rs622591 changes Dbx1, Hoxa4, Ifr, Mef2, Zfp105, rs9295125 Foxo, Pax-8, Pou1f1 and Rox11, rs3818678 DMRT5, Foxo and TCF4 and rs456598 AIRE binding sites.

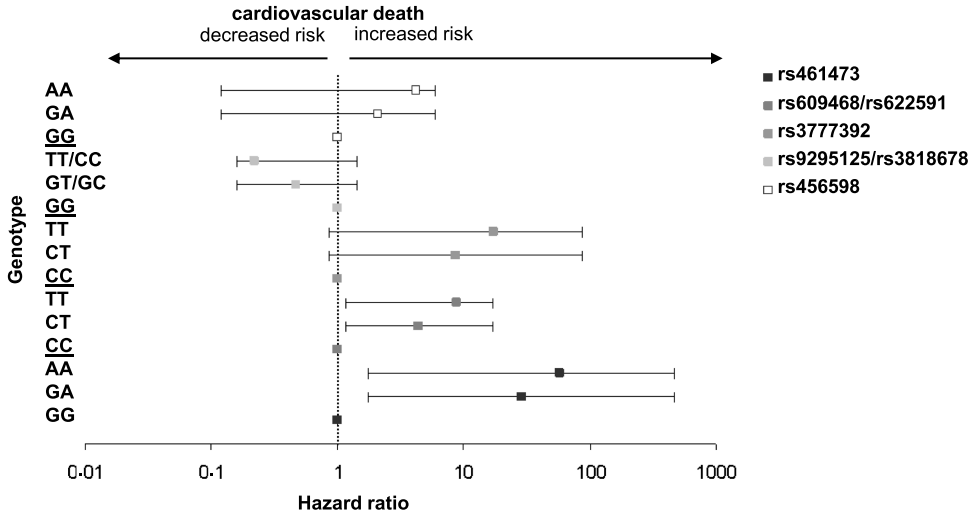

Figure 2 Hazard ratios for cardiovascular death per OCT1 genotype in diabetic metformin users. Squares: hazard ratios calculated with an additive cox proportional hazards regression model adjusted for confounders, error bars: 95% confidence interval, different shades of grey represent different intronic OCT1 SNPs or SNP combinations. Homozygosity for the major allele is set as 1.

Table 7 Association of Intronic OCT1 SNPs with All-Cause and Cardiovascular Death in Metformin Users with T2DM (MUT) After Adjustment for Glycemic Control

rs Number	Major/Minor	All-Cause Death			Cardiovascular Death		
	Allele	p-value	HR	CI	p-value	HR	CI
rs461473	G/A	0.009	14.38	1.94–106.78	0.061	11.25	0.89–141.61
rs609468	C/T	0.046	2.59	1.02–6.61	0.012	13.17	1.78–97.63
rs622591	C/T	0.046	2.59	1.02–6.61	0.012	13.17	1.78–97.63
rs3777392	C/T	0.781	1.23	0.29–5.11	0.070	9.90	0.83–118.41
rs9295125 rs3818678	G/T G/C	0.171	0.49	0.18–1.36	0.077	0.30	0.08–1.14
rs456598	G/A	0.264	2.85	0.45–17.94	0.845	0.80	0.08–17.68

Note: The statistically tested allele is the minor allele. n = 73.
Abbreviations: HR, hazard ratio; CI, 95% confidence interval.

Discussion

In this study, we investigated the effect of intronic *OCT1* SNPs on all-cause and cardiovascular death in 3040 Caucasians with increased cardiovascular risk. We identified 4 SNPs (rs461473, rs609468, rs622591, rs3777392), which were significantly associated with an increased risk per minor allele for all-cause and/or cardiovascular death in 73 individuals with T2DM on metformin therapy. This effect varied between 4.4 and 28.9 fold increased risk of cardiovascular death per minor allele in metformin users with T2DM. After adjustment for parameters of glycemic control, rs609468 and rs622591 were still significantly associated with an increased risk per minor allele for cardiovascular death, implicating that glycemic control does not impact their influence on CVD risk. Rs461473, rs3777392 and rs9295125 showed an association by trend with cardiovascular death after adjustment for parameters HbA1c and HOMA-IR, suggesting that poor glycemic control in our cohort is partly, but not completely, responsible for the association detected.

Intronic OCT1 SNPs might be influencing gene expression by either acting in cis (on the OCT gene cluster), in trans or even by modification of enhancers (influencing other "metformin" transporters or proteins determining metformin efficacy). The alteration of OCT gene expression in "cis" by changing transcription factor binding sites (as predicted for rs622591, rs3777392, rs9295125, rs3818678 and rs456598) would not only directly impact the transport of metformin but also the transport of physiological substrates. The transport of metformin determines its concentrations in blood, hence in end organs, and in the gastrointestinal tract. The first determines the capacity of metformin to inhibit hepatic gluconeogenesis by inhibition of the mitochondrial respiratory chain complex.[6,7] The second changes the microbiota composition in the gastrointestinal tract which might be achieved via inhibition of bacterial complex I homologues.[44] Alterations in microbiota on the one hand decrease gut permeability[45] and alter on the other hand the profile of bacterial products generated. Bacterial products per se might contribute to the effects of metformin on glucose metabolism[11] or have direct effects on inflammation like indole,[46] butyrate[47] and small chain fatty acids.[48] Since inflammation-mediated processes might be one of the main mechanisms the gut microbiota influences T2DM development and progression as well as CVD risk, metformin acts on both by decreasing systemic inflammation. This might be one of the mechanism rs622591 influences the risk of CVD, since Becker and colleagues could not see any effect of its genotypes on HbA1c levels.[32]

An alteration of OCT gene expression also affects the transport of their natural substrates like neurotransmitters, polyamines, prostaglandins and thiamine. All these substances are linked to glucose and lipid metabolism as well as cardiovascular disease either in humans or animal models[49–62] and might thus be responsible for the observed increased cardiovascular risk. Christensen and colleagues could not detect any change of plasma metformin concentrations in individuals with different rs461473 alleles rather implicating a mechanism other than cis-regulation.[22] This might include trans-regulatory processes like creating or destroying enhancers or via non-coding RNAs. Trans regulation may affect other transport proteins using metformin as a substrate[63] like the plasma monoamine transporter (PMAT), the multidrug toxin and extrusion (MATE 1), and MATE 2, again influencing the metformin concentration in gut and thus the gut microbiota Trans regulation may also affect the expression of mediators of metformin action or efficacy like AMPK,[64] LBK1,[65] SRR and BDNF. Another

candidate gene is *SLC2A2*, encoding the metformin target GLUT2 transporter.[66] Rs456598 and rs3777392, ie affect *SOD2* gene expression, which is associated with microvascular complications of diabetic ischemia.[67] Intronic SNPs might also influence epigenetic regulation and thus the accessibility of chromatin as predicted for some of the SNPs.

Based on the results of this study, it might be of interest to monitor several individuals with T2DM currently using metformin, which might have an increased risk of cardiovascular incidents according to their OCT1 genotype. Since metformin is also used for a number of other indications than type 2 diabetes therapy, these findings might also concern other disease groups.

Major limitations of this study are the relatively small sample size of metformin users with T2DM due to lack of safety data for metformin ahead and during the recruitment period and the fact that we performed the analysis without direct access to sample material to measure additional parameters to the given ones. We did not select functional variants in this study, because their effect on metformin efficacy in our patient groups has already been widely investigated. Furthermore, statistical power to investigate some of these functional *OCT1* SNPs known to be associated with decreased metformin efficacy was too low due to a very low allele frequency in our cohort. The selection of intronic SNPs might be another limitation of this study. In contrast to coding SNPs, which directly affect transporter efficacy by changing the protein sequence and thus the ability to transport its substrates, intronic SNPs may change the expression on the genes in their vicinity, or far from their location, enhancing further the complexity of possible ways of action.

Since these data were generated in a cohort with a small number of metformin users it is necessary to replicate our findings in a cohort reflecting presently usage of metformin as first-line therapy in type 2 diabetes treatment and to further include coding SNPs in OCT1 for the analysis of complex intertwining between metformin, glycemic control and thereafter cardiovascular outcomes.

Conclusion

We were able to show in a small number of persons with type 2 diabetes mellitus on metformin therapy a susceptibility to an increased risk of cardiovascular death according to their OCT1 genotype. This unexpected effect was only partly due to impaired glycemic control implicating other pleiotropic effects of metformin. This finding might also be interesting for potential metformin users in other indications than diabetes mellitus.

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