

UNIVERSITY OF LATVIA

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DOCTORAL THESIS

**Pharmacogenetics of efficiency and tolerance  
of the peroral antidiabetic drug metformin**

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*Molecular Biology*

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Declare the past, diagnose the present, foretell the future.

*Hippocrates. Epidemics, Bk. I, Sect. XI*

## ANNOTATION

Metformin is the most widely used peroral antidiabetic drug worldwide prescribed more than 61.6 billion times in the U.S. alone in 2012 and one of only two peroral antidiabetic drugs included in the 18<sup>th</sup> World Health Organisation (WHO) Essential Medicines List. According to FDA (Food and Drug Administration), IDF (International Diabetes Federation) and EASD (European Association for the Study of Diabetes) guidelines, metformin is the recommended first-line medication for newly diagnosed type 2 *diabetes mellitus* (T2D). However, metformin monotherapy is inefficient in ~30% patients and associated with common side-effects in 20–60% users, leading to significant non-compliance (up to 30% of users) and discontinuation of therapy in 5–10% of all cases. Metformin is not metabolised in the human body. Genetic variabilities of 7 metformin transporters in the gut (OCT1, OCTN1, OCT3, and PMAT), liver (OCT1, OCT3) and kidney (OCT1, OCT2, MATE1, MATE2) have been shown to induce alterations in the pharmacokinetics and efficiency of metformin in patients with T2D.

Our group was the first to demonstrate an association between side-effects of metformin and two genetic variants (rs628031 and rs36056065) of the organic cation transporter 1 (OCT1/*SLC22A1*), implying an association between metformin transporters and tolerability of metformin. Subsequently, three novel genetic variations (rs3119309, rs2481030 and rs7757336) in the upstream region of organic cation transporter 2 (OCT2/*SLC22A2*) and organic cation transporter 3 (OCT3/*SLC22A3*) coding genes were shown to be associated with short-term efficiency of metformin therapy. Two of these variants were further analysed in a replication study on 126 T2D patients from Slovakia and a pharmacokinetic study involving 15 healthy participants. Overall, 33 genetic variants of *ATM*, *STK11* and T2D susceptibility genes were investigated, among which only a few showed a nominal association with short-term efficiency of metformin monotherapy.

The results of this research have confirmed significant effects of genetic variations in OCTs on safety and efficiency of metformin therapy, and highlighted a novel mechanism of transporter-associated development of common side-effects of metformin.

## ANOTĀCIJA

Metformīns ir visā pasaulē ļoti plaši lietots perorāls antidiabētisks medicaments; 2012. gadā tas ir ticis nozīmēts 61.6 miljardus reižu Amerikas Savienotajās Valstīs vien, kā arī tas ir viens no tikai diviem perorālajiem antidiabētiskajiem medikamentiem, kas iekļauts Pasaules Veselības organizācijas Esenciālo Medikamentu sarakstā (18. versija). Atbilstoši ASV Pārtikas un Zāļu administrācijas (FDA), Starptautiskās Diabēta Federācijas (IDF) un Eiropas Diabēta Pētniecības asociācijas (EASD) vadlīnijām metformīns ir pirmās izvēles medikaments jaundiagnosticētu 2.tipa cukura diabēta pacientu (T2D) ārstēšanā. Tomēr metformīna monoterapija ir neefektīva aptuveni 30% pacientu un 20-60% gadījumu ir asociēta ar biežu blakņu manifestāciju, kas noved pie būtiskiem zāļu lietošanas režīma pārkāpumiem (līdz 30% lietotāju) un 5–10% pacientu liek pārtraukt terapiju.

Metformīns netiek metabolizēts cilvēka ķermenī un 7 metformīna transportieru, kas lokalizēti zarnās (OCT1, OCTN1, OCT3, un PMAT), aknās (OCT1, OCT3) un nierēs (OCT1, OCT2, MATE1, MATE2) ģenētiskā variabilitāte ir identificēta kā būtisks metformīna terapiju un farmakokinētiku ietekmējošs faktors T2D pacientiem.

Mēs bijām pirmie, kas identificēja asociāciju starp metformīna blakusparādībām un 2 ģenētiskiem variantiem (rs628031 and rs36056065) organisko katjonu transportierī 1 (OCT1/*SLC22A1*) norādot uz saistību starp metformīna transportieriem un metformīna panesamību. Otrkārt, trīs jaunas ģenētiskas variācijas (rs3119309, rs2481030 un rs7757336) reģionā (*upstream*) pirms organisko katjonu transportiera 2 (OCT2/*SLC22A2*) un organisko katjonu transportiera 3 (OCT3/*SLC22A3*) kodējošiem gēniem tika noteiktas kā būtiski asociētas ar metformīna īstermiņa terapijas efektivitāti. Divi no šiem variantiem tika analizēti replikācijas pētījumā ar 126 T2D pacientiem no Slovākijas un farmakokinētikas pētījumā ar 15 veseliem brīvprātīgajiem. Visbeidzot, 33 ģenētiskas variācijas tika izpētītas *ATM*, *STK11* un T2D kandidātgēnos, bet tikai dažas no tām bija nomināli asociētas ar metformīna īstermiņa terapijas efektivitāti.

Pētījuma rezultāti apstiprina OCT kodējošo gēnu ģenētisko variāciju būtisko nozīmi metformīna terapijas panesamības un efektivitātes nodrošināšanā un norāda uz jaunu, ar transportieru saistītu mehānismu, kuram ir nozīme metformīna biežo blakusparādību attīstībā.

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## ABBREVIATIONS

2DG	2-deoxy-d-glucose	DNA	deoxyribonucleic acid
3A1/2	cytochrome P450 3A1/2	DPP	Diabetes Prevention Program
5-HT	serotonin or 5-hydroxytryptamine	DPP-IV	dipeptidyl peptidase-4
6-AS	6-antroyloxystearic acid	EASD	European Association for the Study of Diabetes
<i>ABCB1</i>	p-glycoprotein coding gene	ECR	evolutionary conserved region
<i>ABCC8</i>	ATP-binding cassette transporter sub-family C member 8 coding gene	eGFR	estimated glomerular filtration rate
<i>ABCG2</i>	cancer resistance protein coding gene	eNOS	endothelial nitric oxide synthase
ABO	blood group system discriminating O, A, B, AB blood types	ENT	equilibrative nucleoside transporter
AC	adenylyl cyclase	ERGO	[3H] ergothioneine
ACC	acetyl-CoA carboxylase	ESRD	end-stage renal disease
ACTH	adrenocorticotrophic hormone	<i>EWS/WT1</i>	fusion oncogene gene
ADA	American Diabetes Association	F	bioavailability
<i>ADCY5</i>	adenylate cyclase, type 5 coding gene	<i>FADS1</i>	fatty acid desaturase 1 coding gene
<i>ADIPOR2</i>	adiponectin receptor 2 coding gene	FDA	Food and Drug Administration
ADP	adenosine diphosphate	FEcreat	net tubular creatinine secretion
<i>ADRA2A</i>	alpha-2A adrenergic receptor coding gene	FEX	fexofenadine
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide	FHG	fasting hyperglycaemia
AKI	acute kidney injury	FPG	fasting plasma glucose
Akt	protein kinase B (PKB)	<i>FTO</i>	fat mass and obesity associated gene
ALT	alanine aminotransferase	FXRs	the farnesoid X Receptors (bile acid receptors)
AMP	adenosine monophosphate	G6pc	glucose-6 phosphatase
AMPK	5' AMP-activated protein kinase	G6PC2	glucose-6-phosphatase, catalytic, 2
AP	apical	GCG	preprotein of glucagon
AP-1	activating protein-1	<i>GCK</i>	glucokinase (hexokinase 4) gene
AP-2rep	AP-2rep Transcription Factor	<i>GCKR</i>	glucokinase (hexokinase 4) regulator gene
ASP <sup>+</sup>	fluorescent organic cation 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide)	GFR	glomerular filtration rate, e-estimated
<i>ATM</i>	ataxia telangiectasia mutated (gene)	GI	gastrointestinal
ATP	adenosine triphosphate	GI AE	gastrointestinal adverse events
AUC	area under the curve	<i>GIPR</i>	gastric inhibitory polypeptide receptor gene
B12	vitamin B12 or cobalamin	<i>GLIS3</i>	GLIS family zinc finger 3 gene
BDNF	brain-derived neurotrophic factor	<i>GLP-1</i>	glucagon-like peptide-1 gene
bFGF	basic fibroblast growth factor	GLUT2	glucose transporter 2
BL	basolateral	GoDARTS	Genetics of Diabetes Audit and Research Tayside study
BMA	British Medical Association	GR	glucocorticoid receptor
BMI	body mass index	GWAS	genome-wide association study
C2CD4B	C2 calcium-dependent domain containing 4B	HbA <sub>1c</sub>	glycated haemoglobin
cAMP	cyclic AMP	HCC	human hepatocellular carcinoma
<i>CAPN10</i>	calpain 10 gene	HDL	high-density lipoprotein
CARDS	Collaborative Atorvastatin Diabetes Study	HEK	human embryonic kidney cel lines
CBG	capillary blood glucose	HFD	high fat diet
<i>CDKAL1</i>	CDK5 regulatory subunit associated protein 1-like 1 gene	<i>HHEX</i>	hematopoietically expressed homeobox gene
<i>CDKN2a [2b]</i>	cyclin-dependent kinase inhibitor 2A [2B] genes	HILIC	hydrophilic interaction liquid chromatography
CDV	cardio-vascular disease	<i>HNF1A</i>	hepatocyte nuclear factor 1 homeobox A gene
CGC	cholangiocarcinoma	<i>HNF1B</i>	HNF1 homeobox B gene
CHO	cholesterol	<i>HNF4A</i>	hepatocyte nuclear factor 4a gene
CI	confidence interval	HNSCC	head and neck squamous cell carcinomas
CL/F	oral clearance	holoTCII	bioavailable B12
Cl <sub>r</sub>	creatinine clearance	HWE	Hardy-Weinberg equilibrium
Cl <sub>renal</sub>	renal clearance	ICD-10	International Classification of Diseases (ICD) - 10th revision
Cl <sub>sec</sub>	active secretion clearance	IDF	International Diabetes Federation
C <sub>max</sub>	maximum plasma concentration	IFG	impaired fasting glucose
COSMIC	the Catalogue of Somatic Mutations in Cancer	<i>IGF1</i>	insulin-like growth factor 1 gene
<i>CRTC2</i>	cAMP response element-binding protein-regulated transcription coactivator 2 gene	<i>IGF2BP2</i>	insulin-like growth factor 2 mRNA binding protein 2 coding gene
<i>CRY2</i>	cryptochrome circadian clock 2 gene	IGT	impaired glucose tolerance
CT	computerized tomography	IR	immediate-release
CVR	cardio-vascular risk	<i>IRS1</i>	insulin receptor substrate 1 gene
CYP2C11	cytochrome P450, subfamily 2, polypeptide 11 (rat)	IS	internal standard
CYP2D1	cytochrome P450 2D1	<i>ITLN2</i>	intelectin 2 gene
DA	dopamine	k	constant of the last exponential phase
DCS	Diabetes Care System West-Friesland	<i>KCNJ11</i>	potassium inwardly-rectifying channel, subfamily J, member 11 gene
<i>DGKB</i>	diacylglycerol kinase, beta coding gene	<i>KCNQ1</i>	potassium voltage-gated channel, KQT-like subfamily, member 1 coding gene
DM	<i>diabetes mellitus</i>	KEGG	Kyoto Encyclopedia of Genes and Genomes
DMSO	dimethyl sulfoxide	K <sub>i</sub>	inhibitor constant
DN	diabetic nephropathy	LC-MS/MS	liquid chromatography/tandem mass spectrometry
		LD	linkage disequilibrium
		LDA	Latvian Diabetes association



LDL	low density lipoprotein	<i>PRKAB2</i>	5'-AMP-activated protein kinase subunit beta-2 gene
LGDB	Genome Database of Latvian Population	<i>PRKAG2</i>	protein kinase, AMP-activated, gamma 2 non-catalytic subunit gene
<i>LKB1</i>	liver kinase B1, the same as <i>STK11</i> gene	<i>PROX1</i>	prospero homeobox 1 gene
LXR $\alpha$	liver X receptor alpha	PSCUH	Pauls Stradins Clinical University Hospital
MADD	MAP-kinase activating death domain	PXR	pregnane X receptor
MAF	minor allele frequency	PYY	peptide YY
MAP	methamphetamine	QALY	quality-adjusted life-years
MATE	multidrug and toxic compound extrusion protein (h – human, r – rat, m – mouse)	<i>RETN</i>	resistin coding gene
<i>MEF2A</i>	myocyte enhancer factor 2A	RBC	red blood cells
<i>MEF2D</i>	myocyte enhancer factor 2D	RR	risk ratio
MMA	methymalonic acid	RT-PCR	real-time polymerase chain reaction
MODY	maturity-onset diabetes of the young	<i>SCO2</i>	SCO2 cytochrome c oxidase assembly protein
MPP	1-methyl-4-phenylpyridinium	SCr	serum creatinine
<i>MTNR1B</i>	melatonin receptor 1B gene	SD	standard deviation
mTOR	regulate rapamycin complex 1	SE	standard error
<i>MZF-1</i>	myeloid zinc finger 1 gene	SGLT-1	sodium-glucose transport protein 1
NAD <sup>+</sup>	nicotinamide adenine dinucleotide	<i>SLC22</i>	solute carrier gene family 22
NADPH	nicotine adenine disphosphonucleotide, reduced	<i>SLC2A2</i>	solute carrier family 2 coding gene
NAFLD	non-alcoholic fatty liver disease	<i>SLC30A8</i>	solute carrier family 30 (zinc transporter), member 8 gene
NE	norepinephrine	<i>SLCO1A2</i>	solute carrier organic anion transporter family, member 1A2 gene
<i>NEGR1</i>	neuronal growth regulator 1 gene	SNP	single-nucleotide polymorphism
NO	nitri oxide	Sp1	transcription factor Sp1
<i>NPAT</i>	nuclear protein gene, Ataxia-Telangiectasia locus	SrCLR	net secretion
<i>OAT</i>	organic anion transporter coding gene	SREBP-1	sterol regulatory element-binding protein-1
OCT	organic cation transporter (h – human, r – rat, m – mouse)	<i>STK11</i>	serine/threonine kinase 11, gene
OCTN	carnitine/organic cation transporter	$t_{1/2}$	terminal half-life
OGTT	oral glucose tolerance test	T1DM	type 1 <i>diabetes mellitus</i>
OMI	serine protease	T2D	type 2 <i>diabetes mellitus</i>
OR	odds ratio	tagSNPs	tagging polymorphism
p53	the p53 tumor suppressor protein	TCA cycle	the citric acid cycle
PARGC1A	peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC1alpha)	TCAs	tricyclic antidepressants
<i>Pck1</i>	phosphoenolpyruvate carboxykinase	<i>TCF7L2</i>	transcription factor 7-like 2 (T-cell specific, HMG-box) gene
PCOS	polycystic ovary syndrome	TEA	tetraethylammonium
PCR	polymerase chain reaction	TGF- $\beta$ 1	transforming growth factor beta 1
<i>PCSK1</i>	proprotein convertase subtilisin/kexin type 1	TGR5	the G-protein coupled bile salt receptor
PG	plasma glucose	$T_m$	melting temperature (PCR)
PKA	protein kinase A	$T_{max}$	time to reach $C_{max}$
PKLR	pyruvate kinase	<i>TMEM18</i>	transmembrane protein 18 gene
PMAT	plasma membrane monoamine transporter	TMD	transmembrane domain
POMC	pro-opiomelanocortin	TMH	transmembrane helices
PP2A	protein phosphatase 2 (PP2)	<i>TNF-<math>\alpha</math></i>	tumor necrosis factor gene
<i>PPARG</i>	peroxisome proliferator-activated receptor gamma gene	<i>TSC2</i>	tuberous sclerosis complex 2 gene
<i>PPARGC1B</i>	peroxisome proliferator-activated receptor gamma, coactivator 1 beta gene	<i>Txnip</i>	thioredoxin interacting protein
<i>PPAR<math>\alpha</math></i>	peroxisome proliferator-activated receptor $\alpha$ gene	UKPDS	UK Prospective Diabetes Study
PPHG	postprandial hyperglycaemia	USFs	upstream stimulating factors
PPI	proton pump inhibitors	Vd/F	apparent volume of distribution
<i>PRKAA1</i>	5'-AMP-activated protein kinase catalytic subunit alpha-1 gene	VIF	variant inflation factor
<i>PRKAA2</i>	5'-AMP-activated protein kinase catalytic subunit alpha-2 gene	VLDL	very-low-density lipoprotein
		WFS1	Wolfram syndrome 1 (wolframin)
		WHO	World Health Organisation
		XR	extended-release

## INTRODUCTION

Metformin (1,1-dimethylbiguanide) is prescribed to at least 120 million people worldwide as a first-line drug for type 2 diabetes mellitus (T2D) (ADA, 2014; Nathan et al., 2008; Viollet et al., 2012). Metformin reduces both fasting and postprandial glucose, the surrogate marker of glycaemic control HbA<sub>1c</sub> (1–1.5%), and insulin resistance (Herman SL, 2002; Ou et al., 2006) without significant risk of hypoglycaemia (UKPDS study, 1998; DeFronzo and Goodman, 1995). Major limitations have been reported as inefficiency of therapy in up to one-third of patients within the first year of therapy (HbA<sub>1c</sub>, 7%) and common gastrointestinal side-effects in ~20–60% of metformin users, leading to non-compliance (30%) and discontinuation of treatment (5–10%) (Brown et al., 2010; Donnelly et al., 2009; Esposito et al. 2012; Graham et al., 2011).

Metformin is not metabolized, and transported via limited passive diffusion through the cell membrane (Pentikainen et al., 1979; Proctor et al., 2008). Metformin distribution in the body is facilitated by organic cation transporters (OCTs), multidrug and toxin extrusion antiporters (MATEs) and plasma membrane monoamine transporter (PMAT) (Muller et al., 2005; Nies et al., 2009; Otsuka et al., 2005; Tanihara et al., 2007; Verhaagh et al., 1999; Zhou et al., 2007). Data from genome-wide complex trait analysis (GCTA) showed that heritability in glycaemic response to metformin varies from 20% to 34% (Zhou et al., 2014) and the first GWAS of long-term efficiency of metformin identified the rs11212617 polymorphism in close proximity to the ataxia-telangiectasia mutated gene (*ATM*) as significantly associated with metformin response (OR=1.35), but accounting for only 2.5% of the variability (GoDarts et al., 2011). A number of pharmacogenetic studies have investigated metformin efficiency to date. However, the results have been inconsistent, with limited genetic coverage (Christensen et al., 2011; Tkac et al., 2013; Tzvetkov et al., 2009; Becker et al., 2010a).

The aim of this study was to investigate variability in genes coding for metformin transporters, metformin molecular targets and type 2 *diabetes mellitus* (T2D) susceptibility loci to identify the specific genetic variants associated with intolerance and efficacy of metformin therapy. The main objectives were as follows:

1. Investigate a possible association of common polymorphisms affecting transport activities of OCT1, OCT2 and MATE1 with the presence of metformin side-effects and other phenotypic and clinical measurements in T2D patients;

2. Assess genetic variability in OCT1, OCT2, OCT3, MATE1, MATE2 and PMAT coding genes and determine the genetic predictors of metformin efficacy in a prospective study on a group of newly diagnosed, drug-naive subjects with T2D;
3. Analyse the polymorphisms significantly associated with susceptibility to T2D within the population of Latvia with respect to incidence of T2D and metformin efficacy;
4. Estimate the impact of SNPs identified for metformin nonresponsiveness on the pharmacokinetics of metformin in healthy volunteers.

# 1 LITERATURE REVIEW

## 1.1. *Diabetes mellitus*

In 2012 at least 1.5 million deaths were directly caused by *diabetes mellitus* (DM) and in 2014 the global prevalence of DM was estimated to be 9% among adults (>347 millions of people)(WHO, 2012, 2014). Rising incidence of obesity throughout society, and especially in children, will lead to an increase of the number of DM patients DM will be the 7th leading cause of death by this time (Mathers and Loncar, 2006; Shaw et al., 2010; WHO, 2012). DM is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action (insulin resistance in liver, adipose tissue and muscles), or both (Kahn, 2003). T2D is the most common form of DM (up to 90–95% of all cases), type 1 *diabetes mellitus* is diagnosed in up to 5–10% of DM patients and more rare forms account for MODY (maturity-onset *diabetes* of the young) and gestational *diabetes* (altogether up to 4%) (ECDCDM, 1997; Zimmet et al., 2001). Between year 2008 and 2013 the total number of T2D patients in Latvia has increased from 59 112 to 76 595 and accounts for 94% of all registered DM patients (CSB, 2013 ).

Glucose homeostasis is regulated by interplay of insulin, glucagon, amylin, and incretin hormones and, normally, gluconeogenesis is upregulated in the liver during fasting (Magnusson et al., 1992; Woods et al., 2006). Inability of insulin to suppress hepatic glucose output is a major aetiological factor of hyperglycemia in the T2D (Cherrington, 1999). Initial development of hyperglycaemia is characterized by an insulin resistance leading to impairment of insulin signaling pathways and down-regulation of activity of glucogenic enzymes (Saltiel, 2001). Disease gradually develops when  $\beta$  cells become insufficient to compensate for insulin resistance and thus the glucose level increases to damaging concentrations (ADA, 2014).

Genetic studies have shown that both insulin sensitivity (30–40%) and the insulin response (38%) are heritable and a vast amount of T2D susceptibility genes are involved in the regulation of beta cell functions (Bergman et al., 2003; Elbein et al., 1999). Other T2D risk factors are: early pre- and postnatal environment, birthweight, obesity and central body composition, age, genetic factors, sedentary lifestyle and western diet (high fat, high sugar content and highly processed food) (Berends and Ozanne, 2012; Bi et al.). Symptoms of DM range from mild ones like tiredness, polyuria, polydipsia, weight loss, polyphagia, blurred vision to severe ones, such as hyperglycemia with ketoacidosis or the nonketotic hyperosmolar syndrome (Genuth et al., 2003). The rate of cardiovascular disease is

approximately two times higher and overall mortality risk doubles in diabetic patients in comparison to non-diabetic patients (Morrish et al., 2001; Sarwar et al., 2010).

The main biochemical analysis performed on regular basis for diagnostics and treatment of T2D are measurements of levels of fasting plasma glucose (FPG), glycosylated haemoglobin (HbA<sub>1c</sub>) and results of oral glucose tolerance test (OGTT) (Nakagami et al., 2010; Stevens et al., 1977). FPG is simple and cost-effective biochemical analysis, however, it lacks sensitivity in early T2D, is highly variable and dependent on many other factors (food, physical activity, disorders, injuries, other drugs than ones used in antidiabetic therapy)(Kim et al., 2008a; Somogyi, 1949). OGTT, on the other hand, is suitable for early detection of T2D, however, due its greater cost, inconvenience and lower reproductibility, it is not recommended for a routine clinical use (Baird and Duncan, 1959). HbA<sub>1c</sub> reflects the average plasma glucose level in the last 2-3 months and has become available as routine procedure in most of the counties in the last three decades (Garlick et al., 1983; WHO, 2006). Currently HbA<sub>1c</sub> is not widely recommended for an early screening of T2D (ADA, 2004; Alberti and Zimmet, 1998).

A risk factor for future T2D, as well as cardiovascular disease, is considered “pre-diabetes” which is characterized by an impaired fasting glucose (IFG) 5.6–6.9 mmol/L and/or impaired glucose tolerance (IGT) after 2-h post load glucose 7.8–11.1 mmol/L. Criteria for the diagnosis of T2D according to the guidelines of Latvian Diabetes association (LDA, 2007) are:

- 1) fasting plasma glucose (FPG): 7.0 mmol/l;
- 2) symptoms of hyperglycemia and a casual plasma glucose: 11.1 mmol/l;
- 3) 2 h plasma glucose: 11.1 mmol/l during an oral glucose tolerance test (OGTT);

Biochemical analysis should be repeated in other day to ensure accuracy in the diagnosis of DM (Brown et al., 2010; CDA, 2013; WHO, 2006).

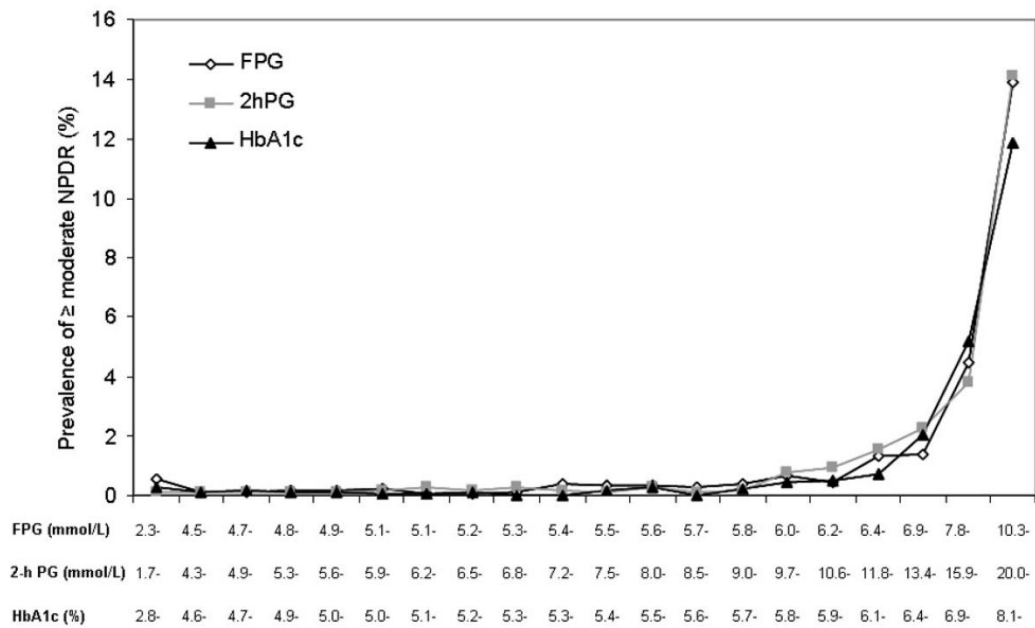
Recent study showed that at cut-off values of FPG  $\geq 6.1$  mmol/l and HbA<sub>1c</sub>  $\geq 6.1\%$  if both criteria were satisfied, the correct prediction of DM was relatively high (82.9%) and if both FPG was  $< 6.1$  mmol/l and HbA<sub>1c</sub> was  $< 6.1\%$ , than there was little likelihood of T2D (1.7%). Such findings reduce the need for OGTT down to 19.4% when only one of the measurements is increased instead of 34.2% according to recommendations by ADA (Kim et al., 2008a). Similarly, a good result of HbA<sub>1c</sub> efficiency as diagnostic tool for early T2D was obtained in another study (Nakagami et al., 2010). In the year 2011 WHO accepted the use of HbA<sub>1c</sub> testing in diagnosing T2D with cut-off value of 6.5% (WHO, 2006).

### ***1.1.1. Goals of antidiabetic therapy***

It is thought that patients with a good blood sugar control, life-style changes and self-care may prevent or delay onset of T2D and, if early diagnosed with T2D, have good outcomes when treated timely and adequately (DCCTR group. 1993; UKPDS, 1998; Gerstein et al., 2008; Holman et al., 2008; van den Berghe et al., 2001). Poor control of T2D (long-term hyperglycemia) is associated with development of macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and wide range of microvascular complications associated with damage of the eyes (retinopathy with potential loss of vision), kidneys (nephropathy leading to renal failure), nerves (peripheral neuropathy with risk of foot ulcers, amputations, Charcot joints and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction)(ADA, 2014; Rosolova et al., 2008). Cardiovascular disease is the cause of death in up to 70 % of diabetic patients, leading to an approximately 10 years shorter life-span in comparison to healthy individuals (Amos et al., 1997; Reaven, 1988).

The diagnostic screening cut-off points should be decided by their relevance for likelihood of complications (Nakagami et al., 2007). T2D has asymptomatic period of 4-5 years and thus newly diagnosed patients are characterized with high frequency hyperlipidemia, hypertension, neuropathy, nephropathy and retinopathy (73.5%, 58.5%, 52%, 10%, and 6%, respectively (Iraj Heydaria, 2010). Cut-off point of 5.6% in study investigating T2D patients from Japan (adequate to 5.9% in U.S.) was found to be the most proper one in respect to screening test and prediction of vascular complications (Nakagami et al., 2007). HbA<sub>1c</sub> had a similar predictability of future retinopathy to 2 h PG or FPG, and the optimal HbA<sub>1c</sub> associated with future retinopathy was 5.7% in a Hisayama study (or 6% in U.S.)(Miyazaki et al., 2004) and between 5.9% and 6.2% in the Third National Health and Nutrition Examination Survey (U.S.). Marker of increased incidence of rethinopathy from longitudinal study in Pima Indians was HbA<sub>1c</sub> >7% (McCance et al., 1994).

Compilation of studies investigating microvascular complications (diabetic retinopathy and microalbuminuria) was reflected in the WHO report in year 2006 and it showed a high variability in optimal thresholds of HbA<sub>1c</sub> (5.7-7.6%), FPG (6.4-8.5 mmol/L) and 2 h PG (OGTT)(9.8-14.4 mmol/L)(Stolar, 2010; WHO, 2006). Correlation between HbA<sub>1c</sub>, FPG, OGTT and incidence of diabetic retinopathy is found to increase exponentially if fasting plasma glucose exceeds >6 mmol/l or HbA<sub>1c</sub> is >5.8% (Figure 1.1.).



**Figure 1.1.** From World Health Organisation report “Prevalence of diabetes-specific retinopathy” by vigintiles of distribution of FPG, 2 h PG and HbA<sub>1c</sub> from DETECT-2” in 2006 (WHO, 2006).

In targeted T2D screening group, the prevalence of macrovascular complications was found to be similar to that of patients detected in general practice, but with a lower degree of hyperglycaemia (Spijkerman et al., 2004). HbA<sub>1c</sub>, FPG and 2 h PG were shown to positively, but not linearly correlation with all of the cause and CVD mortality in population-based study albeit association was less clear in comparison to microvascular complications (Khaw et al., 2004; Nakagami and Group, 2004). Each 1% reduction in HbA<sub>1c</sub> was shown to reduce risk for 21% for any endpoint related to T2D, 21% for deaths related to T2D, 14% for myocardial and 37% for microvascular complications (Stratton et al., 2000). Scientists have assessed that the optimal thresholds may be very low due to association of even lesser impairments of glucose regulation that are associated with an increased CVD risk (Decode Study Group, 2003).

### 1.1.2. Treatment guidelines

At least 7 groups of peroral antidiabetic medications and insulin analogs are available for treatment of T2D with considerable variety in efficiency - ranging from 25.9% to 63.2% of participants reaching for glycaemic goal of HbA<sub>1c</sub> <7% (Esposito et al., 2012). The primary factors for choice of drugs for treatment of T2D include efficacy and safety of antidiabetic drugs. The UK Prospective Diabetes Study (UKPDS) and other studies have found metformin to be more effective than chlorpropamide, glimepiride, glibenclamide and insulin (UKPDS study, 1998; Adler et al., 2000; Stratton et al., 2000;

Zhu et al., 2013). Other factors responsible for the variation in the pattern of utilization of pharmacotherapy are cost, quality of life, and patient preferences (Singh, 2014).

Many studies have demonstrated that lifestyle or pharmacological interventions in subjects with impaired glucose tolerance (IGT) can delay or prevent T2D (Kawamori et al., 2009; Knowler et al., 2002; Tuomilehto et al., 2001). Metformin intervention in comparison to placebo was estimated to delay the development of T2D by 3 years and to reduce the absolute incidence of T2D by 8%, respectively. The cumulative incidence of the complications was reduced and survival was improved by 0.2 years. The cost per quality-adjusted life-years (QALY) was cost-effective (\$31 300) in all age groups, except for persons older than 65 years. It should be noted that lifestyle intervention was more effective and less resource consuming in most of comparisons to metformin therapy (Herman et al., 2005). Data from n=1 073 patients in the standard lifestyle management plus metformin arm from DPP study suggests, that improved postprandial hyperglycaemia (PPHG) was superior with an intensive lifestyle than metformin, whereas both treatments exerted a similar effect on fasting hyperglycaemia (FHG). Metformin was not as efficient as intensive life style intervention, but it had a clinically significant effect in obese individuals and in those with impaired fasting glucose (IFG)(Herman SL, 2002).

The International Diabetes Federation (IDF) states that lifestyle changes after T2D diagnosis can provide control of blood glucose concentrations to safe levels in a minority of patients and usually for a limited period after diagnosis (IDF, 2005). Indeed, approximately 15% of type 2 diabetic subjects fail to lower their fasting plasma glucose to 15.0 mmol/L after 3 months of diet therapy (UKPDS, 1998) and 3 years later 75% of subjects treated with only a dietary intervention failed to maintain an HbA<sub>1c</sub> of 7% (Turner et al., 1999).

The American Diabetes Association (ADA) and European Association for the Study of Diabetes (EASD) recommend metformin to be the first-line medication in all newly diagnosed patients, regardless of age, if lifestyle changes are not sufficient (Nathan et al., 2008). Metformin is moderately favorable in respect to the ease of dosing but it should be adjusted for renal impairment. It is recommended to titrate the dose and apply multi-dosing of metformin in meal-times over early weeks to minimize incidence of side-effects and discontinuation due to intolerance. Evidence or risk of renal impairment (eGFR<60 ml/min/1.73 m<sup>2</sup>) or serum creatinine >1.5 mg/dl (132.60 µmol/L) in males and >1.4 mg/dl (123.76 µmol/L) in females) are contraindication for metformin use. Guidelines

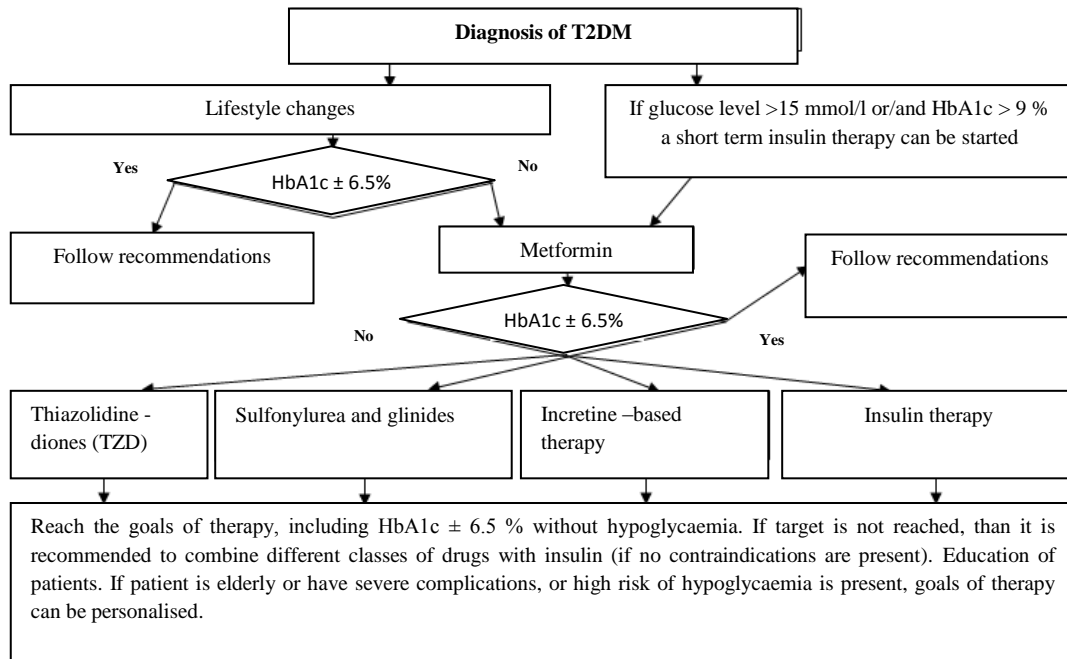


of IDF enlist also a need for regular measurements of HbA<sub>1c</sub>, consultations of healthcare professionals, life-style measures, self-monitoring and education to ensure quality of treatment (IDF, 2005).

Retrospective study in 15 516 patients confirmed the effectiveness of initial selection of metformin as first line medication. Despite guidelines, only 57.8% of participants began an antidiabetic therapy with metformin but this group showed reduced subsequent treatment intensification, without differences in rates of hypoglycaemia or other adverse clinical events in comparison to sulfonylurea, thiazolidinediones, or dipeptidyl peptidase 4 inhibitors (Berkowitz et al., 2014).

Increasing burden of T2D requires economical evaluation of antidiabetic therapy and it favors metformin (Diabetes Prevention Program Research, 2012a), however goals of therapy are open to discussion. Small study in patients using metformin for 9 months showed that HbA<sub>1c</sub> cut-off values dramatically change proportions between responders and non-responders (19% if treatment goal was <7% to 43% if treatment goal was <7.5%), leading to assumption that even minor changes in HbA<sub>1c</sub> goal will change the course of therapy for large portion of patients (Moses, 2008). Economically, threshold <9% in comparison to <6.5% HbA<sub>1c</sub> was associated with a shorter time spent on monotherapy, ranging from 1.1 years to 13 years, respectively. Lowest thresholds were found to increase the total lifetime cost of therapy (McEwan et al., 2015).

Treatment guidelines of T2D in Latvia are in concordance with recommendations developed by ADA, EASD and IDF albeit cut-off values for treatment vary (Figure 1.2.). The glycaemic goal of the therapy in Latvia is to reach <6.5% HbA<sub>1c</sub> but optimal cut-off value can be personalized for elderly or patients with concomitant diseases (LDA, 2007). Similarly, Canadian Diabetes Association Clinical Practice Guidelines recommends HbA<sub>1c</sub> goal to be less than <6.5% and <7 mmol/L for FPG, and ≤11.1 mmol/L for OGTT (or random postprandial glucose measurement)(CDA, 2013). For patients with T2D, the American Diabetes Association (ADA) and European Association for the Study of Diabetes (EASD) in 2008 recommended a glycated haemoglobin (HbA<sub>1c</sub>) target of <7%, and the British Medical Association (BMA) Quality and Outcomes Framework recommends a HbA<sub>1c</sub> target of ≤7.5% (BMA, 2006; Nathan et al., 2008).



**Figure 1.2.** Clinical Guidelines for the treatment of T2D in Latvia. From “Guidelines for Prevention, Diagnostics and Treatment of Type 2 Diabetes”(LDA, 2007).

## 1.2. Metformin and its clinical use

Metformin is a peroral antihyperglycaemic drug which improves glucose tolerance in patients with T2D, lowering both basal and postprandial plasma glucose by reduction of hepatic neogenesis in non-insulin-dependent *diabetes mellitus* patients (Stumvoll et al., 1995). This effect occurs mainly by reducing the rate of gluconeogenesis (Hundal et al., 2000; Stumvoll et al., 1995), but metformin was shown also to down-regulate glycogenolysis (Mithieux et al., 2002). Metformin improves insulin sensitivity in the muscle and liver (Ou et al., 2006), increases glucose utilization in gut and insulin-mediated glucose uptake in muscles (Wiernsperger and Bailey, 1999), enhances insulin receptor expression and tyrosine kinase activity (Gunton et al., 2003).

Metformin can be used as a monotherapy or can be combined with all other peroral antidiabetic drugs and insulin in the treatment of T2D (LDA, 2007). It also can be indicated in case of pre-diabetes (Perreault et al., 2009), type 1 *diabetes mellitus* (Vella et al., 2011) and gestational *diabetes* (Rowan et al., 2008). Metformin has been successfully used in adolescents with a insulin resistance (Love-Osborne et al., 2008). Besides antihyperglycaemic action, metformin exerts beneficial effects on  $\beta$  cells (Hinke et al., 2007), normally does not induce hypoglycaemia and hyperinsulinemia (Garcia, 1950), is slimming or at least weight neutral (UKPDS study, 1998). In clinical trials metformin has

shown beneficial effects in case of metabolic syndrome (Pasquali et al., 2000), non-alcoholic fatty liver disease (NAFLD) (Mazza et al., 2012) and hyperlipidemia in animal studies (Ghatak et al., 2011; Pentikainen et al., 1990) by reducing adipose tissue lipolysis (Ren et al., 2006), levels of circulating plasma triglycerides (DeFronzo and Goodman, 1995), nonesterified fatty acids and diminishing production of very-low-density lipoprotein (VLDL)(Abbasi et al., 1997). Promising results were obtained from studies investigating insulin resistant/obese women with polycystic ovary syndrome (PCOS)(Diamanti-Kandarakis, 2008), cancer (Rizos and Elisaf, 2013) and hypothyroidism (Krysiak and Okopien, 2011). In tissue and animal models metformin has shown a promise regarding anti-ageing (De Haes et al., 2014) and Alheimers disease therapy (Kickstein et al., 2010). However, recent meta-analysis has questioned whether or not metformin indeed is associated with lactic-acidosis and lower CVD risks in T2D patients (UKPDS study, 1998; Boussageon et al., 2012).

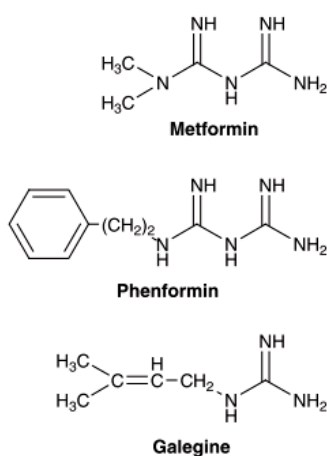
Major weaknesses of metformin are inefficiency of therapy in up to 1/3 of patients in the first year of the therapy (primary failure) and common gastrointestinal side-effects in approximately 20-60% of metformin users contributing to the non-compliance in as many as one-third of patients initially on prescribed therapy (Donnelly et al., 2009; Esposito et al., 2012) and leading to the discontinuation in approximately 5–10% of the cases (Brown et al., 2010; Graham et al., 2011). Recent findings have questioned if restriction of metformin use in all patients with kidney disease is supported by the scientific evidence (GGT2D, 2006; Singh, 2014).

### ***1.2.1. Discovery of metformin***

*Diabetes mellitus* is ancient disease for the first recored at least 3 600 years ago in Ebers Papyrus (ancient Egypt). It was described and classified in Type 1 and 2 by Sushruta and Charaka approximately 500 - 600 BC in India (Algaonker-SS., 1972; Griffith, 1893). The term of disease was derived from the greek verb "*diabainein*" by Aretaeus of Cappadocia (130–200 CE) meaning "a siphon" and implicating polyuria, one of the main symptoms of DM. Rediscovery of the *diabetes* started with Thomas Willis (1675), who also added "*mellitus*" to the term, meaning "honey-sweet" due to specific smell of diabetic urine (Araetus, 1856; McGrew, 1985).

More than 400 traditional plant treatments for *diabetes mellitus* have been recorded providing a useful source for search of new oral hypoglycaemic compounds. While most of the sources have not been evaluated in the clinical trials, excellent findings were obtained

from studies dedicated to the toxic weed *Galega officinalis* (native to Middle East, known also as Goat's rue, French lilac)(Bailey and Day, 1989). Goat's rue is a perennial herb >1 m in height, growing in temperate regions. In Europe, aerial parts (leaves and flowering tops in an infusion) of this plant were used since medieval times to treat symptoms of T2D such as an intense urination; treatment was characterized by mild overall efficiency and associated with pronounced side-effects (Bailey and Day, 1989; Witters, 2001). Goat's rue is rich in compounds with antiglycaemic properties - guanidine and galegine (Bailey and Day, 1989; Dronsfield A, 2011). Metformin and phenformin were developed from galegine (guanidine derivative) (Graham et al., 2011)(Figure 1.3.).



**Figure 1.3.** Metformin, phenformin and galegine. From (Graham et al., 2011).

In the 1917 guanidine hydrochloride was shown to reduce blood glucose levels in rabbits, but was mistakenly associated with tetany of hypoparathyroidism (Watanabe-K., 1918). Guanidine itself was proven to be too toxic for clinical use and, instead, galegine and alkyl diguanides Synthalin A and Synthalin B were introduced in early 1920s as antidiabetic drugs. These compounds were overshadowed by a wide use of insulin and discontinued in the next two decades due to various adverse effects, including liver toxicity (Bailey CJ, 2004; Mixner et al., 1957; Sterne, 1969).

For the first time, metformin was described in 1922 by the Dublin chemists Emil Werner and James Bell (Werner EA, 1922). It was shown to reduce blood glucose levels in rabbits, but, unlike other similar compounds, did not affected blood pressure and heart rate in animals (Dawes and Mott, 1950). Problems experienced with guanidine and diguanides prompted the development of biguanides and the later use of metformin (Bailey, 1988).

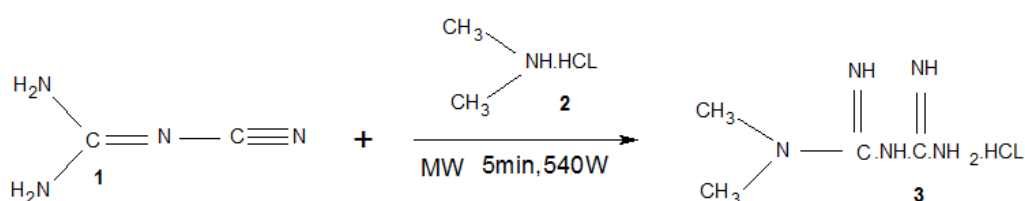
In late 1940s, renamed as *Fluamine*, metformin was investigated by Garcia in the study as treatment against influenza and it was found to lower blood glucose levels to the normal physiological level and appeared to be safe in clinical use (Garcia, 1950). In 1957, Sterne reported antihyperglycaemic properties of metformin in human (Sterne, 1957); soon properties of metformin were investigated in other clinical trials (Gottlieb and Auld, 1962; Supniewski and Chrusciel, 1954). The new antidiabetic drug was well received due to a high demand for peroral drug with strong antihyperglycaemic effect but not associated with severe hypoglycaemia.

In the next few years were published promising results of trials investigating properties of other even more efficient biguanides - phenformin and buformin (Bailey CJ, 2004; Mehnert and Seitz, 1958; Ungar et al., 1957). Phenformin was withdrawn from market in late 1970s and use of buformin currently is outlawed in many countries due to severe and highly lethal side-effect - lactic acidosis (Misbin, 1977).

Nowdays, more lipofilic biguanides and guanidine derivates are being tested as potential peroral antidiabetic compounds in order to improve efficiency and bioavailability of metformin (Rauf et al., 2014).

### 1.2.2. Physicochemical properties

Metformin hydrochloride (1,1-dimethylbiguanide,  $C_4H_{11}N_5 \cdot HCl$ ) belongs to the peroral antidiabetic drug class biguanides (Viollet et al., 2012). The original experiment of metformin synthesis is a reaction between dimethylamine hydrochloride and 2-dicyanodiamide (Werner EA, 1922)(Figure 1.4.).



**Figure 1.4.** Synthesis of metformin in the reaction between dicyanodiamide **1** and dimethylamine hydrochloride **2**. Adapted from (Shalmashi, 2008).

Metformin hydrochloride is freely soluble in water and is practically insoluble in acetone, ether and chloroform (<https://pubchem.ncbi.nlm.nih.gov/> Accessed in January 2015). The dose of metformin is indicated as the hydrochloride salt (molecular weight 165.63) but concentrations in biological fluids are expressed as the free base (molecular

weight 129.16)(Graham et al., 2011). Metformin is a hydrophilic base (acid dissociation constant values pK<sub>A</sub> 2.8 and 11.5) with less than 0.01% unionized in blood and characterized by low logP value of -1.43 (Brittain, 2013). It indicates low lipophilicity of the drug and limited passive diffusion through cell membranes. The hydrophobic tail of metformin extends into the hydrocarbon core of membranes. The protonated biguanide group gives a positive charge to the surface of the membrane, which acts to displace divalent cations and alter membrane potentials. That may affect divalent cation membrane functions and act in general as a calcium channel blocker (Schafer, 1976).

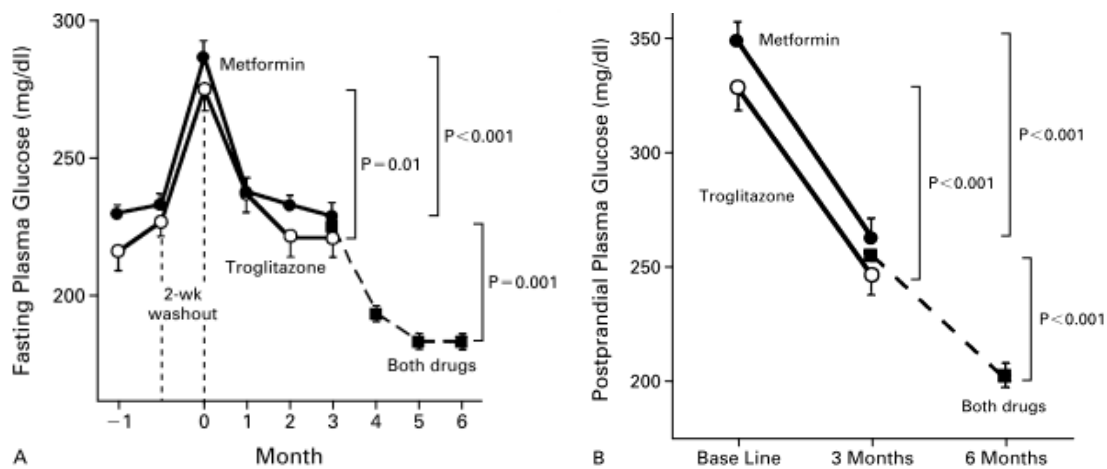
### **1.2.3. Formulations**

Metformin is administered in peroral tablets containing 500, 850 or 1000 mg doses for single or multiple-dosing. For the therapeutic effect it is required to use high doses of metformin - up to 2500 mg per day (Rena et al., 2013). IC<sub>50</sub> of metformin is 275 mmol/L (Davidoff and Carr, 1972) and Bioavailability (F) of 500 mg is approximately 50%-61% and it decreases when dose is increased (not proportionally)(Graham et al., 2011; Pentikainen et al., 1979). Metformin therapy requires repeated administration of doses due to fast elimination of drug to maintain effective plasma concentrations (Pentikainen et al., 1979). In some cases metformin is combined in fixed dose in combination with other peroral antidiabetic drugs like glipmipiride, sitagliptin, rosiglitazone, but the majority of available formulations are simple immediate-release (IR) tablets (Bailey and Day, 2009; Kim et al., 2009). Metformin is not a good candidate for a traditional sustained-release dosage form because its absorption is limited largely to the small intestine (Graham et al., 2011). Sustained- or extended-release formulations are designed to release metformin at a constant rate and may reduce side-effects and allow once a day dosing. Sustained-release (SR) dosage forms of metformin have been prepared to prolong gastric and, possibly, intestinal residence by forming gel-like mass (Davidson J, 2004; Timmins et al., 2005). Metformin is also produced in extended-release single-composition osmotic tablet (XT) with prolonged time to peak plasma concentration (Wagstaff and Figgitt, 2004).

### **1.3. Efficiency of metformin**

The expected average improvement in HbA<sub>1c</sub> with the use of metformin is approximately 1-1.5% (Bennett et al., 2011). Metformin in doses up to 1500 mg per day reduce HbA<sub>1c</sub> levels by approximately 1 % compared to placebo after 3 months of therapy. Higher baseline HbA<sub>1c</sub> levels are associated with greater declines in HbA<sub>1c</sub>; however, there

is little evidence for additional reduction at higher doses. Most of the treatment effect was evident 3 months after the beginning of the therapy and no consistent effect of T2D duration was observed on the change in HbA<sub>1c</sub> (Esposito et al. 2012). Metformin 2000 mg per day in subjects inadequately controlled on diet and exercise resulted in 0.6% decrease in HbA<sub>1c</sub> from baseline 7.2%, and 76% of patients reached therapy goal of <7% HbA<sub>1c</sub> (Aschner et al. 2010). In other study group of patients over 52 weeks decreased HbA<sub>1c</sub> for 1.4 % from a baseline of 8.4% and 45% of patients reached therapy goal of <7% HbA<sub>1c</sub> (Schweizer et al., 2007). Small study investigated changes in fasting plasma glucose concentrations (A) and postprandial plasma glucose concentrations (B)(to convert to the values for glucose to millimoles per liter, multiply by 0.056) if metformin and troglitazone were used as monotherapy or combined. Study was designed as follows - 2 weeks wash-out period when previous therapy was stopped, then 3 months of monotherapy followed by 3 months a combined therapy (Inzucchi et al., 1998). Results reflect a fast and significant decrease of postprandial and fasting glucose level in serum in case of metformin monotherapy (Figure 1.5.).



**Figure 1.5.** Effects of metformin monotherapy on FPG and PPG levels. From (Inzucchi et al., 1998).

The UK Prospective Diabetes Study (UKPDS) demonstrated a progressive loss of glycaemic control with prolonged use of metformin (Turner et al., 1999) due to decline of pancreatic  $\beta$  cell function in the face of persistent insulin resistance, rather than loss of action of the drug itself (UKPDS, 1995; Weyer et al., 1999). Metformin was shown to maintain control for up to 2 years (Charbonnel et al., 2005). Over the 1 year of treatment with metformin drug-naïve patients with T2D decreased HbA<sub>1c</sub> from 8.7% to 7.3%. Most of HbA<sub>1c</sub> reduction was attained by week 12, and the efficacy was sustained through the

treatment. Body weight decreased during treatment ( $-1.9 \pm 0.3$  kg,  $P < 0.001$ ). Any adverse event incidence was 75.4% and incidence of gastrointestinal side-effects was 43.7% (Schweizer et al., 2007).

In 1 year study, secondary failure rate of metformin monotherapy (defined as the addition or switch to other antihyperglycaemics) was 21.8%. The best HbA<sub>1c</sub> achieved within 1 year of metformin initiation was shown to be the most powerful predictor of avoiding secondary failure monotherapy (defined as the addition or switch of antihyperglycaemics) (Boccuzzi et al., 2001). In long term, antidiabetic therapy requires intensification of monotherapy or add-on of other antidiabetic drugs due to deterioration of  $\beta$  cell function (UKPDS, 1998). The 50% secondary failure was identified within 36 months in subjects with best HbA<sub>1c</sub> of 7–7.9% whereas it took  $>60$  months for those with 6–6.9% HbA<sub>1c</sub> (Nichols et al., 2006). A recent study, when compared with sulfonylurea, metformin was associated with a delay in secondary failure in those who had used it for at least 2 years (Eurich et al., 2005). In the U.K. Prospective Diabetes Study approximately 45% of overweight patients attained HbA<sub>1c</sub>  $<7\%$  after 3 years of first-line use of metformin (Turner et al., 1999).

In almost 1000 T2D patients using metformin for 1 year, therapy was associated with decrease of body weight ( $-2.5$  kg). Best results were observed in patients with highest baseline weights. Metformin caused small decreases within the normal range in haemoglobin and hematocrit. It may beneficially affect cardio-vascular system by lowering hemoconcentration and hypercoagulability (Belcher et al., 2005). In Diabetes Prevention Program (DPP) long-term follow-ups metformin was shown to reduce haemoglobin and hematocrit levels during first year and effect stabilized after this time-point. In patients using metformin versus placebo group weight loss was stable (2.0 vs. 0.2%,  $P < 0.001$ ) and durable (up to 10 years), and strongly correlated with drug adherence (Diabetes Prevention Program Research, 2012b).

Recent meta-analysis of metformin efficacy in 13 110 T2D patients resulted with surprising results. Metformin did not significantly affect the primary outcomes of all-cause mortality, risk ratio (RR) = 0.99 (95% CI: 0.75 to 1.31), and cardiovascular mortality, RR=1.05 (95 % CI: 0.67 to 1.64).

The secondary outcomes (all myocardial infarctions, heart failure, peripheral vascular disease, leg amputations, microvascular complications) were also unaffected by the metformin treatment. For all-cause mortality and cardiovascular mortality, there was a significant heterogeneity when including the UK Prospective Diabetes Study subgroups

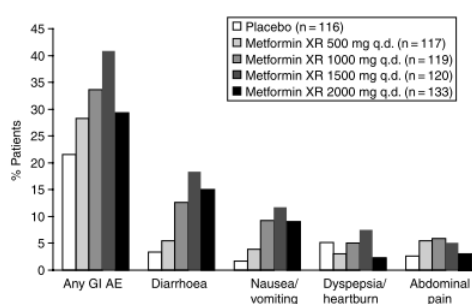


and significant interaction with sulphonylurea as a concomitant treatment for myocardial infarction. Thus study reached, actually, controversial results - a 25% reduction or a 31% increase in all-cause mortality and 33% reduction or a 64% increase in cardiovascular mortality in subgroups. Meta-analysis was performed by including UKPDS subgroup (metformin plus sulphonylurea versus sulphonylurea alone) and low number of randomized controlled trials with limited number of events in analysis. The deteriorious effect of the combination of metformin plus sulphonylurea remains unexplained. Authors of meta-analysis conclude that previous CVR reduction in metformin group may be an effect of concomitant therapy or a study bias (absence of placebo group and double-blinding). They also point out a common weakness of many of studies dedicated to the research of metformin efficacy, which is lack of investigation of patient-relevant outcomes (Boussageon et al., 2012). Scientists have stressed that due to absence of clinical evidence supported by a double-blind randomized controlled trial versus placebo on the clinical efficacy of antidiabetic drugs, it is not possible to prove the ability of HbA<sub>1c</sub> to predict and capture the effect of treatments (Prentice, 1989). For example, previously mentioned subgroup of metformin and sulfonylurea in UKPDS study had the better HbA<sub>1c</sub> reduction in comparison to the group of sulfonylurea alone. However, excess of mortality was found in the group receiving combined therapy (Boussageon et al., 2012).

#### **1.4. Side-effects of metformin**

Metformin therapy irrespectively to the dose is associated with common, usually gastrointestinal (GI) side-effects in the 20–30% of all users, leading to the discontinuation of therapy in up to 5-6% of cases (Garber et al., 1997; Hirst et al., 2012). For the note, titration to the maximal dose in 1 year is reached only in 59% of all metformin users (Belcher et al., 2005; Garber et al., 1997). Common side-effects of metformin therapy are chest pain, allergic reactions, diarrhea, bloating and abdominal pain, vomiting, stomach ache, headache and lethargy (Garber et al., 1997). Use of biguanides for long time was thought to be associated with increased risk of severe and highly lethal side-effect (up to 50%) - lactic-acidosis. While it appears to be true for phenformin, recent studies and meta-analysis have rejected such correlation in case of metformin. GI side-effects remains major limiting factor for optimizing glucose-lowering therapy and negatively affect quality of life and adherence in T2D patients, especially during the uptitration in the beginning of the therapy and (Florez et al., 2010; Hermans et al., 2012).

Sustained and extended release formulations are recommended by National Institute for Health and Clinical Excellence in case if gastrointestinal intolerance prevents continuation of the immediate-release preparation (Adler et al., 2009). In 12-weeks of clinical trial with 529 participants extended-release tablets showed similar efficiency, overall incidence of side-effects and systemic exposure to the metformin as immediate-release tablets, but, for unknown reason, increased triglyceride levels (Schwartz et al., 2006). In meta-analysis of 4 studies (95 patients with adverse events in total) investigating replacement of IR tablets for sustained-release tablets, incidence of GI side-effects varies from 0–38%, however, tolerability seems to be dose dependant (Feher MD, 2007). In a small study 45.9% of patients experiencing metformin side-effects from IR metformin, the gastrointestinal disturbances were reduced to 33% using extended-release (XR) metformin tablets. For the note, this study experienced high (>40%) withdrawal of patients (Levy et al., 2010). In other study of extended-release (XR) formulation of metformin 1 240 patients were randomized in placebo and 2 groups receiving metformin XR and 742 patients were randomized to receive metformin XR in different doses versus placebo group. Gastrointestinal side-effects in all groups of metformin XR formulations were higher than in placebo group – in Group 1 63.5% versus 59.5%; in Group 2-65.8% versus 59.5%, respectively. Discontinuation occurred in 4.4% of metformin XR-treated patients and in 2.5% of placebo-treated patients in Group 1, and in 2.9% and 0.9% of patients, respectively, in Group 2. Gastrointestinal adverse events (GI AE) found in >5% of metformin on placebo users are shown in the picture (Fujioka et al., 2005)(Figure 1.6).



**Figure 1.6.** Gastrointestinal adverse events (GI AE) found in metformin or placebo users. From (Fujioka et al., 2005).

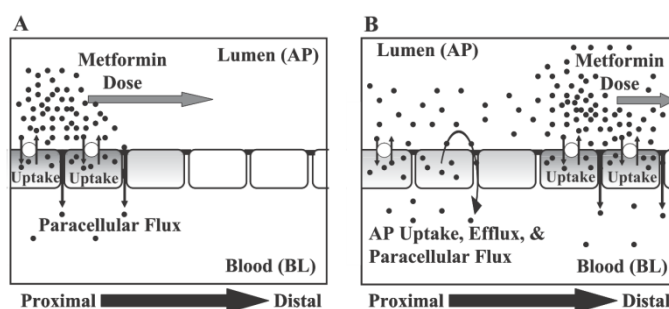
### ***1.4.1. Common side-effects***

Pathophysiology of metformin side-effects is unknown and dose of drug positively correlates with incidence, however, not in all studies (Feher MD, 2007; Garber et al., 1997; Hirst et al., 2012). In a recent publication, 83 cases of metformin discontinuation due to severe gastrointestinal (GI) side-effects were analysed versus a larger group of tolerant patients. Metformin gastro-intestinal intolerance phenotype was characterized by the low rate of ischaemic heart disease, left-handedness, ABO group imbalance, higher ferritine levels and an iron load. Previously, ABO groups have been linked to the cardiometabolic risk (Reilly et al., 2011) and relative proportion of healthy GI microbiota (Makivuokko et al., 2012). Elevated ferritine levels may confer to vascular benefit in metformin intolerant patients, but association of ferritine with metformin gastrointestinal side-effects remains elusive. Such heterogenous parts of phenotype possibly can help to characterize cardiovascular risk (Hermans et al., 2013), albeit lacks specificity and sensitivity to be used as in a clinical setting.

It is unclear whether adverse effects could be attributed to the drug present in the mucosa or in the intestinal lumen. Different hypotheses have been proposed: 1) high local metformin concentration in the intestines (Bailey et al., 2008; Wilcock and Bailey, 1994), 2) metformin effects on glucose-absorption in the gut and changes in microbiota (Napolitano et al., 2014), 3) changes in incretin and glucose metabolism (Bouchoucha et al., 2011), 4) stimulation of intestinal serotonin secretion (Cubeddu et al., 2000) 5) ghrelin and bile salt handling (Carter et al., 2003; Scarpello et al., 1998).

The highest levels of metformin have been detected in the enterocytes and lead to an increase in anaerobic processes and elevated production of lactate (Bailey et al., 1994). Thakker et al. showed in (Caco-2 cells, *in vivo* and *ex vivo* mouse) transporter-dependent accumulation of metformin in the enterocytes and also its absorption through the saturable paracellular route (up to 90%). It can be speculated that a portion of the metformin dose is sequestered in the enterocytes because of the lack of an efficient basolateral (BL) efflux transporter mechanism and thus may lead to adverse-effects (Proctor et al., 2008). Metformin uptake in the gut is hypothesised to have a complicated pattern. Study provides strong evidence supporting an “OCT-like” bidirectional uptake/efflux transport mechanism on the apical (AP) membrane in Caco-2 cells for metformin, possibly, OCT3. In comparison to AL metformin transport, BL transport was quite inefficient and rate-limiting

step leading to a paracellular not transcellular metformin uptake (mediated by OCT1)(Proctor et al., 2008)(Figure 1.7.).



**Figure 1.7.** Mechanisms underlying saturable intestinal absorption of metformin. From (Proctor et al., 2008).

It should be noted that expression of OCT in cancer tissues is different from normal tissues and Caco-2 cells may not be the best model for study. Metformin increase uptake of 2DG (sugar) in the intestinal tissues from humans and, possibly, may increase glucose levels in the enterocytes and affected trafficking of GLUT2 transporters in intestinal epithelia. Oxidative metabolism of glucose in the anaerobic condition of intestinal epithelium may explain lactic acidosis caused by metformin (Bailey et al., 2008; Han T, 2012; Stepensky et al., 2002; Yufeng Xia, 2014).

Research in animal models has shown that modulation of the gut microbiota may contribute to the antidiabetic effects of metformin. Recently it was shown that metformin alters the gut microbiota, disrupts microbial metabolic pathways and promotes longevity in the worm *C. elegans* (Cabreiro et al., 2013). In C57BL/6 mice treated with metformin for 6 weeks it was shown that metformin HFD (high fat diet) fed mice developed higher abundance of the mucin-degrading bacterium *Akkermansia muciniphila* and increase in number of mucin-producing goblet cells than HFD-fed control mice. Interestingly, oral administration of *Akkermansia* to HFD-fed control mice significantly enhanced glucose tolerance and reduced adipose tissue inflammation (*via* T cells). *Akkermansia muciniphila* does not alter gut permeability (Burcelin, 2014; Shin et al., 2014). Further research on composition of gut microbiota was examined in the high-fat diet induced obese mouse model with and without metformin treatment. Use of metformin increased abundance of *Akkermansia muciniphila* and *Clostridium cocleatum* mouse model and *in vitro*. Metformin appeared to act as a growth factor for *Akkermansia muciniphila* which positively correlated with mucus thickness and also may be involved in the regulation of

lipid metabolism. In addition, in total 18 KEGG metabolic pathways were significantly upregulated in the gut microbiota during a treatment with metformin of mice on high fat diet (Lee and Ko, 2014).

Metformin effects on human microbiota were investigated in T2D patients using metformin at least 3 months. Metformin therapy was stopped for 7 days and re-started in the next three weeks, when fasting capillary blood glucose (CBG) had increased for 25% of baseline level. Results of the study showed, that that metformin has effects on bile acid metabolism, entero-endocrine hormone secretion (GLP-1) and concentration of cholic acid and conjugates serum was significantly correlated with Phyla *Firmicutes* and *Bacteroidetes* abundances. Additionally, these two phyla were significantly correlated with circulating concentrations of PYY in patient sera, but correlation of PYY and cholic acid concentrations were non-significant. Results confirm, that metformin may inhibit the reabsorption of bile acids by altering the function of the sodium-dependent intestinal bile acid transporter (Tremaroli and Backhed, 2012).

Underlying causes of metformin effects on PYY and GLP-1 are unknown, but may be explained by effects of drug on bile acid transporters such as FXRs and TGR5 or inhibitory effect on the metabolizing enzyme DPP-IV, in addition to enhanced secretagogue action of the L cell. Gut microbiome changes in patients with T2D on-metformin and off-metformin showed greater inter-individual variability than intra-individual, therefore, alas metformin changed spectrum, and the results in a small group appeared to be non-significant. However, microbiota is constantly changing and diet and physical activity was shown to alter amounts of *Akkermansia Muciniphilia* in humans (Anhe et al., 2014).

Metformin has been associated with malabsorption of nutrients (folates and B12 vitamin) and increased homocysteine level that is cardio-vascular risk factor (Liu et al., 2014). In more than 6 000 patients (in controls and group with T2D) B12 deficiency was significantly increased in the metformin user group. Incidence of deficiency was not decreased in T2D patients using B12 containing supplements (Reinstatler et al., 2012). Currently, there are no guidelines for the supplementation and appropriate dose of vitamin B12 for T2D patients on metformin (Liu et al., 2014). Vitamin B12 is stored in the body in amounts required for 3-6 years; factors influencing B12 absorption are availability of metformin in the meals and presence of intrinsic factor. Metformin induced B12 deficiency incidence was assessed to vary between 5.8–33% (Pflipsen et al., 2009; Reinstatler et al., 2012) and is diagnosed with low total B12 vitamin concentrations in

serum (usually <150 pmol/L). B12 deficiency is associated with development of pernicious anemia or symptoms similar to T2D neuropathy and neurocognitive symptoms appearing as soon as 4 months - 5 years after the beginning of the therapy (Andres et al., 2007; Filioussi et al., 2003).

In recent meta-analysis with >8 000 T2D patients correlation was confirmed between metformin use and higher prevalence of B12 deficiency and reduced serum concentrations (Niafar et al., 2015). A small yet clinically relevant study has shown that levels of biavailable B12 (holoTCII) may be increased if patients are supplemented with calcium. It shows that B12 malabsorption may happen because of antagonism of metformin on a calcium-dependent ileal brush-border membrane and B12-intrinsic factor complex uptake transporter (Bauman et al., 2000). Currently, it is thought that biavailable B12 (holoTCII) is a more precise early marker of B12 deficiency, but with manifestation of symptoms total B12 vitamin will also be reduced. However, other studies investigating methylmalonic acid (MMA) or active B12 vitamin concentrations question whether or not metformin really is associated with total B12 deficiency as previously thought. It seems that metformin-treated objects have improved cellular vitamin B12 metabolism or intensive uptake of B12 vitamin in the tissues that may lead to false impression of deficiency. However, measurements of total B12 is less expensive and still more popular alternative both for clinic and for research (Obeid et al., 2013).

#### ***1.4.2. Lactic acidosis***

In case of metformin, the drug label contains list of contraindications for use of metformin in elderly people and those with renal impairment due to increased risk of lactic acidosis. Lactic acidosis is a rare but extremely severe side-effect which is characterized by a low arterial pH (7.35), elevated arterial lactate levels and mortality of up to 50% (5.0 mEq/l in humans) (Brown, 1998; Kwong and Brubacher, 1998). After withdrawal of phenformin and buformin, metformin use has significantly increased, but for years it was suspected to increase risk of lactic acidosis.

Studies with thousands of patients like COSMIC, UK Prospective Diabetes Study and Diabetes Prevention Program (UKPDS study, 1998; Cryer et al., 2005; Knowler et al., 2002) have found absence of association between metformin use and lactic acidosis. Salpeter et al. reviewed 176 metformin trials lasting >1 month and found no lactic acidosis in 35 619 patient-years (8.4 cases per 100 000 patient-years) of exposure to metformin in comparison to 30 002 patient-years (9 cases per 100 000 patient-years) in the non-

metformin group. There also were similar lactate levels in metformin users compared to placebo or use of other non-biguanides (Salpeter et al., 2003). Even more, no correlation was identified in retrospective analysis between metformin and blood lactate concentrations in 49 metformin-treated patients with lactic acidosis, strongly indicating, that metformin is not the cause of lactic acidosis but rather it is evoked by other underlying causes like renal failure, heart failure, infection or cancer (Lalau and Race, 1999; Misbin, 2004).

### **1.5. Drug-drug interactions**

Drug interactions, involving excretion by addition or removal of concomitant drug therapy, cause marked changes in plasma and intracellular concentrations of the affected drug and can lead to severe adverse effects or unexpected pharmacological effects. T2D patients often use antidiabetic peroral drugs and insulin in addition to metformin, as well as concomitant drug therapy (antihypertensive, antilipidemic), possibly, associated with similar-adverse effects or drug-drug interactions with metformin or altering glucose levels (Dungan K, 2008). A total of 680 drugs (4757 brand and generic names) are known to interact with metformin (14 major drug interactions (82 brand and generic names), 608 moderate drug interactions (4306 brand and generic names) and 58 minor drug interactions (369 brand and generic names). Almost the all major drug interactions listed are contrast agents used only in a hospital or clinic for certain procedures, like X-rays and CT scans (www.drugs.com accessed in January 2015).

Metformin is proven to interact with widely used proton pump inhibitors (PPI), like omeprazole and anticancer therapy drug cimetidine. It was shown that PPIs inhibit metformin uptake by organic cation transporters (Nies et al., 2011a) and cimetidine reduced renal clearance of metformin for 27% (Somogyi et al., 1987). A common treatment is the co-administration of proton-pump inhibitors (PPIs) and metformin (Dujic et al., 2014). Results of the study have shown that proton-pump inhibitors affect metformin pharmacokinetics. For example, lansoprazole increase  $AUC_{0-24}$  after the second dosing by 15 and 17%, respectively, and prolonged the metformin elimination half-life from 3.9 to 4.5 h and decreased its renal clearance by 13%, but has no effect on maximum glucose level and the area under the serum glucose concentration-time curve of metformin (Ding et al., 2014).

Verapamil shown significant reduce in glucose lowering effect of metformin, however with a small impact on pharmacokinetics (Cho et al., 2014). Rifampin was shown

to increase the OCT1 expression and hepatic uptake of metformin in 16 healthy patients, leading to the enhanced glucose-lowering action (Cho et al., 2011).

Analysis of the commonly prescribed drugs PPIs, verapamil, doxazosin, codeine significantly reduced tolerability of metformin therapy (Figure 1.8.).

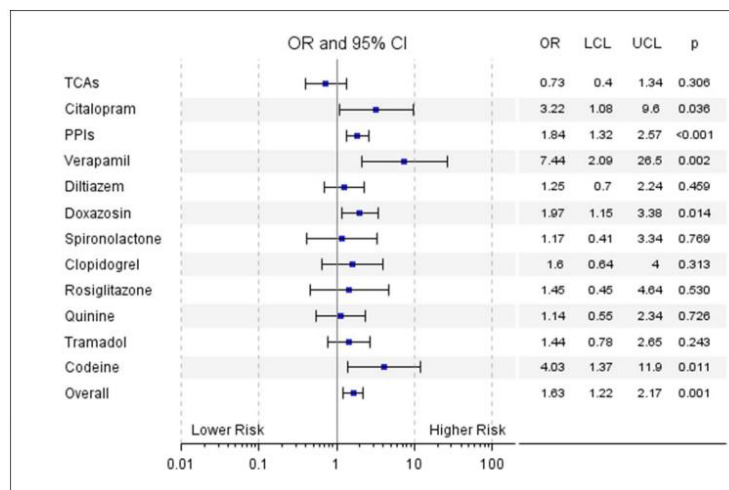


Figure 1 Association of the individual drugs/drug classes with metformin intolerance. The analysis was adjusted for age, sex, weight, and co-treatment with other OCT1 inhibitors. TCAs, tricyclic antidepressants; PPIs, proton pump inhibitors; OR, odds ratio; LCL, lower confidence limit; UCL, higher confidence limit.

**Figure 1.8.** Intolerance to metformin in T2D: A GoDARTS Study. Figure and legend from (Dujic et al., 2014).

## 1.6. Pharmacokinetics

Metformin is characterized by “flip-flop” pharmacokinetics, which are typical for drugs with a slower absorption than elimination, thus terminal portion of slope represents bioavailability rather than excretion (Pentikainen et al., 1979). In different studies, metformin was identified not to be bound to plasma proteins nor *in vivo* nor *in vitro* (Pentikainen et al., 1979; Tucker et al., 1981) or bound in small amounts up about 10–20% (Garrett et al., 1972). Pharmacokinetics in healthy subjects and T2D patients in general are similar and comparable (Tucker et al., 1981).

The gastrointestinal absorption of metformin is a rate-limiting step in metformin pharmacokinetics (Pentikainen et al., 1979). Metformin is mainly absorbed in the small intestine up to 6-10 hours after administration with negligible absorption in the stomach and the large intestine (Tucker et al., 1981). In animal studies, effect of metformin XR dose on dog colonic absorption was small, indicating poor and dose-disproportional absorption (Tajiri et al., 2010). Plasma level of metformin in humans decreases upon arrival of metformin in the colon, but it may decrease faster if the intestinal transit is



significantly slowed (Marathe et al., 2000). Studies have shown that the gastrointestinal tract is an important target organ of metformin and these data support compelling evidence about superiority of peroral administration over intravenous (Bonora et al., 1984; Stepensky et al., 2002).

Intestinal mucosa accumulates much higher concentrations of metformin (up to  $10^{-3}$  mol kg<sup>-1</sup>) compared to other tissues such as skeletal muscle (in the range of  $10^{-5}$  mol kg<sup>-1</sup>) (Bailey et al., 1994). At the level of the intestine, metformin firstly was shown to have a negligible effect on glucose absorption, however, it slightly delays the absorption process (Cuber et al., 1994), enhances insulin-mediated glucose disposal (Nosadini et al., 1987; Riccio et al., 1991). While glucose utilization is sensitive to changes in the regional blood flow (DeFronzo et al., 1981) it does not seem to be altered by metformin.

The gut can contribute to the control of glucose homeostasis by its high glycolytic capacity, gluconeogenesis (up to 25% of endogenous in fasting) and signaling in glucose and energy homeostasis (Mithieux, 2009; Mithieux and Gautier-Stein, 2014). In a more recent view, metformin was shown to increase glucose utilization in the intestine by 69% after intrajejunal administration of metformin in rats (Bailey et al., 1994). Metformin was also shown to induce a fast regulation of the two major intestinal glucose transporters SGLT-1 (decrease in blood glucose) and GLUT2 (increase in blood glucose), through a rapid AMPK phosphorylation and inhibition. Surprisingly, as it means an increase and a reduction of the glucose absorption simultaneously (Sakar et al., 2010). Similar results were obtained in another study. Metformin was shown to increase the number of low affinity insulin receptors in erythrocytes (Holle et al., 1981; Rizkalla et al., 1986) and in obese women with normal glucose tolerance metformin increased the number of insulin receptors and the tyrosine kinase activity in erythrocytes (Santos et al., 1997).

Quantification of metformin in the erythrocytes is proposed as a potential marker of metformin accumulation and efficiency, as it does not change so drastically between the doses, and, possibly, better reflects metformin accumulation in the target tissues (Tucker et al., 1981) and allows to ascertain dose in elderly patients to improve the efficiency and safety of the therapy (Robert et al., 2003). It should be noted that metformin accumulation in erythrocytes is thought to be time-dependent (Scheen, 1996). Membrane fluidity of erythrocytes was also shown to be increased in metformin users *in vitro* and *in vivo* (measured by monitoring changes in the anisotropy of the fluorescent probe 6-anatroxyloxy stearic acid (6-AS) (Muller et al., 1997). Different routes of metformin accumulation in erythrocytes and target organs may lead to misleading conclusions.

Currently, those measurements are not performed on a daily basis in clinics. In animal experiments, metformin has also been shown to accumulate in salivary glands, however, metformin excretion in saliva and plasma concentration are not proportional in humans and thus cannot be used as a precise marker (Wilcock and Bailey, 1994).

### ***1.6.1. Intravenous administration***

The 3-compartment model indicates, that the two first phases (half-lives of 3.3 min and 23 min) represent the distribution of metformin and considerable elimination ( $1/2$  of intravenous dose). The third exponential phase (between 3 and 10 h after administration) represented a phase of elimination. Plasma concentrations of metformin after intravenous administration have a vague elimination half-life ( $t_{1/2}$ ) of 1.7 to 4.5 hours. Concentration of metformin rapidly decreases, and, after about 10 - 12 hours is not detectable. The total plasma clearance is similar to renal clearance and is about five-times the creatinine clearance. No metformin was recovered from faeces. The half-life of metformin in saliva after the intravenous dose was 2.9 h and the volume of distribution ( $V_d$ ) range was from 63 to 276 L (last 8–12 hours after intravenous dosage). Concentrations of metformin in urine are higher than in plasma, terminal  $t_{1/2}$  ranges from 9-19 hours and it is detectable up 48-72 hours (Pentikainen et al., 1979). Longer terminal phase is due to a compartment that metformin enters and leaves slowly – erythrocytes ( $t_{1/2}$  20 h)(Graham et al., 2011). After intravenous administration secretion of metformin from blood to the gut was negligible. Elimination of metformin from human body is fast and in the first 8 h after metformin administration >95% of total urinary output (79% of dose administered) are excreted in the urine (Tucker et al., 1981).

### ***1.6.2. Single peroral dose administration***

Administration of metformin with meal has been reported to decrease the bioavailability of immediate-release (IR) tablets by about 24% and delay peak concentration for about 37 min (Sambol et al., 1996), but the reduced absorption is unlikely to be clinically significant in most patients (Scheen, 1996). Single dose peak plasma concentrations of metformin occur approximately 2.6–3 hours after dosage (in erythrocytes - 4.7 h after 850 mg dose) and range from 1.0 to 1.6 mg/L after a 0.5 g dose, increasing to about 3 mg/L after a 1.5 g dose (for erythrocytes – approximately 6 times lower after 850 mg dose). Area under curve does not differ between plasma and erythrocytes due to the longer elimination half-life in erythrocytes (Pentikainen et al.,

1979; Robert et al., 2003). Metformin concentration in plasma and erythrocytes crossed approximately 16 h after 850 mg administration and metformin was undetectable after 24 h and 48 h for plasma and erythrocytes, respectively (Robert et al., 2003). Short-term studies have shown that metformin is characterized by a long residence time in the liver, erythrocytes and other effect compartments (Lalau and Lacroix, 2003; Wilcock and Bailey, 1994). Renal clearance was similar to the one observed after intravenous drug administration. Urinary excretion and erythrocytes has terminal  $t_{1/2}$  of about 20 hours (Scheen, 1996). The recovery of metformin in feces was 29.4% of the dose and the recovery in urine was 90.5% of the dose (Pentikainen et al., 1979; Tucker et al., 1981). The fecal recovery of metformin was still continuing one week after the administration of the drug. Metformin concentrations in saliva at 1 h were only about one tenth of the corresponding plasma level. The half-life of metformin in saliva after the the oral dose was  $9.2 \pm 1.3$  h (Figure 1.9.). No traces of metformin are detectable in the expired air (Pentikainen et al., 1979).

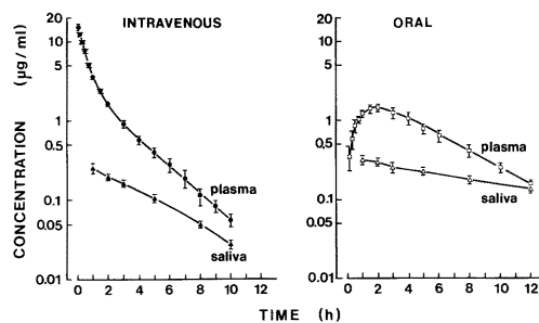


Fig. 6. Plasma and salivary concentration of metformin in healthy volunteers who received 4.4  $\mu\text{Ci}/500$  mg metformin HCl intravenously (N = 3) and orally (N = 5 including those with the intravenous dose); mean  $\pm$  SE

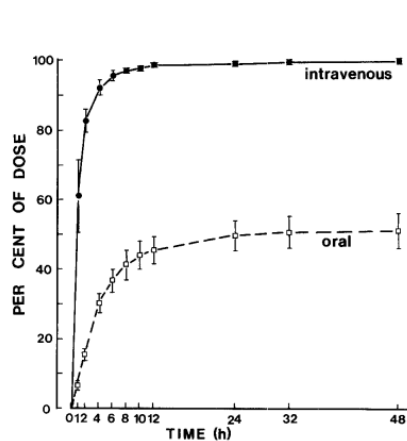


Fig. 4. Cumulative excretion of metformin in urine in healthy volunteers following administration of 4.4  $\mu\text{Ci}/500$  mg  $^{14}\text{C}$ -metformin intravenously (N = 3) and orally (N = 5 including those with the intravenous dose); mean  $\pm$  SE

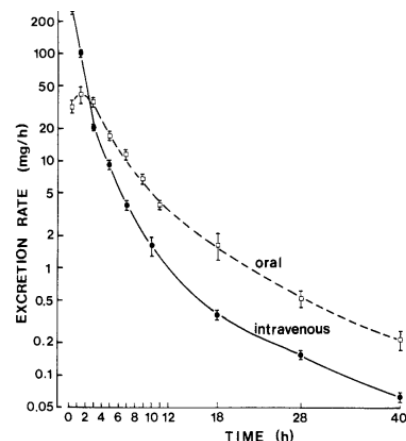


Fig. 5. Excretion rate of metformin in urine in healthy volunteers following 4.4  $\mu\text{Ci}/500$  mg of  $^{14}\text{C}$ -metformin HCl intravenously (N = 3) and orally (N = 5 including those with the intravenous dose); mean  $\pm$  SE

**Figure 1.9.** Pharmacokinetics of metformin after intravenous and oral administration to man. Figure and legend from (Pentikainen et al., 1979).

### ***1.6.3. Multiple-dose peroral administration***

When 1000 mg of metformin are administered twice per day, the mean plasma concentrations vary from 0.4 to 1.3 mg/L ( $t_{1/2}$  is about 5–5.2 h) (Hong et al., 2008). During dosage with 2000 mg metformin daily (immediate-release or sustained-release tablets)  $V_d/F$  is approximately 600 L (actual  $V_d$  is about 300 L due to 50% bioavailability), indicating significant uptake of metformin in the tissues. Fast elimination of metformin in subjects with normal renal function (fraction of dose eliminated with the terminal half-life of about 9 h is less than 5%) in normal setting was thought not to lead to any significant accumulation of metformin in the body (Pentikainen et al., 1979). In a study, investigating metformin pharmacokinetics for a longer period of time, it was evident that in 24 hours additional elimination phase was not detectable, whereas through levels increased slowly in 7 days and 14 days due to slow elimination phase. Multiple-dose administration showed a small variation of AUC values in the individual subjects - only 13% (range 4–23%) if administered for 2 weeks (Tucker et al., 1981). A recent long-term metformin accumulation study has shown up to 80-fold difference in metformin plasma levels between T2D patients using this drug for 1.5 years indicating high interindividual variability of metformin accumulation (Christensen et al.). Slow rises in through plasma drug concentrations may reflect much more dramatic increases in the accumulation of drug in the tissues (Gonda and Harpur, 1980), no information, however, is available on long-term accumulation of metformin in target tissues.

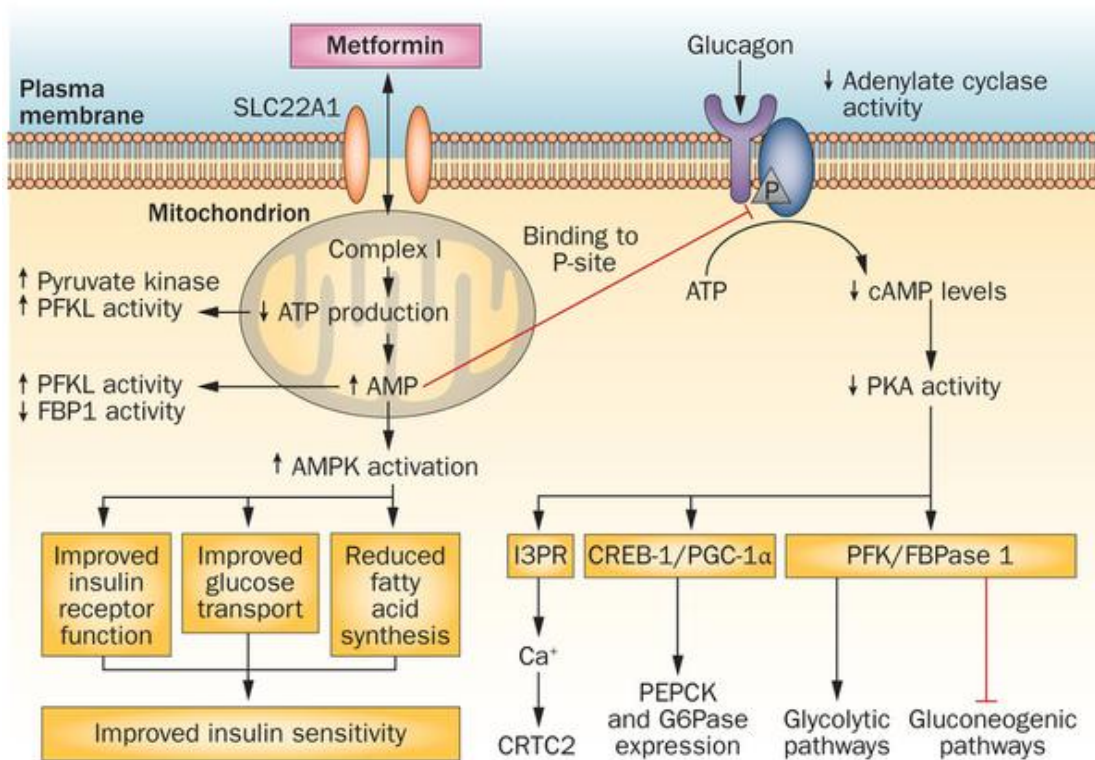
### ***1.6.4. Extended-release tablets***

In general, absorption of extended-release form increases in opposite to immediate-release tablets if administered with meal (Wagstaff and Figgitt, 2004). When 2 g sustained-release tablets are administered once daily the plasma concentrations reach  $T_{max}$  at about 7-8 h and  $C_{max}$  of about 1.8 mg/L (troughs of about 0.16 mg/L)(Graham et al., 2011).

## **1.7. Mechanisms of metformin action**

Metformin exerts its therapeutic effects in people with T2D through pleiotropic mechanisms and physiologic pathways (Pawlyk et al., 2014). Metformin enters hepatocytes via active transport mechanism - organic cation transporter 1 (OCT1) and 3 (OCT3) (Figure 1.10.)(Choi et al., 2007; Jonker and Schinkel, 2004; Wright, 2005). Metformin is known to be a growth inhibitor that mildly inhibits complex I of the

mitochondrial respiratory chain (Owen et al., 2000; Schafer, 1969; Viollet et al., 2012; Zakikhani et al., 2006; Zakikhani et al., 2008) thus reducing aerobic oxidation. It subsequently leads to the decreased ATP production (Todd and Florez, 2014), inhibition of mitochondrial glycerol-3-phosphate dehydrogenase (Madiraju et al., 2014) and activation of AMP-activated protein kinase (Kim et al., 2008b).



**Figure 1.10.** Antihyperglycaemic action of metformin in the hepatocytes. From (Pernicova and Korbonits, 2014).

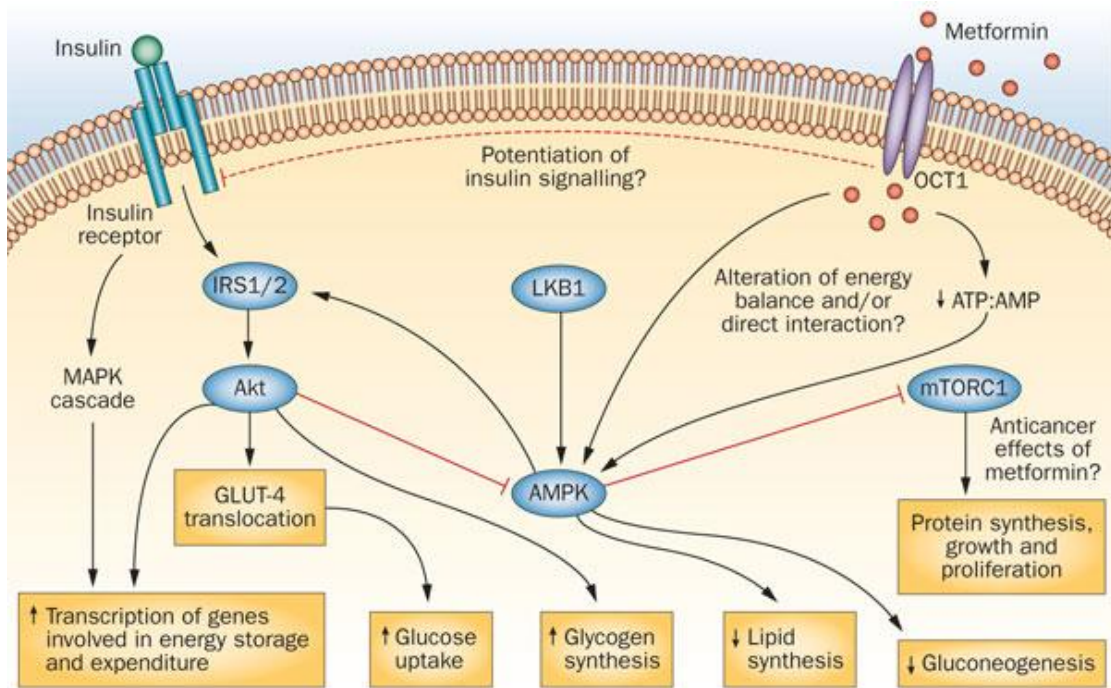
In MIN6 cells, metformin reduced mitochondrial complex I activity (- 44%) and mitochondrial reducing potential (25% net reduction) but methyl succinate (complex II substrate) can bypass metformin blockade and it resulted in a reduced phosphorylation of AMPK which decreased biguanide toxicity to b-cells *in vitro* (Owen et al., 2000). However, it is not clear if mitochondrial complex I is only metformin target as metformin effects on mitochondrial respiration varies between cells (Hawley et al., 2010).

Interestingly, that metformin-induced complex I inhibition is not a consequence of a direct interaction with the respiratory chain but rather acts via complex signaling pathway, as dimethylbiguanide, which is known for being not metabolized (Pentikainen et al., 1979) has no effect on isolated mitochondria. Studies have clearly shown that NO pathway and Ca<sup>2+</sup> homeostasis are not involved in respiratory inhibition, while effects of

oxygen radicals cannot be ruled out. ATP-/ADP-/AMP-independent effects on pyruvate carboxylase may also contribute to the inhibition of gluconeogenesis accompanying mild self-limiting mitochondrial inhibition by metformin (Owen et al., 2000). In conclusion, metformin may act via interaction with membrane receptor; this hypothesis is supported by the observed logarithmic dose-dependent effect of dimethylbiguanide on cellular respiration (El-Mir et al., 2000).

Crystallographic and spectroscopic analysis has shown that metformin effects in mitochondria require binding to metal ions, however, no evidence is available of binding of the drug to recognized metformin-regulated proteins (Sen D, 1969; Ray RK, 1999; Sen\_D., 1969; Zhu M, 2002). Metformin decrease the level of sterol regulatory element-binding protein-1 (SREBP-1) which is a significant lipogenic transcription factor and increases fatty acid oxidation in the hepatocytes (Zhou et al., 2001), however, in humans, metformin therapy resulted with a suppression of the whole body lipid oxidation (Perriello et al., 1994). In isolated preadipocytes metformin (5 mmol/l) decreased the expression of lipogenic genes and lipid droplets accumulation while increasing AMP-activated protein kinase (AMPK) activation, preventing differentiation of human pre-adipocytes (Moreno-Navarrete et al., 2011).

For many years metformin was thought to directly affect AMPK which is a critical sensor and regulator of energy homeostasis in the cell (Hardie et al., 2012; Zhou et al., 2001). Currently, AMPK is thought to be activated by an increased ADP:ATP and AMP:ADP ratio (Figure 1.11.) (Todd and Florez, 2014), and leads to inhibition of gluconeogenesis and lipid and cholesterol biosynthesis in the liver, and increased fatty acid oxidation in the liver. It also increases glucose uptake in muscle and hepatic cells (Klen et al., 2014). Activated AMPK carries the signal to the mTOR pathway (pathway responsible for stimulation of cell growth and proliferation if nutrients are available).



**Figure 1.11.** AMPK dependent and independent action of metformin. From (Pernicova and Korbonits, 2014).

It is supported by a study investigating genetic variants of the AMPK complexes with wild-type  $\gamma 2$  isoform or Arg531  $\rightarrow$  Gly mutation that renders  $\gamma 2$  complexes insensitive to the effects of ADP and AMP on phosphorylation (Hawley et al., 2010).

A study, investigating fat-fed liver-specific *Lkb1* (also known as *Stk11*) knockout mouse model (Shaw et al., 2005), showed that tumor suppressor protein LKB1 (upstream kinase of AMPK) is a significant part of LKB1 – AMPK signalling control over the expression of gluconeogenic genes. Similarly to insulin, inhibition of glucose production is obtained through regulation of cAMP response element-binding protein-regulated transcription coactivator 2 (CRTC2) which enhances expression of peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (*Ppargc1a*), phosphoenolpyruvate carboxykinase (*Pck1*) and glucose-6 phosphatase (*G6pc*)(Lin and Accili, 2011) or act independently of insulin/Akt signalling via LKB1–AMPK pathway.

On the opposite, in another study *LKB1* knockout mouse and *AMPK* knockout mouse model was shown to respond to metformin treatment as good as control (Foretz et al., 2010). Possible explanations of such differences may lay in alternative glucose homeostasis pathways in mice and differences in the study design, however, *Lkb1* have not

convincingly been reported to play a role in the glycaemic response to metformin in T2D (Rena et al., 2013).

In the cell lines, metformin inhibits *Txnip* mRNA and protein expression which is known to inhibit cellular glucose uptake and metabolism and promote hepatic gluconeogenesis; also it was shown to be dependent on AMPK activation (Chai et al., 2012). Metformin was shown to down regulate rapamycin complex 1 (mTORC1) activity and thus prevent the conversion of carcinogen-induced oral dysplasias into head and neck squamous cell carcinomas (HNSCC) in mouse model (Vitale-Cross et al., 2012). Metformin phosphorylates the mTOR inhibitor TSC2 (Inoki et al., 2003) and the mTOR interaction factor raptor (Gwinn et al., 2008), which both lead to a reduction of mTOR kinase activity and an activation of the major mTOR inhibitor PP2A (Janssens and Goris, 2001).

Metformin at pharmacologically relevant concentrations also directly scavenges •OH free radicals which may have protection towards LDL peroxidation which may be beneficial in prevention or delay of diabetic complications. Metformin does not have significant effects on •O<sub>2</sub> free radicals and towards hydrogen peroxide, however it may alter activity of the NADPH oxidase (Bonfont-Rousselot et al., 2003). Metformin was shown to attenuate oxidative stress-induced apoptosis in cardiomyocytes, increase phosphorylation of adenosine monophosphate (AMP)-activated protein kinase (AMPK) and subsequently endothelial nitric oxide synthase (eNOS), and reduced the expression of TGF-β1, basic fibroblast growth factor (bFGF), and tumor necrosis factor (TNF)-α (Ouslimani et al., 2005; Wang et al., 2011).

Treatment of primary neurons with metformin and its derivatives leads to an immediate reduction of the phosphorylation of PP2A-dependent tau epitopes, but, interestingly enough, PP2A activation by metformin seems to be AMPK stimulation independent, as it did not lead to an increase of the phosphorylation of the AMPK target ACC and induced only a weak stimulation of the phosphorylation of AMPK itself. Furthermore, the AMPK activator AICAR did not cause similar effects on tau phosphorylation. These findings indicate that metformin may be beneficial in prevention or treatment of Alzheimer's disease, as hyperphosphorylated tau is a crucial factor in the pathogenesis of disease (Kickstein et al., 2010).

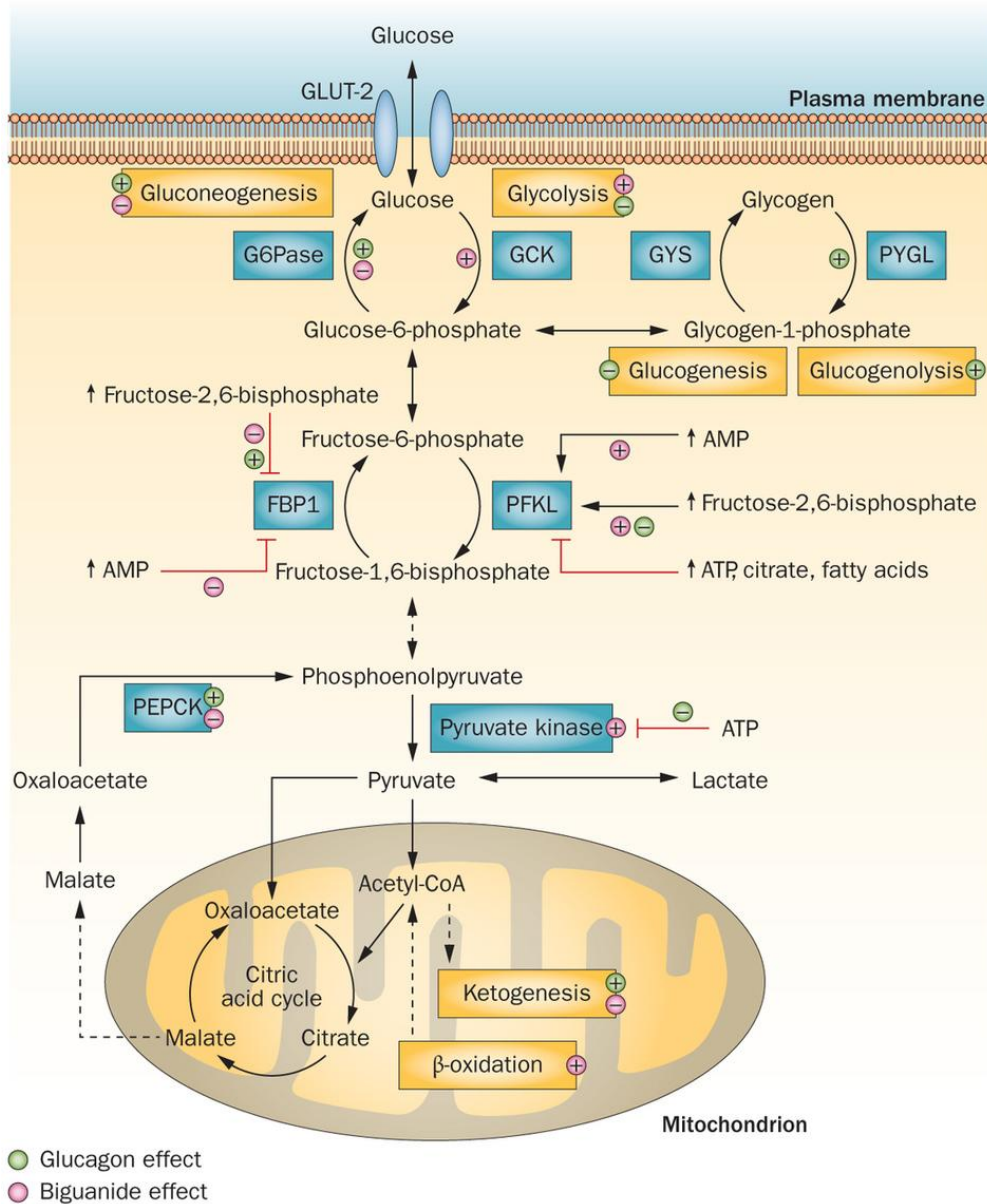
Recent studies in knockout animal models have shown, that mice hepatocytes without AMPK preserve glucose-lowering effects (Todd and Florez, 2014). Metformin was shown to inhibit hepatic gluconeogenesis and increase peripheral glucose uptake by



interfering with glucagon and cortisol signalling (insulin counter-regulatory hormones) (Cho et al. 2015; Miller et al., 2013).

The most recent publication investigated urinary endogenous metabolites and found fluctuation of metabolite cortisol (insulin counter-regulatory hormone) levels which stimulates gluconeogenesis. It seems that metformin induce phosphorylation of LXR $\alpha$  in the pituitaries and suppress pro-opiomelanocortin (POMC)/ adrenocorticotrophic hormone (ACTH)/cortisol levels (Cho et al. 2015). A new discovery also demonstrated that biguanides antagonize the glucose-raising effects of glucagon in the liver. Binding of glucagon to its receptor on the hepatocyte plasma membranes leads to activation of adenylyl cyclase (AC), production of the second messenger cyclic AMP (cAMP), and stimulation of protein kinase A (PKA), which phosphorylates protein targets that work in concert to increase hepatic glucose output (Figure 1.12.) (Miller et al., 2013). The authors suggest that biguanides exert their effect via accumulation of AMP and related nucleotides, which can bind an inhibitory 'P' site on adenylyl cyclase, the enzyme responsible for cAMP production. Subsequent inhibition of adenylyl cyclase, reduce levels of cyclic AMP and protein kinases A (PKA) activity, abrogate phosphorylation of critical protein targets of PKA, and block glucagon-dependent glucose output from hepatocytes. These findings were replicated in AMPK-deficient hepatocytes, indicating that the effects are independent of AMPK. For the note, it should be investigated if all effects can be referred to the metformin action in humans and not restricted to phenformin investigated in particular study (Todd and Florez, 2014).

Metformin has both AMPK-dependent (Shaw et al., 2005; Zakikhani et al., 2006) and AMPK-independent (Foretz et al., 2010; Kalender et al., 2010) antiproliferative actions. Metformin decreased MCF-7 cell number, increased glucose consumption, and increased lactate production, reduced ATP levels, mitochondrial membrane potential, and oxygen consumption, indicating inhibition of oxidative phosphorylation. Metformin also increased apoptosis and necrosis, and the fraction of mitochondrial respiration devoted to uncoupled respiration. Metformin significantly reduced the concentrations of the TCA cycle intermediates fumarate, malate, citrate, alpha ketoglutarate and also succinate. NAD<sup>+</sup> levels are reduced by metformin, probably, by inhibition of complex I. Thus metformin could reduce oxidative phosphorylation, and also inhibit TCA cycle.



**Figure 1.12.** Metformin and glucagone effects on glycolysis and gluconeogenesis. From (Pernicova and Korbonits, 2014).

Metformin was also reported to increase plasma active glucagon-like peptide-1 (GLP-1) in obese nondiabetic subjects after oral glucose loading under an euglycaemic hyperinsulinemic clamp protocol (Mannucci et al., 2001), alas results between studies were not consistent. This incretin has insulin-like or insulin-potentiating characteristics; it stimulates insulin gene expression, inhibits glucagon secretion and promotes satiety,

inhibition of food intake, and slowing of gastric emptying (Drucker, 1998; Holst, 1999). Elevation of plasma active GLP-1 was detected, but the mechanism through which metformin induces GLP-1 secretion from L cells remains to be elusive. In conclusion, metformin directly acts as a glucagone antagonist and indirectly inhibits glucagone secretion via increase in GLP-1 (Mannucci et al., 2001).

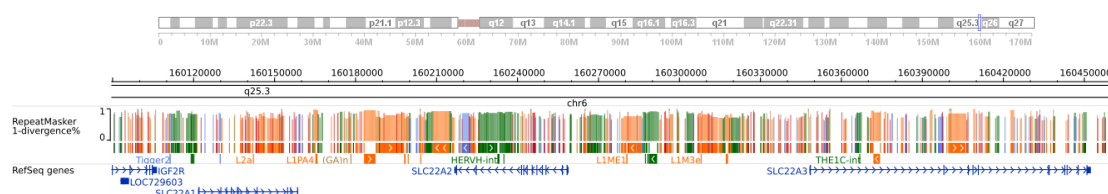
Ataxia telangiectasia mutated protein (ATM) loss of function is associated with increased carcinogenesis (Lavin, 2008; Savitsky et al., 1995), however, it is also involved in insulin signaling through phosphorylation of eIF-4E-binding protein (Ditch and Paull, 2012; Yang and Kastan, 2000). ATM activates p53 (Canman et al., 1998) and p53 upregulates oxidative phosphorylation by increasing SCO2 (a protein required for cytochrome c oxidase assembly) (Matoba et al., 2006). In the muscle, ATM inhibition reduces cytochrome c oxidase activity as consequence to SCO2 reduction which subsequently results in the reduced mitochondrial function. Fibroblasts from a patient with the ataxia telangiectasia syndrome (Ambrose et al., 2007) showed that ATM deficiency could be associated with abnormalities in mitochondrial function independent of DNA repair deficits. ATM has other substrates than p53 (Linding et al., 2007; Matsuoka et al., 2007), including Sp1 (Olofsson et al., 2007), that may alter nuclear gene expression. ATM is present in mitochondria and it may regulate oxidative phosphorylation on-site.

Metformin was shown to diminish selenium supply to extrahepatic tissues by dose-dependently downregulating expression of selenoprotein P (associated with T2D incidence) in such a manner drug might contribute to the improvement of peripheral insulin sensitivity (Hundal et al., 1992).

## 2 DESCRIPTION OF METFORMIN TRANSPORTERS

### 2.1. Organic cation transporters (OCTs) 1-3

Organic cation transporter type 1 (554 amino acids)(OCT1/*SLC22A1*) (Grundemann et al., 1994), organic cation transporter type 2 (555 amino acids) (OCT2/*SLC22A2*) (Gorboulev et al., 1997), organic cation transporter type 3 (556 amino acids) (OCT3/*SLC22A3*) (Grundemann et al., 1998b; Kekuda et al., 1998; Wu et al., 1998) coding genes are localized nearby on chromosome 6 (GRCh38.p2, NC\_000006.12, 6chr:160121789-160159198, chr6: 160216762-160258931, complement and chr6:160348373-160452581, respectively) (Motohashi and Inui, 2013) (Figure 2.1.).



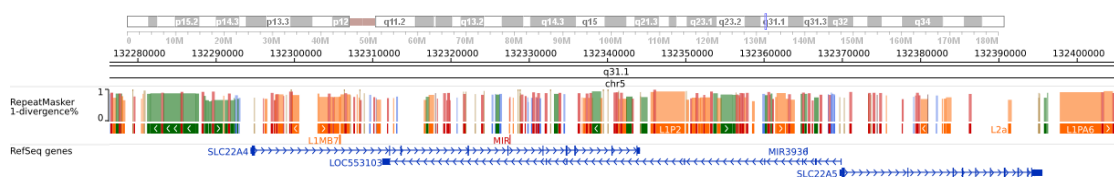
**Figure 2.1.** The human OCT1 (*SLC22A1*), OCT2 (*SLC22A2*) and the OCT3 (*SLC22A3*) are located in cluster on chromosome 6 (6q26). From (Zhou et al., 2011).

OCT 1-3 transporters belong to solute carrier family 22 (SLC 22) of organic ion transporters (> 12 members), characterized by a predicted membrane of epithelial cells and involved in the clearance of xenobiotics and elimination of endogenous cationic compounds like neurotransmitters (Dresser et al., 1999; He et al., 2009; Koepsell, 1998). The human SLC22A family includes 13 well-characterized plasma membrane proteins, including 3 organic cation transporters (OCTs) involved in the uptake of cationic drugs across the sinusoidal membrane of hepatocytes (Koepsell, 2013) and kidneys. OCTs have several highly conserved sequence motifs, localized between TMD2 and TMD3 and between TMD8 and TMD9, and N- and C-terminal halves, suggesting a duplication of genes and significance of certain residues in establishing the secondary structure of these proteins. Hydrophobicity is a principal determinant for substrate recognition by OCTs (Ahlin et al., 2008; Bednarczyk et al., 2003; Suhre et al., 2005; Zolk et al., 2009) and at least one positive charge is required for transport of small hydrophilic compounds, ranging in size from about 60 to 350 Da (Harlfinger et al., 2005; Jonker and Schinkel, 2004). However, positive charge of substrates is not prerequisite for hOCT1 and hOCT2 transport (Kimura et al., 2002). OCT substrates or inhibitors interact less efficiently with OCT3 (also

designated as extra neuronal monoamine transporter (EMT)) than with OCT1 or OCT2 (Koopsell et al., 2007). No tridimensional structure of OCTs is available but analysis of homology has shown that OCT1 and OCT2 are 70% identical. Both transporters have comparable substrate profiles and similarities in binding to predicted binding pockets (Wright and Dantzler, 2004). OCT2 transports phenformin better than metformin and thus may correlate with lactic acidosis (Sogame et al., 2013). OCT3 orthologs share only 50 % of sequence identity with OCT1 or OCT2 has more stringent binding requirements than its counterparts (Sala-Rabanal et al., 2013). The rodent transporter - rOCT1 (rOCT1, gene *SLC22A1*) contains 556 amino acids and is characterized by one large, extra-cytoplasmically localized, hydrophilic loop between TMD1/2 (Koehler et al., 1997; Zhang et al., 1997b). The rOCT2 (rOCT2, gene *SLC22A2*) encodes a 593-amino acid protein with 67% identity with rOCT1 (Okuda et al., 1996) and rOCT3 encodes a 551-amino acid protein with 48% identity with rOCT1 (Kekuda et al., 1998). Hepatocyte Nuclear Factor 1 (HNF1) was found to bind to evolutionary conserved region (ECR) and increase expression of OCT1 transporter in the cell lines and also it is associated with OCT1 expression level in the human liver (O'Brien et al., 2013). OCT1 is indirectly regulated by GR receptor via HNF4 $\alpha$  up-regulation in primary human hepatocytes (Hagos et al., 2014; Rulcova et al., 2013).

## 2.2. Carnitine/organic cation transporter OCTN1

*SLC22A4* solute carrier family 22 (organic cation/zwitterion transporter), member 4 (551 amino acids) is located on chromosome 5 (GRCh38.p2, NC\_000005.10, chr5:132294384-132344206) (Motohashi and Inui, 2013) (Figure 2.2).



**Figure 2.2.** The human OCTN1 coding gene (*SLC22A4*) is located on chromosome 5 (5q31). From (Zhou et al., 2011).

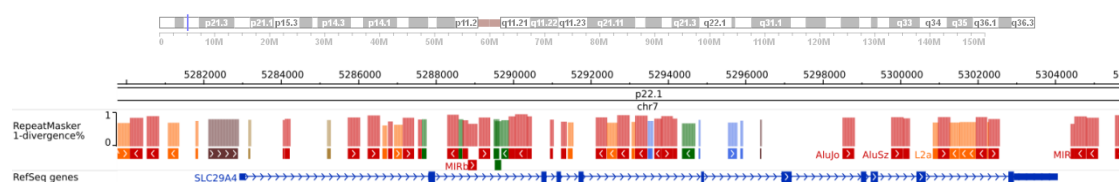
OCTN1 transporter belongs to solute carrier family 22 (SLC 22) of organic ion transporters (>12 members), characterized by a predicted 12-transmembrane-domain (TMD) structure and generally localized in the plasma membrane of epithelial cells

and involved in the clearance of xenobiotics and elimination of endogenous cationic compounds like neurotransmitters (Dresser et al., 1999; Koepsell, 1998).

The human SLC22A family includes 13 well-characterized plasma membrane proteins, including 3 Na<sup>+</sup>-zwitterion/cation cotransporters (OCTNs) involved in the uptake of anionic drugs across the sinusoidal membrane of hepatocytes. OCTs and OCTN have approximately 30% structural homology (Koepsell, 2013). OCTN1 has been proposed to be a bidirectional transporter, possibly affecting oral bioavailability of metformin but not involved in renal secretion or systemic elimination of the drug (Nakamichi et al., 2013). OCTN1 in mice is not functionally expressed on basolateral membranes of hepatocytes, but is localized in non-parenchymal hepatic sinusoidal cells thus with unclear impact on the hepatic uptake of metformin (Sugiura et al., 2010).

### 2.3. The plasma membrane monoamine transporter (PMAT)

The plasma membrane monoamine transporter (PMAT) or solute carrier family 29 (equilibrative nucleoside transporter), member 4 is coded by *SLC29A4* gene located on chromosome 7 (GRCh38.p2, NC\_000007.14, chr7: 5274374-5304073)(Figure 2.3.).



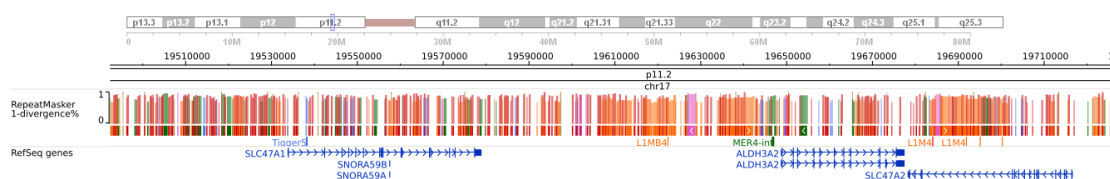
**Figure 2.3.** The human PMAT coding gene (*SLC29A4*) is located on chromosome 7 (7p22.1). From (Zhou et al., 2011).

It belongs to the equilibrative nucleoside transporter (ENT) family (SLC 29) and was alternatively named ENT 4 (Barnes et al., 2006). It shares a similar substrate and inhibitor profile with the OCTs (Engel and Wang, 2005; Engel et al., 2004). PMAT-mediated transport is Na<sup>+</sup> independent and enhanced by an acidic environment (Barnes et al., 2006; Xia et al., 2007). In humans and rodents PMAT is expressed in brain, kidney, heart, and small intestine (Barnes et al., 2006; Engel et al., 2004). Interestingly, that PMAT is expressed in the podocytes (failure of these cells is main reason for kidney diseases) in opposite to other renal OCTs, including OCT2 and MATE1, are primarily localized to tubular epithelial cells (Fujita et al., 2006; Li et al., 2006; Otsuka et al., 2005; Xia et al.,

2009). It was shown that PMAT is a target of the transcription factor, EWS/WT1 (fusion oncogene) (Lee and Haber, 2001; Li et al., 2008).

## 2.4. Human multidrug and toxin extrusion (MATEs) 1 and 2

Human multidrug and toxin extrusion 1 (*MATE1/SLC47A1*) and human multidrug and toxin extrusion 2 (*MATE2/2-k /SLC47A2*) transporter coding genes are located nearby on chromosome 17 (GRCh38.p2, NC\_000017.11, chr17: 19533854-19579033 and chr17: 19678315-19718842, complement, respectively)(Figure 2.4.).



**Figure 2.4.** The human MATE1 gene (*SLC47A1*) and the MATE2 gene (*SLC47A2*) are located in tandem on 17p11.2, both consisting of 17 exons From (Zhou et al., 2011).

Genes encoding mouse homologues are located in tandem on chromosome 11. In mouse gene encoding mMATE1 (53 kDa) was predominantly expressed in kidney, liver, heart but mMATE2 is expressed in testis (Hiasa et al., 2006; Lickteig et al., 2008; Otsuka et al., 2005; Terada et al., 2006). The gene products are designated mMATE1 and mMATE2 and are 78.1 and 38.1% identical to hMATE1 (Damme et al.; Yonezawa and Inui, 2011). As MATE2 is not suitable for experiments *in vitro*, mainly experiments are performed with MATE2-K splicing variant (Damme et al., 2011). Expression of hMATE2-B in kidney is low (Komatsu et al., 2011; Masuda et al., 2006). The Smith–Magenis syndrome is consequence of large deletion of >80 genes in of chromosome 17p11.2, including MATE1. Analysis of phenotypic characteristics of syndrome like distinctive physical features, developmental delay, cognitive impairment, and behavioral abnormalities, however, does not explain functions of MATE1 in human and are thought to be dependent on other genes (<http://www.ncbi.nlm.nih.gov/> Accessed in January 2015).

Transporters belong to multidrug and toxin extrusion (MATE) family (Brown et al., 1999; Masuda et al., 2006; Ohta et al., 2006; Putman et al., 2000) and function as an exchange of H<sup>+</sup> and a variety of organic cations (Tsuda et al., 2007). Driving force of hMATE1 and hMATE2-K is oppositely directed H<sup>+</sup> gradient, not inside-negative membrane potential. These results suggested that [<sup>14</sup>C] TEA transport via hMATE1 and

hMATE2-K is the electroneutral antiport of H<sup>+</sup> and similar mechanism was observed in rats (Tsuda et al., 2009b). In mammals MATE1 consists of 13 transmembrane helices (TMHs) with intracellular NH<sub>2</sub> and extracellular COOH (Zhang et al., 2007), but *MATE2/MATE2-K* consist of 12–13 helices and may have intra- or extracellular COOH terminus (Zhang et al., 2007).

## 2.5. Substrates and inhibitors of metformin transporters

OCTs are involved in the elimination of monoamines and cationic xenobiotics and transports organic cations such as choline, guanidine, dopamine, serotonin, histamine, acetylcholine, norepinephrine, and creatinine as well as drugs such as tetraethylammonium (TEA), 1-methyl-4-phenylpyridinium (MPP), N-methylquinine, N-(4,4-azo-n-pentyl)-21-deoxyajmalinium, procainamide, desipramine, amantadine and cis-diammine-biursodeoxycholate-platinum(II) (Muller et al., 2005). However the affinity of some substrates was shown to depend on expression system, either due to phosphorylation or minor differences in protein folding (Muller et al., 2005). OCT2, OCT3 and PMAT are low-affinity, high-capacity transporters (Zhu et al., 2012). OCT2 has the capacity for uptake of NE, 5-HT, DA, and histamine (Busch et al., 1998), OCT3 transports monoamine neurotransmitters including DA, NE, 5-HT, histamine and epinephrine (Amphoux et al., 2006; Kekuda et al., 1998; Zhu et al., 2010). For discrimination of transporters may be used prazosin for hOCT1 (reversibly inhibited by PbA, not inhibited by SKF550 and OMI), PbA for hOCT2 (reversibly by SKF550 but not affected by prazosin, b-oestradiol and OMI) and hOCT3 is selectively inhibited by corticosterone, OMI and decynium22 (Hayer-Zillgen et al., 2002). A number of compounds have been shown to inhibit transport via OCT1 *in vitro* - tricyclic antidepressants (TCAs), citalopram, proton pump inhibitors (PPIs), verapamil, diltiazem, doxazosin, spironolactone, clopidogrel, rosiglitazone, quinine, tramadol, and codeine (Ahlin et al. 2011; Ahlin et al., 2008; Bachmakov et al., 2008; Li et al., 2014; Nies et al., 2011a; Nies et al., 2011b; Tzvetkov et al., 2013; Tzvetkov et al., 2011). Interestingly, that verapamil and amitriptyline exhibited drug–drug interactions at clinical plasma concentrations of metformin for OCT1-M420del variant (Ahlin et al. 2011; Muller et al., 2005). Transport studies showed that the function of the variants decreased in the following order: OCT1-reference = V408M = M420del > R61C >> G465R. Variants M420del and R61C showed increased drug inhibition and 23 times lower IC<sub>50</sub> values up to in comparison with OCT1-reference (Ahlin et al. 2011).



In general, the  $IC_{50}$  values for inhibition of  $^{14}C$ -metformin uptake in HEK293 cells and polarized MDCK monolayers via OCT2, MATE1 and MATE2-K transporters were similar to or lower than the ones obtained with  $ASP^+$  (fluorescent organic cation 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide) (Wittwer et al., 2013). Cimetidine was identified as weak inhibitor of hOCT2 and strong inhibitor of hOCT2-A variant. In study by Wang et al. was assessed genetic variability of OCT2 transporter coding gene *SLC22A2* in 112 healthy chinese participants and showed significantly lower  $CL_t$  decrease of metformin in presence of cimetidine for carriers of TT genotype of 808G>T (18.7 vs. 48.2%,  $P = 0.029$ ) (in the group of 15 participants; GG n=6; GT n=5 and TT n=4) (Wang et al., 2008b).

A strong overlap of inhibitors (and substrates) among the organic cation transporters in the OCT family and the MATEs is inferred (Wittwer et al., 2013). MATEs was shown to transport more than 40 drugs, including cisplatin, oxaliplatin, and norfloxacin (Damme et al., 2011; Ohta et al., 2009; Yonezawa et al., 2006). MATE1, but not MATE2, substrates are levofloxacin, cephalexin, and cephadrine (Masuda et al., 2006; Tanihara et al., 2007) and inhibitors - amantadine, diltiazem and famotidine (Tsuda et al., 2009b). MATE2 can be inhibited by ciprofloxacin and pramipexole (Tanihara et al., 2007; Tsuda et al., 2009b). While inhibition in mice kidney slices by OCT2 in clinically relevant concentrations was negligible, cimetidine was detected to be potent inhibitor of hMATE1 and hMATE2-k. Even the largest  $K_i$ s for hMATE1 and hMATE2-K, which were determined using metformin, were 25- and 14-fold smaller than those for hOCT2, and could explain observed 50% decrease in tubular secretion by cimetidine (Ito et al., 2012). Mitoxantrone and ondansetron in clinically relevant plasma concentration inhibit MATE1 and MATE2-K over OCTs and irinotecan was a dual inhibitor for OCT2 and MATE1. Pantoprazole and pentamidine was selective for OCT2 over the MATEs whereas nifekalant is clinically significant inhibitor of MATE1 (Wittwer et al., 2013). In study by Matsushima et al. investigated interaction between cimetidine and FEX (fexofenadine) in human kidney slices and human embryonic kidney (HEK293) cells expressing human MATE1 (hMATE1), but no MATE2/k-2 (its specific uptake of FEX was not high enough to examine the inhibitory effect of cimetidine). In result, drug-drug interaction between cimetidine and FEX was found to be inhibition of hMATE1-mediated efflux of FEX rather than the inhibition of its renal uptake process (Matsushima et al., 2009). To investigate drug interactions of cationic drugs in basolateral-to-apical transport and intracellular secretion process, double-transfected Madin-Darby canine kidney (epithelial) cells were

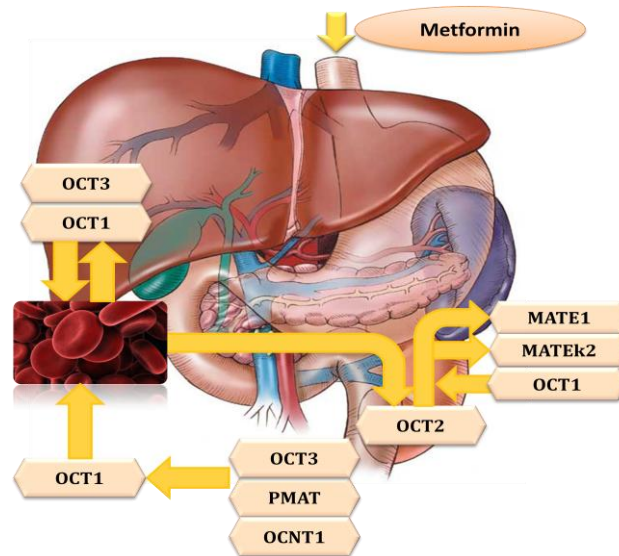
engineered to stably express both hOCT2 and hMATE transporters. Inhibition of apical hMATE1 was significant, but not OCT2, and obtained results were concordant with results in single transport systems. These findings show that interaction of metformin by cimetidine is mainly contributed to the MATE1 not OCT2, but accumulation of metformin mediated by OCT2 could also have impact (Tsuda et al., 2009b).

PMAT uptake is involved in uptake of monoamine neurotransmitters DA, 5-HT and NE in the intestines and the brain (Busch et al., 1998). PMAT transports classic OCT substrates, such as tetraethylammonium, guanidine, and histamine. Prototype OCT inhibitors, including cimetidine, and type II cations (e.g., quinidine, quinine, verapamil, and rhodamine123) are also PMAT inhibitors (Engel and Wang, 2005). OCTN1 is a carnitine and carnitine esters transporter inhibited by organic cations - cimetidine, procainamide, pyrilamine, quinidine, quinine, and verapamil (Yabuuchi et al., 1999).

## **2.6. ADME of metformin**

Metformin is not metabolized in the human body (Hardie, 2007; Pentikainen et al., 1979) but the drug is widely distributed into body tissues by at least 7 transporters (Figure 2.5.). The intestinal absorption of metformin is thought to be mediated by plasma membrane monoamine transporter (PMAT/*SLC29A4*), organic cation transporter 1 (OCT3/*SLC22A1*) and organic cation transporter 3 (OCT3/*SLC22A3*). PMAT is expressed on the luminal side of the enterocytes (Zhou et al., 2007), OCT3 is expressed in the brush boarder of the enterocytes (Muller et al., 2005). Additionally, in smaller amount OCT1/*SLC22A1* is expressed on epithelial cells and neurons of the intestine (Muller et al., 2005). While PMAT and OCT3 are thought to be involved in the uptake of metformin, OCT1 may facilitate transfer of metformin into the interstitial fluid. It could be assumed that common side-effects may be attributed to the local accumulation of metformin in enterocytes and therefore affected by OCT1, OCT3 and PMAT transport activity.

The liver uptake and removal of metformin is primarily mediated by OCT1/*SLC22A1* and OCT3/*SLC22A3* which are expressed on the basolateral membrane of hepatocytes (Nies et al., 2009). MATE1/*SLC47A1* is highly expressed in the liver, kidney and to some extent in skeletal muscle (Otsuka et al., 2005). Biliary excretion of metformin is insignificant in humans (Graham et al., 2011).



**Figure 2.5.** Metformin transporters. Adapted from (Graham et al., 2011).

The mean renal clearance of metformin is estimated to be  $510 \pm 120$  mL/min and is higher than creatinine clearance, indicating active tubular excretion in kidneys (half-life approximately 5 hours) (Graham et al., 2011). Indeed in the human proximal tubules uptake of metformin from circulation into renal epithelial cells is mediated by human organic cation transporter 2 (hOCT2/solute carrier (SLC) 22A2) (Takane et al., 2008). In rodents renal clearance is mediated differently by involving both OCT1 and OCT2 transporters to similar extent (Grundemann et al., 1998a). *In situ* hybridization reveals that, within the kidney, the rOCT2 mRNA is restricted to the outer medulla and deep portions of the medullary rays indicating selective expression in the S3 segment of the proximal tubule. It supports function of rOCT2 („r” for rodents) to maintain renal dopamine handling (Grundemann et al., 1998a). Further metformin is eliminated into the lumen by apical  $H^+$ /organic cation antiporters, human multidrug and toxin extrusion 1 (hMATE1/*SLC47A1*) and hMATE2-K (*SLC47A2*) (Masuda et al., 2006; Otsuka et al., 2005; Sato et al., 2008; Tanihara et al., 2007; Tsuda et al., 2009a; Tsuda et al., 2009b), which are expressed on brush border of proximal tubules and apical membrane of the renal proximal tubule cells in humans, respectively.

Interestingly, that OCT1 and PMAT transporters, which are thought to be involved in metformin uptake in the small intestine, are also expressed in kidneys and may be involved in reabsorption of metformin. OCT1 is expressed on the apical and subapical domain side of both the proximal and distal tubules in kidney (Tzvetkov et al., 2009) and plasma membrane monoamine transporter (PMAT/*SLC29A4*) is expressed on the apical

membrane of renal epithelial cells in the glomerulus with minimal expression in tubular cells (Xia et al., 2009).

Besides the main target organs, metformin is also transported by various transporters in other tissues. OCT1 was shown to be significantly expressed in adipose tissues and thus, possibly, may explain better treatment outcomes for obese patients using metformin (Moreno-Navarrete et al., 2011). In lungs OCT2 appears to be involved in the alveolar epithelium, whereas basolateral localised OCT3 might play a role in alveolar as well as in bronchial epithelial cells (Salomon et al., 2012). Skeletal muscle (OCT3) and intestine (OCT3, PMAT, OCT1 and OCTN1) were identified as important targets of metformin-stimulated glucose utilization (Adnitt PI, 1972; Musi N, 2002; Puah, 1986). However, it seems that metformin accumulation in muscle cells is too low to have a major impact on response to the therapy (Otsuka et al., 2005; Turban et al., 2012; Wilcock and Bailey, 1994). OCT1 and OCT3 are expressed in human heart and hypothetically may alter metformin cardiovascular effects. OCT3 is expressed at high levels in aorta, skeletal muscle, prostate, adrenal gland, salivary gland, liver, placenta, lung (fetal)(Verhaagh et al., 1999). Apical renal transport system for organic cations (OCT2) exists in dopamine-rich tissues such as kidney and brain regions such as the *nucleus accumbens*, *striatum*, and *substantia nigra* (Grundemann et al., 1998a). Expression of rOCT2 in HEK293 cells facilitates transport of dopamine, noradrenaline, adrenaline, and 5-hydroxytryptamine (5-HT) is sensitive to corticosterone as well as to the iso- and pseudoisocyanines (Grundemann et al., 1998a). PMAT and OCT2 transporters are expressed in the endometrial stroma and can potentially regulate re-uptake of monoamines (Bottalico et al., 2007). In the placenta metformin is transported by P-glycoprotein (ABCB1) (58±20%) and breast cancer resistance protein (ABCG2) (25±14%), MATE1 and OCT3 (Hemauer et al., 2010). No differences between OCT1, OCT2 and OCT3 transporters were observed in human placenta from pre-eclamptic and normotensive pregnancies (Bottalico et al., 2004). Calculated infant dose in breast milk is only approximately 0.3% of the weight-adjusted maternal dose and influence of cimetidine on the overall safety of metformin in breastfeeding is minimal (Gardiner et al., 2008).

## 2.7. Animal studies

Metformin is metabolized in rats by CYP2C11, 2D1 and 3A1/2, but in human body no significant metabolization of metformin has been observed (Choi and Lee, 2006; Pentikainen et al., 1979). Also, significant differences in transporter expression patterns exist between human and rodents. The rOCT1 mRNA is expressed in liver (sinusoidal membranes of hepatocytes around the central veins of the hepatic lobuli), kidney, intestine (Grundemann et al., 1994), while hOCT1 is primarily expressed in the liver and to a much lesser extent in the intestines and kidneys (Gorboulev et al., 1997; Zhang et al., 1997b). OCT2 mRNA is expressed in the kidney in rats and humans (Gorboulev et al., 1997; Karbach et al., 2000; Meyer-Wentrup et al., 1998; Okuda et al., 1996). The rOCT1 and rOCT2 are localized in kidneys proximal tubules, unlike analogs in humans (Karbach et al., 2000; Urakami et al., 1998). *In vitro* hOCT2 and rOCT2 was found to have 10- and 100-fold transport capacity in comparison to the hOCT1 and rOCT1, and *in vivo* studies in rats showed, that accumulation of metformin is dependent on rOCT2 transporter rather than rOCT1 (Kimura et al., 2005). Mouse OCT1 is regulated by peroxisome proliferator-activated receptors  $\alpha$  and  $\gamma$  (PPAR $\alpha$  and PPAR $\gamma$ )(Nie et al., 2005) but in rats it was down-regulated by accumulation of bile acids that inhibit hepatocyte nuclear factor-4 $\alpha$  (HNF4 $\alpha$ )(Denk et al., 2004). Overexpression of PXR rat pregnane X receptor increased expression of rOCT1 and rOCT2 (Maeda et al., 2007). The rOCT2 was detected in *substantia nigra*, *nucleus accumbens*, and *striatum* (Grundemann et al., 1997), hOCT2 was detected in the pyramidal cells of the cerebral cortex and hippocampus. Expression of OCT2 in the kidney is gender-dependent (Slitt et al., 2002; Urakami et al., 1999; Urakami et al., 2000) and age-dependent. Testosterone induces the expression of rOCT2, but not of rOCT1 and rOCT3, via the androgen receptor-mediated transcriptional pathway (Asaka et al., 2006). Acute kidney injury (AKI) induced in rat kidneys was showed to decrease renal excretion and disposition of organic cations accompanied by the down-regulation of OCT2 and MATE1 (Matsuzaki et al., 2008). The hOCT3 was detected in brain cortex, heart, liver (Grundemann et al., 1998b) aorta, skeletal muscle, prostate, adrenal gland, salivary gland, liver, placenta, and fetal lung (Ahmadimoghaddam et al., 2012; Verhaagh et al., 1999). The rOCT3 was expressed in the intestine and placenta, *cerebellum*, hippocampus, *pontine nucleus*, and cerebral cortex, heart, low in kidney and lung, but undetectable in liver (Kekuda et al., 1998). The hOCT3 was detected in normal human astrocytes (Inazu et al., 2003; Wu et al., 1998). Rajan et al. (Rajan et al., 2000) found that in mice mOCT3 mRNA

is also expressed in the retinal pigment epithelium. In the mouse mMATE1 is significantly expressed in kidney, liver whereas mMATE2 is specifically expressed in testis, but in human both hMATE1 and hMATE2 are expressed in the kidneys (Otsuka et al., 2005). OCT3 and MATE1 is expressed in rat placenta (Ahmadimoghaddam and Staud, 2013). In summary, metformin transporter protein expression patterns differ significantly between human, rat and mouse, indicating that results of studies investigating knock-out animals and animal models should be interpreted carefully if referred to human being.

Expression changes of compensatory transporters in knockout animals are of concern (Giacomini et al., 2010) (Kusuhara et al., 2011), but metformin pharmacokinetic parameters (oral bioavailability in human 50%-60% versus 59–64% in mice), volume of distribution ( $4.7 \pm 2.6$  l/kg in versus 1.5 l/kg in mice), renal clearance (3.5 times unbound GFR in humans versus 4 times in mice) are comparable (Higgins et al., 2012). In mouse hepatocytes, deletion of OCT1 resulted in a reduction in the effects of metformin on AMPK phosphorylation and gluconeogenesis. In OCT1 (-/-) mice, the hepatic uptake and intestinal excretion of organic cations are greatly reduced (Jonker et al., 2003). Metformin uptake in primary mouse hepatocytes was significantly lower (3.4-fold;  $P \leq 0.0001$ ) in OCT1 -/- cells compared with those in cells with a functional OCT1 allele (OCT1 +/- and OCT1 +/+ hepatocytes). Metformin (1 mM) significantly suppressed glucagon-stimulated glucose production in hepatocytes from wild-type mice (30% suppression;  $P < 0.001$ ) but not in hepatocytes from OCT1 -/- mice. Following a single oral dose (15 mg/kg), the plasma concentrations of metformin were similar in OCT1 -/- and wild-type mice but hepatic accumulation was significantly greater in wild-type mice (4.2-fold;  $P < 0.001$ ; 1 hour after dosing) than in OCT1 -/- mice, but phosphorylation of both AMPK and ACC (acetyl-CoA carboxylase) was substantially reduced in livers from OCT1 -/- mice compared with those from wild-type mice. No accumulation differences were measured in other major organs. OCT1 -/- and OCT1 +/+ mice were on a high-fat diet for 8 weeks and then treated with saline or metformin for 5 days. While baseline fasting blood glucose levels between OCT1 -/- and OCT1 +/+ mice on high-fat diets were similar, metformin significantly reduced fasting plasma glucose levels by more than 30% in wild-type mice fed the high-fat diet ( $P = 0.012$ ) but not in the OCT1 -/- mice on the same diet. These data suggest that OCT1 serves a critical function in metformin's primary therapeutic effect *in vivo*, but significant metformin uptake and metformin-independent AMPK activation in OCT1 -/- primary hepatocytes indicates contribution of other mechanisms, such as passive diffusion or impact of OCT3 transporter (Shu et al., 2007). In the study by Wang there was

identified a marked difference in the response to intravenous metformin administration between wild-type and OCT1  $-/-$  mice, while plasma concentration-time profiles of metformin were similar. The blood lactate concentration in metformin-treated wild-type mice was 2.5-fold greater than that in metformin-treated OCT1  $-/-$  mice. In contrast to the significant reduction of metformin concentration in the liver of OCT1  $-/-$  mice, the concentration of metformin in muscle was similar in both groups. The  $EC_{50}$  for metformin were determined to be  $734 \pm 168 \mu\text{M}$  if calculated from highest blood lactate AUC. In the isolated rat hepatocytes oxygen consumption was decreased in the presence of biguanides in a concentration-dependent manner and 75% reduction correlated with  $EC_{50}$  values determined in vivo in this study for the increase of blood lactate. No significant difference in levels of metformin in skeletal muscle between OCT1  $-/-$  and OCT1  $+/+$  mice, indicates that the uptake of metformin in skeletal muscle may involve another transporter, for example OCT3 (Wang et al., 2003). Study in OCT1 knockout mice have shown that deletion of OCT1 resulted in a more severe off-target toxicities and significantly decreased clearance ( $0.444 \pm 0.0391 \text{ ml/min*kg}$  versus  $0.649 \pm 0.0807 \text{ ml/min*kg}$  in wild-type mice,  $P < 0.05$ ) and volume distribution ( $1.90 \pm 0.161 \text{ L/kg}$  versus  $3.37 \pm 0.196 \text{ L/kg}$  in wild-type mice,  $P < 0.001$ ) when treated intravenously with anticancer drug cis-diammine (pyridine) chloroplatinum (II) (CDPCP) but not oxaliplatin (Li et al., 2011). In the study with mouse knockout model OCT1  $-/-$  there was demonstrated that OCT1 is a major thiamine transporter in the liver, which is inhibited by action of metformin thus thiamine deficiency enhanced the phosphorylation of AMPK and its downstream target, acetyl-CoA carboxylase (Chen et al., 2014). OCT2  $-/-$  and OCT1/2  $-/-$  mice are viable and fertile and display no obvious phenotypic abnormalities, indicating that these transporters are not absolutely essential and may possibly be compensated for by action of redundant transporters. OCT1  $-/-$  and OCT2  $-/-$  knockout versus wild-type mice have 2-fold decrease in steady state of TEA levels in plasma and accumulation in kidneys, but at the same time no significant changes in plasma and urinary levels of TEA were observed. The proposed mechanism was that TEA excretion and accumulation are facilitated separately (apical membranes and basolateral membranes), it is based on finding that the apical membrane is rate-limiting in the secretion of TEA by renal proximal tubules. In OCT1/2  $-/-$  renal secretion of TEA was completely abolished, leaving only glomerular filtration as a TEA clearance mechanism and increasing TEA concentrations in the plasma (Jonker et al., 2003). Despite significant changes in metformin clearance and distribution in OCT1/OCT2 double-knockout mice, hepatic drug exposure was not reduced in expected magnitude, and

metformin pharmacodynamic effects were not diminished due to absence of both renal and hepatic OCT transport. It provided additional evidence that OCT1 is not the sole mechanism of metformin hepatic uptake necessary for inhibition of gluconeogenesis (Higgins et al., 2012).

Controversial results were obtained from a study investigating implications of simultaneously impaired OCT1 and OCT2 transporters (facilitates hepatic uptake and renal clearance in mice and humans) on metformin pharmacokinetic/pharmacodynamic (PK/PD) changes in knockout mice. Liver- and kidney-to-plasma concentration ratios are lower in OCT1/OCT2 knockout mice (4.2- and 2.5-fold). But 2.9-fold increase in oral metformin exposure does not affect metformin concentration in tissues. Absolute kidney exposure was unchanged and liver exposure was only modestly decreased. Oral glucose area under the curve (AUC) lowering by metformin was not impaired in OCT1/OCT2-knockout mice at the five dose levels tested ( $ED_{50}=151$  versus 110 mg/kg in knockout versus wild type), but higher exposure was required to achieve effect. Despite major changes in metformin clearance and volume of distribution in OCT1/OCT2 knockout mice, tissue drug exposure and PD were not affected. Observed 4.5-fold decrease in systemic clearance to approximately GFR and a 3.5-fold decrease in the volume of distribution in knockout mice are consistent with up to 2.6-fold reduction in secretory renal clearance in humans with a functional OCT2 variant (Chen et al., 2009a; Song et al., 2008b), as well as a 2.2-fold decrease in oral volume of distribution in people with a functional OCT1 variant (Shu et al., 2008; Shu et al., 2007). No differences in oral bioavailability were observed.

The present findings are conceptually inconsistent with the presumption that systemic pan-OCT inhibition will affect metformin pharmacology. OCT1 is estimated to mediate 76% of metformin hepatic uptake in mice, whereas OCT1 and OCT2 together account for 60% of metformin renal uptake. These calculations assume as much as 24% of hepatic and 40% of renal metformin uptake to be facilitated by non-OCT1/OCT2 transporters, and thought to be effects of OCT3 and possibly other transporters. Anti-cancer drug cimetidine (Konig et al., 2011; Somogyi et al., 1987; Wang et al., 2008b), affects OCT1 (hepatic uptake) and OCT2 (renal excretion) simultaneously and thus should inhibit uptake both renal and hepatic similarly. Increased metformin exposure and decreased hepatic uptake should minimally affect kinetics of the drug ( $dX_{Liver}/dt = C_{systemic} \times CL_{uptake}$ ). Cimetidine, however did not affect metformin induced lactic acidosis (Somogyi et al., 1987). Possibly enhanced metformin effects due to inhibition of renal clearance would be more relevant for selective OCT2 inhibitors like amantadine or



amphetamine (Amphoux et al., 2006; Higgins et al., 2012). In OCT1 and OC2 double-knockout mice was observed significantly impaired creatinine clearance and no inhibition of cation transport in proximal tubules by creatinine and in 68 cancer patients cisplatin caused acute elevation of serum creatinine (P value=0.0083) (Ciarimboli et al., 2012). *In vivo*, metformin was actively transported with a high level of accumulation in the salivary glands of wild-type mice. In contrast, active uptake and accumulation of metformin in salivary glands were abolished in OCT3  $-/-$  mice (Lee et al., 2014). OCT3-deficient mice show altered monoamine neurotransmission, anxiety-related behavior, stress response, and response to psychostimulants indicating that inhibition of OCT3 may be protective against depression (Cui et al., 2009; Hengen et al., 2011; Vialou et al., 2008; Wultsch et al., 2009).

A defect of MATE1 significantly delayed the systemic elimination of metformin and cephalexin in mice (Tsuda et al., 2009a; Watanabe et al., 2010) and MATE inhibitor, pyrimethamine, significantly decreased the luminal efflux of TEA and metformin in the liver and kidney in mice. It should be noted that MATE1 expression in human liver is significantly lower than in rodent liver and biliary excretion of metformin in human is almost absent (Ito et al., 2010) and this difference between species is significant and should be taken in consideration if data from animal studies are applied to research in humans (Gong et al., 2012).

The maximum plasma concentration ( $C_{max}$ ) after oral administration of metformin to OCTN1 gene knockout mice (OCTN1 $-/-$ ) differs in response to the dose (lower dose leads to higher  $C_{max}$ ) and has similar in terminal half-life. Systemic elimination of metformin after intravenous administration was similar in the two strains. OCTN1-mediated uptake of metformin was observed in human embryonic kidney 293 cells transfected with mouse OCTN1 gene, and metformin uptake in intestinal epithelial cell line Caco-2 was inhibited by [ $^3$ H] ergothioneine (ERGO) (Nakamichi et al., 2013). OCTN1 is expressed on activated intestinal macrophages and could contribute to the altered disposition of ERGO in intestinal inflammation associated with Crohn's disease (Shimizu T, 2015).

The Tsc1  $+/-$  mouse model (tuberous sclerosis) showed no benefit from metformin, possibly due to epigenetic suppression of the organic cation transporters 1–3 in renal tumors (Yang et al., 2013).

### 3 OVERVIEW OF PHARMACOGENETIC STUDIES

#### 3.1. OCT1 genetic variants

The predominant splice isoform, *SLC22A1*-001 (OCT1-a) has eleven exons and results in a functional 554 amino acid protein. Region -141/-69 was found to be essential for the basal core transcriptional activity, has no polymorphisms and as it contained the sequence of a cognate E-box (CACGTG) is bound by upstream stimulating factors (USFs), and the functional involvements of USF1 and USF2, and also stimulated by transcription factor hepatocyte nuclear factor 4 (Kajiwara et al., 2008). Alternative splicing occur in rOCT1 (kidney, truncated protein encoded by rOCT1A), however lack of the first two TMDs has no significant effect transport activity of TEA (Zhang et al., 1997a). Several alternatively spliced variants of OCT1 were detected in human (glioma cell line SK-MG-1, human liver). However even lack the last two C-terminal TMDs or the last six TMD does not affect uptake of MPP<sup>+</sup> in transfected human embryonic kidney 293 cells (Hayer et al., 1999). More interesting finding is *SLC22A1*-006 splicing variant (exon 7 splicing at the duplicate splice site with translated protein of 506 instead of 554 amino acids) that might have functional relevance in antidiabetic and cancer treatment (Grinfeld et al., 2013)(Figure 3.1.).

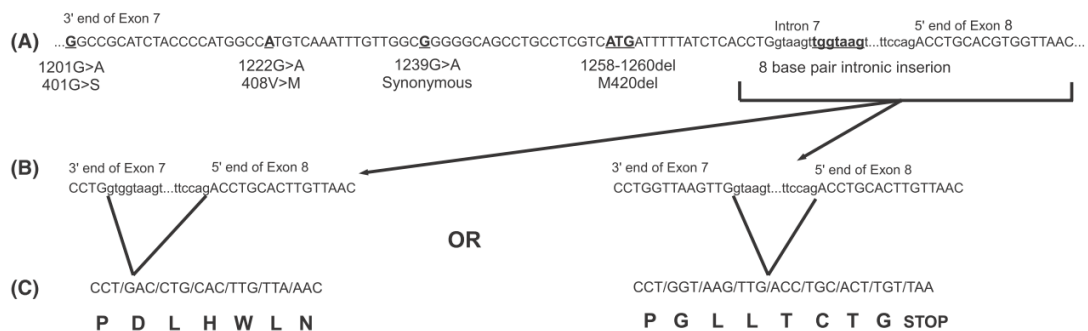


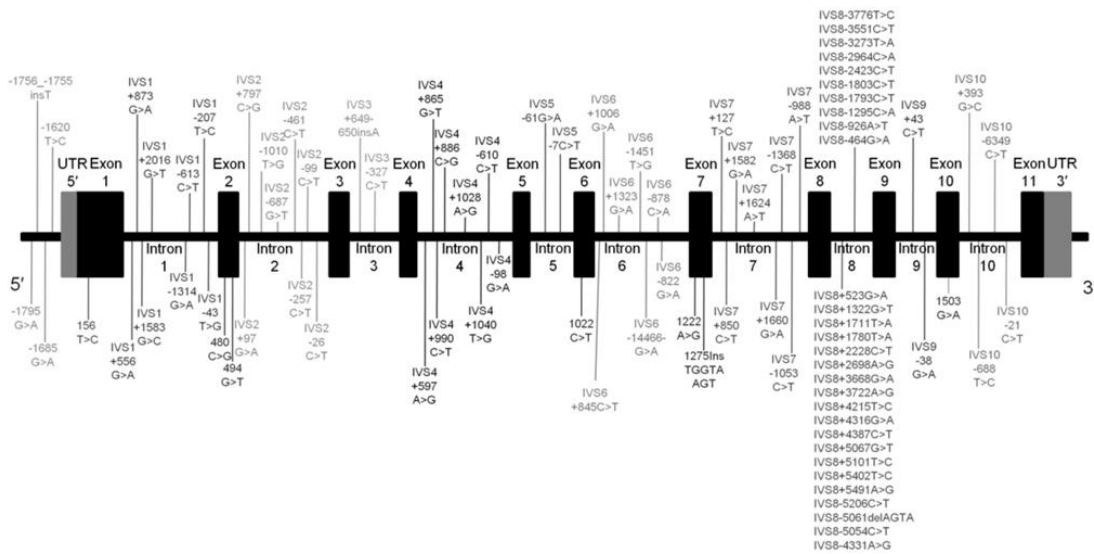
Fig 1. Polymorphisms in *SLC22A1* exon 7. (A) Polymorphism locations, nomenclature and amino acid changes. B and C show alternate splicing at the marked site in A. (B) Alternative splice donor sites at the 3' end of exon 7. (C) cDNA and protein sequences resulting from alternative splicing.

**Figure 3.1.** A common novel splice variant of isoform *SLC22A1*-006. Figure and legend from (Grinfeld et al., 2013).

OCT1 mRNA and protein expression in liver tissue samples from 150 Caucasian subjects varied 113- and 83-fold and transcript levels were on average 15-fold higher compared with OCT3. OCT1 is independent of age and sex but was significantly reduced

in rat kidney with chronic renal failure and human liver donors diagnosed as cholestatic and in the carriers of Arg61Cys variant (rs12208357) ( $P < 0.0001$ ) (Komazawa et al., 2013; Nies et al., 2009). Two variants, -43T>G in intron 1 and 408Met>Val (1222A>G) in exon 7, were predictors of metformin efficiency in 33 patients and mRNA in human liver tended to be lower (non-significantly) in 408 Met>Val genotype carriers (Shikata et al., 2007).

Organic cation transporter OCT1 have more than 1000 genetic variations and most investigated ones are five mutations resulting in the amino acid changes Arg61Cys (rs12208357), Cys88Arg (rs55918055), Gly401Ser (rs34130495), and Met420del (respective MAF of 9.1%, 0.6%, 3.2%, and 16% in Caucasians) and G465R (rs34059508) (Lozano et al., 2013) (Figure 3.2.). Many SNPs in OCT1 have significantly different minor allele frequency between populations, for example, Arg61Cys (rs12208357) was not detected in Xhosa population and in 202 participants of Asian ancestry SNPs rs12208357, rs55918055, rs34130495, rs34059508 were not found at all but incidence of substitution Met420del was low (1.5%) (Jacobs et al., 2014; Perwitasari DA., 2014).



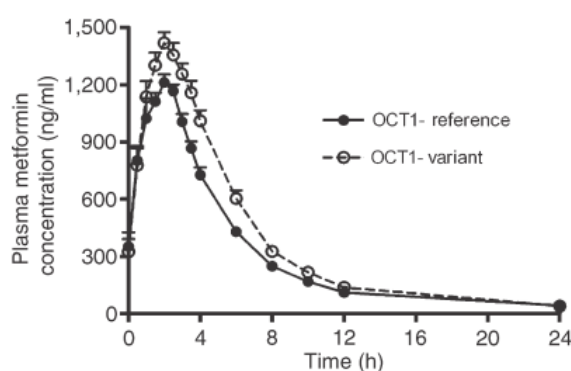
**Figure 3.2.** *SLC22A1* gene structure and location of single nucleotide polymorphisms.

From (Lozano et al., 2013).

Phe160Leu and Met420del exhibited substrate affinities and selectivities identical to hOCT1 wild-type, but Arg61Cys, Cys88Arg and Gly401Ser affinities were reduced to 30%, 1.4% and 0.9% compared to wild-type, respectively (Cys88Arg and Gly401Ser mutants exhibit changed substrate selectivity). These results show that Arg61Cys, Cys88Arg and Gly401Ser may alter the disposition, duration and intensity of effects of

drugs and neurotransmitters which are substrates for hOCT1 (Kerb et al., 2002). In Chinese and Japanese population were identified six nonsynonymous polymorphisms and three of them were previously functionally non-characterized. Study showed that Q97K, P117L, and R206C (MAF 0.017, 0.023 and 0.008) significantly alter transport activity of OCT1 transporter (a relative activity in comparison to OCT1 reference  $62\pm 4.3\%$ ,  $55\pm 6.8\%$ , and  $22\pm 1.5\%$ , respectively) (Chen et al.).

In the study by Shu et al. in comparison to OCT1-reference, 7 OCT1 variants investigated exhibited significantly reduced or lost metformin uptake (S14F, R61C, S189L, G220V, G401S, 420del, G465R), despite similar levels of mRNA in HEK293 (Clone 9, 3T3-L1) cell lines. R61C, G410S, 420del, and G465R exhibited reduced  $MPP^+$  transport activity in cellular assays and were further investigated in pharmacodynamic study in 20 healthy volunteers. Plasma glucose levels and areas under the glucose concentration–time curve (AUCs) after OGTT in volunteers carrying only reference OCT1 alleles (n=8) and those carrying a reduced-function polymorphism (n=12) were similar, however, after metformin treatment volunteers carrying the OCT1 polymorphisms had significantly higher plasma glucose levels for most of the sampling time points during the 180-minute OGTT (polymorphisms as compared with those carrying only reference alleles ( $18,200\pm 1.600$  versus  $21.300\pm 2.290$  min/mg/dl;  $P=0.004$ ). An increase in glucose half-life in individuals with the OCT1 variants was observed, probably due to delay and decrease in glucose absorption (Shu et al., 2007). Similar tendencies were observable in pharmacokinetic study in healthy individuals (Figure 3.3.).



**Figure 3.3.** Figure from (Shu et al., 2008). Pharmacokinetics of metformin in healthy individuals who carry an OCT1-variant allele (n=12) and those who carry only OCT1-reference alleles (n=8).

Individuals who carried a variant OCT1 allele had a significantly higher  $C_{max}$  and 53% higher oral clearance (CL/F), 54% lower oral volume of distribution (V/F) than those who did not ( $P=0.004$ ). Area under the plasma concentration time curve (AUC) of metformin was significantly greater in the OCT1-variant group than that in the OCT1-reference group ( $AUC_A$ ,  $P=0.01$ )(Shu et al., 2008).

In 103 healthy Caucasian males renal versus metformin clearance varied 3.8-fold and was significantly dependent on creatinine clearance ( $r^2 = 0.42$ ,  $P < 0.0001$ ), age ( $r^2 = 0.09$ ,  $P=0.002$ ). Carriers of zero, one, and two low-activity OCT1 alleles (arg61Cys, gly401ser, 420del, or gly465arg) had mean renal clearances of 30.6, 33.1, and 37.1 l/h, respectively ( $P=0.04$ , after adjustment for creatinine clearance and age). Variation in creatinine clearance accounted for 42% and genetic variants in OCT1 accounted for a further 10% of the observed variation in the renal clearance of metformin (Tzvetkov et al., 2009).

Study investigating PCOS found that only the reference allele carriers versus R61C (C>T), G401S (G>A), G465R (G>A), and 420del carriers reduced their total cholesterol, triglycerides and hyperinsulinemia in response to 6 months long metformin therapy (Gambineri et al., 2010). In the GoDARTS study with 1 531 T2D patients using metformin for 6-18 months R61C and 420del variants did not affect the initial A1C reduction in short- and mid-term or reaching the treatment target of  $HbA_{1c} < 7\%$ , and time to monotherapy failure. Similarly, in study with 189 Caucasian patients using metformin for 160±52 days association of R61C, G401S, 420Del, and G465R with relative  $HbA_{1c}$  change was not replicated (Choi et al., 2011). However, many variants in OCT1 coding gene have been investigated (Figure 3.4.)(Lozano et al., 2013).

Reference SNP	Amino acid change	Nucleotide variation	Exon	Protein domain	Transport activity compared with wild type				PML
					MPP <sup>+</sup>	TEA	Metformin	Others	
rs34447885	S14F	c.41C>T	1	NH2 terminus	↑ [30]	= [3]	↓ [36]	= Sorafenib [3]	
rs12208357	R61C	c.181C>T	1	Large EL	↓ [26, 30]	↓ [3, 26]	↓ [36]	↓ Sorafenib [3]	NO [36]
NA	R61S fs*10	c.181delCGinsT	1	Large EL		↓ [3]		↓ Sorafenib [3]	
rs35546288	L85F	c.253C>G	1	Large EL	= [30]				NO [3]
rs55918055	C88R	c.262T>C	1	Large EL	↓ [26]	↓ [3]		↓ Sorafenib [3]	NO [3]
NA	C88A fs*16	c.262delT	1	Large EL		↓ [3]		↓ Sorafenib [3]	NO [3]
NA	Q97K	c.289C>A	1	Large EL			↓ [35]		YES [35]
NA	P117L	c.350C>T	1	Large EL			↓ [35]		YES [35]
rs683369	L160F	c.480G>C	2	TMD2	= [26, 30, 33]	= [3, 33]	= [36]	= Sorafenib [3]	YES [33]
rs34104736	S189L	c.566C>T	3	TMD3	= [30]	↓ [3]	↓ [36]	↓ Sorafenib [3]	NO [3]
NA	P197S	c.589C>T	3	TMD3		= [3]		= Sorafenib [3]	YES [3]
NA	R206C	c.616C>T	3	EL2			↓ [35]		NO [35]
rs36103319	G220V	c.659G>T	3	TMD4	↓ [30]	↓ [3]	↓ [36]	= Sorafenib [3]	
rs4646277	P283L	c.848C>T	5	TMD6	↓ [33, 41]	↓ [32, 33, 41]	= [41]	↓ Lamivudine [41]	YES [33]
rs4646278	R287G	c.859C>G	5	Large CL	↓ [33]	↓ [3, 32, 33]		↓ Sorafenib [3]	YES [33]
rs2282143	P341L	c.1022C>T	6	Large CL	↓ [30, 33, 41]	↓ [32, 33, 41]	= [36, 41]	↓ Lamivudine [41]	YES [33]
rs34205214	R342H	c.1025G>A	6	Large CL	= [30]		= [36]		
rs34130495	G401S	c.1201G>A	7	CL4	↓ [26, 30]	↓ [26] = [3]	↓ [36]	= Sorafenib [3]	
rs628031	M408V	c.1222A>G	7	TMD9	= [30]		= [36]		
rs20222080	M420del	c.1258_1260delATG	7	TMD9	= [26, 30]		↓ [36]		
rs35956182	M440I	c.1320G>A	7	TMD10	= [30]				
rs34295611	V461I	c.1381G>A	7	CL5	= [30]				
rs34059508	G465R	c.1393G>C	8	TMD11	↓ [30]	↓ [3]	↓ [36]	= Sorafenib [3]	YES [3]
rs35270274	R488M	c.1463G>C	8	EL6	= [30]		= [36]		YES [30]

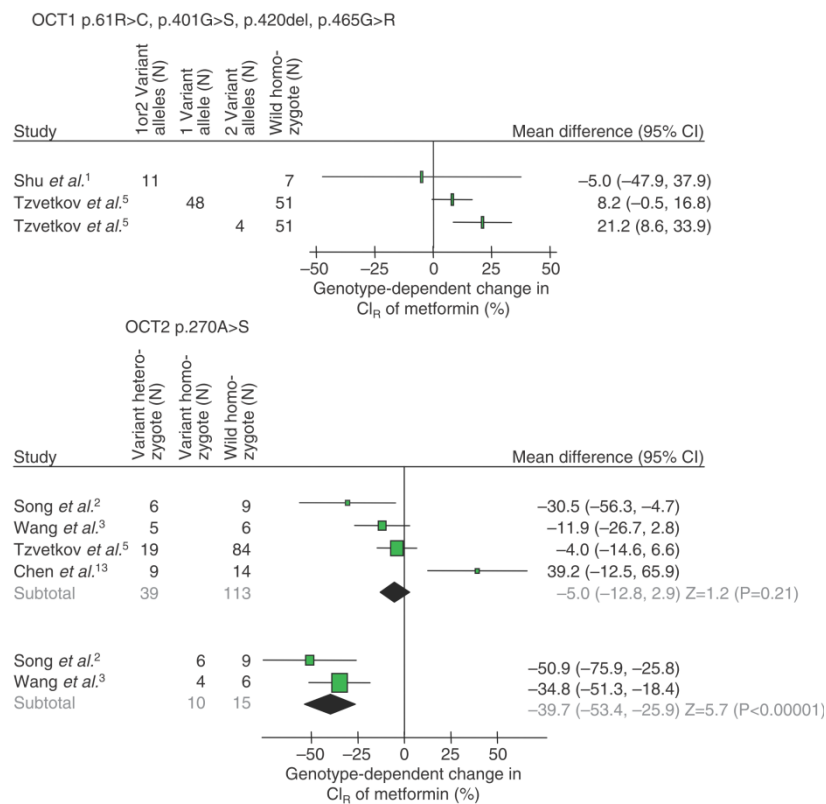
EL: extracellular loop; CL: cytoplasmic loop; NA: not assigned; PML: plasma membrane localization; TMD: transmembrane domain; ↑: increased; ↓: decreased; =: unaltered.

**Figure 3.4.** Role of the Genetic Variants in OCT1, From (Lozano et al., 2013).

Possibly, more clinically relevant is study by Christensen et al. that was published in the year 2011 and showed that metformin exerts high interindividual variability (up to 80-fold) in through steady state metformin plasma concentration after 1.5 years of therapy. The study was performed in a study group of 159 T2D patients and estimated mean through steady-state of metformin plasma concentration was 576 ng/ml and interindividual variability ranged from 54 to 4133 ng/ml in absolute values. Significantly, it correlated with number of reduced function alleles in OCT1 (none, one or two: 642, 542, 397 ng/ml;  $P=0.001$ , respectively). Accumulation of metformin in serum was predicted by OCT1 variant rs72552763 (M420del). OCT1 genotypes rs12208357 (R61C), rs34130495 (G401S), rs72552763 (M420del) and rs34059508 (G465R) resulted in 5 haplotypes showing significant and additive decrease in the through steady-state plasma concentration with increased number of reduced function alleles. PMAT, MATE1, MATE2, OCT2 (rs316019, A270S) was shown as not affecting metformin pharmacokinetics and rs34130495 was associated with  $\Delta$  HbA<sub>1c</sub> in response to metformin therapy. OCT3 was not included in analysis. In the same study in Danish patients, genetic variants in OCT1 were investigated if they were able to predict response to metformin therapy – change in absolute HbA<sub>1c</sub> or ability to reach therapy goals (HbA<sub>1c</sub> <7% or <6.5%). Decrease in Hb1Ac both initially and in long term (6 and 24 months) correlated with the number of reduced function alleles in OCT1 (Christensen et al., 2011).

It should be noted, that associations found by Shu et al. did not reach statistical significance in the Rotterdam study and in study by Christensen and colleagues showed opposite direction (Goswami et al., 2014). A meta-analysis of genetic studies of OCT1 and OCT2 transporter variants found no association between (p.61R>C, p.401G>S, p.420del, p.465G>R and metformin efficiency, showing significant impact of environment (up to 25% on metformin renal clearance) and other genetic factors (metformin transporters like MATEs, probably PMAT and OCTN1 transporter and genetic susceptibility variants affecting T2D and renal diseases) (Zolk, 2009)(Figure 3.5.). Patients with OCT1 R61C and rs622342 polymorphism showed increased HOMA-IR (Berstein et al., 2013). In 122 South Indian T2D patients, carriers of two copies of allele (AA) for rs622342 had 5.6 times greater chance of responding to metformin treatment (Umamaheswaran et al., 2014). In the group of women with polycystic ovary syndrome (PCOS) and controls, references versus carriers of polymorphisms R61C (C>T), G401S (G>A), G465R (G>A), and 420del, had good effect on total cholesterol (-14 mg/dl (-22 to -5);  $P=0.002$ ) and triglycerides (-17

mg/dl (-29 to -5);  $P=0.008$ ). An effect on insulin was positive in references and carriers of 1 risk allele but not in carriers of 2 or more polymorphisms (Gambineri et al., 2010).



**Figure 3.5.** Meta-analysis of genetic studies of OCT1 and OCT2 transporter variants. From (Zolk, 2009).

Metformin has demonstrated antineoplastic effects and OCT1 was shown to transport antitumor drugs like cisplatin, irinotecan, mitoxantrone, sorafenib, and paclitaxel. (Gupta et al., 2012; Li et al., 2011; Segal et al., 2011). Hypermethylation of *SLC22A1* promoter down-regulates expression of OCT1 in human hepatocellular carcinoma (HCC) and was associated with tumor progression and a worse patient survival (Heise et al., 2012; Schaeffeler et al., 2011). OCT1 is less expressed in case of HCC and cholangiocarcinoma (CGC), and R61S fs\*10 and C88A fs\*16 encode truncated proteins that may reduce response of to sorafenib (Herraez et al., 2013). Results of the studies in cancer patients and cell lines proves the concept but effects of OCT1 genetic variants differ depending on the substrate (Urban et al., 2006). For example P283L and P341L variants do not affect metformin uptake, but reduce uptake of methylpyridinium (Kerb et al., 2002; Shu et al., 2003) and lamivudine (Choi and Song, 2012), but S14F with impaired metformin uptake was shown to increase uptake of methylpyridinium. In respect to primary tumors it was

shown that OCT1 expression varies significantly and thus may decrease response to concomitant metformin therapy (Segal et al., 2011). OCT1, due to the expression on leukocytes, has become an extensively studied in respect to the efficiency of cancer drug imatinib (Eechoute et al., 2011). Although it is worth noting that OCT1 was showed as uncapable to transport imatinib in nonleucemic animals and cell lines (Hu et al., 2008). *In vitro* sensitivity to imatinib was correlated with intracellular uptake in mononuclear cells from chronic myeloid leukemia (CML). OCT1 inhibitor prazosin leads to requirement of increased dose of imatinib to maintain effects in patients (Gardner et al., 2008; White et al., 2006). However prazosin was shown to be non-specific OCT1 inhibitor and hypothetically may affect imatinib uptake indirectly (Burger et al., 2013). In the first study investigating 6 patients with CML was shown that inhibition of OCT1 decreased intracellular imatinib uptake in the blood leukocytes (Thomas et al., 2004). It was validated in other 2 studies showing that OCT1 mRNA expression correlates with imatinib uptake in CML cell lines (Kim et al., 2014). Results obtained from short and long term studies with cancer patients revealed that increased OCT1 expression is good marker of as predictors of outcomes of CML (Crossman et al., 2005; Wang et al., 2008a; White et al., 2010). Increased dose of imatinib in such patients increased response, however finding remains elusive as it does not explain mechanism of action (White et al., 2007). Lately SNPs M420del and M408V were show to alter imatinib uptake and M420del modifies clinical outcome in imatinib-treated chronic myeloid leukemia (Giannoudis et al. 2013). Many mistakes, including impossible haplotype and incorrect three-dimensional model explaining interaction between genetic variations, were noted by other research group ( 2014). However, association with genetic factors in OCT1 were replicated in other studies with CML patients (Grinfeld et al., 2013; Singh et al., 2012), but not with OCT1 (*SLC22A1*) R61C polymorphism (Zach et al., 2008). Possible explanation may be in fact that OCT1 expression is found to be linked to expression level of at least three other transporters ABCB1, ABCG2, and SLCO1A2 and therefore may OCT1 may be potential surrogate marker (Hu et al., 2008).

OCT1 was found to be associated with low-density lipoprotein levels, development of primary biliary cirrhosis and prostate cancer as well as to alter pharmacokinetics and efficacy of drugs like tropisetron, morphine, O-demethyltramadol (Fukuda et al., 2013; Herraes et al., 2013; Ohishi et al., 2014; Teslovich et al., 2010; Tzvetkov et al., 2013; Tzvetkov et al., 2012).



### 3.2. OCT2 genetic variants

OCT2 is responsible for up to 80% of metformin clearance (Kashi et al., 2015). The hOCT2-A, alternatively spliced variants from human kidney (Urakami et al., 1999). Due to premature stop codon this transcript codes for shorter protein with 81% amino acid identity with hOCT2 but it lacks the last three C-terminal TMDs. In human embryonic kidney 293 cells hOCT2-A exhibited normal transport activity of TEA, but reduced transport of MPP<sup>+</sup> and cimetidine, and no transport activity of guanidine. The pattern of expression differs as OCT2 is detected in kidney, brain, testis, and placenta, but hOCT2-A was found primarily in kidney, but liver, colon, skeletal muscle. Age and testosterone alter OCT2 expression in the kidneys (Urakami et al., 1999). Cirrhosis upregulates expression of OCT2 in rats and in humans increases tubular secretion of creatinine (Lopez-Parra et al., 2006; Sansoe et al., 2002). Population-genetic analysis demonstrated that OCT2 have lower diversity because natural selection has acted against accumulation of non-synonymous variants (Leabman et al., 2002). Analysis of regulatory polymorphisms revealed that -578/-576delAAG (ss94002365, MAF 8.7% in Asians) reduced promoter activity for 14% but it does not significantly affected levels of mRNA (Ogasawara et al., 2008). Region spanning -91 to -58 base pairs was found to be essential for basal transcriptional activity and contained a CCAAT box and an E-box. Upstream stimulating factor 1 (USF-1) was found to act as a basal transcriptional regulator of the hOCT2 gene via the E-box. USF-1 is widely expressed transcriptional factor, but OCT2 is expressed only in several tissues (Sirito et al., 1994). Possibly, methylation of the E-box in the kidney or unidentified kidney-specific transcription factor may explain kidney-specific expression of OCT2 (Asaka et al., 2007). *In vitro* studies showed that the stable expression of hOCT2 is linked to a significant accumulation of creatinine in HEK293 cells and in 590 patients polymorphisms including rs2504954 (P=0.000873) were associated with creatinine levels (Ciarimboli et al., 2012). Previously also SNPs rs2279463 (located in the OCT2 gene *SLC22A2*) and rs3127573 (nearby *SLC22A2*) were associated with creatinine clearance (Chambers et al., 2010; Kottgen et al., 2010) Genetic variants of OCT2 (T199I, T201M (rs145450955), and A270S) decreased the transport activity of metformin in oocytes (Song et al., 2008b) and also Lys432Gln, 134-135insA, Met165Ile, and Arg400Cys exhibited functional differences from the reference OCT2 (Hayer-Zillgen et al., 2002).

In 40 patients, OCT2-T201M minor allele T (n=8) was shown to have higher HbA<sub>1c</sub> values, fasting glucose and insulin resistance (HOMA-IR and HOMA-BCF) due to

decreased response to metformin therapy (Kashi et al., 2015). Variants A270S (808G>T) variant was shown to affect mainly tubular excretion and renal clearance, and increase the plasma metformin level. In TT genotype carriers versus reference  $C_{max}$  of metformin was increased 1.62-fold,  $AUC_{last}$  was increased 1.74-fold, but active secretion clearance ( $Cl_{sec}$ ) (from equation =creatinine clearance ( $Cl_{cr}$ ) - renal clearance ( $Cl_{renal}$ )) was decreased by 61.8% (Song et al., 2008a). Genetic polymorphism OCT2-808 G>T had a significant ( $P<0.05$ ) effect on metformin pharmacokinetics, yielding a higher peak concentration with a larger area under the serum time–concentration curve in Korean patients (Yoon et al., 2013). Non-synonymous single-nucleotide polymorphism 808G>T (rs316019) in the OCT2 coding gene *SLC22A2* exon 4 (Serine to Alanine at codon 270) was associated with decreased expression of *SLC22A2* in a gene-dosage dependent fashion and reduced cisplatin-induced nephrotoxicity in patients (Filipski et al., 2009). In a study investigating metabolomics in groups of GG, GT and TT genotype carriers, tryptophan and uridine in urine were found to be decreased. *In vitro* functional studies showed significant OCT2 inhibition potential by tryptophan. Thus, it could be used as a potential biomarker of genotype-dependent OCT2 transport activity (Song et al., 2012). 14 tagSNPs (tagging polymorphisms) with minor allele frequency  $\geq 5\%$ , including A270S, were not associated with renal clearance in 103 healthy Caucasian males. However, in 23 healthy volunteers of Caucasian and African American ancestries significantly higher renal clearance ( $CL_R$ ) and the net secretion ( $SrCL_R$ ) of metformin were observed in the volunteers heterozygous for the variant allele (808G/T) in comparison with the volunteers homozygous for the reference allele (808G/G) ( $P<0.005$ ) (Chen et al., 2009a; Tzvetkov et al., 2009). In three studies with participants of Asian ancestry renal clearance of metformin was shown to be significantly lower (opposite direction to that found in Caucasians) in homozygous OCT2 serine 270 carriers in comparison to homozygous alanine 270 carriers in opposite to previous results (Song et al., 2008a). In 33 patients OCT2 transporter variants 201Thr>Met and 270Ala>Ser were not associated with response to metformin therapy (Shikata et al., 2007). In study with 189 Caucasian patients using metformin for  $160 \pm 52$  days association of 270Ala>Ser with relative  $HbA_{1c}$  change was not replicated (Choi et al., 2011).

Minor alleles of single nucleotide polymorphism (SNP) rs316009 was associated with lower net tubular creatinine secretion ( $FE_{creat}$ ), and rs316009 and rs3127573 minor alleles associated with increased risk of end-stage renal disease (ESRD) in 1 142 ESRD patients receiving renal transplantation and 1 186 kidney donors as controls (Reznichenko et al., 2013).

OCT2 is involved in blood pressure homeostasis and variant Ser270 allele was associated with a lower incidence of hypertension than homozygous carriers of the wild type allele Ala270 ( $P=0.028$ ) in 607 patients (Lazar et al., 2006). In other study in 1 086 Finnish men with T1DM SNPs rs653753, rs596881, rs316019 (OCT2) and rs376563, rs2048327, rs2457576, rs1567438 (OCT3) were associated with diabetic nephropathy (DN) and hypertension; however data were not replicated in the group of 1 252 patients (Sallinen et al., 2010).

### 3.3. OCT3 genetic variants

In human *OCT3* coding gene reduced variability reflects selective mechanisms against accumulation of certain amino acid changes or may be associated with population subdivision (Lazar et al., 2003). *OCT3* mRNA expression in liver tissue samples from 150 Caucasian subjects varied 27-fold but on average transcript levels were 15-fold lower compared with *OCT1*. *OCT3* expression is independent of age and sex but was significantly reduced in rat kidney with chronic renal failure and human liver donors diagnosed as cholestatic ( $P=0.01$ ), and carriers of rs2292334, rs2048327, rs1810126, rs3088442 SNPs (Komazawa et al., 2013; Nies et al., 2009). Three polymorphisms in *OCT3* coding gene *SLC22A1* (rs8187717 A116S, rs8187725 T400I, and rs12212246 A439V) exhibited reduced uptake of both [ $^3\text{H}$ ] histamine and [ $^3\text{H}$ ] MPP<sup>+</sup> (Sakata et al., 2010). Six tagSNPs in *OCT3* was shown not to be associated with renal clearance in 103 healthy Caucasian males (Tzvetkov et al., 2009). *OCT3* is involved in salt-intake regulation and removal catecholamine and histamine and thus may contribute to processes such as hypertension, allergic diseases, and neuropsychiatric diseases (Ogasawara et al., 2006; Sakata et al., 2010; Schneider et al., 2005; Vialou et al., 2004). Study in mice suggested that inhibition of *OCT3* may be a target for the treatment of depression (Kitaichi et al., 2005). In the *OCT3*-transfected cells transport activity of *OCT3* was inhibited by antidepressants (desipramine, sertraline, paroxetine, amitriptyline, imipramine and fluoxetine) (Zhu et al., 2012). SNPs rs3088442, rs4709426 and rs3106164 in *hOCT3/SLC22A3* correlated with polysubstance abuse in Japanese individuals with dependence on the amphetamine derivative methamphetamine (MAP) (Aoyama et al., 2006). But the same SNPs does not correlated with depression in small case-control study (Hengen et al., 2011).

*SLC22A3* is downregulated in human hepatocellular carcinoma but not associated with tumor progression and patient survival (Heise et al., 2012). In a number of head and neck squamous cell carcinomas (HNSCC) cell lines, in human oral epithelial dysplasias and moderately differentiated HNSCC tumors, only OCT3 is highly expressed. When OCT3 activity was inhibited, treatment of HNSCC cells with metformin did not induced AMPK activation and mTORC1 pathway inhibition and thus showed lack of anti-tumor effects (Patel et al., 2013). In six colorectal cancer-derived cell lines, hOCT3 mRNA levels were markedly higher than mRNA levels of hOCT 1-2, hMATE 1-2K. The level of hOCT3 mRNA in the colon was 9.7-fold higher in cancerous than in normal tissues in six Japanese patients (P=0.0247), similar results were found in Caucasian patients and the cytotoxicity of oxaliplatin was associated with the expression level of transporter (Yokoo et al., 2008). In cells expressing T400I (located in pore lining) and V423F (located in proximal membrane-spanning helices) uptake of metformin is significantly reduced, but the uptake of metformin by T44M was significantly increased for more than 50% (Chen et al., 2010).

### **3.4. MATE1 genetic variants**

The human *SLC47A1* gene is located in tandem with the *SLC47A2* gene on chromosome 17p11.2 and encodes MATE1 transporter protein of 570 amino acids. Analysis of MATE1 promoter revealed regions -65/-25 and -146/-38 to be essential for the maintenance of basal transcriptional activity both of the hMATE1 and rMATE1 promoter. Single nucleotide polymorphism in the promoter region of hMATE1 (G-32A) belongs to a Sp1-binding site (MAF 3.7%) and is associated with decreased Sp1-binding and promoter activity (Kajiwara et al., 2007). Variant C -44C>T, c.-53C>G increased promoter activity *in vitro* but c.-66T>C reduced expression in kidneys, possibly due to altered binding of transcription factors AP-1, AP-2rep; c.1490G>T (or C) was shown to change substrate-specific activity (Chen et al., 2009b; Ha Choi et al., 2009; Meyer zu Schwabedissen et al., 2010). A lot of variants affecting transporter activity have been reported in recent years but lack information about clinical impact on pharmacokinetics or efficiency (Damme et al., 2011). Variants c.1557G>C, c.-118G>A, c.191G>A, c.929C>T, c.983A>C, c.1012G>A, c.1421A>G, c.1438G>A, c.373C>T, c.476C>T, c.922-158G>A were shown to reduce or abolish transport activity *in vitro* (Becker et al., 2009; Chen et al., 2009b; Ha Choi et al., 2009; Kajiwara et al., 2009; Toyama et al., 2010; Tzvetkov et al., 2009). Variants c.983A>C (p.D328A, ss104806857), c.191G>A (p.G64D, rs77630697) and c.373C>T

(p.L125F, rs77474263) were analyzed in clinical trials but showed no impact on metformin pharmacokinetics (Toyama et al., 2010; Tzvetkov et al., 2009).

Rs2289669 was shown to not be associated with renal clearance in 103 healthy Caucasian males (Tzvetkov et al., 2009) albeit this SNP is not functional *per se* and may act as enhancer or be in LD with causal variant. In the group of 148 T2D patients 22% were homozygous for A allele of *SLC47A1* rs2289669 (c.922-158G>A) and had twofold reduction in HbA<sub>1c</sub> in comparison with the patients carrying G allele (GG+GA: 0.55±0.09% vs. AA: 1.10±0.18%, P=0.018 after 6 months of treatment with metformin (Tkac et al., 2013). In the Rotterdam Study MATE1 intronic variant rs2289669 for each minor A allele showed HbA<sub>1c</sub> reduction of 0.30% (95% CI -0.51 to -0.10; P=0.005) larger (Becker et al., 2009). In other study in 189 recently diagnosed DM patients association of rs2289669 with relative HbA<sub>1c</sub> change after 160±52 days of treatment almost reach significance (Choi et al., 2011).

### 3.5. MATE2 genetic variants

The human *SLC47A2* gene is located in tandem with *SLC47A1* gene on chromosome 17p11.2 and encodes MATE2 transporter protein of 602 amino acids. MATE2 nonsynonymous variants c.485C>T and c.1177G>A correlate with reduced protein expression. Its basal promoter haplotypes containing the most common variant, g.-130G>A (28% allele frequency in Caucasians), reduced binding to the transcriptional repressor myeloid zinc finger 1 (MZF-1) and resulted in decreased response to metformin intervention for 160±52 days in patients with newly diagnosed DM (relative change of HbA<sub>1c</sub> in AA genotype carriers -0.027 (95% CI -0.076, 0.033) versus GG and GA genotype carriers -130G (95% CI -0.15 [-0.17, -0.13], P=0.002)(Choi et al., 2011). Transporter variants K64N (rs111060529 G>T) and G211V (rs111060532 GC>TT) were shown to decrease transport activity of MATE2 (Kajiwara et al., 2009; Toyama et al., 2010).

### 3.6. PMAT genetic variants

A cluster of intron SNPs in PMAT could be associated with decreased metformin absorption. Interestingly, complete LD was seen for SNPs in OCT1 and PMAT - deletion rs72552763 and cSNP rs34059508 (Christensen et al.).

### 3.7. OCTN1 genetic variants

OCTN1 transporter binds ergothioneine with low affinity and was reported to be driven by the proton gradient and inhibited by levofloxacin (Shitara et al., 2013). In the Caco-2 cells expressing mOCTN1 metformin efflux was inhibited by ERGO, thus implicating possible involvement of OCTN1 in intestinal uptake of metformin (Nakamichi et al., 2013). The OCTN1 mRNA is highly expressed in the liver of rodents but not detectable in the human adult liver (Kato et al., 2010; Sugiura et al., 2010; Tamai et al., 2000; Tamai et al., 1997; Wu et al., 2000). Organic cation/carnitine transporter 1 (OCTN1) was reported to be localized on mitochondria and at least partially affect mitochondrial complex I inhibition in case of phenformin (Lamhonwah and Tein, 2006). Besides OCT1 may be contributing to the toxicity by phenformin (Wang et al., 2003). OCTN1 L503F was shown to not be associated with renal clearance of metformin (Tzvetkov et al., 2009). Genetic polymorphism of OCTN1 -917C>T had a significant ( $P<0.05$ ) effect on metformin pharmacokinetics, yielding a higher peak concentration with a larger area under the serum time–concentration curve in Korean patients (Yoon et al., 2013). OCTN1 variant (*SLC22A4* 1672C>T) (53.6% vs 43%;  $P=0.0008$ ) and homozygosity for the OCTN1/2-TC haplotype (28.4% vs 16%;  $P=0.0042$ ) were associated with Crohn's disease versus healthy controls (Noble et al., 2005). OCTN1 facilitates the Na<sup>+</sup>-independent active tubular secretion of gabapentin and contributes for 56% of the variation in renal clearance. This effect is diminished or absent in individuals carrying the OCTN1-L503F polymorphism but oral bioavailability of gabapentin is not affected (Urban et al., 2008). D165G and R282X result in complete loss of transport function, and M205I cause a reduction in activity to approximately 50% of the reference sequence protein. L503F showed altered substrate specificity (minor allele frequency 42% in the European-Americans)(Urban et al., 2007).

### 3.8. Interactions between genetic variants in transporters

In double-transfected cells with MATE1 and OCT2 cellular accumulation of compounds was significantly increased in presence of MATE1 specific inhibitors. Two genetic variants c.404T>C (p.159T>M) and c.1012G>A (p.338V>A) resulted in a loss of transport activity for metformin (Meyer zu Schwabedissen et al., 2010). However, in 53 patients serum creatinine (SCr) levels in the patients with OCT2 808GG and 808GT were

increased by 1.43- and 1.19-fold and during treatment 12 patients (27%) with 808GG experienced over grade 2 S<sub>Cr</sub> elevation (assessed by Common Terminology Criteria for Adverse Events), but GT genotype carriers do not experienced adverse effects. Whereas rs2289669 G>A SNP in MATE1 was not associated with adverse effects (Iwata et al., 2012).

Additive decrease in HbA<sub>1c</sub> (-0.52; 95% CI:-0.94 to -0.11; P=0.015) was demonstrated in patients carrying the minor SNPs rs2289669 (MATE1) and rs622342 CA and CC genotypes (OCT1)(Becker et al., 2010a), but association was not replicated in Danish patients (Christensen et al.). Metformin therapy treatment outcome for rs622342 CC genotype and rs2289669 carriers was shown to be better (-0.68; 95% CI: -1.06 to -0.30; P=0.005) but for each C allele prescribed doses of levodopa and other antiparkinson drugs were higher (95% CI 0.064, 0.62; P=0.017) and correlated with increased mortality (Becker et al., 2010a).

### 3.9. T2D susceptibility genes

In opposite to few genetic variants coding for monogenic *diabetes mellitus* (MODY, neonatal mitochondrial *diabetes*, Wolfram syndrome) (Gardner and Tai, 2012; Polak and Cave, 2007; Tanabe et al., 2015), the T2D is associated with more than 70 susceptibility genes (45 identified in Caucasians, 29 in Asians, GWAS,  $P < 5 \times 10^{-8}$ )(Sun et al., 2014). Mostly genes appear to be linked to glucose metabolism with majority related to the  $\beta$ -cell functions and to lesser extent related to insulin resistance, obesity and height (Kahn et al., 2012). Altogether known genetic loci account for only about 10% of the approximately 40% of T2D overall heritability (Markowitz et al., 2011; Sun et al., 2014). Genetic risk scores have been developed to identify individuals with high risk for DM and to improve motivation and adherence to preventive interventions (Johansen Taber and Dickinson, 2015). However, even detection of all linked genetic variants with additional variables like age, sex, family history, obesity and other risk factors are not economically effective, because calculated effects for T2D are small to modest (OR 1.06-1.10)(Talmud et al., 2010) and genetic variants show population specificity (Fesinmeyer et al., 2013). It leads to the conclusion that T2D is a complex disease characterized by a unique combination of genetic variants, clinical risk factors, and behavior in each individual (Malandrino and Smith, 2011).

First T2D susceptibility gene *PPAR $\gamma$* , was discovered in year 2000, which alters insulin sensitivity, later *GCKR*, *IGF1* and *FTO* were added to this list (Burton et al., 2007;

Altshuler et al., 2000; Dupuis et al., 2010; Qi et al., 2010; Rung et al., 2009; Scott et al., 2007; Voight et al., 2010; Zeggini et al., 2008; Zeggini et al., 2007). Most of the genes associated with DM are linked to the  $\beta$ -cell disturbances - *TCF7L2*, *SLC30A8*, *GIPR*, *C2CD4B*, *PCSK1*, *MTNR1B*, *FADS1*, *DGKB*, *GCK*, *KCNJ11/ABCC8*, *WFS1*, *CDKAL1* and other (Bouatia-Naji et al., 2009; Diabetes Genetics Initiative of Broad Institute of et al., 2007; Gloyn et al., 2003; Gudmundsson et al., 2007; Sandhu et al., 2007; Sladek et al., 2007; Steinthorsdottir et al., 2007; Voight et al., 2010; Zeggini et al., 2008). *TCF7L2* is regarded as the most significant T2D susceptibility gene identified to date as it was replicated in the DPP and many populations (Damicott et al., 2006; Groves et al., 2006; Scott et al., 2006). Impact of insulin resistance and  $\beta$ -cell dysfunction were studied in a relation to the development of T2D in a group of participants with impaired glucose tolerance in the Diabetes Prevention Program at baseline and after intensive life-style modifications (n=1 079), placebo (n =1 082) and metformin therapy (850 mg twice a day) (n=1 073). Results showed that life-style interventions are more effective than metformin, and placebo had no significant change in insulin sensitivity and  $\beta$ -cell function after 1 year (Kitabchi et al., 2005). Carriers of the Q risk allele at *ENPP1* K121Q (rs1044498) have an increased incidence of T2D and lifestyle or metformin intervention arms of the DPP abolished this effect (HR, 1.08 (95% CI, 0.81–1.43; P=0.60 for metformin arm) (Moore et al., 2009). In the study with 2 994 participants from DPP was investigated preventive intervention with metformin in respect to >1 500 tagSNPs in some 40 genes selected in two ways: 1) SNPs in high-likelihood candidate genes and 2) SNPs identified by ongoing GWAS for T2D or related metabolic traits (monogenic forms of *diabetes*, T2D drug targets or drug-metabolizing/transporting enzymes, involved in cellular metabolism, hormonal regulation, or response to exercise). Only three of 7 metformin transporter genes were included in the analysis (*OCT1*, *OCT2* and *MATE1*) and association of variants in the metformin transporter gene *SLC47A1* (minor, but not major allele of rs8065082 is associated with lower T2D incidence in the metformin arm (HR 0.78, 95% CI 0.64–0.96, P=0.02)), and a missense SNP in *SLC22A1* (rs683369, encoding L160F, 31% risk reduction in T2D incidence but only under the action of metformin), were replicated. Also many SNPs from genes altering insulin secretion like *ABCC8-KCNJ11* region (22 SNPs including rs5215), *HNFA4*, *HNFB1* were identified as interacting with metformin. Additional associations were detected in genes affecting insulin sensitivity - *ADIPOR2*, *GCK*, *CAPN10* and genes involved in the regulation of energy metabolism - *MEF2A*,



*MEF2D*. Interestingly, that *ITLN2*, *GCG*, *PKLR*, *PPARGC1B* also were showed to be associated with progression to T2D in metformin arm (Jablonski et al., 2010).

Further study revealed that the preventive effect of metformin on T2D progression was abolished in *KCNJ11* E23K K/K homozygotes. It possibly can be explained by a suppression of the beneficial effect of metformin on insulin sensitivity at 1 year (HR 0.89 (95% CI 0.66–1.19) for E/K heterozygotes; 0.95 (0.54–1.67) for K/K homozygotes and 0.55 (0.42–0.71) for for E/E (nominal P value <0.0001 vs. placebo)(Florez et al., 2007). In other study list of genes, including genes affecting insulin secretion (*GLIS3*, *G6PC2*, *MADD*, *MTNR1B*, and *ADCY5*), insulin sensitivity (*GCK*, *IRS1*, *IGF1* and *GCKR*), and energy metabolism (*CRY2*) as well as other genes like *PROX1*, *DGKB*, *ADRA2A*, *FADS1*, *C2CD4B* were showed to not interact with metformin at all, albeit in review article they appeared as affecting FG levels (Florez et al., 2012a).

In 3 234 participants from the DPP study group was investigated potential association between 16 obesity-predisposing single nucleotide polymorphisms (SNPs) with weight loss in short (up to 6 months) and long term (up to 2 years) and weight regain in the Diabetes Prevention Program. Ala12 allele at *PPARG* associated with short- and long-term weight loss (-0.63 and -0.93 kg/allele,  $P \leq 0.005$ , respectively) in lifestyle, placebo and metformin groups. In long term metformin weight-loss effect was altered by *NEGR1* rs2815752 and weight regain was affected by *NEGR1* rs2815752, *BDNF* rs6265, *PPARG* rs1801282 (Delahanty et al., 2012).

Later in this group (2 993 participants from DPP) were tested for 32 single-nucleotide polymorphisms (SNPs) associated with dyslipidemia and concentrations of lipids and lipoprotein particle sizes and numbers. Higher genetic risk score (GRS) was associated with adverse lipid profile and attenuated response in LDL-C levels and small LDL particle number to dietary and physical activity interventions in the lifestyle intervention arm but not in the placebo or metformin groups (Pollin et al., 2012).

In 148 recently diagnosed T2D patients *PRKAA1* rs249429, *STK11* rs741765, *PCK1* rs4810083, *PPARGC1A* rs10213440, *HNF1A* rs11086926, and *CAPN10* rs3792269 variants were investigated in respect to the metformin treatment defined by achieving HbA<sub>1c</sub> <7% and absolute reduction in HbA<sub>1c</sub> after 6-month metformin therapy. Only *CAPN10* rs3792269 A>G polymorphism was significantly associated with reduced treatment efficiency (OR 0.27 95% CI 0.12–0.62,  $P=0.002$  per G allele) (Tkac et al., 2015).

In 402 patients with T2D and 171 healthy controls (all of Asian ancestry) serine racemase (*SLC30A8*) rs391300 G/A polymorphism was associated with better

improvements in respect to the levels of serum fasting plasma glucose (FPG), postprandial plasma glucose (PPG), and cholesterol (CHO) ( $P < 0.05$ ), but not HbA<sub>1c</sub> in GA or AA genotype versus GG genotype carriers after metformin therapy (Dong et al., 2011).

Not all investigated T2D susceptibility variants influence outcomes of intervention differently in the lifestyle or metformin group in the Diabetes Prevention Program. Possible correlation between T2D susceptibility alleles and metformin therapy outcomes were analysed in the GoDARTS and DPP studies however *TCF7L2* SNPs were not associated with therapy goal of HbA<sub>1c</sub>  $< 7\%$  (Pearson et al., 2007) or T2D incidence (Florez et al., 2006). Similarly, no effects were observed in case of *SLC30A8* missense polymorphism R325W (Majithia et al., 2011). After discovery of  $> 10$  new T2D susceptibility genes additional study was performed in DPP study sample of 3 548 participants, but for the most part associations with T2D were not replicated and also had no interactions with metformin treatment (Moore et al., 2008).

### **3.10. Genetic variants in metformin targets**

In the Diabetes Prevention Program (DPP) SNPs encoding putative drug targets for metformin were identified in the gene encoding the AMPK kinase *STK11* and the AMPK subunit genes *PRKAA1*, *PRKAA2*, *PRKAB2* and in genes coding for proteins involved in AMP-activated pathway/gluconeogenesis. *PPARA*, *PARGC1A*, *PCK1* was found to influence a response to metformin intervention. The most significant association with T2D incidence occurred in the AMPK subunit gene *PRKAG2* (hazard ratio 1.24, 95% CI 1.09 – 1.40,  $P < 7 \times 10^{-4}$ ) (Jablonski et al., 2010). The Peutz-Jegher syndrome tumor-suppressor gene encodes a protein-threonine kinase, *LKB1*, which phosphorylates and activates AMPK (adenosine monophosphate (AMP)-activated protein kinase). In mice, *LKB1* absence results with hyperglycemia, increased gluconeogenic and lipogenic gene expression (Shaw et al., 2005).

In a study, investigating metformin use in women with Polycystic Ovary syndrome (PCOS), C allele of *STK11* rs8111699 was significantly associated with a significantly lower chance of ovulation (CC versus GG was 0.30) and results were confirmed in other studies (Goldenberg N, 2008; Lopez-Bermejo et al., 2010).

In the first GWAS locus on 11 chromosome was associated with the response to metformin (GoDarts et al., 2011), probably, *Npat* and *Atm* genes as the likely causal genes.

In meta-analysis of three studies with 4 443 patients the the odds of treatment success (HbA<sub>1c</sub>  $< 7\%$  or 6.5%) with the presence of the C allele resulted with OR of 1.25

(95% CI 1.13, 1.38),  $p=7.8\times 10^{-6}$ ). In the metformin monotherapy group OR was 1.33 (95% CI 1.16, 1.50),  $p=1.4\times 10^{-5}$  (van Leeuwen et al., 2012). Contrary to the results of first GWAS investigating metformin efficiency, analysis in 2 994 DPP metformin users revealed that the C allele carriers of rs11212617 showed no preventive advantage on T2D incidence, insulin sensitivity, fasting glucose, glycated haemoglobin (Florez et al., 2012b). This may be explained by different designs and study outcomes as DPP investigated patients with pre-diabetes but GWAS analyzed T2D patients if they reached glycaemic goal in 0.5-1.5 years of metformin treatment.

## **4 MATERIALS AND METHODS**

### **4.1. The study design**

In this work, we applied combined prospective-retrospective design to study the metformin intolerance (METFOGENE) and efficiency (OPTIMED), and used prospective design in pharmacokinetic study.

All study participants were recruited by medical personnel in hospitals or general practices in Latvia for participation in the Latvian Genome Data Base (LGDB), a government-funded biobank with a cross-sectional prevalence. Participants were enrolled voluntarily and were not paid for participation, biosamples, clinical and phenotypic data. The requirements for participants enrolled in LGDB were the following - 18 years and older, signed informed consent, obtained venous blood sample (30 ml). Data of anthropometric measurements (including weight and stature) were obtained by direct measurements, and ethnic, social, and environmental information and familial health status were obtained in the questionnaire-based interview. Information about their health status was confirmed by physicians using International Classification of Diseases (ICD)-10 codes.

The biobank protocol was approved by Central Medical Ethics Committee of Latvia (Protocols No. A-30, 2005 and A-7, 2007), the use of control group in study was approved by Central Medical Ethics Committee of Latvia (Protocol No. A-3,2008).

### **4.2. Study group**

#### ***4.2.1. Intolerance study***

Study (METFOGENE) was performed in the framework of EEA and Norway Grants (grant number EEZ09AP-34/01). Patients for the intolerance study were selected retrospectively from 1473 participants with T2D (ICD-10: E11) from the LGDB. All metformin users, using 500 mg metformin/day or more, with a clearly registered metformin tolerance status, were further selected for the study, resulting in a group of 235 T2D patients without side effects of metformin therapy and 16 T2D patients with metformin side effects. To increase the study sample, 58 metformin intolerant patients from the Pauls Stradins Clinical University Hospital (PSCUH) were recruited by endocrinologists. For the metformin intolerance study we used the outcome - metformin intolerance phenotype versus metformin tolerance phenotype.

The side effects of metformin were defined (questionnaire of side-effects filled by doctor) as the presence of at least one of the following gastro-intestinal symptoms: diarrhea, nausea, flatulence, abdominal pain, asthenia, and vomiting within the year after the start of the therapy. 19 patients from cases were excluded due to idiosyncratic symptoms to metformin therapy. We excluded 42 controls and 2 cases using OCT1-inhibiting medications (verapamil, amitriptyline, omeprazole, esomeprazole, rosiglitazone, and spironolactone). No users of other PPIs or prazosin, quinidine, disopyramide, repaglinide were detected in the study group (Ahlin et al., 2011; Ahlin et al., 2008; Nies et al., 2011a; Nies et al., 2011b).

All patients were involved in LGDB and had normal creatine levels. METFOGENE project protocol was approved by Central Medical Ethics Committee of Latvia (Protocol No. 01 - 29.1/16) and Committee of Ethics (PSCUH) (Nr. 281009 - 15L).

#### ***4.2.2. Metformin efficiency and genetic variability of metformin transporters***

OPTIMED project was performed in the framework of the Latvian National Research Programme 2010–2013, ‘Development of new prevention, treatment, diagnostics means and practices and biomedicine technologies for improvement of public health’ (VPP Biomedicine). The 22 endocrinologists from health care centers and hospitals located in Latvia were involved in the recruitment of patients with T2D in order to maintain up to 3 years long prospective follow-up study. Inclusion criteria were as follows: patients with ICD-10 E11 diagnosis (fasting blood glucose test result  $\geq 7$  mmol/L and/or OGT test result  $\geq 11$  mmol/L), drug naïve or not using antidiabetic medications for at least 3 months, over 18 years old, signed consent, women who are not pregnant.

Baseline data about other diagnoses, history of gestational diabetes, anthropometric measurements (height, weight, waist circumference and blood pressure), intolerance of antidiabetic drugs and available biochemical analysis were gathered on the first visit. All information about drugs prescribed during visit and co-medications was collected. Samples of blood were collected for testing of HbA<sub>1c</sub>, C-peptide, triglycerides, cholesterol, ALAT, HDL, LDL, creatinine levels and for the DNA extraction. One week following the drug prescription, the doctors consulted their patients via phone in order to collect information about possible episodes of drug intolerance. Patients were scheduled for planned follow-up visits to their endocrinologists at periodic intervals (second visit after 3 months, all following visits - every 6 months), information from unplanned visits was also collected and biochemical tests were performed (HbA<sub>1c</sub>, ALAT, C-peptide and creatinine).

Overall 315 patients were participating in a follow-up study on May 2015. 102 patients that had received metformin monotherapy for 3 months and had HbA<sub>1c</sub> measurements were selected for study. Additionally recruitment of 171 T2D patient with known history of metformin use and available HbA<sub>1c</sub> measurements was performed (RetroOPTIMED study) and altogether 486 patients were included in the LGDB in May 2015.

In the Study group from Slovakia T2D was diagnosed in patients according to the criteria of the American Diabetes Association. Study was conducted in a university hospital setting. 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<sub>1c</sub> in the range of 6.5-11% were included. Baseline HbA<sub>1c</sub> measurement was done within one week prior the treatment initiation and second measurement after 6 months of metformin monotherapy.

HbA<sub>1c</sub> is widely accepted as the gold standard for glycaemic control monitoring (Rahbar, 1968) (WHO, 2006). Treatment efficiency was estimated based on the change in HbA<sub>1c</sub> levels measured 3 or 6 months after the beginning of the therapy.

OPTIMED project protocol was approved by Central Medical Ethics Committee of Latvia (Protocol No. 01-29.1/22) and Committee of Ethics (PSCUH)(Nr.3000610 - 18L).

#### ***4.2.3. Pharmacokinetic study***

Recruitment of all participants in the pharmacokinetic study was prospective. The ages of these healthy volunteers in the study group ranged between 22 and 41 years, and they were previously evaluated by doctors to be healthy. To be eligible for this study, subjects were not taking any medications other than vitamin. Level of liver enzymes of participants (alanine aminotransferase, g-glutamyltransferase) were less than double of respective normal value, none had renal failure (mean serum creatinine 73.5±13.8 mmol/l). Women were asked to provide a urine sample to confirm a negative pregnancy test before the study.

Pharmacokinetic research was performed in framework of VPP Biomedicine and has permission from Committee of Ethics (PSCUH) Nr. 2012.1212 - 10L.

#### ***4.2.4. Association of other genetic variants with metformin efficiency***

Design for study investigating metformin efficiency was prospective. In previously described group (OPTIMED group) of 102 participants we investigated possible association between genetic susceptibility factors for the T2D and metformin efficiency. The Project protocol was approved by Central Medical Ethics Committee of Latvia (Protocols No. 01-29.1/10 and No. 01-29.1/22), Committee of Ethics (PSCUH)(Nr.3000610 - 18L).

#### ***4.2.5. Analysis of T2D susceptibility variants in respect to the incidence of T2D***

Recruitment of participants for the investigation of incidence of T2D has a combined prospective - retrospective design. Criteria for enrolment of participants in the study were as follows – all patients in LGDB with the data on age, weight, height, and information on disease (until year 2010), resulting in 987 T2D patients (cases) and 1080 controls, and we added a 466 genotyped T2D patients (from all 486 RetroOPTIMED and OPTIMED participants) to investigate genetic susceptibility factors for the T2D.

The Project protocol was approved by Central Medical Ethics Committee of Latvia (Protocols No. 01-29.1/10 and No. 01-29.1/22), Committee of Ethics (PSCUH)(Nr.3000610 - 18L).

### **4.3. Preanalytical sample handling**

Biological materials for this study were provided by LGDB. LGDB maintains a collection of human plasma, serum, white blood cells and DNA from participants. Blood sample (30 ml) is collected and plasma and white blood cells are separated within 2 days from collection. DNA is prepared using phenol extraction method. Sample processing, storage, retrieval was performed manually.

All samples are labeled with 1D barcode, 2D barcode and printed labels (certified for intended use). For sample and data administration Nautilus, Delphi IT technology is used. For each patient there is an IT based patient record, containing phenotypic and genotypic data, and information on available biosamples associated with metadata. Nautilus 8.01 LIMS system provides input of samples, data tracking and management, label printing, export from database with MS Access.

DNA samples were stored in -80°C in storage facility equipped with alarm for storage temperature and aliquots of samples are stored in independent storage units. For

reaction were aliquoted from storage tubes into 96-well PCR plates and normalized according to protocol using Tecan Freedom Evo (Tecan Group Ltd, Mannedorf, Switzerland) with disposable filter tips.

#### 4.5. Genetic analysis

##### 4.5.1. SNP selection and genotyping for intolerance study

We selected 6 polymorphisms in OCT1-2 and MATE1 previously associated with the altered transport activity of metformin to investigate association with metformin intolerance. We selected four polymorphisms: rs12208357 (Arg61Cys), rs34059508 (Gly465Arg), rs628031 (Met408Val), and rs72552763 (420del) in the OCT1 gene and one SNP from the OCT2 (rs316019, Ala270S) and MATE1 (rs2289669, intronic) genes (Becker et al., 2009, 2010a, 2011; Joerger et al., 2015; Tzvetkov et al., 2012; Tzvetkov et al., 2009). Only SNPs with minor allele frequency (MAF) above 0.05 in a Caucasian race were selected due to a small study sample.

##### 4.5.2. Real-Time PCR system based genotyping with TaqMan SNP assays

Genotyping of rs12208357, rs34059508 (OCT1/*SLC22A1*), rs316019 (OCT2/*SLC22A2*), and rs2289669 (MATE1/*SLC47A1*), rs7903146 (*TCF7L2*), rs12255372 (*TCF7L2*) and rs7561317 (*TMEM18*) was carried out using the Applied Biosystems TaqMan SNP (Applied Biosystems, Foster City, California, USA) Genotyping assay (Table 4.1.).

**Table.4.1.**

#### RT-PCR genotyping probes

NR	SNP code	Gene	SNP position	Applied Biosystems	Allele 1/2
1.	rs12208357	SLC22A1	Chr6:60463138	C__30634096_10	C/T
2.	rs34059508	SLC22A1	Chr6:160495827	C__30634080_20	A/G
3.	rs316019	SLC22A2	Chr6:160590272	C__3111809_20	A/C
4.	rs2289669	SLC47A1	Chr17:19403935	C__15882280_10	A/G

RT-PCR reaction was performed using modified protocol: 4.75 ml TaqMan Genotyping Mix, 0.25 ml SNP genotyping assay, and 5 ml Millipore H<sub>2</sub>O on a 7500 Real-Time PCR system (Applied Biosystems). Following conditions were set: 1 cycle: 95°C, 15 min, 40 cycles: 95°C–15 s, 60°C–60 s. The RT-PCR was carried out on a ViiA™ 7

Real-Time PCR System (Life Technologies) with settings for VIC fluorescent dye (B filter) 552 and for FAM fluorescent dye (filter A) 518 nm. 7500 Software v2.0.1



(Applied Biosystems, ASV) and AutoCaller 1.1 (Applied Biosystems) software was used to assign genotype calls for all samples simultaneously.

#### 4.5.3. Direct DNA sequencing

Genotyping of indel rs72552763 was performed by direct sequencing of PCR products in 96-well PCR plates or 8-well PCR strips. Optimization of PCR reaction was performed by using variable  $T_m$  (47°C–65°C),  $MgCl_2$  concentrations from 1 to 2.5 mmol and addition of DMSO.

PCR was performed using previously described primers: 5' -GCA TTC TAA ACC CAG TGA T-3' and 5'-CAT TCC AGA GGC TTA TCA A-3' (Shu et al., 2008). The following PCR reaction setup was used: 1 mmol/l DB buffer, 2.5 mmol/l  $MgCl_2$ , 0.5 U Hot FirePol, 0.2 mmol/l dNTP mix (SolisBioDyne, Tartu, Estonia), 0.3 mmol/l primers, and 28 ng of template DNA (Table 4.2.).

**Table. 4.2.**

**The standard PCR protocol**

The reagents	Amount for reaction, 25 $\mu$ l
10xPCR buffer BD	2.5 $\mu$ l
$MgCl_2$ ( 25 mM )	2.0 $\mu$ l
dNTP mix, 10mM	0.5 $\mu$ l
Oligonucleotide (100mM)	0.075 $\mu$ l
Oligonucleotide (100mM)	0.075 $\mu$ l
DNA (28 ng)	1 $\mu$ l
<i>HotFIREPol</i> polymerase	0.125 $\mu$ l
Deionized H <sub>2</sub> O	18.73 $\mu$ l

PCR reagents were mixed according to protocol and DNA was added later in a separate room to avoid contamination, sealed before reaction. PCR was carried out on a Veriti96ThermalCycler (AppliedBiosystems)(Table 4.3).

**Table. 4.3.**

**PCR conditions SLC22A1**

No.	Temperature	Time	Cycles
1.	95°C	15 min	1 cycle
2.	95°C	30 sek	40 cycles
	55°C	30 sek	
	72°C	1 min	
3.	72°C	5 min	1 cycle
4.	4°C		

The PCR product was confirmed by agarose gel (1.5%) electrophoresis. Dephosphorylation of remaining dNTPs was performed with shrimp alkaline phosphatase

(ExoI, SAP)(Fermentas UAB, Vilnius, Lithuania) according to the manufacturer's protocol. Amplification products had both strands directly sequenced using the primers 5' -TTTCTT CAG TCT CTG ACT CAT GC-3' and 5' -TCC CCACAC TTC GAT TGC-3' (Table 4.4.).

**Table. 4.4.**

**Sequencing reaction protocol**

The reagents	Amount for reaction, 10 µl
5x seq buffer	2 µl
BD ( Big Dye)	0.5 µl
Primer 1	0.5 µl
DNA (afterdephosphorylation)	2 µl
Deionized H <sub>2</sub> O	5 µl

The following sequencing reaction setup as used: 1 ml BigDye, 2 ml 5 X Big Dye sequencing buffer, 0.5 mmol/l corresponding primer, 150–250 ng of template DNA (from the previously described PCR reaction), and 5.5 ml H<sub>2</sub>O. Plate was sealed and the reaction was carried out on a Veriti96ThermalCycler (AppliedBiosystems) (Table 4.5.).

**Table.4.5.**

**Sequencing reaction conditions**

No.	Temperature	Time	Cycles
1.	95°C	15 min	1 cycle
2.	95°C	30 sek	25 cycles
	55°C	30 sek	
	72°C	1 min 30 sek	
3.	72°C	5 min	1 cycle
4.	4°C		

The products were purified using Sephadex G50 (Sigma-Aldrich, St Louis, Missouri, USA)(centrifuge *Universal 23*), Hi-Di formamide was added and DNA was sequenced using an ABI Prism 3100 (AME Bioscience, Toroed, Norway) capillary electrophoresis sequencer.

All chromatograms were inspected manually using Contig Express software of Vector NTI Advance 9.0 package (Invitrogen Corporation, Carlsbad, California, USA). The presence of polymorphisms was confirmed by an opposite strand analysis. Two additional polymorphisms were found (rs34130495 and indel rs36056065). A minor allele of rs34130495 was found only in three samples and this SNP was not considered for further association analysis.

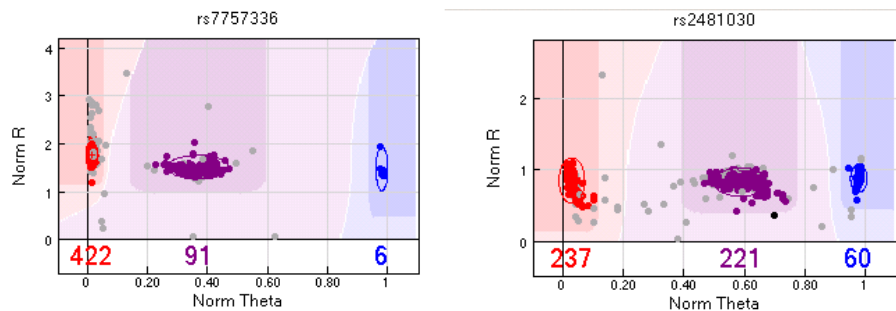
#### **4.5.4. SNP selection and genotyping with 192-plex SNP genotyping panel**

For the aim of the study we developed a genotyping panel by using HaploView 4.2 (27 genome release) (Barrett et al., 2005) for SNP selection from first GWAs of metformin efficiency (van Leeuwen et al., 2012) and largest studies investigating genetic factors of metformin therapy in participants with T2D and obesity (Assmann et al., 2014; Barros et al., 2014; Chauhan et al., 2011; Kalnina et al., 2012; Kalnina et al., 2013; Park et al., 2013; Shokouhi et al., 2014; Wang et al., 2013; Xi et al., 2014), pre-diabetic or PCOS (Christensen et al., 2011; Goldenberg N, 2008; Jablonski et al., 2010). Due to the relatively small sample size only variations exceeding minor allele frequency (MAF) of 0.05 in Caucasian race were included. In the BeadXpress primer design (Illumina, San Diego, CA) the designability rank score (0 to 1) was calculated for each SNP by Illumina and SNPs with scores <0.5 were excluded and list of tagSNPs was updated in Haploview v4.2 until tagSNPs with best designability rank score were obtained. Altogether 192 SNPs and tagSNPs from 52 genes were selected for analysis. One hundred and eight tagSNPs of the pharmacogenetic panel covered OCT1 (*SLC22A1*), OCT2 (*SLC22A2*), OCT3 (*SLC22A3*), MATE1 (*SLC47A1*), MATE2 (*SLC47A2*), PMAT (*SLC29A4*) regions and 37 SNPs were associated with T2D susceptibility or accounted for genes possibly involved in metformin action.

Genotyping was performed in all patients from OPTIMED and pharmacokinetic studies by using GoldenGate Genotyping Assay with VeraCode technology (Illumina, Inc.). 250 ng of genomic DNA was used for each subject, with control DNA on each plate. Genotyping was performed according to the manufacturer's protocol (Hyten et al., 2008). Workflow consists of 1) activation which enables genomic DNA samples to bind to paramagnetic particles ,2) hybridization of 2 allele-specific and one locus-specific oligonucleotides with the template DNA, 3) allele-specific extension/ligation, 4) PCR with three universal primers (P1 (Cy3-labelled), P2 (Cy5-labelled), and P3); 5) binding of PCR product labeled with Cy3 or Cy5 depending on the allele, and containing an Illumicode address sequence, 6) hybridization single-stranded, dye-labeled PCR products on VeraCode BeadPlate to their complementary bead type through their unique address sequences and 7) Scan address sequence and fluorescence in VeraCode BeadPlate with BeadXpress®Reader. Genotypes for each locus are detected with signal in either the Cy3 or Cy5 channels (homozygotes) or both (heterozygotes).The automatic allele calling was

done using the Illumina GeneCall software with a GeneCall threshold of 0.25 which analyses fluorescence and assigns 3 genotypes for each SNP (Deulvot et al., 2010).

Each SNP was evaluated also manually and 15 SNPs were removed from analysis due to failed genotyping, weak genotype separation due to low fluorescence and very low minor allele frequency. We obtained allelic data for 92.2% of the SNPs (177 out of 192) included in the OPTIMED panel. Genotype clusters of rs7757336 and rs2481030 (*SLC22A2* – *SLC22A3*) after automatic allele calling and manual corrections of obtained clusters (Figure 4.1.).



**Figure 4.1.** Genotype clusters of rs7757336 and rs2481030

One positive control sample in each assay plate was used; a concordance rate of 19 randomly distributed duplicate samples was 99.7%. Samples with call rates lower than 0.9 were excluded. Primary data analyses were performed using Illumina GenomeStudio software as previously reported.

#### **4.5.5. SNP selection and genotyping for investigation of incidence of disease**

SNPs rs7903146 (*TCF7L2*), rs12255372 (*TCF7L2*), rs7561317 (*TMEM18*) were strongly associated with susceptibility to the T2D (Assmann et al., 2014; Barros et al., 2014; Chauhan et al., 2011; Park et al., 2013; Shokouhi et al., 2014; Wang et al., 2013; Xi et al., 2014) and were selected for genotyping in 987 T2D patients (cases) and 1080 controls from LGDB but not participants from OPTIMED project.

All procedures and reagents were used according to the intolerance study protocol described above (Table 4.6.).

**Table.4.6.**

**RT-PCR genotyping probes**

Nr.	SNP code	Gene	SNP position	<i>Applied Biosystems</i>	Allele 1/2
1.	rs12255372	TCF7L2	Chr10:113049143	C__291484_20	G/T
2.	rs7561317	TMEM18	Chr2:644953	C__11804554_10	A/G

SNP rs7903146 (TCF7L2) was custom made (B biotin cap, L\* photosensitive linker):

*Fw* ACCGTTGGATGTGTTATTTACTGAACAATTAGAGAGCTAAG  
*Rs* ACGTTGGATGGCCCAAGCTTCTTCAGTCAC  
*B-CAATAGAGAGCTL\*AGCACTTTTATAGATA\_[T/C]*

#### 4.6. Quantification of metformin

Venous blood samples (0, 1, 2, 3, 4, 6, 10, 24 h after oral administration of metformin) and urine samples (4, 6, 10, 24 h after oral administration of metformin) were taken within 24 hours. Whole blood samples were centrifuged immediately and the pellet was washed three times with 0.9% sodium chloride. All plasma, urine and pellet samples were stored at -20°C until determination of metformin. After acetonitrile-induced (internal standard – phenformin containing) protein precipitation of the biological samples, metformin and IS were analyzed on hydrophilic interaction liquid chromatography (HILIC) using multiple reaction monitoring (MRM) in positive ion electrospray mode. The assay was validated for quantitative determination of metformin in human RBC and plasma samples. The obtained calibration curves were characterized by correlation coefficient  $R^2 > 0.99$  over the concentration range of from 5ng/ml to 500 ng/ml for RBC, from 5ng/ml to 1500 ng/ml for blood plasma and from 2.5µg/ml to 250 µg/ml for urine samples (APPENDIX V, Supplementary methods).

#### 4.7. Statistical methods

Statistical analysis was performed using the PLINK 1.07 open software (<http://pngu.mgh.harvard.edu/purcell/plink/>)(Purcell et al., 2007), HaploView 4.2 open software, and SPSS 13.0 (Standard version, Chicago, IL, USA).

Tidwell-Box linearity test, Cook's distance, standartized residual values, leverage points, Hosmer-Lemeshow test, standard errors (SE) of independent variables and Pearson's r coefficient were obtained using SPSS 13.0 (Standard version, Chicago, IL, USA) to analyse quality of data and confirm the use of samples in logistic and linear

regressions performed. Durbin-Watson test, Kalmogorov-Smirnov test and Shapiro-Wilkson test, VIF (variant inflation factor) and tolerance, standardized residuals were obtained using SPSS 13.0 (Standard version, Chicago, IL, USA) to analyse quality of data retrieved from pharmacokinetic study.

LD was determined using the program Haploview v4.2, considering all the patients included in intolerance study and used separately for determination of TagSNPs in metformin transporters coding genes as described previously (OPTIMED panel). Statistical power was calculated using Quanto v1.2.3. software (Natara Software, Naperville, Illinois, USA) according to allele frequencies published in NCBI database (<http://www.ncbi.nlm.nih.gov/> Accessed in January 2015) for Caucasian race. Variables and outcomes were tested for normality by using Kolmogorov-Smirnov test (Neter J, 1988) and Shapiro-Wilk test (Royston-P, 1995; Shapiro SS, 1965) and if it was required, were normalised according to Box-Cox power transformations (Osborne, 2010). All SNPs included in the study were tested for deviation from the Hardy–Weinberg equilibrium by the exact test described by Wigginton et al. (Wigginton et al., 2005). Significance level for all associations were set as  $\alpha=0.05$  and results were corrected for multiple testing using Bonferroni correction and permutation test with 100 000 permutations for each analysis (Purcell et al., 2007). Impute2 software was used to perform genotype imputation and haplotype phasing (Howie et al., 2012).

#### ***4.7.1. Intolerance study***

Logistic regression assuming an additive, dominant, or recessive mode of inheritance was used to test the difference between the metformin intolerant participants and the controls and analysis was adjusted for other non genetic factors - sex, age, body mass index (BMI), use of other antidiabetic drugs, and use of co-medications as cofactors. Estimated glomerular filtration rate (eGFR) was calculated by using Cocroft-Gault equation ( $eGFR_{cocroft}=(140-age)*(mass,kg)*[1.23 \text{ if male or } 1.04 \text{ if female}]/(\text{serum creatinine, } \mu\text{mol/L})$ ). BMI was derived from the mass (weight) and height of participants ( $BMI=mass,kg/(\text{height,m})^2$ ). Waist measurement, BMI, and HbA<sub>1c</sub> levels showed abnormal distribution and were used as outcomes in linear regression analysis using the sex, age, and metformin intolerance status as cofactors. Our sample size provided 80% power (at  $\alpha=0.05$ ) to detect an odds ratios from 1.60 to 2.15 depending on MAF.

#### **4.7.2. Metformin efficiency and genetic variability of metformin transporters**

Logistic regression of responders versus non-responders (defined as positive versus no change or negative change of HbA<sub>1c</sub>) was performed using PLINK 1.07 open software assuming an additive mode of inheritance to estimate association of SNPs with non-responsiveness after 3 or 6 months metformin monotherapy (discovery and replication group, respectively) and using number of transformed and non-transformed cofactors (age, sex, BMI, time between HbA<sub>1c</sub> measurements, dose of metformin, eGFR) to adjust the analysis for other non-genetic factors. Creatine clearance was estimated by using Cocroft-Gault equation ( $eGFR_{cocroft} = (140 - \text{age}) * (\text{mass, kg}) * [1.23 \text{ if male or } 1.04 \text{ if female}] / (\text{serum creatinine, } \mu\text{mol/L})$ ). BMI was derived from the mass (weight) and height of participants ( $BMI = \text{mass, kg} / (\text{height, m})^2$ ). The 1000 Genomes project browser (<http://browser.1000genomes.org/index.html> Accessed in January 2015) predicted high linkage disequilibrium ( $D' = 1.0$ ) between rs7757336, rs3119309 and rs2481030 in the Caucasians with variable  $r^2$  due to differences between MAFs ( $r^2 = 0.182 - 0.657$ ). Statistical power of sample size was calculated using Quanto software to provide 80% power (at  $\alpha = 0.05$ ) to detect an odds ratios (ORs) from 1.55 to 2.9 depending on MAF of different SNPs.

#### **4.7.3. Pharmacokinetic study**

Creatinine clearance ( $Cl_{cr}$ ) was calculated from creatinine measurements from 24 hours urine and corrected for BSA by using the Mosteller formula. The maximum observed concentration ( $C_{max}$ ) and the time point of observed  $C_{max}$  ( $t_{max}$ ) were both obtained directly from the measured data. Estimation of under the curve ( $AUC_{0-24}$ ) was calculated by trapezoid method and corrected for infinity using the rate constant of the last exponential phase ( $k$ ) (Robert et al., 2003). Extrapolation to infinity was computed  $AUC_{\infty} = AUC_{0-24} + C_{last}/k$ . Ratio of  $AUC_{0-24}$  to  $AUC_{\infty}$  is  $\leq 3\%$ . The  $k$  value was calculated from final 2 concentrations of the concentration time-curve  $k = (\ln C_{10h} - \ln C_{24h})/t$  (Bardin et al., 2012). The elimination half-life ( $t_{1/2}$ ) was calculated as  $0.693/k$ . Clearance/bioavailability ( $CL/F$ ) was calculated as  $\text{dose}/AUC_{\infty}$ , where bioavailability was estimated as 50% as reported previously (Bailey and Turner, 1996). Volume of distribution/bioavailability ( $V/F$ ) was estimated by dividing  $CL/F$  by  $k$ . Linear regression was performed using PLINK 1.07 open software to estimate association of rs7757336 and rs2481030 polymorphisms (100% genotype rate for both variants) with absolute changes in

obtained pharmacokinetic parameters and to corrected for covariates (weight, age, creatinine clearance).

#### ***4.7.4. Association of other genetic variants with metformin efficiency***

Analysis of correlation between metformin efficiency (responders and non-responders defined as positive versus no change or negative change of HbA<sub>1c</sub>) and 33 genetic variants in T2D susceptibility genes and metformin targets was performed using logistic regression with covariates - age, sex, BMI, time between HbA<sub>1c</sub> measurements, dose of metformin, eGFR. Statistical power of sample size was calculated using Quanto software to provide 80% power (at  $\alpha=0.05$ ) to detect an odds ratios (ORs) from 1.55 to 2.9 depending on MAF of different SNPs.

#### ***4.7.5. Analysis of T2D susceptibility variants in respect to the incidence of T2D***

Three SNPs - rs7903146 (*TCF7L2*), rs12255372 (*TCF7L2*) and rs7561317 (*TMEM18*) previously associated with T2D incidence in Latvia, were used in the logistic regression with covariates: sex, age, BMI (transformed if required). BMI was derived from the mass (weight) and height of participants ( $\text{BMI} = \text{mass, kg} / (\text{height, m})^2$ ).



## 5 RESULTS

### 5.1. Intolerance study

The baseline characteristics of the group of T2D patients divided into case and control groups based on the presence of metformin-induced side-effects are shown in Table 5.1. In the group of T2D patients with available biochemical and metformin prescription information, metformin intolerance status was assigned based on details of side-effects (patients with non-specific “gastrointestinal side-effects”, “allergy” or similar were excluded) in cases diagnosed with at least one of the following: diarrhoea, nausea/vomiting, asthenia and abdominal pain and flatulence, leading to a total of 53 cases.

**Table 5.1.**

<b>Characteristics of the metformin-tolerant T2D patient group (Controls) versus T2D patients displaying metformin side-effects (Cases)</b>			
<b>Characteristic<sup>a</sup></b>	<b>Cases (n=53)</b>	<b>Controls (n=193)</b>	<b>P value</b>
Male, n (%)	13 (24.5)	61 (31.6)	0.4069
Female, n (%)	40 (75.5)	132 (68.4)	0.4069
Mean age $\pm$ SD, years	63.8 $\pm$ 8.2	58.9 $\pm$ 9.9	<b>0.0011</b>
Mean BMI $\pm$ SD, kg/m <sup>2</sup>	34.8 $\pm$ 7.2	34.7 $\pm$ 6.6	0.9542
Duration of diabetes, years	8.3 $\pm$ 6.9	9.0 $\pm$ 7.5	0.5172
Onset of diabetes, years	55.7 $\pm$ 8.3	50.13 $\pm$ 10.0	0.0003
Presence of hypertension, n (%)	40 (75.5)	157 (81.4)	0.4478
Smoking, n (%)	4 (7.55)	25 (13.0)	0.3963
Regular alcohol consumption, n (%)	17 (32.1)	147 (76.2)	<b>&lt; 0.0001</b>
Physically active, n (%)	2 (3.8)	14 (7.25)	0.5568
Insulin, n (%) <sup>b</sup>	3 (7.1)	123 (63.7)	<b>&lt;0.0001</b>
Other antidiabetic drugs, n (%) <sup>b</sup>	15 (35.7)	72 (37.3)	0.9854
Other drugs, n (%) <sup>b</sup>	35 (83.3)	174 (90.2)	0.3069
Dose of metformin $\pm$ SD, mg/per day <sup>c</sup>	1759.8 $\pm$ 605.6	2122.0 $\pm$ 337.9	<b>&lt;0.0001</b>
Creatinine level in blood $\pm$ SD, mmol/l <sup>d</sup>	71.63 $\pm$ 13.75	74.89 $\pm$ 18.11	0.3108
HbA <sub>1c</sub> $\pm$ SD, % <sup>e</sup>	7.8 $\pm$ 1.3	9.0 $\pm$ 2.1	<b>0.0008</b>

SD—standard deviation; BMI—body mass index; HbA<sub>1c</sub>—glycated haemoglobin, %; P<0.05 are marked in bold. <sup>a</sup>- data selected at the time of patient enrolment in the study. <sup>b</sup> data available from 235 subjects (193 controls, 42 cases). <sup>c</sup> data available from 234 subjects (193 controls, 41 cases). <sup>d</sup> data available from 219 subjects (184 controls, 35 cases). <sup>e</sup> data available from 189 subjects (150 controls, 39 cases)

We observed significant differences in age, mean dose of metformin, HbA<sub>1c</sub> levels, alcohol consumption, use of insulin and onset of T2D between the case and control groups (n=193). The presence of side-effects correlated positively with age (P=0.0011), but negatively with alcohol consumption (P<0.0001) between cases and controls. The control group included 63% insulin users, while only 3 patients (7.1%) from the case group used insulin. From the case group, 41 patients continued to use metformin therapy at the time of

recruitment. Mean daily dose of metformin and HbA<sub>1c</sub> levels were significantly lower in patients displaying side-effects ( $P < 0.0001$ ,  $P = 0.0008$ , respectively).

No significant differences in the duration of diabetes ( $9.0 \pm 7.5$  years in the control group ( $n = 189$ ) versus  $8.3 \pm 6.9$  years in the case group ( $n = 52$ ),  $P = 0.52$ ) or blood creatinine level ( $71.63 \pm 13.75$  mmol/l in the intolerance group ( $n = 35$ ) versus  $74.89 \pm 18.11$  mmol/l in the tolerance group ( $n = 184$ ),  $P = 0.31$ ) were evident. However, onset of diabetes was later in the case group ( $55.7 \pm 8.3$  versus  $50.13 \pm 10.0$ , respectively,  $P = 0.0003$ ). Since lack of insulin use and lower alcohol consumption are unlikely to be factors that trigger side-effects, further association analysis was adjusted using age, sex, and use of other peroral antidiabetic drugs and co-medications as covariates in logistic regression (Table 5.2).

**Table 5.2**

<b>Characteristics of T2D patients with metformin side-effects</b>	
<b>Biochemical analysis</b>	
eGFR $\pm$ SD, ml/min/1.73 m <sup>2</sup>	81.7 $\pm$ 23.6
HbA <sub>1c</sub> $\pm$ SD, %	7.8 $\pm$ 1.3
Cholesterol $\pm$ SD, mmol/L	5.5 $\pm$ 1.1
HDL $\pm$ SD, mmol/L	1.3 $\pm$ 0.4
LDL $\pm$ SD, mmol/L	3.3 $\pm$ 0.9
Triglyceride $\pm$ SD, mmol/L	2.2 $\pm$ 1.1
Albumin/Creatinine $\pm$ SD, mg/mmol	7.7 $\pm$ 25.9
<b>Dose of metformin</b>	
Mean starting dose $\pm$ SD, mg/per day	822.8 $\pm$ 439.6
Mean side-effect-inducing dose $\pm$ SD mg/per day	1311.8 $\pm$ 695.1
<b>Symptoms of intolerance</b>	
Diarrhoea	19
Nausea	14
Vomiting	3
Abdominal Pain	10
Asthenia	7
Flatulence	13

SD – standard error, eGFR – estimated glomerular filtration rate (Cockcroft-Gault equation), HbA<sub>1c</sub> – glycated haemoglobin; HDL – high-density lipoproteins; LDL – low-density lipoproteins

Allelic frequencies of detected polymorphisms ranged from 0.04 to 0.38, and did not differ substantially from the minor allele frequencies reported previously (<http://www.ncbi.nlm.nih.gov/> Accessed in January 2015). Genotyping success rate was  $>95\%$ , ranging from 95.5% to 100%. No significant deviation from Hardy-Weinberg equilibrium was observed for any of the SNPs. The characteristics of SNPs used in this study are presented in Table 5.3.

Table 5.3.

## Characteristics of SNPs used for study

Gene	SNP code	Position <sup>a</sup>	AA change	Genotype call rate %	Minor allele	Major allele	MAF <sup>b</sup>	MAF <sup>c</sup>	HWE
OCT1	rs12208357	c.181C>T	R61C	96.7	T	C	0.10	0.07	0.45
OCT1	rs34059508	c.1393G>A	G465R	95.5	A	G	0.04	0.05	1
OCT1	rs628031	c.1222A>G	M408V	97.2	A	G	0.39	0.42	0.79
OCT1	rs72552763	c.1260-1262delGAT	M420del	95.5	del	GAT	0.18	0.25	1
OCT1	rs36056065	c.1276+9_1276+16del GTAAGTTG	-	98.4	GTAAG TTG	del	0.39	0.41	0.69
OCT2	rs316019	c.808T>G	A270S	100	T	G	0.08	0.09	0.20
MATE1	rs2289669	c.922-158G>A	-	97.2	A	G	0.39	0.45	0.892

AA – amino acid; <sup>a</sup> nucleotide position relative to the gene start codons; MAF minor allele frequency, <sup>b</sup> – study sample, <sup>c</sup> – NCBI; HWE -Hardy-Weinberg P value.

We tested the association of all polymorphisms with HbA<sub>1c</sub> level, BMI and waist circumference measurements in metformin users (patients using metformin for at least 3 months before enrolment) (Table 5.4).

Table 5.4.

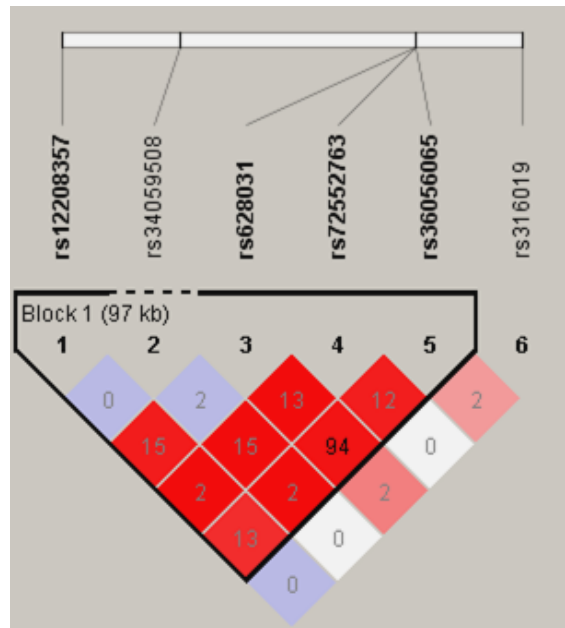
## SNP association with quantitative variables in regular metformin users

SNP	Mean ± SE per genotype <sup>a</sup>			P value <sup>b</sup>		
	11	12	22	Additive	Dominant	Recessive
<b>BMI, kg/m<sup>2</sup>, n=274</b>						
rs12208357	38.75±15.54	34.18±7.12	35.00±7.74	0.85	0.65	0.43
rs34059508	-	35.00±5.57	35.03±7.95	1.00	1.00	-
rs628031	34.38±6.75	35.45±8.35	34.72±7.51	0.98	0.72	0.61
rs72552763	36.00±3.90	34.81±7.60	35.10±7.32	0.78	0.89	0.60
rs36056065	34.12±6.81	35.53±8.30	34.52±7.36	0.94	0.54	0.51
rs316019	33.33±7.03	36.87±6.82	34.78±7.84	0.36	0.24	0.62
rs2289669	33.32±6.41	34.50±6.71	36.87±8.12	0.0033*	0.0055*	0.07
<b>HbA<sub>1c</sub>, %, n=238</b>						
rs12208357	10±0.00	8.84±2.03	8.33±2.08	0.12	0.13	0.53
rs34059508	-	7.92±1.68	8.49±2.07	0.26	0.26	-
rs628031	8.73±2.40	8.51±1.95	8.27±2.08	0.45	0.45	0.66
rs72552763	7.80±3.27	8.56±2.02	8.43±2.08	0.66	0.48	0.50
rs36056065	8.71±2.36	8.54±1.95	8.19±2.10	0.27	0.23	0.62
rs316019	8.00±1.41	8.15±2.20	8.48±2.05	0.19	0.20	0.51
rs2289669	8.72±2.17	8.22±2.06	8.64±2.01	0.59	0.22	0.48
<b>Waist, cm, n=262</b>						
rs12208357	132.0±59.7	111.6±14.3	112.1±15.5	0.50	0.89	0.033
rs34059508	-	112±13.3	112.3±16.6	0.73	0.73	-
rs628031	113.7±20.4	113.9±15.5	110.2±15.8	0.22	0.17	0.63
rs72552763	115.2±5.1	110.3±16.0	112.6±16.7	0.72	0.56	0.58
rs36056065	113.5±20.1	114±15.5	109.8±15.6	0.17	0.09	0.70
rs316019	125.3±21.0	116.7±15.6	111.9±16.2	0.08	0.12	0.22
rs2289669	111.1±15.8	110.5±15.6	115.2±17.3	0.044	0.024	0.48

<sup>a</sup> Minor allele=1; Major allele=2. SE – standard error. <sup>b</sup> - P-values estimated using linear regression adjusted for age, sex and metformin intolerance status\* P-values that remained significant (P<0.05) in the permutation test corrected for multiple testing-adjusted (EMP2)

Carriers of the SLC47A1 rs2289669 A allele were characterized by significantly lower mean BMI ( $P=0.0033$ ) in the additive genetic model and reduced waist circumference in the dominant genetic model ( $P=0.024$ ). Homozygous A allele carriers of another SNP, rs12208357, from the OCT1 gene displayed higher mean waist circumference ( $P=0.033$ ). Associations with waist circumference did not reach the significance level when adjusted for multiple testing, and none of the SNPs examined were associated with HbA<sub>1c</sub> level.

The minor allele frequencies (MAF) of rs628031 and rs36056065 polymorphisms in the control group (0.42 for both polymorphisms) were similar to those reported previously in the HapMAP project (0.417 for rs628031 and 0.412 for rs36056065 in the NCBI SNP database reported by Marshfield et al.). MAF of the same SNPs in the case group were significantly lower (0.275 and 0.274 for rs628031 and rs36056065, respectively). Estimation of the level of LD between OCT1 and OCT2 polymorphisms using HaploViewer software (Figure 5.1) revealed almost complete LD between rs628031 and rs36056065 ( $r^2=0.94$ ).



**Figure 5.1.** Graphical representation of LD between analysed SNPs. Pairwise  $r^2$  coefficients are shown in each box (expressed as a percentage), with red shading corresponding to stronger linkage between polymorphisms.

The A allele of rs628031 as well as an 8 bp insertion (rs36056065) were significantly associated with the absence of side-effects when tested independently using

the logistic regression test, and remained associated with the presence of metformin side-effects after adjustment for multiple comparisons using a permutation test (Table 5.5).

**Table 5.5.**

<b>SNP associations in T2D patients with metformin side-effects</b>						
SNP	Genetic model	Genotype number		OR [95% CI] <sup>a</sup>	P <sup>a</sup>	P <sub>perm</sub> <sup>b</sup>
		Intolerance group	Controls			
rs12208357	Additive (AA/AG/GG)	0/5/48	3/34/148	0.34[0.10–1.16]	0.085	0.38
	Dominant (AA/AG+GG)	5/48	37/148	0.34[0.10–1.18]	0.089	0.41
	Recessive (AA+AG/GG)	0/53	3/182	–	0.999	1
rs34059508	Additive (TT/TC/CC)	0/1/52	0/15/167	0.31[0.04–2.53]	0.28	0.86
	Dominant (TT/TC+CC)	1/52	15/167	0.31[0.04–2.53]	0.28	0.86
	Recessive (TT+TC/CC)	0/53	0/182	NA	NA	1
rs628031	Additive (AA/AG/GG)	3/22/26	34/89/65	0.47[0.26–0.82]	<b>0.009</b>	<b>0.042</b>
	Dominant (AA/AG+GG)	25/26	123/65	0.39[0.19–0.82]	<b>0.012</b>	0.062
	Recessive (AA+AG/GG)	3/48	34/154	0.32[0.09–1.18]	0.088	0.31
rs72552763*	Additive (DD/ID/II) <sup>c</sup>	0/21/32	7/47/128	1.25[0.66–2.34]	0.50	0.99
	Dominant (DD/ID+II)	21/32	54/128	1.62[0.78–3.39]	0.20	0.72
	Recessive (DD+ID/II)	0/53	7/175	–	1	0.99
rs36056065*	Additive (II/ID/DD)	3/23/27	35/89/65	0.41[0.22–0.72]	<b>0.002</b>	<b>0.010</b>
	Dominant (II/ID+DD)	26/27	124/65	0.36[0.17–0.74]	<b>0.006</b>	<b>0.027</b>
	Recessive (II+ID/DD)	3/50	35/154	0.20[0.04–0.89]	<b>0.035</b>	<b>0.078</b>
rs316019	Additive (TT/TG/GG)	0/7/46	3/27/163	0.86[0.33–2.25]	0.75	1
	Dominant (TT/TG+GG)	7/46	30/163	0.93[0.33–2.64]	0.89	1
	Recessive (TT+TG/GG)	0/53	3/190	–	0.99	1
rs2289669	Additive (AA/AG/GG)	5/26/22	30/89/67	0.71[0.42–1.22]	0.22	0.76
	Dominant (AA/AG+GG)	31/22	119/67	0.77[0.37–1.59]	0.48	0.98
	Recessive (AA+AG/GG)	5/48	30/156	0.41[0.11–1.45]	0.17	0.47

a – OR and P values estimated from logistic regression adjusted for age, sex, use of other peroral antidiabetic drugs and co-medications. b – P value obtained from 100 000 permutations and corrected for multiple testing (EMP2). \* – D deletion, I insertion

We did not identify specific haplotypes or pairwise interactions between investigated SNPs with stronger association effects than those of individual SNPs (data not

shown). The additive genetic model provided the best fit for association of both polymorphisms with presence of side-effects.

## 5.2. Metformin efficiency and genetic variability of metformin transporters

Metformin efficiency was analyzed in 102 T2D patients from the OPTIMED project and 126 patients from Slovakia that served as discovery and replication groups, respectively. The baseline HbA<sub>1c</sub> level was similar in both groups. However, the discovery group showed significantly better treatment results over a shorter time-period, compared to the replication group ( $\Delta$ HbA<sub>1c</sub>, 0.9% versus 0.5%). BMI and metformin dose were markedly different between the groups (Table 5.6).

**Table 5.6.**

### Characteristics of T2D patients in the discovery group (Latvia) and replication group (Slovakia)

Characteristics	Discovery group (n=102)	Replication group (n=126)	P values
Male, n (%)	33 (32.4)	58 (46.0)	0.0513
Female, n (%)	69(67.6)	68 (54.0)	0.0513
Mean age $\pm$ SD, years	59.7 $\pm$ 10.6	58.3 $\pm$ 9.5	0.295
Mean BMI $\pm$ SD, kg/m <sup>2</sup> , baseline	33.8 $\pm$ 4.8	30.6 $\pm$ 3.3	<b>&lt;0.0001</b>
eGFR $\pm$ SD, mL/min	120.1 $\pm$ 43.7	98.1 $\pm$ 24.2	<b>&lt;0.0001</b>
Dose of metformin $\pm$ SD, mg/per day	1525.0 $\pm$ 533.5	1382.1 $\pm$ 532.6	<b>0.0453</b>
Non-responders to treatment (decrease of HbA <sub>1c</sub> , balance ) n, %	18 (17.6)	26 (20.6)	0.6875
Days between HbA <sub>1c</sub> measurements $\pm$ D	95.5 $\pm$ 9.0	6 months <sup>c</sup>	NA
HbA <sub>1c</sub> $\pm$ SD, %, baseline	7.4 $\pm$ 1.5	7.5 $\pm$ 0.8	0.5203
HbA <sub>1c</sub> $\pm$ SD, %, after treatment, %	6.5 $\pm$ 0.6 <sup>a</sup>	7.0 $\pm$ 0.7 <sup>b</sup>	<b>&lt;0.0001</b>
Decrease of HbA <sub>1c</sub> $\pm$ SD, after treatment, %	0.9 $\pm$ 1.3	0.5 $\pm$ 0.7	0.0034

SD, standard deviation; BMI, body mass index; P values < 0.05 are marked in bold. <sup>a</sup> After 3 months of treatment; <sup>b</sup> After 6 months of treatment; <sup>c</sup> 180 days between HbA<sub>1c</sub> measurements. P values assessed using *t*-test and comparison of proportions. Nonresponders were defined as subjects with no change or increase in HbA<sub>1c</sub> after treatment.

In the discovery group, Tidwell-Box linearity test ( $P > 0.05$ ), Cook's distance values <1, unstandardized residual values <2.58 and under maximum leverage value confirmed the applicability of logistic regression to ascertain the effects of age, eGFR, BMI, sex, length of therapy, dose of metformin and 102 SNPs of the six metformin transporters (OCT1, OCT2, OCT3, MATE1, MATE2 and PMAT) on the likelihood of non-responder phenotype (no changes versus increased HbA<sub>1c</sub> level) after 3 months of metformin monotherapy, as described previously (Ichimori et al., 2012). In total, 26 SNPs were nominally ( $P \leq 0.05$ ) (APPENDIX V) associated while 3 SNPs remained significantly associated after correction for multiple testing. The 1000 Genomes project browser

(<http://browser.1000genomes.org/index.html>, Assessed in January 2015) predicted high linkage disequilibrium ( $D'=1.0$ ) between rs7757336, rs3119309 and rs2481030 in Caucasians with variable  $r^2$  due to differences between MAFs ( $r^2=0.182-0.657$ ).

Table 5.7 depicts the three SNPs (rs2481030, rs7757336 and rs3119309) significantly associated with non-responder phenotype after Bonferroni correction.

**Table 5.7**

**Characteristics of SNPs associated with non-response phenotype following metformin monotherapy**

Gene	SNP code	Position <sup>a</sup>	Call rate, %	Minor allele	Major allele	MAF, AFF	MAF, NA	MAF <sup>b</sup>	HWE
<b>SNPs associated with non-response to metformin in a study sample of 102 T2D patients from Latvia</b>									
SLC22A2-SLC22A3	rs7757336	160689558	100	G	T	0.36	0.08	0.17	0.68
	rs3119309	160685072	100	T	C	0.28	0.05	0.12	1
	rs2481030	160756435	100	G	A	0.75	0.32	0.40	0.42
<b>SNPs from a replication study on 126 T2D patients from Slovakia</b>									
SLC22A2-SLC22A3	rs7757336	160689558	100	G	T	0.10	0.11	0.10	0.36
	rs2481030	160756435	100	G	A	0.31	0.29	0.29	0.83

AFF – affected, NA – non-affected; HWE – Hardy-Weinberg equilibrium, *P* value

The results of adjusted logistic regression for each SNP are shown in Table 5.8.

**Table 5.8.**

**SNPs associated with efficiency of metformin monotherapy in patients from Latvia and Slovakia**

SNP		Genotype number		OR [95% CI] <sup>a</sup>	<i>P</i> <sup>a</sup>	<i>P</i> <sub>perm</sub> <sup>b</sup>
		Non-responders	Responders (non-aff)			
rs7757336	Discovery	2/9/7	0/14/70	50.360 [5.998-422.900]	<b>3.06*e<sup>-4</sup></b>	<b>0.004</b>
	Replication	0/5/21	0/21/79	1.241 [0.368-4.191]	0.728	0.980
	Combined	2/14/28	0/35/149	3.594 [1.670-7.737]	<b>0.001</b>	<b>0.002</b>
rs3119309	Discovery	1/8/9	0/9/75	26.580 [4.631-152.500]	<b>2.34*e<sup>-4</sup></b>	<b>0.003</b>
	Replication	NA	NA	NA	NA	NA
	Combined	NA	NA	NA	NA	NA
rs2481030	Discovery	10/7/1	8/38/38	13.700 [3.435-54.670]	<b>2.09*e<sup>-4</sup></b>	<b>0.002</b>
	Replication	4/8/14	6/46/48	1.225 [0.612-2.454]	0.566	0.917
	Combined	14/15/15	14/84/86	1.635 [1.256-2.129]	<b>1.00*e<sup>-4</sup></b>	<b>0.005</b>

Discovery group, Latvia, n=102 patients. a – OR (odds ratio) and *P* values estimated from logistic regression adjusted for age, sex, baseline BMI, number of treatment days, dose of metformin (mg/day), eGFR (Cockcroft-Gault equation). b – *P* values obtained from 100 000 permutations and corrected for multiple testing (EMP2). Replication group, Slovakia, 126 participants. a – OR and *P* values estimated from logistic regression adjusted for age, sex, baseline BMI, dose of metformin (mg/day) (transformed, ln), eGFR (Cockcroft-Gault equation)(Box-Cox power transformation, lambda -2). b – *P* values obtained from 100 000 permutations and corrected for multiple testing (EMP2). Combined analysis, n=228 participants. a – OR and *P* values estimated from logistic regression adjusted for age, sex, baseline BMI, number of treatment days (transformed, lambda -1), dose of metformin (mg/day), eGFR (Cockcroft-Gault equation)(transformed, lambda 2), rs2481030 (transformed, lambda 2) b – *P* values obtained from 100 000 permutations and corrected for multiple testing (EMP2)

Associations of all 102 SNPs in the discovery group are shown in a LD plot of the 102 tagSNPs in the OCT region (n=72) in APPENDIX I. Linkage disequilibrium of

tagSNPs in MATE (n=25) and PMAT (n=5) are shown in APPENDIX II and III, respectively.

The logistic regression model of combined negative alleles in the discovery group (number of minor alleles of rs3119309, rs2481030 and rs7757336) was statistically significant ( $\chi^2(4)=57.979$ ,  $P<0.000$ ). The model explained 71.5% (Nagelkerke  $R^2$ ) variance in response to metformin, and carriers of negative alleles were 8.4 times more likely to exhibit non-responder phenotypes than those of wild-type alleles. Longer time of the therapy and increased age were associated with a lower likelihood of exhibiting non-responder phenotype ( $p<0.05$ ).

Wide confidence intervals for models including covariates rs2481030, rs7757336, rs3119309 or a combination of negative alleles can be explained by small sample size and not as a consequence of high standard errors, as SE values for all logistic regression models were  $\leq 1.01$  and Pearson's  $r<0.8$ . Two SNPs, rs7757336 and rs2481030, were selected for replication in the group of 126 Slovakian T2D patients (replication group). Minor allele frequency analysis in the replication group disclosed up to 30% decreased frequency of rs7757336 (MAF 0.10, 0.13, 0.17) and rs2481030 (MAF 0.29, 0.40, 0.40), compared to the discovery group and Caucasian race, respectively.

We found no significant association of rs7757336 and rs2481030 SNPs with non-responder phenotype in the group from Slovakia after 6 months of metformin monotherapy using the model with the following covariates: sex, age, BMI, length of therapy and transformed variables of dose and eGFR (natural and Box-Cox power transformation, lambda-2, respectively).

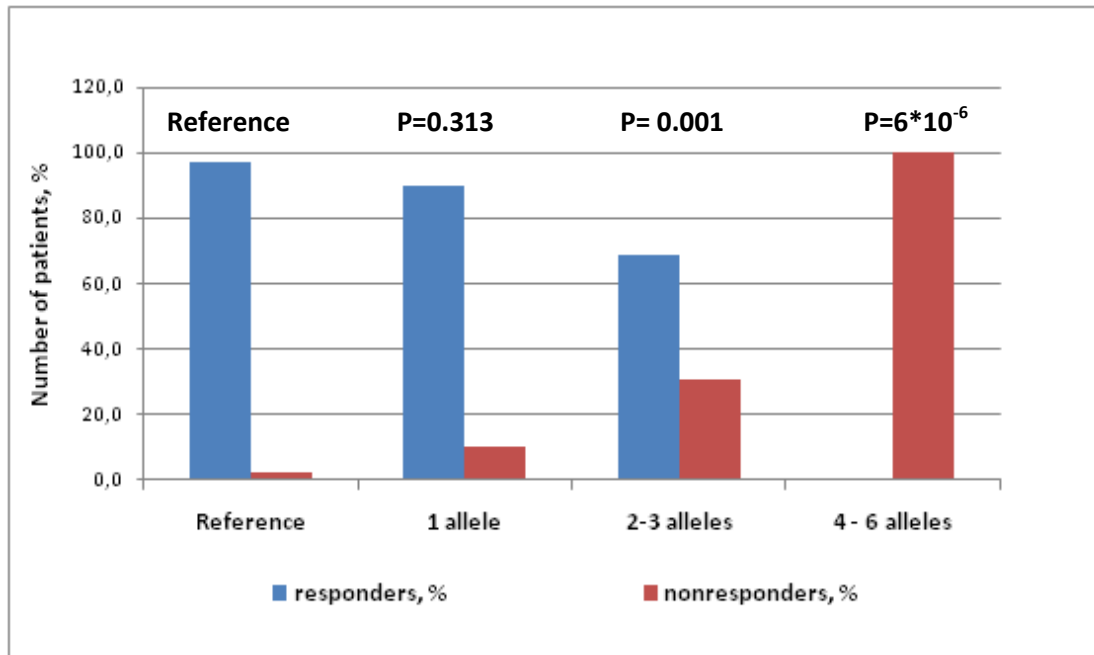
In combined group analysis (228 participants), the logistic regression model was statistically significant ( $\chi^2(4)=43.514$ ,  $P<0.001$ ) for squared number of minor alleles of rs2481030 and rs7757336 (Box-Cox power transformation, lambda 2) and the following variables: age, sex, baseline BMI, number of treatment days (Box-Cox power transformation, lambda-1), dose of metformin (mg/day), eGFR (Box-Cox power transformation, lambda 2). Carriers of increased negative alleles (transformed as  $n^2$ ) were 1.34 times more likely to exhibit non-responder phenotypes than those with wild-type alleles. Lower creatinine clearance, higher dose of metformin and increased age were significantly associated with decreased likelihood of non-responder phenotype ( $P<0.05$ ).

The number of risk alleles for rs7757336, rs3119309 and rs2481030 polymorphisms was assessed for each individual, and the reference group (0 risk alleles) analyzed against three groups, specifically, 1, 2-3 or 4-6 risk variant carriers (38 versus 30,



29 and 5 subjects, respectively). The number of risk alleles was positively correlated with prevalence of non-responders in each group, ranging from 2.6% in the reference group to 10.0%, 31.0% and 100.0% for 1, 2-3 or 4-6 risk variant carriers, respectively.

P values were calculated against the reference with 0 risk alleles (REF) (Figure 5.2).



**Figure 5.2.** Correlation between the number of OCT2-OCT3 transporter risk alleles and metformin efficiency

### 5.3. Pharmacokinetic study

The influence of rs2481030 and rs7757336 on metformin pharmacokinetic parameters was validated in a group of 15 participants. Four heterozygotes (AG) and 3 homozygotes (GG) of the rs2481030 risk allele were identified. Two heterozygotes (AC) of the rs7757336 risk allele were identified, both of which were also 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, 8 participants with 0 risk alleles were assigned to the ‘reference’ group and 7 with at least one risk allele to the ‘risk’ group.

Age was higher in the risk group with borderline significance ( $30.14 \pm 6.04$  versus  $24.88 \pm 3.27$ ,  $P=0.05$ ), while no significant differences in sex, weight and creatinine

clearance were observed between groups. Phenotypic and biochemical data on these subjects are presented in Table 5.9.

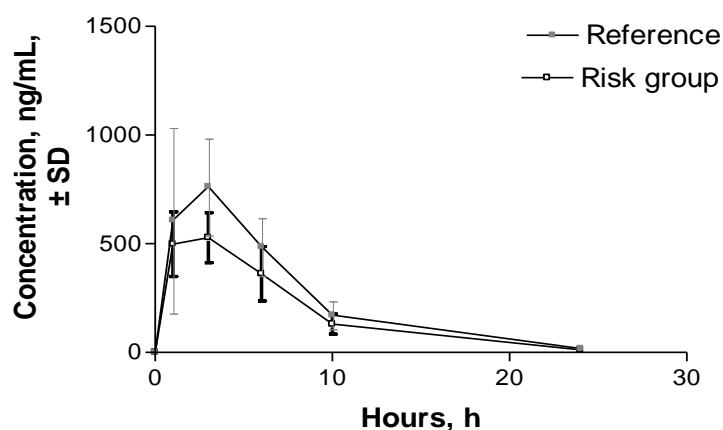
**Table 5.9**

**Characteristics and pharmacokinetic parameters of 15 healthy participants in relation to combined alleles of rs2481030 and rs7757336-rs3119309 associated with metformin inefficiency after oral administration of a single dose of metformin (500 mg)**

Characteristics	Study sample	Comparison of groups		
		Reference, n=8	Risk group, n=7	P value <sup>a</sup>
Men n, %	6 (40.00)	2 (25.00)	4 (57.14)	0.460
Weight, kg, ± SD	73.93±13.73	69.13±15.38	79.43±9.88	0.154
Age, ± SD	27.33±5.33	24.88±3.27	30.14±6.04	0.052
Creatinine clearance, mL/min/BSA, SD	127.03±12.21	127.65±13.26	126.33±11.91	0.843
AUC <sub>0-∞</sub> , µg/h/mL, ± SD	5.45±1.66	6.32±1.84	4.67±1.09	0.006
C <sub>max</sub> , µg/mL, plasma, ± SD	0.72±0.28	0.84±0.32	0.57±0.13	0.077
T <sub>max</sub> , plasma, h, ± SD	2.20±1.01	2.5±0.93	1.86±1.07	NS
C <sub>max</sub> , µg/mL, erythrocytes, ± SD	0.17±0.07	0.17±0.07	0.16±0.06	NS
T <sub>max</sub> , erythrocytes, h, ± SD	9.47±1.41	9.00±1.85	10.00±0.00	NS
Estimated half-life, h, ±SD	4.20±0.62	4.24±0.8	4.15±0.34	NS
CL/F, L/h ± SD	49.09±15.25	42.51±12.02	56.6±15.85	0.029
V/F, L ± SD	292.74±89.63	250.71±44.3	340.78±106.79	NS
Metformin excreted in the urine, % of dose <sup>c</sup>	39.88±16.14	43.7±11.97	43.05±6.1	NS

Data are shown as mean values ± standard deviation. <sup>a</sup> – P value derived from t-test. <sup>b</sup> – P values by linear regression with covariates – number of negative alleles, age, sex, weight, Clcr (corrected for body surface area). – 14 participants included in the analysis. NS – linear regression model is not significant.

The concentration of metformin in plasma was higher in individuals from the reference group, compared to those from the risk group at 4 sampling points (Figure 5.3).



**Figure 5.3.** Concentration of metformin in plasma of 15 healthy individuals grouped based on the presence of risk alleles after oral administration of a single dose of 500 mg metformin

Quality measures for regression analysis including combined risk alleles of rs7757336 and rs2481030 polymorphisms and covariates (weight, age, creatinine clearance

(calculated from 24 h urine samples)) were as follows:  $R^2=83\%$ , Durbin-Watson test=2.78, VIF (variant inflation factor) 1.2-1.8, and tolerance 0.55 - 0.86. Standardized residuals of linear regression with values less than  $<\pm 1.5$  were normally distributed ( $P=0.2$ ).

$AUC_{\infty}$  of metformin plasma was significantly lower ( $P=0.006$ ) in the risk group ( $4.67\pm 1.09$   $\mu\text{g/h/mL}$ ) versus reference group ( $6.32\pm 1.84$   $\mu\text{g/h/mL}$ ) in linear regression analysis. The model including number of risk alleles, age, sex and weight significantly predicted metformin  $AUC_{\infty}$  in plasma (sig. *F change* (5, 9)=8.803,  $P<0.003$ ). Weight ( $P=0.001$ ) and number of negative alleles ( $P=0.006$ ), but not sex ( $P=0.254$ ) or creatinine clearance ( $P=0.746$ ) were significant variables for prediction, while age had borderline significance ( $P=0.051$ ).

Apparent clearance (CL/F) was higher in the risk group ( $56.6\pm 15.85$  L/h versus  $42.51\pm 12.02$  L/h,  $P=0.029$ ). No significant differences were observed between groups in respect to  $T_{\text{max}}$  and  $C_{\text{max}}$  in plasma and erythrocytes, estimated half-life and apparent volume of distribution, as well as dose of metformin excreted in the urine 24 h after drug administration.

All rs7757336 heterozygotes are also carriers of rs2481030 minor alleles. Comparison of rs7757336/rs2481030 carriers versus reference allele/rs2481030 allele carriers in a linear logistic model with covariates (age, sex, weight and creatinine clearance) revealed significant association of rs7757336/rs2481030 carriers with reduced  $AUC_{\infty}$  ( $P=0.002$ ), CL/F ( $P<0.001$ ) and V/F ( $P<0.001$ ) while association of rs2481030 risk variant carriers versus reference allele carriers was of borderline significance ( $P=0.052$ ).

#### **5.4. Association of other genetic variants with metformin efficiency**

A significant proportion of SNPs in the OPTIMED genotyping panel include polymorphisms previously identified in GWAS studies. Among these are variants associated with T2D and obesity susceptibility (*CDKN2a [CDKN2b]*, *HNF4A*, *HHEX*, *PPARG*, *HNF1B*, *TCF7L2*, *PPARG*, *SLC30A8*, *IGF2BP2*, *ABCC8*, *RETN*, *KCNQ1*, *GCK*, *CAPN10*, *IGF2BP2*, *SLC2A2*, *KCNJ11*, *TMEM18*, *CDKAL1*, *FTO*) as well as metformin efficiency (*ATM* and *LKB11*). In this investigation, we attempted to determine whether these variants influence metformin efficiency in the group of 102 T2D patients from the OPTIMED study. Overall, 37 variants were genotyped, but 4 SNPs were excluded from analysis due to low genotyping quality (rs151290 (*KCNQ1*), rs1801261 (*ABCC8*), rs1801278 (*IRS-1*) and rs880663 (*PPARG*)). Rs290487 (*TCF7L2*) was additionally

excluded due to significant deviation from Hardy-Weinberg equilibrium. The average genotype call rate of the 33 SNPs was 98.9%. Minor allele frequency of the detected polymorphisms in responders and non-responders (defined as positive versus no or negative change of HbA<sub>1c</sub>) ranged from 0 to 0.56 and thus substantially differed from previously reported minor allele frequencies (<http://www.ncbi.nlm.nih.gov/>, Assessed in January 2015) (Table 5.10).

**Table 5.10.**

**Characteristics of SNPs used for study**

Gene	SNP	Chr	Position <sup>a</sup>	Minor allele	MAF non-responders/responders	MAF <sup>c</sup>	Hardy-Weinberg P value	Genotype call rate, %
<i>CDKN2a</i> [ <i>CDKN2b</i> ]	rs10811661	9	22134094	G	0.22/0.18	0.21	1.00	100.00
<i>HNF4A</i>	rs11086926	20	43058697	C	0.00/0.04	0.12	1.00	100.00
<i>HHEX</i>	rs11111875	10	94462882	A	0.22/0.41	0.44	1.00	100.00
<i>ATM</i>	rs11212617	11	108283161	C	0.56/0.45	0.47	0.43	100.00
<i>PPARG</i>	rs1152003	3	12477055	G	0.36/0.30	0.48	0.82	100.00
<i>HNF1B</i>	rs11868513	17	36052692	A	0.14/0.23	0.17	0.55	100.00
<i>TCF7L2</i>	rs12255372	10	114808902	A	0.25/0.21	0.17	1.00	100.00
<i>PPARG</i>	rs13073869	3	12353993	A	0.44/0.30	0.20	1.00	84.31
<i>PPARG</i>	rs13088205	3	12487806	C	0.19/0.26	0.28	1.00	99.02
<i>SLC30A8</i>	rs13266634	8	118184783	A	0.33/0.32	0.28	0.17	100.00
<i>IGF2BP2</i>	rs1470579	3	185529080	C	0.22/0.37	0.41	0.27	99.02
<i>ABCC8</i>	rs1799854	11	17448704	G	0.39/0.47	0.42	0.07	100.00
<i>PPARG</i>	rs1801282	3	12393125	G	0.17/0.19	0.07	1.00	100.00
<i>RETN</i>	rs1862513	19	7733793	G	0.19/0.27	0.32	1.00	96.08
<i>KCNQ1</i>	rs2237892	11	2839751	A	0.06/0.06	0.17	1.00	100.00
<i>KCNQ1</i>	rs2237895	11	2857194	C	0.42/0.41	0.32	0.68	100.00
<i>KCNQ1</i>	rs2237897	11	2858546	A	0.03/0.02	0.16	1.00	100.00
<i>KCNQ1</i>	rs2283228	11	2849530	C	0.06/0.07	0.18	0.34	100.00
<i>GCK</i>	rs2908289	7	44223942	A	0.06/0.07	0.21	1.00	100.00
<i>CAPN10</i>	rs3792269	2	241531479	G	0.09/0.11	0.11	1.00	87.25
<i>PPARG</i>	rs4135263	3	12423266	G	0.22/0.11	0.12	0.20	100.00
<i>ABCC8</i>	rs4148609	11	17484731	A	0.53/0.35	0.35	0.67	100.00
<i>IGF2BP2</i>	rs4402960	3	185511687	A	0.22/0.38	0.34	0.39	100.00
<i>TCF7L2</i>	rs5210	11	17408251	A	0.28/0.42	0.47	1.00	98.04
<i>SLC2A2</i>	rs5400	3	170732300	A	0.06/0.08	0.19	1.00	100.00
<i>PPARG</i>	rs6806708	3	12487650	A	0.19/0.26	0.29	1.00	100.00
<i>KCNJ11</i>	rs7124355	11	17412960	A	0.39/0.30	0.26	1.00	100.00
<i>TMEM18</i>	rs7561317	2	644953	A	0.25/0.20	0.17	1.00	100.00
<i>KCNJ11/ABCC8</i>	rs757110	11	17418477	C	0.44/0.36	0.29	0.40	100.00
<i>CDKALI</i>	rs7754840	6	20661250	C	0.31/0.36	0.41	0.83	100.00
<i>TCF7L2</i>	rs7903146	10	114758349	A	0.31/0.22	0.22	0.58	99.02
<i>FTO</i>	rs8050136	16	53816275	A	0.36/0.46	0.34	0.07	100.00
<i>STK11</i>	rs8111699	19	1209714	C	0.36/0.38	0.36	0.41	100.00

MAF minor allele frequency; Chr—chromosome; <sup>a</sup> - nucleotide position <http://www.ncbi.nlm.nih.gov/>.<sup>c</sup> Global MAF obtained from the 1000 genomes project (<http://www.biomart.org/>) Accessed in January 2015.

In a final analysis, all 33 SNPs were included, leading to the identification of nominal association ( $P < 0.05$ ) of rs1111875 (*HHEX*) and rs4148609 (*ABCC8*) with metformin efficiency. All phenotypes (positive or negative changes in HbA<sub>1c</sub> after 3 months of treatment) and logistic regression analysis settings were as described previously in the analysis of transporter gene SNPs (Table 5.11).

**Table 5.11.**

SNP association with metformin efficiency								
Gene	SNP	P nominal	P <sup>a</sup>	OR <sup>a</sup>	Lower limit <sup>a</sup>	Upper limit <sup>a</sup>	Genetic additive model	P <sup>b</sup>
<i>CDKN2a</i> [ <i>CDKN2b</i> ]	rs10811661	0.54	0.48	1.50	0.49	4.57	3/32/67	1.00
<i>HNF4A</i>	rs11086926	0.21	0.10	1.9*10 <sup>-9</sup>	0.00	∞	0/7/95	1.00
<i>HHEX</i>	rs1111875	<b>0.04</b>	<b>0.03</b>	0.29	0.09	0.89	14/48/40	0.57
<i>ATM</i>	rs11212617	0.26	0.30	1.61	0.66	3.91	20/56/26	1.00
<i>PPARG</i>	rs1152003	0.45	0.08	2.38	0.90	6.26	9/45/48	0.91
<i>HNF1B</i>	rs11868513	0.24	0.14	0.43	0.14	1.33	3/37/62	0.99
<i>TCF7L2</i>	rs12255372	0.64	0.47	1.44	0.53	3.91	5/35/62	1.00
<i>PPARG</i>	rs13073869	0.13	0.13	2.21	0.80	6.06	9/39/38	1.00
<i>PPARG</i>	rs13088205	0.42	0.69	0.80	0.27	2.39	6/38/57	1.00
<i>SLC30A8</i>	rs13266634	0.84	0.87	0.92	0.35	2.41	7/51/44	1.00
<i>IGF2BP2</i>	rs1470579	0.10	<b>0.03</b>	0.28	0.09	0.89	9/51/41	0.57
<i>ABCC8</i>	rs1799854	0.37	0.94	1.03	0.47	2.28	26/41/35	1.00
<i>PPARG</i>	rs1801282	0.80	0.70	0.79	0.26	2.35	3/31/68	1.00
<i>RETN</i>	rs1862513	0.36	0.25	0.57	0.22	1.49	6/38/54	1.00
<i>KCNQ1</i>	rs2237892	0.93	0.16	4.21	0.57	31.32	0/12/90	0.99
<i>KCNQ1</i>	rs2237895	0.90	0.70	0.84	0.36	2.00	18/47/37	1.00
<i>KCNQ1</i>	rs2237897	0.70	0.41	3.11	0.21	46.63	0/4/98	1.00
<i>KCNQ1</i>	rs2283228	0.83	0.30	2.39	0.46	12.53	1/11/90	1.00
<i>GCK</i>	rs2908289	0.73	0.73	1.37	0.23	8.18	0/14/88	1.00
<i>CAPN10</i>	rs3792269	0.79	0.86	0.87	0.20	3.89	1/17/71	1.00
<i>PPARG</i>	rs4135263	0.06	0.10	2.49	0.85	7.29	3/20/79	0.94
<i>ABCC8</i>	rs4148609	<b>0.04</b>	0.11	2.06	0.85	4.99	13/51/38	0.97
<i>IGF2BP2</i>	rs4402960	0.08	<b>0.03</b>	0.28	0.09	0.89	10/51/41	0.56
<i>TCF7L2</i>	rs5210	0.11	0.13	0.44	0.16	1.26	15/49/36	0.98
<i>SLC2A2</i>	rs5400	0.57	0.34	0.40	0.06	2.67	0/16/86	1.00
<i>PPARG</i>	rs6806708	0.40	0.67	0.79	0.26	2.37	6/39/57	1.00
<i>KCNJ11</i>	rs7124355	0.32	0.31	1.62	0.64	4.09	10/45/47	1.00
<i>TMEM18</i>	rs7561317	0.53	0.62	0.77	0.27	2.16	4/35/63	1.00
<i>KCNJ11/</i> <i>ABCC8</i>	rs757110	0.36	0.42	1.45	0.58	3.61	12/53/37	1.00
<i>CDKAL1</i>	rs7754840	0.56	0.38	0.67	0.27	1.66	13/45/44	1.00
<i>TCF7L2</i>	rs7903146	0.29	0.19	1.91	0.73	4.96	7/34/60	1.00
<i>FTO</i>	rs8050136	0.26	0.36	0.68	0.30	1.54	25/41/36	1.00
<i>STK11</i>	rs8111699	0.88	0.62	0.78	0.30	2.06	12/52/38	1.00

a – OR and P estimated from logistic regression adjusted for age, sex, baseline BMI, number of treatment days, dose of metformin mg/day, eGFR (Cockcroft-Gault equation), b – P obtained from 100 000 permutations and corrected for multiple testing (EMP2).

After adjustment for age, sex, baseline BMI, number of treatment days, dose of metformin (mg/day) and eGFR, only rs1111875 (*HHEX*) remained statistically significant. Moreover, two other SNPs, rs1470579 and rs4402960 (*IGF2BP2*), were identified as

significantly associated with metformin efficiency in logistic regression adjusted for covariates. However, none of the SNPs were significant after Bonferroni correction and permutation test.

### 5.5. Analysis of T2D susceptibility variants in relation to incidence of T2D

Among the polymorphisms, rs7561317 from the *TMEM18* locus and rs12255372 and rs7903146 from the *TCF7L2* locus were included in the OPTIMED genotyping panel. Association of these SNPs with obesity and T2D was previously reported in the Latvian population by our group (Kalnina et al., 2012; Kalnina et al., 2013). We combined the genotyping results from these studies with the group of 466 genotyped T2D patients from the OPTIMED study (486 participants) to determine potential associations with T2D. Age and BMI were significantly different between case and control groups ( $P < 0.05$ ), and included as covariates in logistic regression (Table 5.12).

**Table 5.12.**

<b>Characteristics of the study group</b>				
<b>Characteristic</b>	<b>Controls (n=1075)</b>	<b>OPTIMED (n=466)</b>	<b>Combined T2D (n=1449)</b>	<b>P value</b>
Male, n (%)	344 (32.0)	178 (38.2)	496 (34.2)	0.02/0.26
Female, n (%)	731 (68.0)	288 (61.8)	953 (65.8)	0.02/0.26
Mean age $\pm$ SD, years	53.7 $\pm$ 12.9	59.2 $\pm$ 10.3	60.0 $\pm$ 10.7	$<1 * 10^{-4} / <1 * 10^{-4}$
Mean BMI $\pm$ SD, kg/m <sup>2</sup>	27.2 $\pm$ 5.2	33.6 $\pm$ 5.9	33.0 $\pm$ 6.5	$<1 * 10^{-4} / <1 * 10^{-4}$

SD – standard deviation. P – comparison of control and OPTIMED groups/comparison of control and Combined groups.

The allelic frequencies of detected polymorphisms and genotyping success rate in the combined and control groups ranged from 0.17 to 0.23 and 97.0 to 99.2%, respectively. We observed a significant deviation from Hardy-Weinberg equilibrium in the case of rs7561317 in the OPTIMED and control group comparison, which was thus excluded from analysis. Minor allele frequencies of SNPs were similar to those found in Caucasians (Table 5.13).

**Table 5.13.**

<b>Characteristics of SNPs used for study</b>								
Gene	SNP code	Position <sup>a</sup>	Genotype call rate <sup>b</sup> , %	Minor allele	Major allele	MAF <sup>b</sup>	MAF, dbSNP <sup>c</sup>	Hardy-Weinberg P value
<i>TMEM18</i>	rs7561317	Chr2:644953	99.0/99.0	A	G	0.17/0.17	0.14	0.03*/0.10
<i>TCF7L2</i>	rs12255372	Chr10:113049143	99.2/99.2	T	G	0.20/0.21	0.22	0.38/0.59
<i>TCF7L2</i>	rs7903146	Chr10:112998590	97.0/97.3	T	C	0.22/0.23	0.28	0.82/0.46

<sup>a</sup> According to Genome Reference Consortium Human genome build38 (GRCh38); <sup>b</sup> OPTIMED and control group/combined group and control group; <sup>c</sup> <http://www.ncbi.nlm.nih.gov/> Accessed in January 2015; AA – amino acid; MAF minor allele frequency; <sup>a</sup> nucleotide position relative to the gene start codon

Logistic regression was performed for the rs12255372 and rs7903146 SNPs and incidence of T2D in 466 patients from OPTIMED and 1075 controls, and corrected for sex, 1/BMI and natural logarithm of age to meet the assumptions of logistic regression (Tidwell-Box test P value for continuous variables >0.05) (Table 5.14).

**Table 5.14.**

<b>SNP association with incidence of T2D in the OPTIMED study</b>						
SNP	Genetic model	Genotype number		OR [95% CI]	P <sup>a</sup>	Pperm <sup>b</sup>
		Cases	Controls			
rs7903146* ( <i>TCF7L2</i> )	Additive (TT/TC/CC)	34/165/245	38/339/673	1.58 [1.27-1.97]	3.47*10 <sup>-5</sup>	1.1*10 <sup>-4</sup>
	Dominant (TT/TC+CC)	199/245	377/673	1.72 [1.32-2.25]	6.52*10 <sup>-5</sup>	3.6*10 <sup>-4</sup>
	Recessive (TT+TC/CC)	34/410	38/1012	1.94 [1.11-3.40]	0.02	0.01
rs12255372* ( <i>TCF7L2</i> )	Additive (TT/TG/GG)	31/162/271	36/317/709	1.59 [1.28-1.98]	3.45*10 <sup>-5</sup>	1.1*10 <sup>-4</sup>
	Dominant (TT/TG+GG)	193/271	353/709	1.67 [1.28-2.18]	1.61*10 <sup>-4</sup>	1.4*10 <sup>-4</sup>
	Recessive (TT+TG/GG)	31/433	36/1026	2.30 [1.27-4.14]	5.66*10 <sup>-3</sup>	0.04

a – OR and P values estimated from logistic regression adjusted for sex, 1/BMI and natural logarithm of age. b – P value obtained from 100 000 permutations and corrected for multiple testing (EMP2); \*significant after Bonferroni correction (P=0.002). After filtering for the presence of genotypes and covariates, 1526 participants were analyzed.

Logistic regression was performed for rs12255372, rs7903146 and rs7561317 SNPs and incidence of T2D in the combined group of 466 patients from OPTIMED and 1085 T2D patients from LGDB, along with 1075 controls, and corrected for sex, natural logarithm of BMI and natural logarithm of age to meet assumptions (Tidwell-Box test P value for continuous variables >0.05) (Table 5.15).

**Table 5.15.**

<b>SNP association with incidence of T2D in the combined group</b>						
SNP	Genetic model	Genotype number		OR [95% CI]	P <sup>a</sup>	Pperm <sup>b</sup>
		Cases	Controls			
rs7561317 ( <i>TMEM18</i> )	Additive (AA/AG/GG)	34/396/1013	22/317/723	0.96(0.81-1.15)	0.69	0.95
	Dominant (AA/AG+GG)	430/1013	339/723	0.95 (0.78-1.16)	0.63	0.92
	Recessive (AA+AG/GG)	34/1409	22/1040	1.03(0.55-1.93)	0.92	1.00
rs7903146* ( <i>TCF7L2</i> )	Additive (TT/TC/CC)	96/511/798	38/339/673	1.46(1.25-1.72)	2.84*10 <sup>-6</sup>	1.0*10 <sup>-5</sup>
	Dominant (TT/TC+CC)	607/798	377/673	1.53(1.27-1.85)	1.22*10 <sup>-5</sup>	5.0*10 <sup>-5</sup>
	Recessive (TT+TC/CC)	96/1309	38/1012	1.90(1.23-2.93)	3.73*10 <sup>-3</sup>	8.42*10 <sup>-3</sup>
rs12255372* ( <i>TCF7L2</i> )	Additive (TT/TG/GG)	80/507/851	36/317/709	1.44(1.22-1.69)	1.10*10 <sup>-5</sup>	1.0*10 <sup>-5</sup>
	Dominant (TT/TG+GG)	587/851	353/709	1.50(1.24-1.82)	3.26*10 <sup>-5</sup>	1.2*10 <sup>-4</sup>
	Recessive (TT+TG/GG)	80/1358	36/1026	1.82(1.16-2.85)	9.55*10 <sup>-3</sup>	2.32*10 <sup>-2</sup>

a – OR and P value estimated from logistic regression adjusted for sex, natural logarithm of BMI and natural logarithm of age. b – P value obtained from 100 000 permutations and corrected for multiple testing (EMP2); \* significant after Bonferroni correction (P=0.002). After filtering for the presence of genotypes and covariates, 2505 participants were analysed in total.

In all patients, age and BMI were positively correlated with incidence of T2D ( $P < 0.001$ ). Men were more susceptible to T2D ( $P = 0.01$ ), and association with rs7903146 and rs12255372 polymorphisms was significant after the permutation test (100 000 permutations) and Bonferroni correction. Estimated LD between rs7903146 (*TCF7L2*) and rs12255372 (*TCF7L2*) was as follows:  $R^2 = 0.758$  and  $D' = 0.921$ .

The rs7561317 polymorphism was analyzed in all participants in relation to incidence of T2D, as described previously. Without BMI as a covariate, no significant association with outcome was observed ( $P = 0.09$ ).



## 6 DISCUSSION

Metformin pharmacogenetics is an extensively studied field due to worldwide use of the drug in antidiabetic therapy and its potential utility in other serious disorders, such as PCOS, metabolic syndrome, some cancer types, and delay of aging. In the current study, we investigated the correlation between safety and efficiency of this first-line peroral antidiabetic drug and genetic variabilities in organic cation transporters 1–3 (OCT1/*SLC22A1*, OCT2/*SLC22A2*, and OCT3/*SLC22A3*), multidrug and toxin extrusion protein 1-2 (MATE1/*SLC47A1*, MATE2/*SLC47A2*) and plasma membrane monoamine transporter (PMAT/*SLC29A4*) in T2D patients. To our knowledge, our study appears to be first analysis of these genetic variants in association with metformin side-effects. Genetic variants associated with nonresponsiveness to metformin were additionally validated in a pharmacokinetic study in healthy volunteers.

The possibility that genetic factors other than transporter genes, including those associated with risk of T2D, influence responsiveness to metformin cannot be excluded. To analyze the impact of other genetic factors, we investigated 33 polymorphisms of 21 genes identified from previously published GWAS and other studies, the majority of which are associated with susceptibility to T2D and obesity, including two variants associated with altered response to metformin. T2D patients included in the above analysis were selected from the larger OPTIMED cohort. To elucidate the genetic background of the study sample and validate genetic associations of well-known T2D variants (*TCF7L2* and *TMEM18*) in this cohort, we performed another association study on a large group of T2D patients and controls from LGDB that were previously genotyped in the frame of other studies.

### 6.1. Genetic variants influencing metformin side-effects

To our knowledge, at the time of publication, the work described in this section of the thesis was the first ever investigation to specifically target the pharmacogenetics of metformin side-effects. Common side-effects of metformin are mainly gastrointestinal disturbances like diarrhoea, nausea, flatulence, abdominal pain, asthenia and vomiting of unknown pathophysiology. High incidence of intolerance in T2D patients leads to poor adherence to therapy, and in most severe cases, discontinuation of metformin use (Florez et al., 2010; Hermans et al., 2012). The clinical phenotype of patients with gastrointestinal side-effects is unclear, and characterized by a low rate of ischaemic heart disease, left-handedness, ABO group imbalance, higher ferritine levels and iron load. While these traits

may aid in characterizing cardiovascular risk, they do not provide stringent criteria in the clinical setting (Hermans et al., 2013). We observed significant disproportion among the groups regarding insulin use and alcohol consumption. However, lower insulin use or alcohol consumption are unlikely to be the factors eliciting side-effects. The recruitment criteria for the group of T2D patients with side-effects are not identical to those for patients recruited to the biobank on a general basis, which may explain the differences in factors such as alcohol consumption and use of insulin between groups. However, we believe that these factors are not causal and do not influence the presence or absence of side effects.

Our earlier results (Tarasova et al., 2012)(APPENDIX IV) are consistent with those of two subsequent studies. Age was positively correlated with drug tolerance and women were more likely to suffer from side-effects than men, similar to our findings (Dujic et al., 2014; Hermans et al., 2013). This may be an effect of reduced metformin excretion in the elderly and proportionally higher exposure to metformin in relation to body mass. However, we found no differences in serum creatinine measurements between cases and controls, suggesting normal renal functioning in both groups. There may be a simple explanation regarding the low HbA<sub>1c</sub> and smaller metformin doses in patients with side-effects. Firstly, this may be attributable to later onset of T2D in these cases. Secondly, these patients continued to use metformin, regardless of side-effects, but at smaller doses to avoid further increase in severity of the effects.

In this study, we have shown an association of the A allele of rs628031 and 8 bp insertion (rs36056065) in the OCT1 gene with absence of common gastrointestinal side-effects of metformin therapy for the first time. The rs628031 (Met408>Val) polymorphism has been identified in all ethnic groups (Perwitasari-DA., 2014) and shown to elicit normal metformin uptake *in vitro*, as it does not alter protein expression (Shu et al., 2008; Shu et al., 2007). In a small clinical study, Met408>Val was identified as a weak positive predictor of metformin efficacy (Becker et al. 2010b; Shikata et al., 2007) and slightly increased OCT1 mRNA expression in human liver samples (Nies et al., 2009). However, the polymorphism did not alter renal clearance in healthy male Caucasians (Tzvetkov et al., 2009). The rare allele of rs36056065 is characterised by presence of a 8 nt insertion at the 3' end of exon 7 and does not change the amino acid sequence of OCT1, but was recently shown to generate an alternative splice site leading to a premature stop codon after codon 429. This transcript had not been described previously. Additionally, many studies investigating OCT1 expression and 420del appeared to use probes designed to bind sequences lacking 420del and assuming normal exon-intron junction (Grinfeld et al.,

2013). The hOCT1G/L506 isoform expressed in human liver carries a deletion between positions 1385 and 1498 of hOCT1 cDNA and exhibits an altered sequence from aa 462 to aa 506 due to a frameshift following the deletion. Transfection of hOCT1G/L506 resulted only in marginal (but not significant) decynium-22 (D22)-sensitive uptake of tritiated 1-methyl-4-phenylpyridinium-sensitive phenylpyridinium ( $[^3\text{H}]$ -MPP+), compared to the predominant splice isoform. The lack of specific transport by the splice variant is presumably not caused through insufficient mRNA expression, but rather due to a change in the C-terminal region of the protein and loss of the last two transmembrane domains (Hayer et al., 1999). In a recent publication, six alternative splice variants of human *SLC22A1* mRNA were detected in healthy liver and tumor tissues. At least two of these variants were characterized by the c.1276+1insGTAAGTTG mutation. However the study focused on the effects of SNPs on sorafenib transport, and further investigation of the splice variants was not performed (Herraez et al., 2013).

The OCT1 transporter is proposed to participate in hepatic uptake of metformin (Kimura et al., 2005; Nies et al., 2009), but can also found in the luminal membranes of proximal and distal tubules in kidneys (Tzvetkov et al., 2009), basolateral membrane of intestinal cells (Muller et al., 2005) and apical membranes of Caco-2 cancer cells and enterocytes (Han et al., 2013). Metformin bioavailability is low and highly variable (Pentikainen et al., 1979), and highest levels of metformin can be detected in enterocytes (Sakar et al., 2010). Metformin uptake in the gut is hypothesized to have a complicated pattern and basolateral (BL) transport identified as the rate-limiting step, leading to paracellular and not transcellular metformin uptake (Proctor et al., 2008). The “bottleneck” of metformin uptake in the gastrointestinal tract is the basolateral membrane where the OCT1 transport protein is expressed (Bailey et al., 1994; Proctor et al., 2008).

We hypothesized that reduced OCT1 transport function in the small intestine leads to locally increased metformin concentrations and evokes gastrointestinal disturbance, possibly via increased lactate production or altered glucose uptake in cells (Bailey et al., 1994; Sakar et al., 2010). Horie and co-workers showed that apical uptake of metformin in human cancer intestinal epithelial cells is mediated at least partly by OCTs (Horie et al., 2011). Since OCT1 and OCT2 were expressed in addition to OCT3 in these cells, it is possible that OCT1 polymorphisms influence intestinal metformin uptake and trigger gastrointestinal side-effects. OCT1 functions in liver are similar to those of OCT3, and OCT3 can therefore compensate for the reduced function of OCT1 (Muller et al., 2005). In the kidneys, OCT1 reabsorbs metformin, converse to OCT2, MATE1 and MATE2, and is

expressed to a lower extent (Grundemann et al., 1998a; Masuda et al., 2006; Otsuka et al., 2005; Sato et al., 2008; Tanihara et al., 2007; Tsuda et al., 2009a; Tsuda et al., 2009b).

Metformin is a superior substrate for OCT2 (Kimura et al., 2005). OCT2 is expressed in the kidneys and supports the metformin excretion process (Motohashi et al., 2002). The genetic diversity of OCT2 is significantly lower than that of OCT1 (Fujita et al., 2006), implying more critical functions in the human body. In previous studies, the OCT2 allele, Ser270, was shown to be associated with anti-cancer cisplatin-induced nephrotoxicity (Filipski et al., 2009), positive and negative findings on lower prevalence among patients with essential arterial hypertension (Lazar et al., 2006) (Sallinen et al., 2010), and alterations in renal clearance. However, opposite effects were reported in other studies (Chen et al., 2009a; Song et al., 2008a; Tzvetkov et al., 2009). The OCT2 allele, Ser270, displayed no association with side-effects, BMI, waist circumference or metformin efficiency. The rs2289669 A allele in the MATE1 coding gene, *SLC47A1*, was found to be correlated with increased efficiency of metformin therapy, and pairwise interactions with SNPs in OCT1 metformin were identified (Becker et al., 2009; Tkac et al., 2013), although this was not consistently reported in all studies (Choi et al., 2011). No relationship between this SNP and pharmacokinetics of metformin has been detected (Tzvetkov et al., 2009). The SNP was associated with BMI changes after metformin therapy in our study.

One of the major limitations of the current study is the lack of data representing biochemical and phenotypic measures in patients before the start of metformin therapy to analyse changes in BMI, waist circumference and HbA<sub>1c</sub>. For determination of side-effects, small sample sizes and limited numbers of SNPs and genes were analysed. For example, PMAT, OCTN1 and MATE2 transporter coding gene variants were not included. These factors may have an impact on metformin tolerability, as the first two are localized in enterocytes (Nakamichi et al., 2013; Zhou et al., 2007), and the last, together with MATE1, ensures excretion of metformin into urine (Masuda et al., 2006; Otsuka et al., 2005; Sato et al., 2008; Tanihara et al., 2007; Tsuda et al., 2009a; Tsuda et al., 2009b). Gastrointestinal disturbances may also be evoked by other diseases and concomitant drug therapy, diet and lifestyle factors.

Two years ago, our colleagues from the MetGen consortium investigated OCT1 genetic variants in relation to metformin intolerance (described as discontinuation of therapy due to side-effects) in 2 166 patients from GoDARTS (Dujic et al., 2014). The most interesting finding was that that OCT1 genetic variants R61C (rs12208357), C88R (rs55918055), M420del (rs72552763), G401S (rs34130495) and G465R (rs34059508)

were significant markers of metformin intolerance (in case of two risk alleles for reduced function, OR=2.41, 95% CI 1.48-3.93, P<0.001). Interestingly that negative effect of OCT1 inhibitors is more pronounced in carriers of reduced function alleles (OR=4.13, 95% CI 2.09 - 8.16, P<0.001) (Dujic et al., 2014). We found similar odds ratios for Met408>Val and rs36056065, but did not perform analysis including OCT1 inhibitors. The issue of whether rs36056065 and Met408>Val are good intolerance markers remains to be established, as Dujic and colleagues did not investigate the variants identified by our group. However, one of the variants included in analysis, 420del, is less than 50 bp away from Met408>Val and the insertion-deletion polymorphism rs36056065. Both associated SNPs are in almost complete LD, but 420del was found in 33% patients and never in *cis* with the 8+ allele in four cell lines and patient groups (Grinfeld et al., 2013). In our study, 2.2% of all 8+ carriers (n=45) were also carriers of 420del.

The Met408>Val and rs36056065 variants were established as significant markers in two other studies investigating therapy outcomes in cancer patients using imatinib (Grinfeld et al., 2013; Singh et al., 2012). However, these results are of limited importance, as OCT1 was shown to be incapable of transporting imatinib in nonleukemic animals and cell lines (Hu et al., 2008). In another study, OCT1 expression was found to correlate with the expression patterns of the imatinib transporters ABCB1, ABCG2, and SLCO1A2, supporting its potential utility as an effective surrogate marker (Hu et al., 2008).

We conclude that age, sex and genetic susceptibility factors in the OCT1 coding gene are significant modulators of metformin safety. The specificity and sensitivity of the described genetic markers in the OCT1 coding gene with regard to prediction of metformin side-effects requires further investigation in a large prospective study.

## **6.2. Metformin efficiency and genetic variability of metformin transporters**

Minor alleles of SNPs rs3119309, rs7757336 and rs2481030 located in the intergenic region between OCT2 and OCT3 coding genes (*SLC22A2* and *SLC22A3*) were significantly associated with metformin inefficiency in the 102 newly diagnosed T2D patients.

Logistic analysis was performed using robust segregation of outcomes in the responder group showing decreased HbA<sub>1c</sub> versus the non-responder group displaying no change or even increased levels of HbA<sub>1c</sub> three months after the commencement of metformin monotherapy. The main reason for selection of this type of outcome was our intention to perform robust analysis better suited for the investigation in the small, well-

phenotyped group (Ichimori et al., 2012). A number of studies investigating the efficiency of metformin, including the only GWAS performed to date (GoDarts et al., 2011), have used the treatment goal of HbA<sub>1c</sub> level <7% or 6.5% as an outcome. Patients with high baseline HbA<sub>1c</sub> levels usually have larger  $\Delta$ HbA<sub>1c</sub> in comparison to other participant groups. Nevertheless, these patients are often included in the non-response group if treatment goals are set as criteria, even if absolute decrease in HbA<sub>1c</sub> is substantial. For the same reason, use of treatment goals or even  $\Delta$ HbA<sub>1c</sub> as a quantitative dependent variable can be misleading in patients with extreme baseline HbA<sub>1c</sub> values. Thus patients with high baseline HbA<sub>1c</sub> display a larger HbA<sub>1c</sub> decrease over those with HbA<sub>1c</sub> close to normal levels, and patients with low baseline HbA<sub>1c</sub> have lower  $\Delta$ HbA<sub>1c</sub> independent of individual metformin response. This may explain the significant deviation of  $\Delta$ HbA<sub>1c</sub> from normal distribution in our research group.

GWAS identified an association between metformin efficiency and rs11212617 near *ATM*, but no association was evident in loci of known metformin transporter genes (GoDarts et al., 2011). Our detection of an association of SNPs in metformin transporter genes with metformin efficiency in contrast to GWAS findings may be explained by the relatively short time-period used to estimate the efficiency of metformin. Factors influencing the pharmacokinetics (e.g., organic cation transporters) affect efficiency mainly at the beginning of the treatment, especially in the case of metformin where gradual accumulation of drug is observed over treatment time (Christensen et al., 2011). A shorter time-period is also less dependent on factors such as diet, physical activities and adherence to drug, compared with long-term treatment where 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 three months in patients who fail to show decreased HbA<sub>1c</sub>. A study investigating metformin therapy outcomes evaluated initial non-adherence as 17% in participants starting metformin therapy. A third of the patients attempted another pharmacotherapy regime within 6 months, and side-effects were found to be one of the major limiting factors (Florez et al., 2010; Hermans et al., 2012; Nichols et al., 2010). Thus patients with treatment inefficiency caused by defective metformin transport may be eliminated from studies involving longer observation times, such as GWAS (Florez et al., 2012b; GoDarts et al., 2011; Tkac et al., 2013). This may also explain our failure to reproduce the association of identified SNPs in the replication group comprising 126 T2D participants from Slovakia administered metformin for six months. Rs7757336, rs2481030, age, sex, BMI, metformin dose and eGFR were not

associated with non-responder phenotype, indicating a strong influence of other unidentified genetic variants in metformin transporters or environmental factors like diet, physical activity, and adherence to metformin. Notably, MAF of investigated SNPs in the replication group was >30% lower, compared to MAF reported in the Caucasian race (<http://www.ncbi.nlm.nih.gov/> Accessed in January 2015). The absence of association and lower prevalence of minor alleles in patients from Slovakia may, at least partially, be caused by elimination bias of primary non-responders to metformin therapy or up-titration of metformin at the 3 month time-point according to standard antidiabetic treatment guidelines.

Surprisingly, the minor alleles of associated SNPs rs3119309, rs7757336 and rs2481030 were also consistently identified as markers of lower concentrations of metformin and  $AUC_{\infty}$  of plasma in a small independent group of 15 nondiabetic volunteers. In view of the limited number of participants, confirmation of these findings is necessary in larger scale studies. Validation of these findings should support the influence of OCTs in the pharmacokinetics of metformin and the importance of short-term study design in cases where factors influencing bioavailability are investigated. No significant differences were observed in  $AUC_{0-24}$  and  $C_{max}$  in erythrocytes between controls and carriers of risk alleles, indicating that changes in metformin pharmacokinetics cannot be explained by exposure time to metformin. Analysis of other pharmacokinetic measures revealed that oral clearance (CL/F) and apparent volume of distribution (V/F) are higher in subjects with at least one inefficiency allele, while the half-life of metformin and total dose excreted within the first 24 h after administration are not different. However, it should be noted that calculation of renal clearance and apparent volume of distribution were performed assuming no differences in the bioavailability of 500 mg metformin ingested orally. This assumption may not be true, as a number of factors, including genetic variations in transporter genes, may alter bioavailability and absorption. Thus the calculated CL/F and V/F values are strongly related to  $AUC_{\infty}$  measurements and their differences among groups should be interpreted with caution.

Variations in bioavailability and volume of distribution are considered a major source of deviation in the pharmacokinetics of metformin, as it is not metabolised in the human body, and slow absorption is thought to be the rate-limiting factor in metformin disposition (Pentikainen et al., 1979). A number of studies support the existence of an “OCT-like” bidirectional uptake/efflux transport mechanism on the AP membrane in Caco-2 cells for metformin, possibly involving OCT3. Limited transport activity of OCT1 on the

basolateral membrane leads to transporter-dependent accumulation of metformin in enterocytes as well as its absorption through the paracellular, yet saturable route (up to 90%) (Proctor et al., 2008). The proportion of metformin sequestered in enterocytes remains to be established due to inward net flow and entering blood via OCT-related mechanism versus the paracellular route in human small intestine. If the latter case occurs, uptake and excretion remain the major factors influencing metformin plasma levels. OCT1 and OCT3 are responsible for hepatic uptake and excretion, while OCT3 specifically ensures uptake and excretion in the small intestine and skeletal muscle. Excretion of metformin is maintained by OCT2, which undergoes reduced expression in kidney with age and low testosterone level, while the presence of certain diseases and co-medications may also play a role (Lopez-Parra et al., 2006; Sansoe et al., 2002; Urakami et al., 1999). Previous studies have demonstrated tissue specificity of expression of the OCT2 transporter, but not OCT1. OCT3 expression in liver tissue samples from 150 Caucasians were shown to be independent of age and sex, but significantly reduced in liver donors diagnosed as cholestatic (Chen et al., 2013). Lower  $AUC_{\infty}$  of metformin in plasma may indicate lower metformin uptake in the gastrointestinal tract by the altered OCT3 transporter. On the other hand, lower  $AUC_{\infty}$  may be explained by the OCT2 variant that eliminates the drug more quickly, compared to individuals containing the reference alleles.

While rs3119309, rs7757336 and rs2481030 are located in the genomic region separating *SLC22A2* and *SLC22A3*, the possibility that causal SNPs associated with metformin efficiency potentially occur in *SLC22A1* and influence OCT1, known as a major hepatic uptake transporter, cannot be excluded. The polymorphisms investigated in this study are non-coding, and could be in linkage disequilibrium with causal SNPs within the coding/regulatory regions of *SLC22A2* (OCT2), resulting in increased transport activity or expression in target tissues and/or opposite effects on *SLC22A3* (OCT3) if its role in gastrointestinal absorption is considered significant. In large GWAS, minor allele A of rs3127573 (MAF 0.13) located near *SLC22A2* ( $r^2=0.96$  with rs3119309 investigated in our study) was shown to be associated with higher serum creatinine and lower eGFR (Chambers et al., 2010). Creatinine is mainly eliminated via glomerular filtration and its tubular secretion varies between 10-40%, while metformin is excreted mainly via active tubular transport mediated by OCT2 (Lepist et al., 2014). While rs3127573 has been convincingly associated with the higher creatinine level in GWAS, and predicted to alter BRCA1, NF-Y and SP1 motifs with high LD with SNPs involved in binding of CEBPB and FOXA1 proteins, the polymorphism was not correlated with renal clearance of



metformin in 103 healthy males (Tzvetkov et al., 2009). Significant correlations were identified only between renal clearance of metformin and creatinine clearance, combined alleles of OCT1 and age. In our study, both rs3119309 and rs7757336 minor allele heterozygotes relative to major allele carriers were characterized by similar metformin half-life and percentage of metformin excreted in urine in the first 24 h after peroral administration, while metformin AUC<sub>∞</sub> in plasma was significantly lower in minor allele carriers. The relatively small number of rs3119309 and rs7757336 carriers and lack of recessive homozygotes in our study limit data interpretation.

Recent studies have demonstrated that the OAT2 transporter is characterized by three-fold higher RNA expression than OCT2. Moreover, its transport activity is higher than that of OCT. These results suggest that OAT2 transporter is a better main creatinine transporter candidate than OCT2 (Eisner et al., 2010). Interestingly, OCT1 and OCT2 absence in mice did not affect creatinine clearance (Eisner et al., 2010). Transporter tissue expression differs between species and cell lines, and metformin renal clearance in humans is several times higher than creatinine clearance, indicating significant influence on active transport in the kidney, compared to creatinine (Lepist et al., 2014). Thus, it is unclear how variants in OCT2 or regulatory regions are associated with increased creatinine levels and reduced metformin concentrations in plasma. On the other hand, these findings may be explained by altered transport activity of creatinine and the metformin transporter, OCT3 (*SLC22A3*), which is expressed in liver, small intestine and muscle tissue and located on Chr6 near the OCT2 coding gene. Indeed, in earlier experiments with hOCT2- and hOCT3-expressing HEK293 cells (Ciarimboli et al., 2012), increased creatine uptake, compared to cells transfected with the null vector (5.8-fold and 3.7-fold, respectively), was reported, which was not observed for hOCT1. GWAS linked *OCT3* with risk of prostate and colorectal cancer as well as coronary artery disease, indicating a pleiotropic role of the transporter in human physiology and pathophysiology, in view of its involvement in the transportation of a spectrum of monoamines, including serotonin, histamine and norepinephrine (Cui et al., 2011; Eeles et al., 2008; Lazar et al., 2008; Tomlins et al., 2007; Tregouet et al., 2009; True et al., 2006). Previous studies demonstrated that in skeletal muscle, metformin significantly enhances AMPK  $\alpha$ 2 activity via increasing phosphorylation of AMPK at Thr172 (Musi et al., 2002). Moreover, the effect of metformin on AMPK phosphorylation in cultured skeletal muscle cells was substantially inhibited by cimetidine (widely used in previous experiments with OCT2)(Somogyi et al., 1987) as well as OCT3 shRNA, suggesting that OCT3 plays a major role in the therapeutic

action of metformin (Chen et al., 2010). The polymorphisms rs2292334 (MAF 0.37), rs2048327 (MAF 0.35), rs1810126 (MAF 0.36), and rs3088442 (MAF 0.33) were associated with reduced OCT3 mRNA levels ( $P = 0.03$ ), while the common variant (MAF 50%), g.-2G>A (rs555754), was associated with higher transcription rate and expression of OCT3 in liver (Chen et al., 2013). Although none of the SNPs altering OCT3 transporter efficiency were in strong linkage disequilibrium with those investigated in our study, rs2481030 could be used to predict Pou2f2, Pou5f1, and Sox motif changes and Pax-4, and Spz1 motif changes for SNP rs2481031 (LD with rs2481030  $r^2=0.99$ ) (HaploReg v2). The rs2481030 polymorphism may be an effective marker of the reduced uptake OCT3 variant or lower transporter expression, subsequently characterized by slower metformin absorption from the gastrointestinal tract and decreased uptake in liver, muscle, heart tissues.

A major limitation of our study was the small number of participants. Therefore, these findings should be evaluated in larger subject groups. Further research is required to confirm whether rs7757336, rs3119309 and rs2481030 are valid markers of metformin inefficiency and their utility in prediction of clinical response to metformin in T2D patients.

### **6.3. Association of other genetic variants with metformin efficiency**

In the OPTIMED group alone, we investigated potential associations between 33 SNPs and metformin efficiency (defined as positive or negative changes in HbA<sub>1c</sub> after 3 months of treatment). In our study, *HHEX* rs1111875 showed significant nominal and adjusted association that was not significant after correction for multiple testing. The *HHEX* (haematopoietically expressed homeobox) gene encodes a transcription factor protein expressed in the embryonic ventral-lateral foregut (Bogue et al., 2000) that is associated with T2D in many populations (Cai et al., 2011; Omori et al., 2008; Scott et al., 2007). The 3'-UTR variant, rs1111875 (10q23.33), was associated with T2D independently of body fat (Schulze et al., 2007; van Vliet-Ostapchouk et al., 2008). Baseline insulin secretion was lower in subjects with the risk genotype at *HHEX* rs1111875 (Moore et al., 2008). Conversely, another study showed no impact on glucose and insulin concentrations in the fasting state and during OGTT or measurement of insulin sensitivity in non-diabetic participants (Staiger et al., 2008). The *STK11* rs741765 GG and *SLC30A8* rs1326634 TT genotypes, but not *HHEX* variants, were shown to be associated with response rate to metformin (Chauhan et al., 2010). Metformin is known to increase insulin sensitivity in

tissues, and negative effects of *HHEX* T2D risk variants on  $\beta$  cells may reduce efficiency of therapy (Giannarelli et al., 2003).

Another variant showing nominal association with metformin efficiency in our study was rs4148609 (*ABCC8*). However, after adjustment for covariates and Bonferroni correction, the significance of this association was lost. The *KCNJ11* gene (encoding the islet ATP-sensitive potassium channel, Kir6.2) variant harbouring a substitution of glutamate to lysine at position 23 (E23K) is associated with T2D (OR near 1.15)(Florez et al., 2004; Gloyn et al., 2001; Gloyn et al., 2003; Hani et al., 1998; van Dam et al., 2005) and characterized by reduced insulin secretion (Riedel and Light, 2005; Schwanstecher et al., 2002). This variant is in strong linkage disequilibrium with A1369S in *ABCC8* (encoding sulfonylurea receptor SUR1)(Inoue et al., 1997), and thus lysine carriers (*KCNJ11* E23K) almost always contain the alanine allele (*ABCC8* A1369S). While *ABCC8* A1369S/*KCNJ11* E23K (high LD) displayed reduced baseline insulin secretion, they were less likely to develop T2D. However, protection by metformin was abolished in lysine carriers (K/K homozygotes) in a dose-dependent manner, possibly due to suppression of the beneficial effect of metformin on insulin sensitivity or beta cells at one year of treatment (Florez et al., 2007; Marchetti et al., 2004). In Diabetes Prevention Program, 22 SNPs in the *ABCC8-KCNJ11* region exhibited nominally significant interactions with metformin (Jablonski et al., 2010). Thus correlation of rs4148609 (*ABCC8*) with the ability of metformin to prevent T2D has been confirmed in two large-scale studies. However, its mechanism of action as a therapeutic target for T2D remains to be established.

Finally, rs1470579 and rs4402960 (*IGF2BP2*) showed a correlation with metformin efficiency when adjusted for covariates, but this association was not significant after correction for multiple testing. The SNPs rs1470579 and rs4402960 of *IGF2BP2* (coding for insulin-like growth factor 2 mRNA-binding protein 2) are associated with development of T2D and therapeutic efficacy of repaglinide in Chinese T2D patients (Huang et al., 2010). The effects of repaglinide were reduced in patients with rs1470579 C allele carriers while increased treatment efficiency was observed in patients with the rs4402960 T allele (Jacobs et al., 2004). The *IGF2BP2* rs1470579 SNP has significantly different allele frequencies in distinct populations, and a paradoxical trend is observed towards higher insulin secretion in participants with this high-risk genotype (Moore et al., 2008).

We were not able to replicate results with the *CAPN10* rs3792269 A>G polymorphism shown to be significantly associated with reduced metformin treatment

efficiency (Tkac et al., 2015) and the serine racemase rs391300 G/A polymorphism correlated with better response to metformin therapy regarding the levels of serum fasting plasma glucose (FPG), postprandial plasma glucose (PPG) and cholesterol (CHO), but not HbA<sub>1c</sub> in T2D patients with Asian ancestry (Dong et al., 2011).

No correlation was observed between metformin efficiency and 2 SNPs in *ATM* and *STK11*. In the first GWAS and subsequent replication studies, common SNPs near *ATM* (rs11212617) were significantly associated with altered metformin efficiency. However, these data were not replicated in the DPP study (Florez et al., 2012b; GoDarts et al., 2011). The outcome for GWAS was defined as lowest HbA<sub>1c</sub> 0.5–1.5 years after the start of therapy, and the identified genetic marker accounted for only 2.5% variability in response to metformin therapy. *ATM* is involved in DNA repair and cell cycle control, and plays a role in the effects of metformin upstream of AMP-activated protein kinase. An inhibitor of *ATM*, KU-55933, reduced metformin-stimulated phosphorylation of AMPK in H4IIE rat hepatoma cells. Subsequent investigations disclosed that KU-55933 is an inhibitor of OCT1, and its effect on metformin uptake via OCT1 is independent of *Atm* with no detectable influence of *Atm* on OCT1 activity (Woods et al., 2012; Yee et al., 2012). The *STK11* rs8111699 SNP was identified from studies on metformin efficiency in women with polycystic ovary syndrome (PCOS) (Lopez-Bermejo et al., 2010). We could not replicate the association between metformin efficiency and rs8111699, possibly due to the significantly different design and outcomes of the PCOS study. Moreover, rs11212617 appeared to be associated with secondary failure and not primary failure of metformin therapy in our experiments.

The major limitations of our study include lack of tagSNPs in *OCTN1* and *AMPK* coding genes and small sample size, which may underlie the absence of significant associations between the 33 investigated SNPs and metformin efficiency.

#### **6.4. Analysis of T2D susceptibility variants with respect to incidence of T2D**

We performed analysis of the known T2D-associated SNPs, rs12255372, rs7903146 (*TCF7L2*) and obesity-associated SNP rs7561317 (*TMEM18*), in a previously described group of T2D patients from LGDB (Kalnina et al., 2012; Kalnina et al., 2013). We characterized the genetic background of 466 T2D patients from the whole OPTIMED study with the aim of determining associations of genetic variants with T2D. To this end, we compared the OPTIMED group alone and in combination with other T2D patients from LGDB with previously genotyped controls. In our study group, *TCF7L2* rs12255372 and

rs7903146 polymorphisms displayed a significant association with T2D risk (Kalnina et al., 2012; Tong et al., 2009; Vaxillaire et al., 2008). The rs12255372 and rs7903146 polymorphisms demonstrated the strongest association with T2D risk. Thus, as expected, our results agree with previous findings in European populations and other ethnic groups (Assmann et al., 2014; Barros et al., 2014; Chauhan et al., 2011; Park et al., 2013; Shokouhi et al., 2014; Wang et al., 2013; Xi et al., 2014). The ability to replicate this association in the OPTIMED group (e.g., OR and significance values), compared to our previous analysis on T2D patients selected from the Latvian Genome Database, provides additional confidence that the OPTIMED cohort represents a general population of T2D patients, at least with respect to genetic architecture. Accordingly, in the case-control analysis of 2 524 participants (OPTIMED T2D patients were added to the study sample described by Kalnina et al.), we observed an association between increased incidence of T2D and rs7903146 (*TCF7L2*), rs12255372 (*TCF7L2*), but not rs7561317 (*TMEM18*), when logistic regression was adjusted for sex, age and BMI. Even when logistic regression was not corrected for BMI, rs7561317 was not associated with T2D risk, suggesting population-specific differences in the genetics. Addition of the OPTIMED group did not change the results obtained previously with variants of the *TCF7L2* gene.

## CONCLUSIONS

1. The rs628031 and rs36056065 genetic variants in organic cation transporter 1 (*OCT1/SLC22A1*) are correlated with the presence of metformin side-effects, possibly due to altered distribution of metformin in enterocytes.
2. A allele of the intronic variant, rs2289669, in *SLC47A1/MATE1* of metformin users is associated with significantly lower mean BMI and reduced waist circumference.
3. Minor alleles of rs3119309, rs2481030 and rs7757336 located in the intergenic region between *OCT2* and *OCT3* coding genes are associated with non-responsiveness to short-term metformin therapy.
4. The SNPs rs3119309, rs2481030 and rs7757336 are associated with reduced total body exposure to metformin, possibly due to reduced gastrointestinal uptake and/or increased excretion of metformin from blood into kidney cells.
5. The impact of the identified pharmacogenetic variants is the most significant in the first few months of metformin therapy, and may not be detected in cohorts where long-term therapy outcomes are analyzed.
6. No significant correlations are evident for the 33 polymorphisms in genes encoding metformin molecular targets and T2D susceptibility loci with metformin efficacy.

## MAIN THESIS FOR DEFENSE

1. Genetic variabilities in the loci of OCT coding genes contribute significantly to the safety and efficiency of metformin therapy.
2. A transporter-associated mechanism is involved in the development of common side-effects of metformin.
3. Polymorphisms in the OCT1 coding gene are significant pharmacogenetic biomarkers of metformin intolerance.
4. Polymorphisms in the intergenic region between OCT2 and OCT3 are associated with reduced total body exposure to metformin and non-responsiveness to short-term metformin therapy.
5. Associations of identified genetic variants with metformin efficacy vary with different populations and time-points of antidiabetic therapy.
6. Genetic variabilities in *ATM*, *LKB11* and type 2 diabetes mellitus susceptibility genes do not contribute significantly to the efficacy of short-term metformin therapy.

## LIST OF ORIGINAL PUBLICATIONS

1. Tarasova L, Kalnina I, Geldnere K, Bumbure A, Ritenberga R, Nikitina-Zake L, Fridmanis D, Vaivade I, Pirags V, Klovins J. *Association of genetic variation in the organic cation transporters OCT1, OCT2 and multidrug and toxin extrusion 1 transporter protein genes with the gastrointestinal side effects and lower BMI in metformin-treated type 2 diabetes patients*. *Pharmacogenet Genomics*. 2012 Sep;22(9):659-66. doi: 10.1097/FPC.0b013e3283561666.



## APPROBATION OF THE RESEARCH, PUBLISHED THESIS

1. "Copy Number and Sequence Variation in Mendelian and Complex Traits  
Welcome Trust Conference Centre, Hinxton, UK, 24-27 March 2010,"
2. "Replication study of polymorphisms associated with type 2 diabetes in Latvian population", Janis Klovins, Ineta Kalnina, Linda Tarasova, Kristine Geldnere, Valdis Pirags.
3. The 1st OpenGENE Workshop on functional genomics and complex phenotypes in the Baltic Region. "GWAS: from genotyping to sequencing" August 22-26 2010, Tartu, Estonia. Poster presentation "Genetic markers of intolerance to antidiabetic drug metformin OCT1, OCT2 and MATE1 used for treatment of second type diabetes". L. Tarasova, J. Klovins, K. Geldnere, I. Kalnina, V. Pirags
4. The 1st OpenGENE Workshop on functional genomics and complex phenotypes in the Baltic Region. "GWAS: from genotyping to sequencing" August 22-26 2010, Tartu, Estonia, poster presentation "Association of Type 2 Diabetes Risk Alleles in the Latvian Population", Ineta Kalnina, Linda Tarasova, Kristine Geldnere, Valdis Pirags, Janis Klovins
5. Endocrine Abstracts (2011) 26 OC4.3. "Association of genetic polymorphisms in the organic cation transporters OCT1, OCT2 and multidrug and toxin extrusion 1 transporter protein with the metformin intolerance and weight lowering in patients of type 2 diabetes", L Tarasova, J Klovins, I Kalnina, A Bumbure, R Ritenberga, K Geldnere, L Nikitina-Zake & V Pirags
6. 4<sup>th</sup> BBBB International Conference on Pharmaceutical Sciences. „Influence of genetic variations in Organic Cation Transporter 1 and 2 and MATE1 on efficiency and safety of antidiabetic peroral drug metformin therapy”, Linda Tarasova, Ineta Kalnina, Alda Bumbure, Kristine Geldnere, Rota Ritenberga, Liene Nikitina-Zake, Iveta Vaivade, Valdis Pirags and Janis Klovins (Poster presentation)
7. Up Close and Personalized, International Congress on Personalized Medicine Florence, Italy, oral presentation "Association of genetic variation in the organic cation transporters OCT1, OCT2 and multidrug and toxin extrusion 1 transporter protein genes with the gastrointestinal side-effects in metformin treated type 2 diabetes patients", Linda Tarasova, Ineta Kalnina, Kristine Geldnere, Alda

- Bumbure, Rota Ritenberga, Liene Nikitina-Zake, Davids Fridmanis, Iveta Vaivade, Valdis Pirags and Janis Klovins, 02.02.12.-05.02.12.
8. LU 70. Scientific Conference, 2012.01.30., 2. tipa cukura diabēta farmakoģenētisks pētījums personalizētās medicīnas principu ieviešanai klīniskā praksē”, L.Zariņa, L.Tarāsova, I.Kempa, M.Cirse, L.Ļikitina-Zaķe, K.Geldnere, I.Konrāde, A.Lejnieks, J.Kloviņš, V.Pīrāgs
  9. “MODERN ASPECTS AND APPLICATIONS OF CHROMATOGRAPHY IN EDUCATION, RESEARCH AND INDUSTRY”, Daugavpils, 2013.g.16-18.october. “Quantitative determination of metformin in biological samples by liquid chromatography-mass spectrometry”, Dace Hartmane, Solveiga Grinberga, Osvalds Pugovics, Linda Zaharenko. Poster presentation.
  10. 9<sup>th</sup> Congress of Central European Diabetes Association. Organizer: Central European Diabetes Association Federation of International Danube Symposia on Diabetes mellitus. 26 – 28.06.2014 Latvia, Jurmala. Poster presentation. “Pharmacokinetics and pharmacogenetics of metformin in 21 healthy participant after 500 mg single-dose administration”, Linda Zaharenko, Ilze Konrade, Aivars Lejnieks, Valdis Pirags, Dace Hartmane, Solveiga Grinberga, Osvalds Pugovics, Ilze Radovica, Raitis Peculis, Janis Klovins.

## EDUCATION AND RESEARCH EXPERIENCE OF THE AUTHOR

### Education

2005 – 2008	Bachelor degree in Biology, University of Latvia
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2010 – 2013	PhD studies in Biology, University of Latvia

### Employment

2005 - 2009	Latvian Biomedical Research and Study Centre, database operator
From year 2009 till now	Latvian Biomedical Research and Study Centre, scientific assistant

### Research experience

2008 – 2010	European Economic area project EEZ09AP-34/01, „Definition of pharmacogenetic markers for the prediction of efficacy and side-effects of metformin therapy of type 2 diabetes”
2010 – 2014	State Research Programme "Development of advanced prevention strategies, treatment, diagnostic tools and methods, biomedical technologies for improving public health" subproject „Pharmacogenetics of diabetes and cardiovascular diseases, testing of therapeutic target-receptors”
2014 – till now	The National Research programme „Biomedicine for Public Health” (BIOMEDICINE), <i>Project</i> „Molecular Mechanisms, Pharmacogenetics and New Medicines for Treatment of Diabetes and Cardiovascular Complications”

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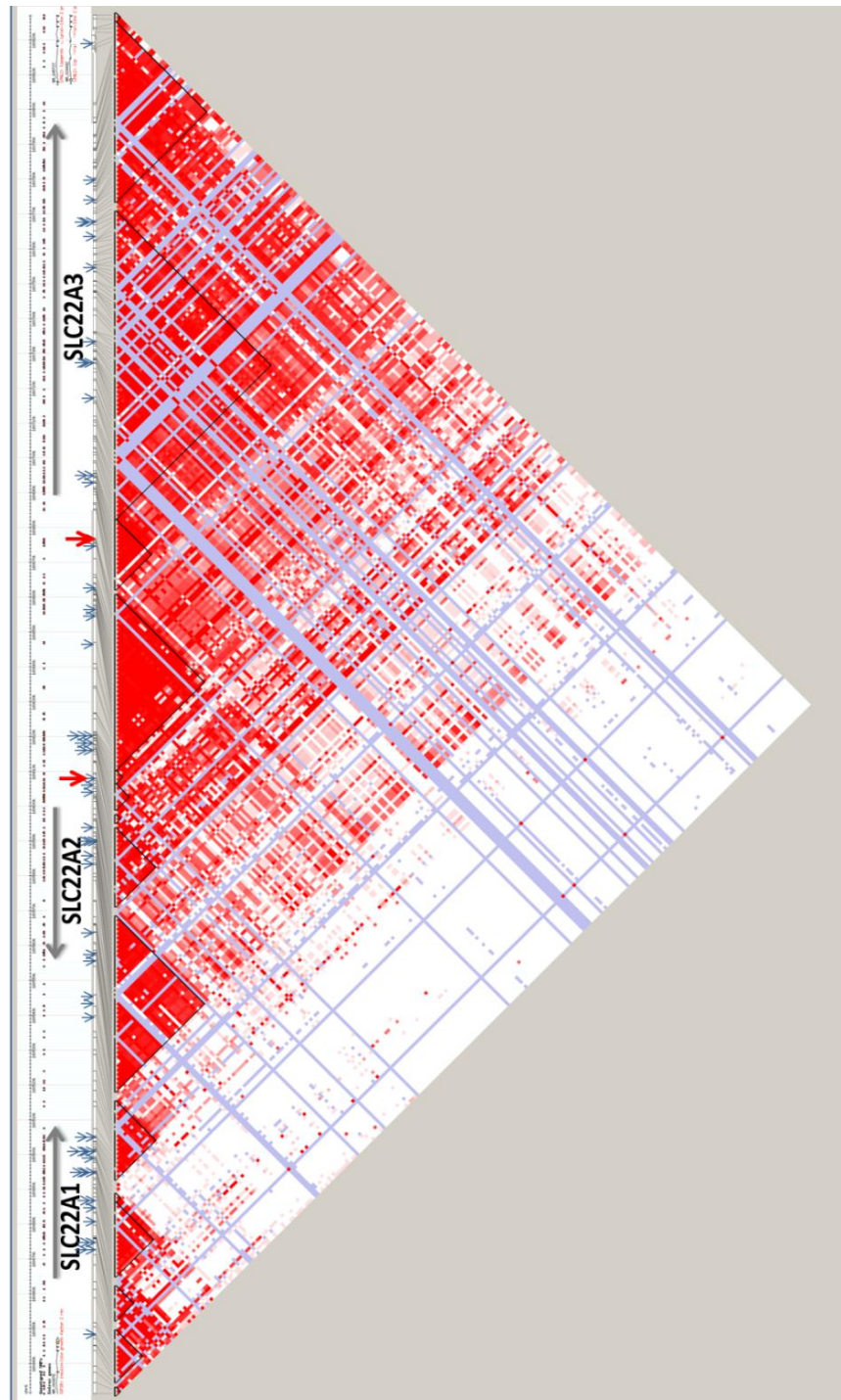
<http://www.ncbi.nlm.nih.gov/> Accessed in January 2015

<https://pubchem.ncbi.nlm.nih.gov/> Accessed in January 2015

# APPENDIX

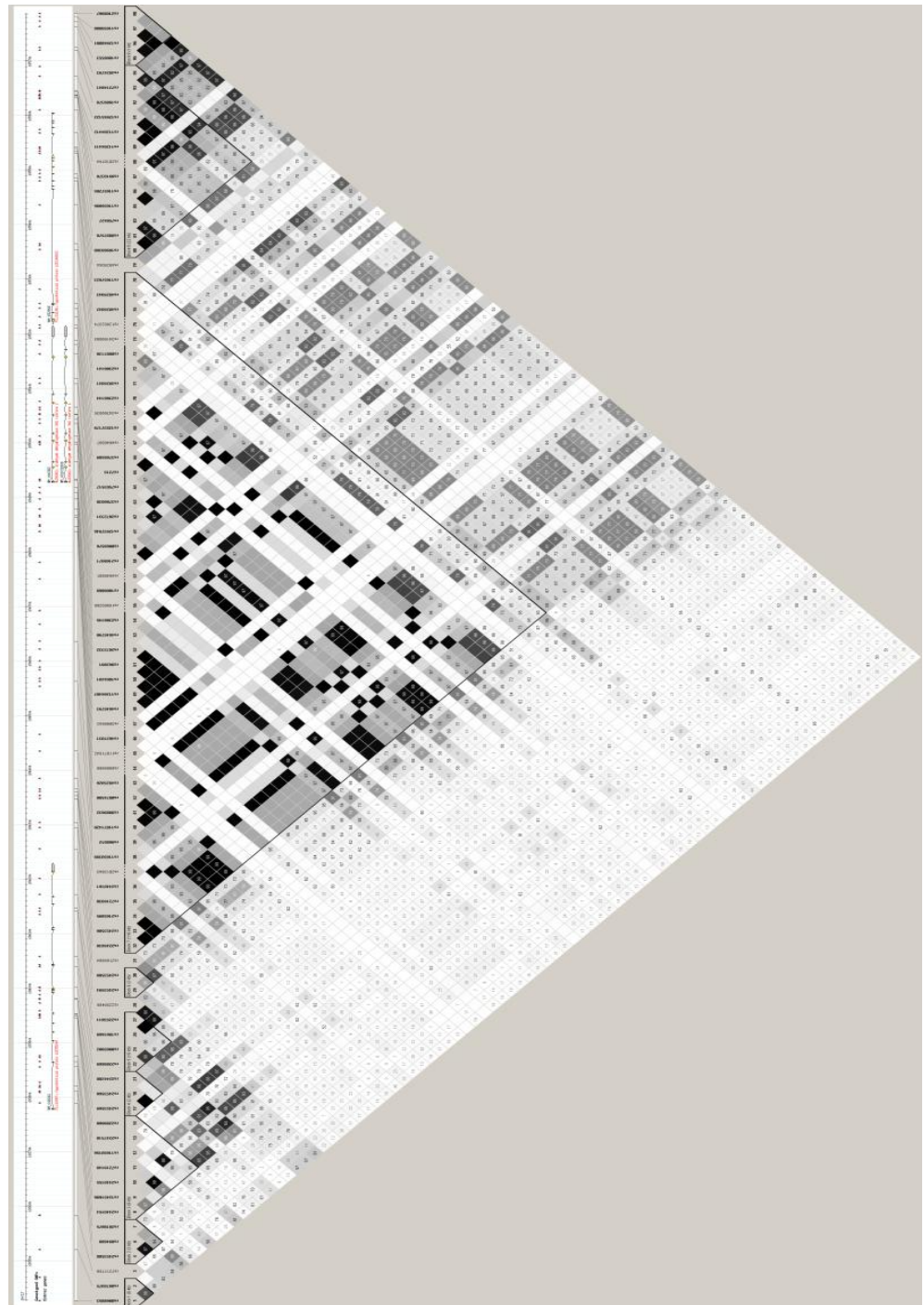
## APPENDIX I

Graphical representation of LD between analysed SNPs in OCT1/SLC22A1, OCT2/ SLC22A2, OCT3/SLC22A3 region. Red shades corresponding to the stronger linkage between polymorphisms. Blue and red arrows represent approximate localization of and 3 most significantly associated tagSNPs in the region, respectively.



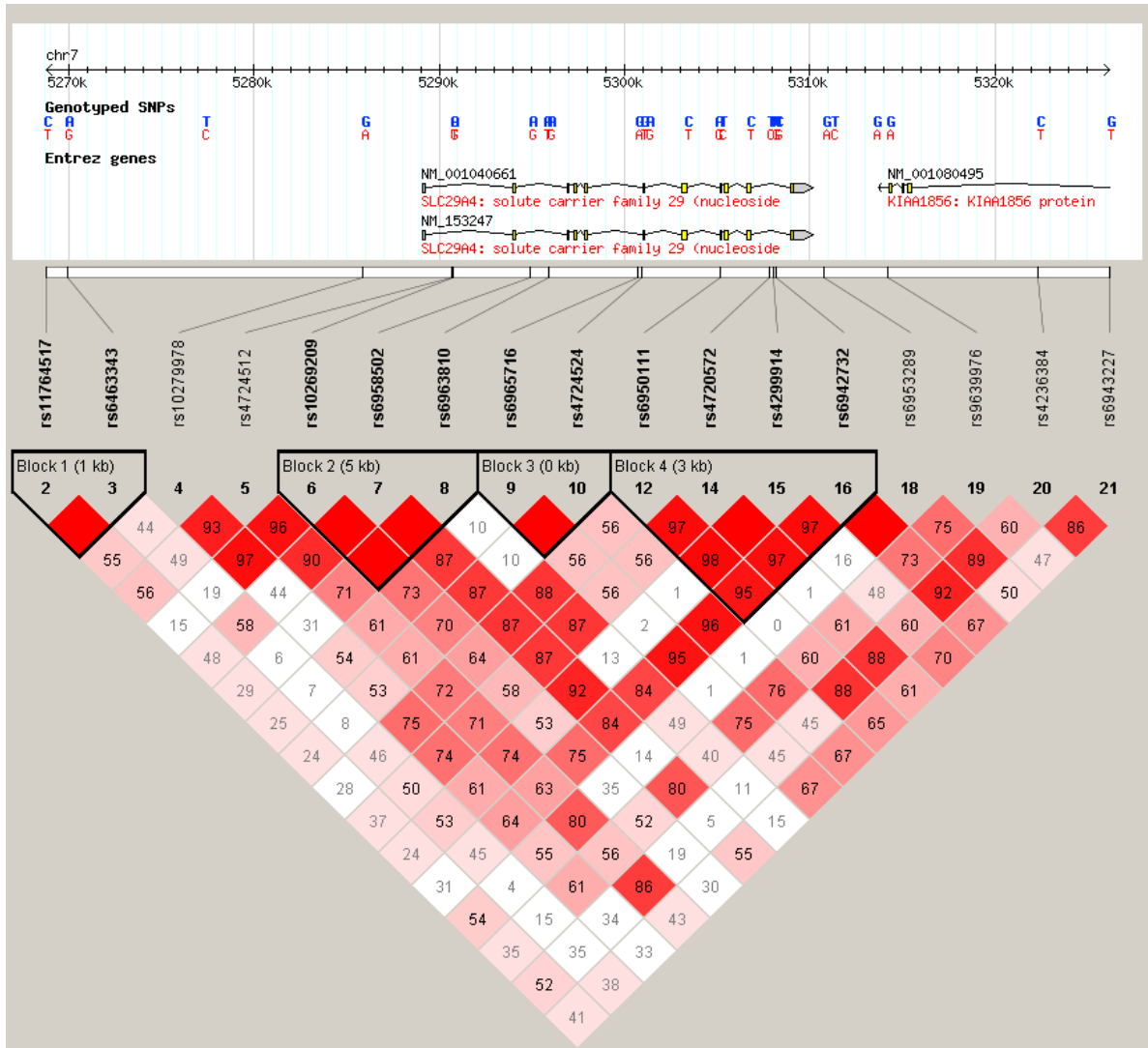
## APPENDIX II

Graphical representation of LD between analysed SNPs in MATE1/SLC47A1, MATE2/SLC47A2 region. Dark shades corresponding to the stronger linkage between polymorphisms.



### APPENDIX III

Graphical representation of LD between analysed SNPs in PMAT/ SLC29A4 region. Red shades corresponding to the stronger linkage between polymorphisms.





## APPENDIX IV

Pharmacogenet Genomics. 2012 Sep;22(9):659-66. doi: 10.1097/FPC.0b013e3283561666.

**Association of genetic variation in the organic cation transporters OCT1, OCT2 and multidrug and toxin extrusion 1 transporter protein genes with the gastrointestinal side effects and lower BMI in metformin-treated type 2 diabetes patients.**

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## **APPENDIX V**

Nominal association of TagSNPs in metformin transporters with metformin inefficiency defined as no change or increase of HbA<sub>1c</sub> levels in comparison to baseline HbA<sub>1c</sub> 3 months after metformin monotherapy.

Chromosome	Gene	SNP	Gene region	Alleles (minor/major)	Position, build 37	Distance between SNPs (bp)	Call rate, %	MAF	Hardy-Weinberg p-value	P-value, nominal	OR, CI 95 %
7	SLC29A4	rs4724512	intron	G/A	5324203	-	97.06	<b>0.26</b>	0.44	<b>0.98</b>	1.01 (0.44 - 2.34)
7	SLC29A4	rs6958502	intron	A/G	5328429	<b>4226</b>	100.00	<b>0.17</b>	0.29	<b>0.62</b>	1.26 (0.50-3.17)
7	SLC29A4	rs4724524	intron	C/A	5334469	<b>10266</b>	100.00	<b>0.44</b>	0.69	<b>0.49</b>	0.77 (0.37-1.61)
7	SLC29A4	rs4299914	intron	G/A	5341570	<b>17367</b>	96.08	<b>0.45</b>	0.07	<b>0.84</b>	0.92 (0.43-1.98)
7	SLC29A4	rs6942732	intron	G/C	5341735	<b>17532</b>	99.02	<b>0.19</b>	0.52	<b>0.36</b>	0.62 (0.23-1.73)
17	SLC47A1	rs2453580	intron	G/A	19438321	-	97.06	<b>0.34</b>	0.51	<b>0.19</b>	0.58 (0.26-1.32)
17	SLC47A1	rs2018675	intron	A/G	19441531	<b>3210</b>	99.02	<b>0.38</b>	0.14	<b>0.84</b>	0.92 (0.44-1.95)
17	SLC47A1	rs2440154	intron	A/G	19444987	<b>6666</b>	100.00	<b>0.40</b>	0.54	<b>0.58</b>	0.81 (0.39-1.71)
17	SLC47A1	rs11650156	intron	G/A	19454374	<b>16053</b>	100.00	<b>0.03</b>	1	<b>0.21</b>	NA
17	SLC47A1	rs2247518	intron	C/A	19454575	<b>16254</b>	99.02	<b>0.18</b>	1	<b>0.45</b>	1.41 (0.58-3.41)
17	SLC47A1	rs2289668	intron	A/G	19454640	<b>16319</b>	100.00	<b>0.06</b>	0.29	<b>0.38</b>	0.41 (0.05-3.26)
17	SLC47A1	rs2453568	intron	A/G	19457509	<b>19188</b>	100.00	<b>0.37</b>	0.39	<b>0.64</b>	0.83 (0.39-1.78)
17	SLC47A1	rs2244280	intron	A/G	19459537	<b>21216</b>	100.00	<b>0.21</b>	0.55	<b>0.47</b>	1.36 (0.59-3.17)
17	SLC47A1	rs2289669	intron	A/G	19463343	<b>25022</b>	100.00	<b>0.40</b>	0.41	<b>0.84</b>	1.08 (0.52-2.24)
17	SLC47A1	rs8065082	intron	A/G	19465191	<b>26870</b>	100.00	<b>0.43</b>	0.42	<b>0.90</b>	0.95 (0.46-1.98)
17	SLC47A1	rs2250486	intron	G/A	19476588	<b>38267</b>	100.00	<b>0.17</b>	1	<b>0.69</b>	1.21 (0.48-3.03)
17	Intergene	rs2453594		G/A	19484951	<b>46630</b>	100.00	<b>0.16</b>	1	<b>0.68</b>	0.81 (0.29-2.25)
17	Intergene	rs2453589		G/A	19488851	<b>50530</b>	89.22	<b>0.39</b>	0.51	<b>0.77</b>	1.12 (0.52-2.39 )
17	Intergene	rs2165894		G/A	19489796	<b>51475</b>	100.00	<b>0.17</b>	0.73	<b>1</b>	1.00 (0.38-2.63)
17	Intergene	rs2440161		G/A	19497890	<b>59569</b>	99.02	<b>0.26</b>	1	<b>0.59</b>	0.79 (0.34-1.87)
17	Intergene	rs968012		A/G	19523055	<b>84734</b>	100.00	<b>0.34</b>	0.52	<b>0.95</b>	0.97 (0.45-2.09)
17	Intergene	rs9889632		A/C	19544138	<b>105817</b>	100.00	<b>0.42</b>	0.69	<b>0.66</b>	0.85 (0.41-1.77)
17	SLC47A2	rs2386141	intron	A/G	19595936	<b>157615</b>	100.00	<b>0.36</b>	0.52	<b>0.98</b>	0.99 (0.47-2.10)
17	SLC47A2	rs4925042	intron	A/G	19608773	<b>170452</b>	70.59	<b>0.33</b>	1	<b>0.58</b>	0.79 (0.34-1.82)
17	SLC47A2	rs4925043	intron	A/G	19612496	<b>174175</b>	98.04	<b>0.07</b>	1	<b>0.29</b>	1.93 (0.57-6.52)
17	SLC47A2	rs11657623	intron	G/A	19612945	<b>174624</b>	57.84	<b>0.17</b>	0.66	<b>0.08</b>	0.28 (0.06-1.28)

Chromosome	Gene	SNP	Gene region	Alleles (minor/major)	Position, build 37	Distance between SNPs (bp)	Call rate, %	MAF	Hardy-Weinberg p-value	P-value, nominal	OR, CI 95 %
17	SLC47A2	rs9897570	intron	G/A	19616245	177924	98.04	0.33	0.65	0.96	1.02 (0.47-2.19)
17	LOC100129125	rs11657205	upstream variant	A/G	19623023	184702	100.00	0.40	0.84	0.86	0.94 (0.45-1.96)
17	Intergene	rs11204411		G/A	19623902	185581	99.02	0.30	0.35	0.73	0.87 (0.39-1.93)
17	Intergene	rs1989379		A/G	19631420	193099	71.57	0.42	0.81	0.94	0.97 (0.44-2.11)
6	IGF2R	rs7753051		G/A	160528057	-	100.00	0.28	0.34	0.47	1.33 (0.61-2.87)
6	SLC22A1	rs12208357	61 Arg>Cys	A/G	160543148	15091	84.31	0.09	1	0.88	1.10 (0.29-4.14)
6	SLC22A1	rs683369	160 Leu>Phe	C/G	160551204	23147	100.00	0.16	0.29	0.11	2.00 (0.84-4.77)
6	SLC22A1	rs3798174	intron	A/G	160552803	24746	100.00	0.08	0.47	0.21	0.29 (0.04-2.28)
6	SLC22A1	rs806383	intron	A/G	160556147	28090	99.02	0.34	0.27	0.20	0.59 (0.26-1.34)
6	SLC22A1	rs3777392	intron	A/G	160556642	28585	100.00	0.16	0.72	0.16	0.42 (0.12-1.45)
6	SLC22A1	rs9457843	intron	A/G	160560933	32876	100.00	0.08	0.47	0.90	1.08 (0.29-4.02)
6	SLC22A1	rs662138	intron	C/G	160564476	36419	100.00	0.20	1	0.37	1.47 (0.63-3.44)
6	SLC22A1	rs3798167	intron	A/C	160567928	39871	100.00	0.24	1	0.52	0.75 (0.31-1.83)
6	SLC22A1	rs2197296	intron	A/G	160568997	40940	100.00	0.33	1	0.48	0.75 (0.34-1.66)
6	SLC22A1	rs650284	intron	C/A	160574535	46478	100.00	0.30	0.82	0.04	2.12 (1.01-4.45)
6	SLC22A1	rs3798164	intron	A/G	160575202	47145	100.00	0.36	0.83	0.27	0.64 (0.29-1.42)
6	SLC22A1	rs11753995	intron	A/G	160575366	47309	100.00	0.19	0.76	0.32	1.53 (0.65-3.59)
6	SLC22A1	rs34059508	465 Gly>Arg	A/G	160575837	47780	100.00	0.05	1	0.39	1.82 (0.46-7.22)
6	SLC22A1	rs609468	intron	A/G	160578914	50857	100.00	0.34	0.52	0.40	0.71 (0.32-1.57)
6	Intergene	rs651164		A/G	160581374	53317	99.02	0.32	0.18	0.87	0.94 (0.43-2.05)
6	Intergene	rs4646283		G/A	160581502	53445	100.00	0.11	1	0.27	0.44 (0.10-1.20)
6	Intergene	rs6455682		A/G	160581911	53854	100.00	0.09	0.16	0.04	NA
6	Intergene	rs9456505		A/G	160582340	54283	100.00	0.09	1	0.24	1.92 (0.64-5.78)
6	Intergene	rs3101826		A/G	160584853	56796	100.00	0.29	0.81	0.87	0.93 (0.42-2.08)
6	Intergene	rs10455864		A/T	160585398	57341	100.00	0.34	0.66	0.22	0.60 (0.27-1.36)

Chromosome	Gene	SNP	Gene region	Alleles (minor/major)	Position, build 37	Distance between SNPs (bp)	Call rate, %	MAF	Hardy-Weinberg p-value	P-value, nominal	OR, CI 95 %
6	Intergene	<b>rs1871389</b>		A/G	160587521	<b>59464</b>	32.35	<b>0.41</b>	0.73	<b>0.79</b>	1.46 (0.09-24.43)
6	Intergene	<b>rs677985</b>		G/A	160619906	<b>91849</b>	100.00	<b>0.26</b>	1	<b>0.54</b>	1.28 (0.58-2.82)
6	Intergene	<b>rs476235</b>		A/G	160622637	<b>94580</b>	100.00	<b>0.29</b>	0.63	<b>0.81</b>	0.91 (0.41-2.02)
6	Intergene	<b>rs2619268</b>		A/C	160625978	<b>97921</b>	100.00	<b>0.21</b>	1	<b>0.24</b>	1.64 (0.72-3.73)
6	SLC22A2	<b>rs10945656</b>		A/G	160635886	<b>107829</b>	100.00	<b>0.10</b>	0.28	<b>&gt;0.01</b>	4.33 (1.67-11.27)
6	SLC22A2	<b>rs694812</b>	UTR-3	G/A	160637890	<b>109833</b>	99.02	<b>0.06</b>	1	<b>0.38</b>	0.40 (0.05-3.22)
6	SLC22A2	<b>rs316002</b>	Intron	A/G	160643819	<b>115762</b>	100.00	<b>0.23</b>	1	<b>0.21</b>	1.67 (0.75-3.73)
6	SLC22A2	<b>rs17588242</b>	Intron	G/A	160663230	<b>135173</b>	100.00	<b>0.19</b>	0.35	<b>0.18</b>	0.48 (0.16-1.43)
6	SLC22A2	<b>rs315996</b>	Intron	A/G	160666365	<b>138308</b>	100.00	<b>0.19</b>	0.51	<b>0.28</b>	1.60 (0.68-3.75)
6	SLC22A2	<b>rs2279463</b>	Intron	G/A	160668389	<b>140332</b>	100.00	<b>0.11</b>	0.61	<b>&gt;0.01</b>	3.67 (1.44-9.31)
6	SLC22A2	<b>rs3912161</b>	Intron	G/A	160669718	<b>141661</b>	100.00	<b>0.06</b>	0.33	<b>0.83</b>	0.84 (0.18-3.96)
6	SLC22A2	<b>rs316019</b>	270 Ser> Ala	A/C	160670282	<b>142225</b>	100.00	<b>0.08</b>	1	<b>0.21</b>	0.29 (0.04-2.28)
6	SLC22A2	<b>rs316012</b>	Intron	A/G	160673884	<b>145827</b>	100.00	<b>0.19</b>	0.52	<b>0.15</b>	1.84 (0.80-4.24)
6	SLC22A2	<b>rs624249</b>	synonymous codon	A/C	160679400	<b>151343</b>	78.43	<b>0.34</b>	0.21	<b>0.05</b>	0.37 (0.13-1.03)
6	Intergene	<b>rs316025</b>		A/G	160683381	<b>155324</b>	100.00	<b>0.23</b>	1	<b>0.21</b>	1.67 (0.75-3.73)
6	Intergene	<b>rs3119309</b>		A/G	160685072	<b>157015</b>	100.00	<b>0.09</b>	1	<b>&gt;0.01</b>	6.80 (2.52-18.31)
6	Intergene	<b>rs316030</b>		C/A	160687866	<b>159809</b>	96.08	<b>0.44</b>	0.84	<b>0.23</b>	1.55 (0.75-3.21)
6	Intergene	<b>rs7757336</b>		C/A	160689558	<b>161501</b>	100.00	<b>0.13</b>	0.68	<b>&gt;0.01</b>	6.22 (2.60-14.88)
6	Intergene	<b>rs492315</b>		A/G	160692694	<b>164637</b>	99.02	<b>0.22</b>	0.56	<b>0.01</b>	0.17 (0.04-0.75)
6	Intergene	<b>rs478112</b>		A/C	160696318	<b>168261</b>	100.00	<b>0.40</b>	0.15	<b>0.63</b>	0.83 (0.39-1.75)
6	Intergene	<b>rs316035</b>		A/G	160696544	<b>168487</b>	100.00	<b>0.13</b>	1	<b>0.05</b>	0.16 (0.02-1.25)
6	Intergene	<b>rs662301</b>		A/G	160696919	<b>168862</b>	99.02	<b>0.04</b>	1	<b>0.15</b>	NA
6	Intergene	<b>rs532440</b>		A/C	160697628	<b>169571</b>	100.00	<b>0.32</b>	1	<b>0.01</b>	0.28 (0.11-0.77)
6	Intergene	<b>rs533452</b>		A/G	160697762	<b>169705</b>	100.00	<b>0.43</b>	0.69	<b>0.20</b>	1.60 (0.78-3.30)
6	Intergene	<b>rs578560</b>		G/A	160699698	<b>171641</b>	100.00	<b>0.16</b>	1	<b>0.02</b>	0.12 (0.02-0.92)

Chromosome	Gene	SNP	Gene region	Alleles (minor/major)	Position, build 37	Distance between SNPs (bp)	Call rate, %	MAF	Hardy-Weinberg p-value	P-value, nominal	OR, CI 95 %
6	Intergene	rs3119312		G/A	160700301	172244	100.00	0.24	0.27	0.27	1.56 (0.70-3.46)
6	Intergene	rs3127583		A/G	160727131	199074	100.00	0.11	0.32	>0.01	4.00 (1.55-10.21)
6	Intergene	rs3125056		G/A	160735281	207224	100.00	0.10	1	>0.01	4.76 (1.80-12.56)
6	Intergene	rs415639		A/C	160736497	208440	99.02	0.18	0.73	0.03	0.23 (0.05-1.00)
6	Intergene	rs367213		G/A	160741894	213837	100.00	0.43	0.16	0.90	0.95 (0.46-2.00)
6	Intergene	rs316167		G/A	160754752	226695	100.00	0.32	0.82	0.03	0.37 (0.15-0.94)
6	Intergene	rs2481030		G/A	160756435	228378	100.00	0.40	0.41	>0.01	6.33 (2.79-14.39)
6	SLC22A3	rs2484241	intron	A/G	160771618	243561	99.02	0.28	0.13	0.01	2.52 (1.19-5.32)
6	SLC22A3	rs512077	intron	A/G	160774928	246871	100.00	0.14	0.42	0.95	0.97 (0.34-2.73)
6	SLC22A3	rs518295	intron	A/C	160775578	247521	100.00	0.50	0.33	0.46	1.31 (0.64-2.70)
6	SLC22A3	rs394468	intron	A/C	160780912	252855	96.08	0.24	0.05	0.02	0.26 (0.08-0.89)
6	SLC22A3	rs6905958	intron	A/C	160797625	269568	100.00	0.48	0.70	0.97	0.98 (0.48-2.02)
6	SLC22A3	rs394352	intron	A/G	160808395	280338	100.00	0.25	0.11	0.01	0.23 (0.07-0.78)
6	SLC22A3	rs2221750	intron	A/G	160808447	280390	100.00	0.22	1	0.73	0.86 (0.35-2.11)
6	SLC22A3	rs7740824	intron	A/C	160808901	280844	100.00	0.26	0.80	0.79	1.12 (0.50-2.51)
6	SLC22A3	rs1567441	intron	G/A	160812262	284205	100.00	0.18	0.74	0.03	0.22 (0.05-0.98)
6	SLC22A3	rs2457571	intron	G/A	160834828	306771	100.00	0.47	0.69	0.01	0.37 (0.17-0.81)
6	SLC22A3	rs9456537	intron	A/G	160842211	314154	100.00	0.16	1	0.41	0.63 (0.21-1.91)
6	SLC22A3	rs4708867	intron	G/A	160842725	314668	67.65	0.06	1	0.21	2.52 (0.56-11.25)
6	SLC22A3	rs17593921	intron	A/G	160846929	318872	100.00	0.03	1	0.03	5.00 (1.00-25.86)
6	SLC22A3	rs2504929	intron	A/G	160847986	319929	100.00	0.26	0.12	0.02	2.44 (1.15-5.20)
6	SLC22A3	rs2665357	intron	C/A	160848167	320110	99.02	0.49	0.69	0.84	1.10 (0.52-2.21)
6	SLC22A3	rs2504928	intron	A/C	160853995	325938	100.00	0.24	0.18	>0.01	2.92 (1.36-6.27)
6	SLC22A3	rs2504927	intron	A/G	160860430	332373	100.00	0.39	1	>0.01	0.26 (0.10-0.66)
6	LPAL2	rs2504920	intron	C/A	160897872	369815	98.04	0.49	0.69	>0.01	3.32 (1.51-7.33)
6	LPAL2	rs6914575	intron	A/G	160898831	370774	99.02	0.21	0.55	0.26	0.56 (0.20-1.55)

## SUPPLEMENTARY METHODS

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was used to determine metformin in human RBC, blood plasma and urine using phenformin as an internal standard (IS). The Acquity UPLC (Waters) ultra performance liquid chromatography system was used in the assay. The chromatographic separation of metformin and IS (phenformin) was achieved on Acquity UPLC BEH HILIC (1.7  $\mu\text{m}$ , 2.1x75 mm) (Waters) column. The LC separation was performed using a gradient elution. Mobile phase A was acetonitrile and mobile phase B was 10 mM ammonium acetate solution (pH 4.0). The elution gradient program was: 0-3.0 min (85 % A), 3.5-5.0 min (50 % A), 5.2-7.0 min (85 % A). The flow rate of the mobile phase was 0.40 ml/min and injection volume was 5  $\mu\text{l}$ . The column and autosampler temperatures were 30 °C and 10 °C, respectively. The MICROMASS QUATTRO micro<sup>TM</sup> API (Waters) triple quadrupole mass spectrometer in MRM mode (multiple reaction monitoring) was used for metformin quantification. The ion source temperature was 120 °C, the desolvation temperature was 400 °C, the desolvation gas flow was 600 l/h, the cone gas flow was 50 l/h and the capillary voltage was 3.3 kV. Nitrogen was used as desolvation gas and argon as collision gas. Metformin was detected using sum of parent to daughter ion transitions  $m/z$  130.1 $\rightarrow$ 60.1 and  $m/z$  130.1 $\rightarrow$ 71.0 (collision energy 12 eV and 20 eV, respectively) and cone voltage 10 V. IS was detected using transition of  $m/z$  206.1 $\rightarrow$ 105.0 (collision 25 eV) and cone voltage 23 V. Biological samples were thawed at room temperature; urine samples were diluted 500 times with deionized water before extraction. Then all samples were extracted using acetonitrile-induced protein precipitation (sample:ACN, 1:30, v/v), vortexed and centrifuged (10000 rpm, 10 min). Supernatants were chromatographed on hydrophilic interaction liquid chromatography (HILIC) analytical column to obtain better retention of polar metformin. The assay was validated for quantitative determination of metformin in human RBC and plasma samples. Quality control samples were prepared at four concentration levels. Accuracy (recovery) ranged from 91,6 to 106,6 % and from 98,3 to 105,7 %, but deviations for precision ranged from 0,6 to 6,0 % and from 1,6 to 7,7 % for RBC and plasma samples, respectively. The limit of quantitation was 5 ng/ml for RBC and plasma and 2,5  $\mu\text{g}/\text{ml}$  for urine. Calibration samples were prepared in the matrix, except for urine, where calibration was prepared in deionized water. The calibration curve was derived from the peak area ratios (metformin/internal standard) using 1/x weighted linear least-squares regression of the area ratio versus the concentration of metformin standard. The obtained six-point calibration curves were characterized by correlation coefficient  $R^2 > 0.99$  over the concentration range of 5-500 ng/ml for RBC, 5-1500 ng/ml for plasma and 2,5-250  $\mu\text{g}/\text{ml}$  for urine samples.



