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Analysis of the potential role of the GLUT1 and the ABCG2 transporters in type 2 diabetes

PhD Thesis

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1. List of abbreviations

ABC	ATP-binding cassette
ADME-Tox	absorption, distribution, metabolism, excretion and toxicity
AF	Alexa-Fluor
ATP	adenosine triphosphate
BBB	blood brain barrier
BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
DN	diabetic nephropathy
DR	diabetic retinopathy
DRC	Drug Research Center
EDTA	ethylene diamine tetraacetic acid
FBS	fetal bovine serum
FSC	forward scatter
GLUT	glucose transporter
GWAS	genome-wide association study
HbA1c	hemoglobin A1c
HEK-293T	human embryonic kidney 293 cell line
HepG2	hepatoma cell line
HOMA	Homeostatic Model Assessment for Insulin Resistance
LD	linkage disequilibrium
MAF	minor allele frequency
Mt	mutant type
NC	negative control
Ns	not significant
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PWM	position weight matrices
RBC	red blood cell
SD	standard deviation
SE	standard error
SLC	solute carrier

SNP	single nucleotide polymorphism
SSC	side scatter
T1DM	type one diabetes mellitus
T2DM	type two diabetes mellitus
TF	transcription factor
TFBS	transcription factor binding sites
TK	thymidine kinase
WGA	wheat germ agglutinin
Wt	wild type

2. Introduction

2.1. Membrane proteins

Membrane proteins are essential components of the cells, comprising approximately 30% of the human proteome (1). They play diverse roles in cellular processes, from transport and signal transduction to the formation of cell-connecting structures. My research is centered around membrane transporter proteins in the context of type two diabetes (T2DM) and the potentially associated genetic variants.

Transporters are a class of integral membrane proteins which enable the passage of substances across the membrane. While channels passively allow the diffusion of small molecules down their concentration gradient, carriers specifically bind the substance and often undergo a conformational change during the process. Carriers can be classified into passive (facilitated) and active (primary, secondary) transporters based on their energy utilization. Facilitative transporters, such as GLUT1 (glucose transporter 1), mediate the movement of membrane-impermeant solutes down their concentration gradient (2,3). In contrast, active transporters have the capability to transport solutes against their concentration gradient by investing energy in the process. Primary active transporters, such as ABCG2 (ATP-binding cassette super-family G member 2), directly obtain the energy necessary for the transport through their ATPase activity. Secondary active transporters, on the other hand, carry out transport using the electrochemical ion gradient resulting from the operation of primarily active pumps. In this process, the ion can move along its gradient, which provides free energy for the transport of the other substance against its gradient. Based on the number of substances and the direction of the transport, carriers can further be categorized as uniporters, symporters, and antiporters (3).

The significance of the membrane transporters in the drug development is substantial, since they can impact the ADME-Tox properties (absorption, distribution, metabolism, excretion, and toxicity) of the drugs. Members of the SLC (solute-carrier) and ABC (ATP-binding cassette) transporter superfamily hold particular importance in this regard (4).

2.1.1. The SLC superfamily – the SLC2 family

One of the largest groups of transporters is the so-called solute carrier (SLC) superfamily. Although the members of this superfamily are grouped together based on evolutionary homology, they differ significantly in terms of their mechanism of operation. There are members with secondary active transport function (e.g. SGLT2), while other SLC proteins carry out passive, facilitated diffusion (e.g. GLUT1) (5). According to the simultaneous transport of substances, there are both uniporters, symporters, and antiporters in this group.

The human SLC superfamily includes more than 400 proteins responsible for the transport of a wide variety of substances (ions, amino acids, sugars, etc.). In recent years, SLC transporters have been increasingly recognized as key pharmacological targets (3,5). Currently, 66 SLC protein families can be distinguished (6), based on 20-25% sequence identity (3,7). In addition, a family is usually characterized by the range of transported substrates.

Due to their essential role in the metabolism and the proper functioning of the cell, mutations in the genes encoding SLC proteins can be linked to the development of different diseases. Some mutations – through the reduced or inappropriate function or expression of the proteins – result in an imbalance of ions and metabolites (7,8). According to OMIM (Online Mendelian Inheritance in Man database), nearly half of the members of the superfamily can be linked to hereditary diseases (e.g. S2538 (*SLC25A38*) - anemia) (9), and their polymorphisms are also associated with complex diseases (e.g. MOT11 (*SLC16A11*) – T2DM; GLUT9 (*SLC2A9*) - gout) (10,11). In addition, the SLC proteins can also influence the outcome of the diseases through the absorption and distribution of the drugs, therefore medicines targeting directly SLC proteins are already available (12,13). Such target proteins are mostly found in the SLC5, SLC6, SLC12 and SLC22 families (14).

Currently three SLC families (SLC2, SLC5, SLC50) are known to play a role in maintaining glucose homeostasis, essential for the energy supply of the cells (15,16).
The 12 members of the SLC5 family carry glucose with secondary active transport against the gradient, enabled by the transport of sodium ions along their gradient. In addition to glucose, the family has many other substrates, such as other mono- and

disaccharides (galactose, mannose, fructose), anions, vitamins and short chain fatty acids (15). The members specialized to glucose transport are SGLT1 and SGLT2 (sodium glucose cotransporter 1/2; *SLC5A1/2*) proteins. As their main function, SGLT1 performs glucose absorption in the small intestine, while SGLT2 is responsible for the reabsorption of the glucose in the kidney, through the cells of the proximal tubule (17). Both of these transporters show strong association with diabetes, and are already targets of available drugs (18,19).

The SLC50 family is a recently discovered group of proteins with glucose transport function. In humans only one member, SWEET1 (*SLC50A1*) has been described. It is expressed in the intestine, oviduct, epididymis and mammary gland (15). According to some assumptions, in the mammary gland SWEET1 may contribute to lactose synthesis (20), while in the intestine it may contribute to the release of glucose into the bloodstream (15,21).

The glucose transporter (GLUT, SLC2) family with its 14-members is generally characterized as uniporters, that facilitate the passive transport of glucose and other monosaccharides (also some other cyclic compound, such as polyols) along the concentration gradient (22). GLUT13 (*SLC2A13*) is an exception, since it is a proton-linked symporter (also known as HMIT; proton-coupled myoinositol transporter) (23). The members of the family are structurally similar, consisting of approximately 500 amino acids and 12 transmembrane helices (24). The amino- and carboxyl-ends face the cytoplasm. These proteins have a central cytoplasmic linker domain and a single N-linked oligosaccharide chain on the extracellular side (25).

Based on sequence similarity, GLUT proteins can be classified into three groups (22). The Class I (GLUTs 1–4, 14) and Class II (GLUTs 5, 7, 9, and 11) members differ structurally from the Class III (GLUTs 6, 8, 10, 12, and HMIT) proteins in terms of the location of N-linked glycosylation. While in case of Class I and II glycosylation is located on the first extracellular linker domain, in case of Class III it is located on the fifth. Although the mechanism and the structure of the proteins are similar, there are distinct tissue-specific expressions and affinities for substrates (see **Figure 1.**) (22).

Without claiming completeness, the followings section outlines the tissue-specific functions of GLUT transporters. In the small intestine glucose absorption occurs via GLUT7 (along with SGLT1), while fructose enters the enterocytes through GLUT5

(26,27). In the basolateral membrane of the enterocytes GLUT2 is responsible for the export of the monosaccharides to the bloodstream (28). Blood glucose is transported to the red blood cells (RBC) mostly via GLUT1 (29). The circulating glucose crosses the blood brain barrier (BBB) via GLUT1 (30) and reaches the neurons through the GLUT3 transporter (31). In the pancreas, the beta cells sense blood glucose levels by glucose uptake via GLUT1 or GLUT2 (32,33), regulating insulin secretion. In response to the insulin signal, liver cells take up glucose via GLUT2, contributing to its breakdown (glycolysis) or storage as glycogen (34). When insulin reaches muscle and fat cells, glucose uptake is carried out mainly by GLUT4 (22,35). Glucose is efficiently reabsorbed, and little is lost to urinary excretion, due to the combined actions of various GLUT type (GLUT1 (36), GLUT2 (22), GLUT5 (37), GLUT9 (38,39)) and other transporters in the kidney.

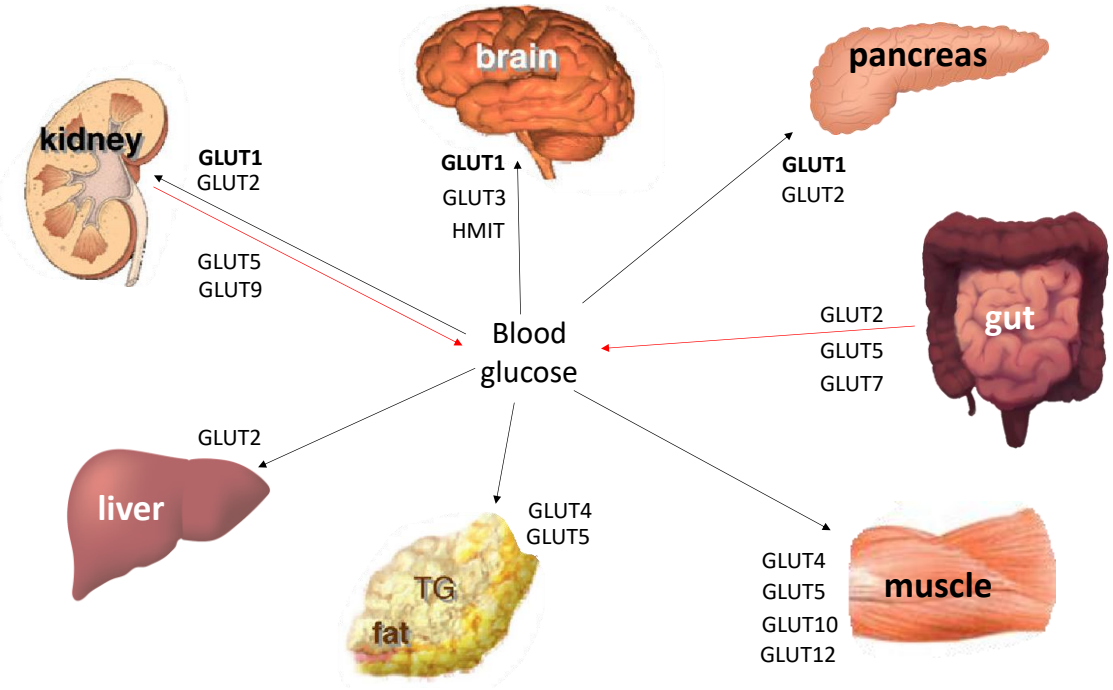

Figure 1. Key GLUT proteins in different organs.

The entry of glucose (and other monosaccharides) through the intestine into the bloodstream and its subsequent delivery to the highlighted organs via the specified glucose transporters (modified version of a figure from Muecler et al. (22)).

2.1.1.1. The GLUT1 membrane protein

GLUT1 is one of the most intensively researched transporters, not only within the SLC2 family but also among all membrane transporters. Studies of this protein began in 1977, with its extraction from the membrane of human RBCs (40).

GLUT1, being the primary glucose transporter in RBCs, creates approximately 5% of the plasma membrane proteins in these cells (29). This allows for the rapid equalization of glucose levels between the blood plasma and the RBCs and increases the overall glucose-carrying capacity of the blood (22). GLUT1 also plays a crucial role in maintaining basal glucose levels for various other cell types. For instance, it is found in the endothelial cells of the BBB, thereby ensuring the glucose uptake to the brain (30). In the placenta, GLUT1 is particularly important in providing glucose, as the vital energy source, for the developing fetus (29). GLUT1 can also be found in the endothelial cells of the kidney (36) and the beta cells of the pancreas, among other cell types (41).

Although the principal substrate of GLUT1 is glucose, the transport of several other monosaccharides (e.g. mannose, galactose, fructose) and vitamin C are also facilitated by this transporter (24). The affinity of GLUT1 to glucose is higher at the outer substrate-binding domain, allowing more efficient glucose uptake from the environment.

The 55 kDa GLUT1 protein, consisting of 492 amino acids, is the most conserved member among GLUT transporters, showing about 97-98% sequence identity with homologous rat, mouse, rabbit and pig proteins (29,42). Similarly, to the other GLUT members, it contains 12 transmembrane domains, and both the N (amino) and C (carboxyl) ends are localized in the cytoplasm. As a Class I member, GLUT1 has a glycosylation (N45) on the first extracellular linker domain (**Figure 2/A**) (25).

GLUT1 is encoded by the *SLC2A1* (solute carrier family 2-facilitated glucose transporter member 1) gene, located in the 34.2 position of the short (p) arm of chromosome 1 [1p34.2; https://ghr.nlm.nih.gov/gene/SLC2A1] (43). The gene includes 33,802 base pairs, with 3 putative enhancer regions and 10 exons (**Figure 2/B**).

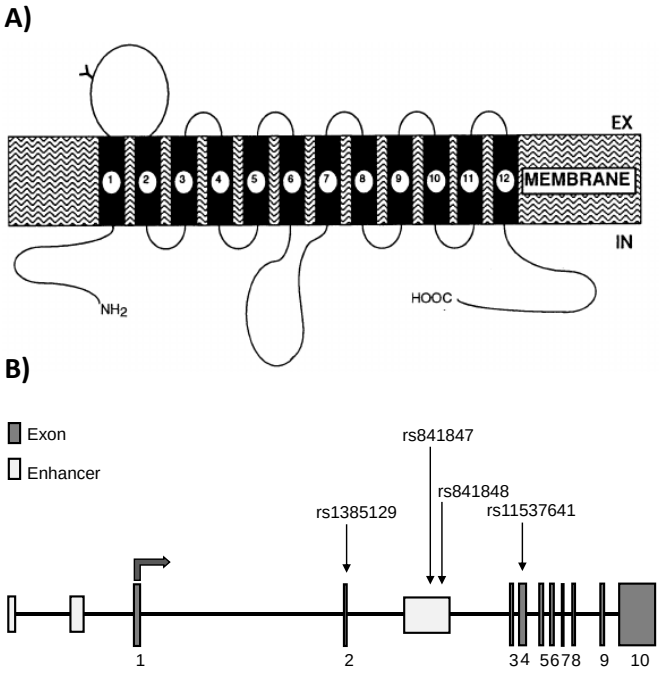

Figure 2. Schematic representation of the GLUT1 membrane protein and the coding *SLC2A1* gene. The protein consists of 12 transmembrane helix domains, both the N (amino) and C (carboxyl) ends are localized in the cytoplasm, while there is a glycosylation (N45) on the first extracellular linker domain. Figure from Barrett et al. (44). The coding *SLC2A1* gene consists of 10 exons (indicated as 1–10) and three putative enhancers (indicated with light grey bars). The 4 examined SNPs are also presented. Figure from Kulin et al. (45).

Abnormal function or expression of this protein are linked to serious disease conditions. Missense and nonsense mutations are very rare and, since GLUT1 is a major glucose transporter in the BBB, these changes cause serious problems in the nervous system. For instance, functional mutations in the *SLC2A1* gene lead to the development of the rare disease, GLUT1 deficiency syndrome. The limited brain glucose availability results in symptoms such as epileptic seizure, coordination disorder (ataxia), and cognitive impairment (22,46,47).

Changes in gene regulation driven by frequent genetic variations (polymorphism) occur more frequently and are associated with several diseases. Reduced GLUT1 expression at the BBB was found to be associated with Alzheimer's disease, characterized by the decreased glucose transport in the early stage of the disease (48,49). In various tumor cells, however (especially under hypoxic conditions), the GLUT1 transporter is overexpressed and this has an important role in the elevated glucose metabolism of these cells (50–52).

Although GLUT1 cannot be linked directly to the development of diabetes, it may strongly influence the progression of the disease. According to a study in nephropathic rats, GLUT1 expression is higher in the glomerular cells of the kidney (53). Moreover, in humans, an increased GLUT1 expression level in the placenta was observed, during gestational diabetes, T1DM (54) and T2DM (55).

2.1.1.2. Genetic variants in the *SLC2A1* gene

GWA (genome-wide association) studies have identified thousands of risk loci linked to various human diseases, including diabetes mellitus. However, only a small proportion of these variants are located within exons and can directly modify protein function by altering the amino acid sequence. The majority of the variants are located in non-coding regions, and their significance stems from being enriched in regulatory regions. These GWAS variants can influence enhancer/promoter activities by altering transcription factor binding sites. However, their functional activity and contribution to disease susceptibility is questionable. Within risk loci, genetic variants exhibit high correlation due to the linkage disequilibrium. These correlated variants move together from generation to generation, forming so called haploblocks. Within these haploblocks, variants are indistinguishable based solely on genetic analysis. In many cases, the statistically significant genetic variant serves merely as a marker of the disease due to the high LD (linkage disequilibrium), rather than being the causal variant. The causal variant drives the observed changes, such as alterations in gene expression levels or the risk of disease development. In eQTL (expression quantitative trait loci) studies, it is the specific variant that alters gene expression by affecting regulatory elements, while in genetic association studies of diseases, a causal variant directly contributes to the altered risk of the condition. By combining these two analyses could be a powerful tool in the study of complex diseases. It is crucial to identify the causal variant that functionally contributes to disease development, and to analyze them individually using various molecular biology tools, enabling the understanding of underlying molecular disease mechanisms.

GWAS studies are not necessarily suitable for uncovering the effect of all genetic variants, because single nucleotide polymorphisms (SNPs) often have small effect sizes on disease risk. In large-scale studies, these small effect sizes can become challenging

to uncover, however in case-control studies conducted on smaller cohorts, the effect of these genetic variants can also be found.

According to case-control studies, T2DM and the complications of this disease (e.g. diabetic nephropathy and diabetic retinopathy) are associated with some *SLC2A1* SNPs, including rs1385129, rs841847, and rs841848 (56–58). Given their potential risk in the disease I chose these SNPs to analyze their role in T2DM. In addition, during my investigations, I found rs11537641 SNP, which I also included in the further analyses (details about the reason of the selection are described in the results section).

The four SNPs in the *SLC2A1* gene examined in my studies have minor allele frequencies between 0.19–0.25 in Europe, thus are present in a relatively large number of the populations (**Table 1.**).

Table 1. Key data of the examined SNPs.

G/C: wild-type allele (wt); A/T: mutant allele (mt); MAF: Minor Allele Frequency; Enh-2: enhancer 2. Table from Kulin et al. (45).

SNP	Alleles (wt>mt)	MAF (EUR)	Location
rs1385129	G>A	0.22	Exon 2
rs841847	C>T	0.25	Intron 2, Enh-2
rs841848	G>A	0.20	Intron 2, Enh-2
rs11537641	G>A	0.19	Exon 4

Variant rs1385129, as one of the most studied *SLC2A1* SNPs in diabetes, was found to increase the risk of diabetic nephropathy in Tunisian (56) and Kurdish (57) cohorts. This synonymous SNP is in exon 2 and is a member of a large haplotype block (**Table 2.**). Variants rs841848 and rs841847, located in intron 2, were also associated with T2DM complications (56,59). SNP rs841847 has been described as a risk factor in diabetic retinopathy and nephropathy (58), while results for rs841848 have been controversial (56,60,61). These intronic variants are in a putative enhancer (enh-2) region of *SLC2A1*, which further increases the suspicion that these are causal SNPs. Besides, variant rs841848 is a member of a relatively large haplotype block (**Table 3.**).

Table 2. Haplotype block of the rs1385129 variant. The alleles shown here are the parts of the haplotype. The table contains the frequency of the minor alleles (MAF), the distance from the rs1385129 SNP and the localization (gene, region) of the variants. Linkage disequilibrium (LD) among the included SNPs is at least 0.8 (R^2/D'). This LD matrix was made with LD Link database from European population. R^2/D': linkage indicators, R^2 is sensitive to allele frequency; IGR: intergenic region; *SLC2A1-AS1*: *SLC2A1* antisense long non-coding RNA.

RS_Number	Allele	MAF	Distance	R2	D'	Gene	Region
rs1385129	G	0.22 (A)	0	1.00	1.00	*SLC2A1*	exon 2
rs710223	C	0.21 (G)	2072	0.93	0.99	*SLC2A1*	intron 1
rs1622805	C	0.21 (T)	2401	0.93	0.99	*SLC2A1*	intron 1
rs1681859	G	0.21 (A)	2863	0.93	0.99	*SLC2A1*	intron 1
rs900834	G	0.21 (A)	3377	0.93	0.99	*SLC2A1*	intron 1
rs900836	C	0.21 (T)	3761	0.91	0.97	*SLC2A1*	intron 1
rs900837	G	0.21 (A)	3827	0.91	0.97	*SLC2A1*	intron 1
rs2297977	G	0.21 (T)	6322	0.91	0.97	*SLC2A1*	intron 1
rs710220	G	0.23 (T)	6380	0.92	1.00	*SLC2A1*	intron 1
rs10890232	C	0.23 (T)	7548	0.92	1.00	*SLC2A1*	intron 1
rs4660691	G	0.21 (T)	8184	0.91	0.97	*SLC2A1*	intron 1
rs2297972	T	0.23 (C)	9060	0.92	1.00	*SLC2A1*	intron 1
rs3754223	T	0.21 (A)	10125	0.91	0.97	*SLC2A1*	intron 1
rs3768043	A	0.23 (C)	10388	0.92	1.00	*SLC2A1*	intron 1
rs3768044	T	0.23 (C)	11188	0.92	1.00	*SLC2A1*	intron 1
rs710219	G	0.21 (A)	13038	0.91	0.97	*SLC2A1*	intron 1
rs11537640	T	0.21 (G)	15553	0.90	0.97	*SLC2A1*	5'UTR
rs28365848	T	0.21 (-)	15745	0.90	0.97	IGR	-
rs3806401	A	0.21 (C)	17246	0.90	0.97	*SLC2A1-AS1*	intron 1
rs3738514	C	0.21 (T)	17625	0.90	0.97	*SLC2A1-AS1*	intron 1
rs710218	T	0.23 (A)	18252	0.91	0.99	*SLC2A1-AS1*	intron 1
rs710216	A	0.21 (G)	19791	0.91	0.98	*SLC2A1-AS1*	intron 1
rs710215	T	0.21 (C)	20139	0.90	0.98	*SLC2A1-AS1*	intron 1
rs841563	C	0.21 (T)	20554	0.90	0.98	*SLC2A1-AS1*	intron 1
rs859511	C	0.21 (T)	21294	0.90	0.99	*SLC2A1-AS1*	intron 1
rs12039898	G	0.21 (A)	21469	0.90	0.99	*SLC2A1-AS1*	intron 1
rs9439065	G	0.21 (A)	23159	0.90	0.99	*SLC2A1-AS1*	intron 1
rs9439066	C	0.21 (T)	23170	0.90	0.99	*SLC2A1-AS1*	intron 1
rs12041787	G	0.21 (C)	23309	0.90	0.99	*SLC2A1-AS1*	intron 1
rs2886876	G	0.21 (A)	23775	0.89	0.98	*SLC2A1-AS1*	intron 1
rs841570	C	0.20 (G)	26605	0.90	0.99	*SLC2A1-AS1*	intron 1
rs841573	C	0.20 (T)	27403	0.90	0.99	*SLC2A1-AS1*	intron 2
rs710214	C	0.22 (T)	30316	0.83	0.91	*SLC2A1-AS1*	intron 2
rs844501	G	0.22 (A)	31817	0.83	0.91	*SLC2A1-AS1*	intron 3

Table 3. Haplotype block of the rs841848 variant. The alleles shown here are the members of the haplotype. The table contains the frequency of the minor alleles (MAF), the distance from the rs841848 SNP and the localization (gene, region) of the variants. Linkage disequilibrium (LD) among the included SNPs is at least 0.8 (R^2/D'). This LD matrix was made with LD Link site from European population. R^2/D': linkage indicators, R^2 is sensitive to allele frequency.

RS_Number	Allele	MAF	Distance	R2	D'	Gene	Region
rs841858	G	0.17 (T)	-3378	0.82	0.99	*SLC2A1*	intron 2
rs841857	C	0.19 (T)	-2529	0.91	0.99	*SLC2A1*	intron 2
rs841855	G	0.19 (A)	-2369	0.92	0.99	*SLC2A1*	intron 2
rs841854	C	0.19 (T)	-2060	0.93	0.99	*SLC2A1*	intron 2
rs1770811	A	0.20 (T)	-1998	0.99	1.00	*SLC2A1*	intron 2
rs841852	C	0.20 (T)	-1046	1.00	1.00	*SLC2A1*	intron 2
rs841851	A	0.20 (G)	-716	1.00	1.00	*SLC2A1*	intron 2
rs841850	T	0.20 (C)	-491	1.00	1.00	*SLC2A1*	intron 2
rs841849	G	0.19 (A)	-340	0.94	1.00	*SLC2A1*	intron 2
rs841848	G	0.20 (A)	0	1.00	1.00	*SLC2A1*	intron 2
rs841845	T	0.20 (C)	2168	1.00	1.00	*SLC2A1*	intron 2
rs56935581	C	0.20 (T)	2919	1.00	1.00	*SLC2A1*	intron 2
rs59042803	C	0.20 (T)	3001	1.00	1.00	*SLC2A1*	intron 2
rs1770810	G	0.19 (A)	3568	0.94	1.00	*SLC2A1*	intron 2

2.1.2. The ABC superfamily – the ABCG subfamily

The ATP-binding cassette (ABC) transporters are evolutionary conserved proteins belonging to one of the largest superfamilies, and are found in all organism from bacteria to humans (62,63).

The ABC transporters are generally consisting of 4 subunits, two of which are the transmembrane domains (TMD), responsible for the substrate transport, and two nucleotide binding domains (NBD), responsible for ATP binding and hydrolyzation (64). The transmembrane domains typically contain six membrane-spanning alpha-helices with variable amino acid sequences. Some ABC proteins (members of the ABCE and ABCF subfamily) do not have membrane spanning domains (65,66). However, there is no ABC protein without the sequentially conserved nucleotide binding domains (67). The three main unit of the NBD are the Walker A and B domains, found in all ATP-binding proteins and the C domain (signature motif), specific to the ABC proteins (68,69). Generally, these transporters must contain two TMDs and two NBDs for functional activity. Full transporters, such as the ABCB1

protein have these four elements in a single polypeptide chain, while half-transporters, such as the ABCG2 protein, contains only one of these domains. To generate a functional transporter, they must form hetero- or homodimers.

ABC transporters carry out primarily active transport with the help of the energy released after ATP binding and hydrolyzation (70). The range of transported substrates is very broad, from small ions to larger polar, amphipathic and hydrophobic organic substances (e.g. lipids, carbohydrates, amino acids) (69). These substrates can be transported in two directions (import, export) but importers are specific only to the bacterial cells (and rarely to endosymbiotic organelles) (71).

The main function of some ABC transporters is the removal of xenobiotics from the cell, which is important in the protection of the cell from the toxic compounds (chemo-immunity). As they can influence the ADME-Tox properties of the drugs, ABC transporters have a significant role in the clinical pharmacology. They are highly expressed in the epithelial cells of the BBB, the intestinal enterocytes (absorption barrier), the hepatocytes, and the epithelial cells of the proximal tubules of the kidney (excretory barriers). In cancer cells, overexpression of certain ABC transporters (e.g. ABCB1, ABCC1, ABCG2) can be observed, which results in the development of multidrug resistance. In addition, they are able to remove many other active ingredients (e.g. antibiotics, antidepressants, anti-epileptics, etc.). The members of this superfamily also play a role in lipid metabolism, and other cellular metabolic processes (4,64,72).

According to the Human Genome Organization, there are 51 ABC genes (6), of which 48 encode proteins (the remaining three are pseudogenes). These genes are classified into 7 subfamilies (ABCA-G) based on domain structure and sequence identity (63). The ABCG subfamily has five members, forming four proteins, as ABCG5 and ABCG8 form a heterodimer. These proteins has a role in lipid homeostasis, with the exception is ABCG2, which main role is the elimination of endo- and xenobiotics (e.g. drugs) out of the cell (73).

2.1.2.1. The ABCG2 membrane protein

ABCG2 (BCRP, MXR) homodimer is found in various physiological barriers such as the intestine, blood-brain barrier, and blood-placental barrier. It is a multispecific protein, responsible for the elimination of xeno- and endobiotics from the cell. Since these chemicals are usually toxic for the cells, ABCG2 contributes to chemo immunity. ABCG2 plays a pivotal role in the ADME-Tox properties of drugs and is implicated in multidrug resistance (4). In addition, ABCG2 also serves a crucial role in the uric acid transport, both in the intestine and in the kidney (74).

The ABCG2 protein with a molecular weight of 72 kDa consists of 655 amino acid residues. The monomer structure features six transmembrane helix domains, and the nucleotide-binding domain situated in the cytoplasm, along with both the N (amino) and C (carboxyl) termini. The N-glycosylated residue and the cysteinyl disulfide bridges are located on the extracellular side of the membrane (**Figure 3/A**)(75).

The *ABCG2* gene is located on chromosome 4 and consists of 16 exons, the first of which is part of the 5'UTR (**Figure 3/B**). The general START site of the protein is located in exon 2, but there are several splicing variants (76). The gene has several mutations that cause changes in the amino acid sequence and affect the protein's expression level, function, or trafficking.

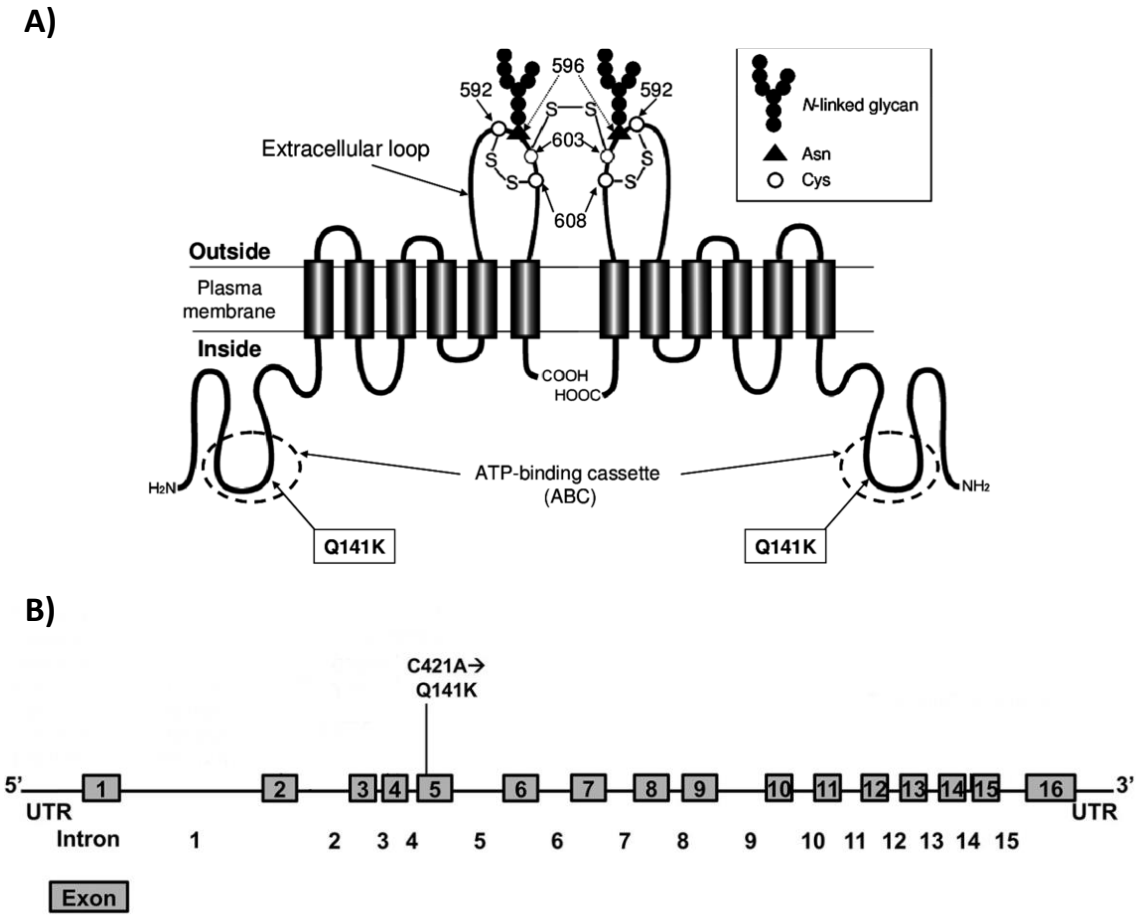

Figure 3. Schematic representation of the ABCG2 membrane protein and the coding gene. The monomer consists of 6 transmembrane helix domains, both the N (amino) and C (carboxyl) ends are facing the cytoplasm. The functional protein is a homodimer, linked via cysteinyl disulfide bridges (circles). The N-glycosylated residue depicted with a triangle (75). The *ABCG2* gene consists of 16 exons, and the general start site of the protein is in exon 2, while the Q141K variant is located in the 5th exon. Modified figure of Bircsak et al.(77).

The decreased expression of ABCG2 results in significantly higher uric acid level, which is a risk factor in the development of gout (78). Besides, uric acid level shows association with other diseases, such as hypertension, T2DM and chronic kidney failure (79,80). Wei et al. found that in patients with T2DM, serum uric acid and HbA1c (hemoglobin A1c) are negatively correlated, but in control samples these parameters showed a positive correlation (79). Although there are contradictions regarding these correlations (81), the question arises as to whether ABCG2 plays a role in these conditions.

2.1.2.2. The ABCG2-Q141K variant

One of the most researched polymorphisms of the coding gene is rs2231142 (C421A), resulting in the ABCG2-Q141K protein variant. GWA studies and molecular genetic investigations showed significant association between the presence of this polymorphism and the decreased level of the protein (79,82,83). The variant can be found in 10% of the European population (MAF=0.1), while in some Asian populations it has a much higher frequency (MAF=0.3).

This variant negatively influences the folding and processing of ABCG2, resulting in the decreased expression level of the protein. Based on the correlations observed so far, it is not surprising that this variant contributes to elevated uric acid levels and thus to the development of gout. In addition, it has been also reported that the Q141K variant affects the pharmacokinetics of several drugs (84).

2.2. Type two diabetes mellitus

According to the IDF's 2022 estimate (International Diabetes Federation, Diabetes Atlas 10th edition), diabetes mellitus is one of the most common metabolic diseases nowadays, affecting more than 500 million adults worldwide (85).

When the human body is functioning normally, it responds to high blood glucose levels with negative feedback. In the pancreas, beta cells of the islets produce insulin in response to high blood glucose concentrations, which gives a signal for the liver cells to take up glucose and store it in the form of glycogen (86). Additionally, it also serves as a signal for muscle and fat cells, to stimulate the translocation of GLUT4 transporter, thereby increasing the glucose uptake, resulting in lower blood glucose levels. The two main forms of diabetes are type one diabetes, characterized by an initial deficiency in insulin secretion, and type two diabetes, where insulin-sensitive cells become resistant and then secretion is also impaired. It is important to note however, that there is no sharp distinction between the two types, rather a transition can be observed between them (87). In addition, according to the current classification, there are several other types (pl. gestational diabetes, maturity onset diabetes of the young (MODY), etc.) of the disease (85).

Type two diabetes accounts for the vast majority (over 90%) of diabetes worldwide (85). The rapid growth can be attributed to environmental (industrialization) and

lifestyle (carbohydrate-rich diet and the sedentary lifestyle) changes, which together with the genetic predisposition can all contribute to the development of the disease, making it difficult to apply an appropriate treatment (86,88). It is also a significant challenge that in several cases the diagnosis is made decades after the onset of the disease, when complications may already appear (87). High blood sugar may cause the beta cells to produce more and more insulin until depletion, resulting in a condition similar to type one diabetes (88). As the disease progresses, a number of other complications may develop (e.g. cardiovascular diseases), which can cause damage in entire organs (kidneys, heart, brain) (58). The two main types of cardiovascular complications are microvascular and macrovascular diseases. The microvascular type includes diabetic retinopathy (DR) and nephropathy (DN), while the macrovascular complications include stroke and coronary artery disease. (89).

There are several membrane transporters which play a role in the development, progression and treatment of the disease (19,90,91). In my thesis I investigated two membrane proteins (GLUT1 and ABCG2) in this concept.

3. Objectives

The aims of my Ph.D. research were the following:

1. Studying the possible association between the expression level of GLUT1 membrane protein and type two diabetes mellitus.
2. Searching for genetic variants underlying the expression pattern of GLUT1.
3. Investigation of the exact molecular mechanism and regulatory background of the association between the identified variants and GLUT1 expression.
4. Examining the possible role of the *SLC2A1* SNPs and the ABCG2-Q141K variant in T2DM.

4. Methods

4.1. Samples and laboratory data

The GLUT1 study was conducted involving 207 Hungarian individuals (87 males and 120 females) with the average age of 60 (±13). 120 samples were obtained from type two diabetic (T2DM) patients, 59 samples from age-matched controls and 28 samples from healthy volunteers. The age-matched healthy subjects were selected from visitors at the clinic not suffering from diabetes or related metabolic diseases. The samples were obtained from the Drug Research Center (DRC, Balatonfüred, Hungary) and the 2nd Department of Internal Medicine, Semmelweis University (SE, Budapest, Hungary). Besides, to increase the sample size we also analyzed samples from in-house volunteers. The disease-related laboratory data (HbA1c, glucose, and insulin levels, HOMA etc.) and anthropometric data (weight, height, BMI, and waist circumference) of the participating patients were provided by the two collaborators (there were no such data for the in-house volunteers) (**Table 4.**). In addition, we received information about the disease-related complications of 23 cases (albuminuria (n=16), neuropathy (n=11), nephropathy (n=7) and retinopathy (n=4)), the family medical history (hypertension, obesity, gout, etc.) and the medication parameters.

Table 4. Summary of some key data of the control and T2DM groups from the GLUT1 study. The values are expressed as means +/- SD. The p values were calculated by the Student's t-test. Here, due to the lack of data, values for the in-house volunteers are not presented. Modified table from Kulin et al. (45).

Variable	Control group	T2DM group	p-value
N	59	120	-
HbA1c (%)	5.3 ± 0.5	6.9 ± 1.3	<0.0001
Blood glucose (mmol/L)	5.0 ± 0.5	6.5 ± 2.1	<0.0001
Insulin (mlU/l)	7.8 ± 4.8	9.9 ± 4.8	0.0234

The study about the ABCG2-Q141K variant was conducted involving 203 Hungarian individuals (87 males and 116 females) with the average age of 67 (±12). The samples from T2DM patients were obtained from the Drug Research Centre and the age-matched healthy volunteers were selected from visitors at the clinic not suffering from diabetes or related metabolic diseases. The key laboratory diagnostic parameters

analyzed here for T2DM patients, and the healthy volunteers included HbA1c, glucose, and uric acid levels (see **Table 5.**). The T2DM group can be further divided into two subgroups. Patients with insulin resistance (n=36), as first-time visitors at the DRC diabetic clinic ("untreated"), and long-time successfully managed ("treated") T2DM patients (n=63) with dietary supervision and with metformin treatment. In some cases, this treatment was supplemented with oral antidiabetics and with allopurinol (milurit), to reduce uric acid levels. The clinical diagnosis of T2DM was established according to the criteria of the American Diabetes Association (ADA) (92). Written informed consent was obtained from all patients, and the study was approved by the Scientific and Research Committee of the Medical Research Council, Hungary (ETT TUKEB references: 19680-3/2019/EKU, 2367-1/2019/EKU).

Table 5. Summary of some key data of the control and T2DM groups from the ABCG2-Q141K study. The values are expressed as means ± SD. The p values were calculated by Student's t-test. Modified table from Szabo et al. (93).

Variable	Control group	T2DM group	p-value
N	104	99	-
HbA1c (%)	5.6 ± 0.8	6.7 ± 1.2	<0.0001
Blood glucose (mmol/L)	5.4 ± 2.0	7.1 ± 3.5	<0.0001
Uric Acid (µmol/L)	314.9 ± 88.7	334 ± 88.5	0.1260

4.2. Flow cytometry

The expression level of GLUT1 was measured in RBCs with a method developed and patented earlier by our research group (49,94,95). Based on the protocol, the EDTA-anticoagulated blood samples stored at 4°C, can be processed within 24 hours. During this period the relative fluorescence signal of the membrane proteins does not change significantly. As the first step of this method, a weak fixation process was performed to produce the ghosts (RBC membranes). For this process we needed only a drop (50 µl) of blood which was added to a 1% formaldehyde/PBS solution and incubated at 37°C with shaking for 5 minutes. After centrifugation (1000g, 5 minutes), the samples were washed with 4 mL of PBS, and spun down (1000g, 5 minutes). The remaining pellet (ghosts and intact cells) was labelled by Alexa Fluor 647 conjugated wheat germ agglutinin (1:200, WGA-AF647, Thermo FS). WGA-conjugates are general membrane dyes, that bind nonspecifically to membrane oligosaccharide chains. This is

necessary because at the end of the fixation process, only a transparent cell membrane (ghost) remains from the RBCs, which is difficult to separate from the background signal due to its low light refraction. With the same reason the ghosts appear much smaller than the intact cells.

After we completed the samples to 500 µL with PBS, we continued the work in a microplate system. The samples were labelled with GLUT1 rabbit monoclonal antibody (1:20, Abcam, ab115730), diluted in 0.5 m/v% BSA/PBS with a final volume of 5 µL. After 40 minutes of shaking incubation at 37°C, samples were diluted in 20 µL PBS. It was followed by the fluorescence labeling with the secondary (Alexa Fluor 488-labeled goat anti-rabbit (H+L) antibody, 1:200, Thermo FS) antibody diluted in 0.5 m/v% BSA-PBS in a final volume of 10 µL. Incubation with shaking on 37°C was lasted for 30 minutes then the samples were diluted in 90 µL PBS. For the measurement we used 40 µL antibody labelled sample in the final volume of 300 µL. The antibodies were previously titrated to obtain the most reliable result and the largest difference in signal intensity compared to the control. To measure the nonspecific signals, we used samples labelled only with WGA and secondary antibody. Instead of centrifugation, serial dilution was used during labeling to remove non-specifically bound antibodies. This was necessary because the specific density of ghosts is close to that of water, so we would lose a lot of ghosts during centrifugation.

The measurement was performed with a 4-laser Attune NxT (Thermo FS) flow cytometer equipped with an automatic plate sampling system. The first step was the gating to the WGA-A647 positive events (RL1 channel, excitation wavelengths: 637 nm, emission filter: 670/14), then the separation of the erythrocyte ghost from the intact erythrocytes based on the forward and side scatter (FSC/SSC) parameters. The antibody marked with AF488 was detected in the BL1 channel (excitation wavelengths: 488 nm, emission filter: 530/30 nm) (see **Figure 4.**).

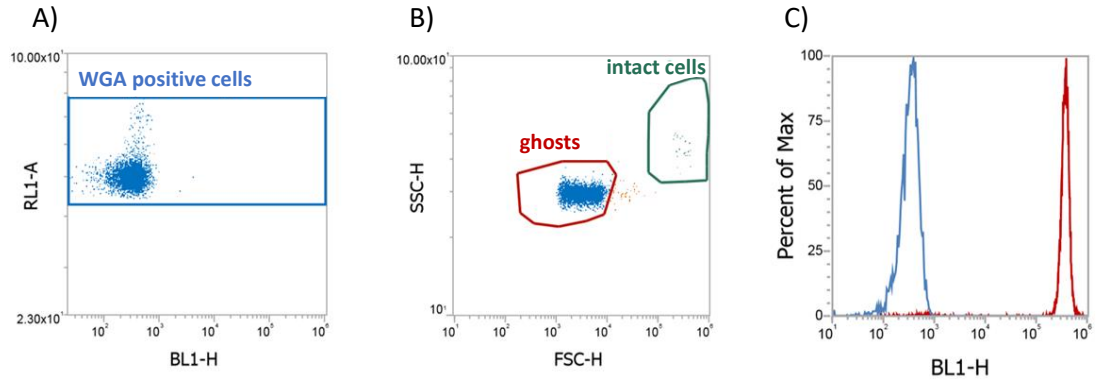

Figure 4. Signal filtering during flow cytometric measurement. Gating to WGA-A647 positive cells (A), separation of ghosts (red) and intact cells (green) (B) and signal intensity of the GLUT-1 antibody (red) compared to the control (blue) (C); RL1: red laser channel 1, BL1: blue laser channel 1, SSC: side scatter, FSC: forward scatter, -A/-H: area/height.

The relative expression values (arbitrary unit) were obtained by dividing the median fluorescence values of the samples labelled with the primary and secondary antibodies together by the median values of the secondary antibody control. For further analysis the mean values of the parallels were used.

This method allows to explore the alterations in the expression of membrane proteins and the related disease conditions (95). We have documented that by using this erythrocyte ghost forming method and applying well established, specific monoclonal antibodies - in contrast to other technologies (e.g. Western blotting or direct chemical labeling) - even small differences in the RBC expression levels can be properly quantified (83,96,97).

4.3. SNP selection and genetic analysis

Genomic DNA was isolated from whole blood using Gentra Puregene Blood Kit (Qiagen) according to the manufacturer's protocol. The DNA concentration of the samples was measured with Nanodrop (Thermo FS) spectrophotometer.

Exon sequencing was performed in nine samples, selected from both low, medium and high expression levels. The primers required for the amplification and the sequencing of the *SLC2A1* exon regions (with the exon-intron boundaries) were designed using the reference sequence and the Primer Blast tool of NCBI (**Table 6.**). After amplifying, the PCR products were run in a 1 w/v% agarose gel and purified by using Wizard SV Gel and PCR Clean-Up System (Promega). Sanger sequencing was

performed by Eurofins. For the further analyzes, the SNPs were selected based on sequencing and previous literature data. The allele discrimination genotyping experiments (both in case of the *SLC2A1* SNPs and the *ABCG2* SNP) were performed on all samples carried out with TaqMan-based qPCR analysis and were performed by a Step One Plus device (Applied Biosystems) with premade assay mixes (Thermo FS, (C___1166185_1_ (rs1385129); C___1166180_10 (rs841847); C___8365112_10 (rs841848); C__30716206_10 (rs11537641); C__15854163_70 (rs2231142)) and master mix (Thermo FS). The specificity of the probes was verified by sequencing. These experiments, together with flow cytometry analysis, provided information on the effect of the tested SNPs and the related haplotype.

Table 6. Sequencing primers. Sequence of the designed forward and reverse primers of the exons in the *SLC2A1* gene. Table from Kulin et al. (45).

Exons	Forward primer (5'→3')	Reverse primer (5'→3')
1	GTCCCAACGCAGAGAGAACG	CGGCCCGCTAGATCCGAA
2	AAAGACTGGTGTGGTGCCAA	AGAAAACTGGCTGGAGAGGC
3-4	TCTCTTAGGGAGGGGTGCAA	TGGTGCTGTGTTCTCTGGAC
5-6	AACAGGGCTCATGCTAGTGG	TTCTTCGGCAGAGGCGTATC
7-8	CAGTGTCCCTTCTGCCTGAG	GGAGCCAGAAAGTCAGACCC
9	ATAGCTCTGCTCTGGCCTCT	CCCTCAGTTTCCTCCTCAGC
10	TAGGTACAAGCGTGGTCTCAG	AGGTTTGGAAGTCTCATCCAGC

4.4. Design of reporter vector constructs and cloning

To generate recombinant luciferase vector constructs, I used the basic pGL3 vector (Promega) modified with a mini-TK promoter (previously designed by Boglárka Zámbó). In this experiment, I analyzed inserts that contained the minor allele only for the tested SNP (for insert sizes and primers see **Table 7.**). The DNA fragments of interest were amplified by PCR and were inserted in the mini-TK promoter vector by using T4 ligase (Thermo FS), after digestion by NheI (NEB) and XhoI (NEB) restriction enzymes. The resulting constructs were introduced to E. coli Dh5α ultra-competent cells by transformation and purified using the PureYield Plasmid Miniprep System (Promega). To ensure whether the ligation was correct, I sent the constructs for Sanger sequencing.

Table 7. Size of the inserts cloned to the modified pGL3 vector and sequence of the designed forward and reverse primers. The inserts contain the minor allele only for the tested SNP. Restriction enzyme cut sites are included and marked with italics. Table from Kulin et al. (45).

SNP	Insert size	Forward primer (5'→3')	Reverse primer (5'→3')
rs1385129	372 bp	ACT*GCTAGC*-AAAGACTGGTGTGGTGCCAA	AGTA*CTCGAG*-AGAAAACTGGCTGGAGAGGC
rs841847, rs841848	577 bp	GAC*GCTAGC*-CCATCTCCGTGCTGAGGTTT	ATGC*CTCGAG*-CCTCCCGGTAGACAGAGGAA
rs11537641	518 bp	CAT*GCTAGC*-GTGGGAGGTAGGGGAGACTT	ATCG*CTCGAG*-TGGTGCTGTGTTCTCTGGAC

4.5. Cell culture, transfection, and treatments

For the cellular experiments, I used human embryonic kidney 293T cells (HEK-293T) and human hepatoma cells (HepG2 – frequently used in diabetes related studies), which were mycoplasma-free cell lines. HEK-293T and HepG2 cells were grown in DMEM/high glucose/GlutaMAX medium (Gibco) completed with 10% heat-inactivated fetal bovine serum (FBS), Gibco and 0.1% gentamicin (Gibco) at 37°C and 5% CO_2.

For the following experiments, 40,000 cells/well were plated in 48-well plates both for HEK-293T and HepG2 cells. The transfection of HEK-293T cells was carried out with Turbofect reagent (Thermo FS) while HepG2 was transfected by using JetOptimus (Polyplus). In both cases 450 ng of pGL3 TK-mini (vector with thymidine kinase promoter) constructs were used together with 50 ng of pRL-TK (Renilla luciferase vector with HSV-Thymidin kinase promoter) plasmid.

For examining the regulatory effects of various incubation conditions, HepG2 liver-related cells, potentially more relevant to metabolic diseases, were treated for 24 hours after transfection. Treatments with glucose and insulin required FBS free medium because the exact composition of FBS is not known, and it is conceivable that it contains these compounds. When testing the effect of glucose concentration, I exchanged the medium to an FBS- and glucose-free medium (Gibco), while for insulin treatment I used a medium without FBS and a low amount (1 g/L) of glucose (Gibco), then, 6 hours before the measurements, 100 nM insulin (Sigma) was added to the samples. This experimental setting was based on previous literature data (98,99). For hypoxia treatment, cells were placed in a humidified, hypoxic incubator (37°C), flushed with a gas mixture of 5% CO_2, 92% N_2 and 3% O_2. Physiological hypoxia is likely to be in the range 2–6% oxygen (100), which suggests that with 3% of oxygen, the hypoxia

response elements may well upregulate, and this condition is also acceptable for the cells.

4.6. Dual luciferase assay

To determine the potential regulatory effects on the expression, including the potential enhancer/suppressor activity of the surrounding region of the examined SNPs, I cloned their respective wild-type and mutant regions into the pGL3 vector (**see Figure 5.**) and measured luciferase activity in the transfected HEK-293T and HepG2 cells.

For the analysis, I used a Dual Luciferase Reporter Assay System kit (Promega), according to the manufacturer's guideline. Luciferase reaction was measured 48 hours after transfection, with VICTOR X3 Multilabel Plate Reader (Perkin Elmer). Firefly luciferase activity was normalized to Renilla luciferase activity in all experiments. Each DNA fragment containing the specific SNPs and the surrounding regions were tested in triplicates, and at least three independent experiments were performed.

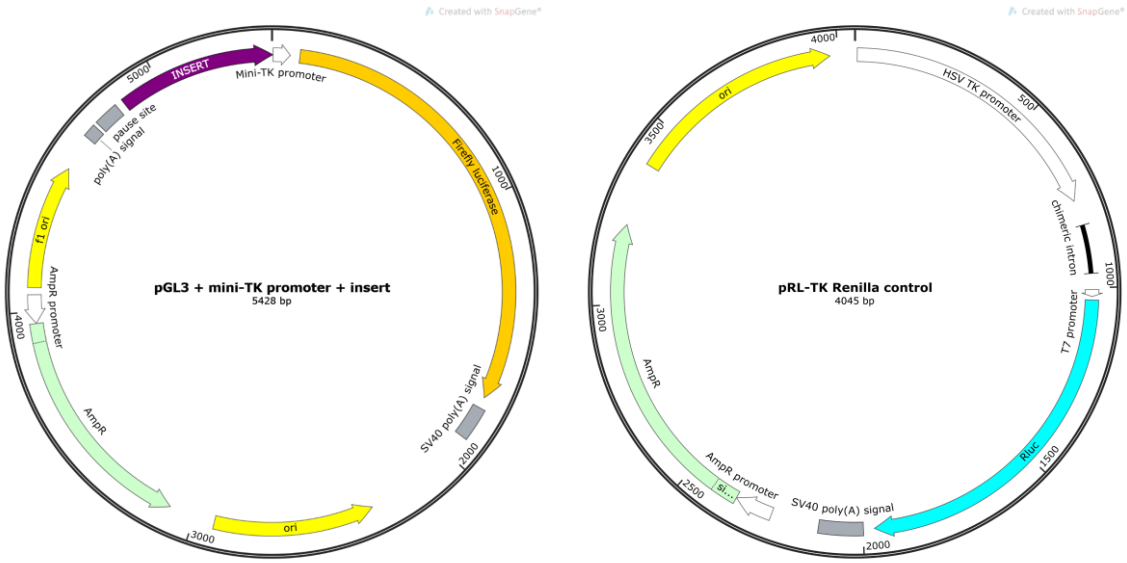

Figure 5. Vector Maps. The modified basic pGL3 vector containing the inserts with variants rs1385129, rs841847, rs841848 or rs11537641, and the pRL-TK vector (Renilla luciferase vector with HSV-Thymidin kinase promoter). Figure from Kulin et al. (45).

4.7. Bioinformatic analysis

In order to predict transcription factor binding sites (TFBS) affected by the examined SNPs we used the matrix scan function of the Regulatory Sequence Analysis Tools (RSAT, http://rsat.sb-roscoff.fr/index.php). With this tool, we can predict and analyze the transcription factor binding sites. For the usage of this tool, on one hand we had to filter the 100 nucleotides long regions around the SNP's - upstream and downstream. For each SNP, we had two different sequences. The first contains the original/reference sequence (with the reference allele of the SNP in the middle of it) and the other one contains the alternative sequence (with the alternative allele of the SNP in the middle of it). On the other hand, we downloaded the newest version of the position weight matrices (PWM) of the transcription factors (TF) from the Jaspar database (http://jaspar.genereg.net). This matrix contains the representation of different motifs or patterns in biological sequences. A position weight matrix is a model for the binding specificity of a TF and can be used to scan a sequence for the presence of DNA words that are significantly more similar to the PWM than to the background.

With our sequences and with the position weight matrix, we could predict the binding sites of different transcription factors in the region around the SNPs, both for the reference sequences and for the sequences, which are containing the SNPs alternative alleles. The matrix scan module can calculate a p-value for each binding site (referring to the probability of binding to the given sequence) and we worked with only those, which were less than 0.0005 ($p<0.0005$). After the matrix scan module calculated the TFBS's (Transcription Factor Binding Sites), we had two different results. The first contains only those TFBS's, which came from the reference sequences with the reference allele of the SNP, and the other part, which contains only those TFBS's, which came from sequences, with the alternative alleles in them. Then we compared the differences between the two results, and we kept only those TFBSs, which were unique. This means that we worked only with those TFBS's, which were only present in one of the results.

4.8. Statistical analysis

Statistical analyses were performed using the GraphPad Prism 8.0.1 software. The potential association between the expression levels of GLUT1 and the occurrence of the disease was analyzed with the Mann-Whitney test. To compare genotypes and protein levels, I chose the Kruskal-Wallis test with a Dunn post-hoc test. The distribution of the SNPs for all variants in the examined samples conformed to the Hardy-Weinberg equilibrium. I examined the SNP frequency in the control and case group and searched for possible differences with $\chi 2$ and Fisher tests. The p values for the comparison of the laboratory parameters between the groups were calculated by Student's t test. To compare relative luciferase activities between the constructs with different alleles, I used Welch's t-test. All dual-luciferase experiments were conducted at least three times, and each of these measurements included three replicates. In the statistical analyses, $p<0.05$ was considered as statistically significant difference.

5. Results

5.1. GLUT1 expression and type two diabetes

In my work I first measured the expression level of GLUT1 on RBCs by flow cytometry, looking for possible differences between the patients with type two diabetes and the healthy individuals. As a result, no difference was found between the control and case groups (p=0.45).

However, we observed that expression levels of certain samples showed big deviation from the mean level (**Figure 6.**). Therefore, my next step was to investigate the genetic (polymorphisms, mutations) and regulatory (metabolic factors, transcription factors) background of this phenomenon. Since there was no significant difference in the mean values of the RBC membrane GLUT1 levels in these relatively small cohorts, in order to find statistically relevant SNP-dependent differences, I used the combined values of the expression levels measured in the control and T2DM patients.

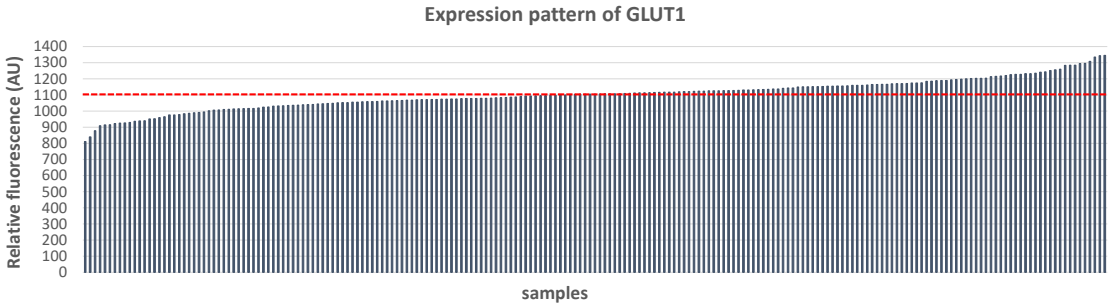

Figure 6. GLUT1 expression pattern in the total (control and T2DM samples) population measured by flow cytometry. Samples with a large deviation from the mean (red dashed line) level required further investigation.

5.2. Genetic background of GLUT1 expression

In parallel with the expression level measurement, I started the genetic analysis of the samples. For sequencing, I prepared a total of nine samples representing varying levels of RBC GLUT1 expression. Consequently, I found 3 SNPs and an insertion. (see **Table 8.**).

Table 8. Sequencing results – Genetic variants in the *SLC2A1* gene of samples with low, medium, and high (indicated by color strength) RBC GLUT1 expression levels (Relative fluorescence, mean ± SD). Minor allele frequency refers to the European population. G: wild type allele; A: mutant allele. Table from Kulin et al. (45).

	Relative fluorescence		Genetic variants			
Sample	**Mean**	**SD**	**rs1385129** MAF=0.22	**rs11537641** MAF=0.19	**rs2229682** MAF=0.19	**rs11282849** MAF=0.47
8	878	53	GG	GA	GA	-
5	927	160	GG	GG	GG	insAAATGGTGAG
13	1058	5	GA	GG	GG	-
33	1075	95	GA	GA	GA	-
26	1085	25	GG	GG	GG	-
29	1127	71	GA	GA	GA	-
37	1174	40	GG	GA	GA	-
24	1226	163	GA	GA	GA	insAAATGGTGAG
36	1296	18	AA	AA	AA	insAAATGGTGAG

Considering the MAF (minor allele frequency) of these genetic variations in the European population, the insertion was excluded from the further investigation due to its high frequency occurrence (MAF(EUR)=0,46). Besides, by checking LD disequilibrium between the variants it revealed that two SNPs (rs11537641, rs2229682) show strong correlation (R^2=0,93), which implies that only one of them is sufficient for subsequent analysis. I selected rs11537641 along with rs1385129, which appeared to accumulate at higher expression levels. Interestingly, previous studies showed that rs1385129 was associated with diabetic nephropathy, a complication of type two diabetes. In addition, these case-control studies mentioned two other SNPs (rs841847 and rs841848) in association with T2DM or its complications, located in the 2nd intron, in a putative enhancer region. Therefore, genotyping analysis was conducted for the variants rs1385129, rs841847, rs841848, and rs11537641 on the entire population.

In our experiments on RBCs, we found that the expression level of the GLUT1 membrane protein was significantly higher in the presence of the mutant allele in case of rs1385129 ($p < 0.0001$), rs841848 ($p<0.0001$) and rs11537641 ($p=0.0024$) SNPs, respectively. On the contrary, the mutant allele of rs841847 was associated with decreased expression level ($p = 0.0178$) (**Fig. 7. A-D/1**).

Two out of the four examined SNPs (rs1385129 and rs841848) were identified as members of haplotype blocks. Consequently, it is crucial to consider that the observed

effects on GLUT1 expression may not necessarily be attributed to the specific SNPs analyzed, but rather to the effects of another SNP within the haplotype. To validate our previous results, I performed dual-luciferase reporter assay. Two different human cell lines were used in these studies. The immortalized human embryonic kidney derived (HEK) cells, which may reflect some of the kidney-type regulatory features, and a hepatoma cell line (HepG2) which may be informative for a more complex, hepatocyte-like regulatory mechanism. In the experiments, we used the modified pGL3 vector with the TK mini promoter (TK) as a benchmark, and the vector supplemented with a random sequence of similar size to the tested inserts was used as negative control (NC).

The luciferase reporter assay revealed that DNA fragments containing the rs1385129, rs841847 and rs841848 variants, had higher luciferase activity compared to the negative control. Thus, these sequences show enhancer/promoter effects.

As to the effect of the rs1385129 variant, the mutant allele increased luciferase activity more strongly than the wild-type allele in the HEK-293T cells (p=0.012, **Fig.7. A/2**). However, in HepG2 cells (**Fig.7. A/3**) there was no such difference between the effects of the two alleles.

In case of the rs841847 variant, we found that luciferase activity was significantly lower in the presence of the mutant allele (HEK-293T: p=0.0039, **Fig.7. B/2**; HepG2: p=0.0243, **Fig.7. B/3**), which results are consistent with the expression level of GLUT1 in the RBC membrane.

Examining rs841848 variant, we did not find substantial difference in the luciferase expression caused by the presence of the minor allele in HEK-293T cells, while in HepG2 cells there is a small decrease in the luciferase activity which is an opposite result compared to the GLUT1 expression data on RBC (HEK-293T: p=0.514, **Fig. 7. C/2**, HepG2: p=0.0354, **Fig.7. C/3**).

The region of rs11537641 slightly reduces luciferase expression driven by the TK promoter in HEK-293T cells but the mutant allele had no additional effect on this suppressed expression. In HepG2 cells, however, it significantly increased the luciferase activity (HEK-293T: p= 0.1015, **Fig.7. D/2**, HepG2: p <0.0001, **Fig.7. D/3**).

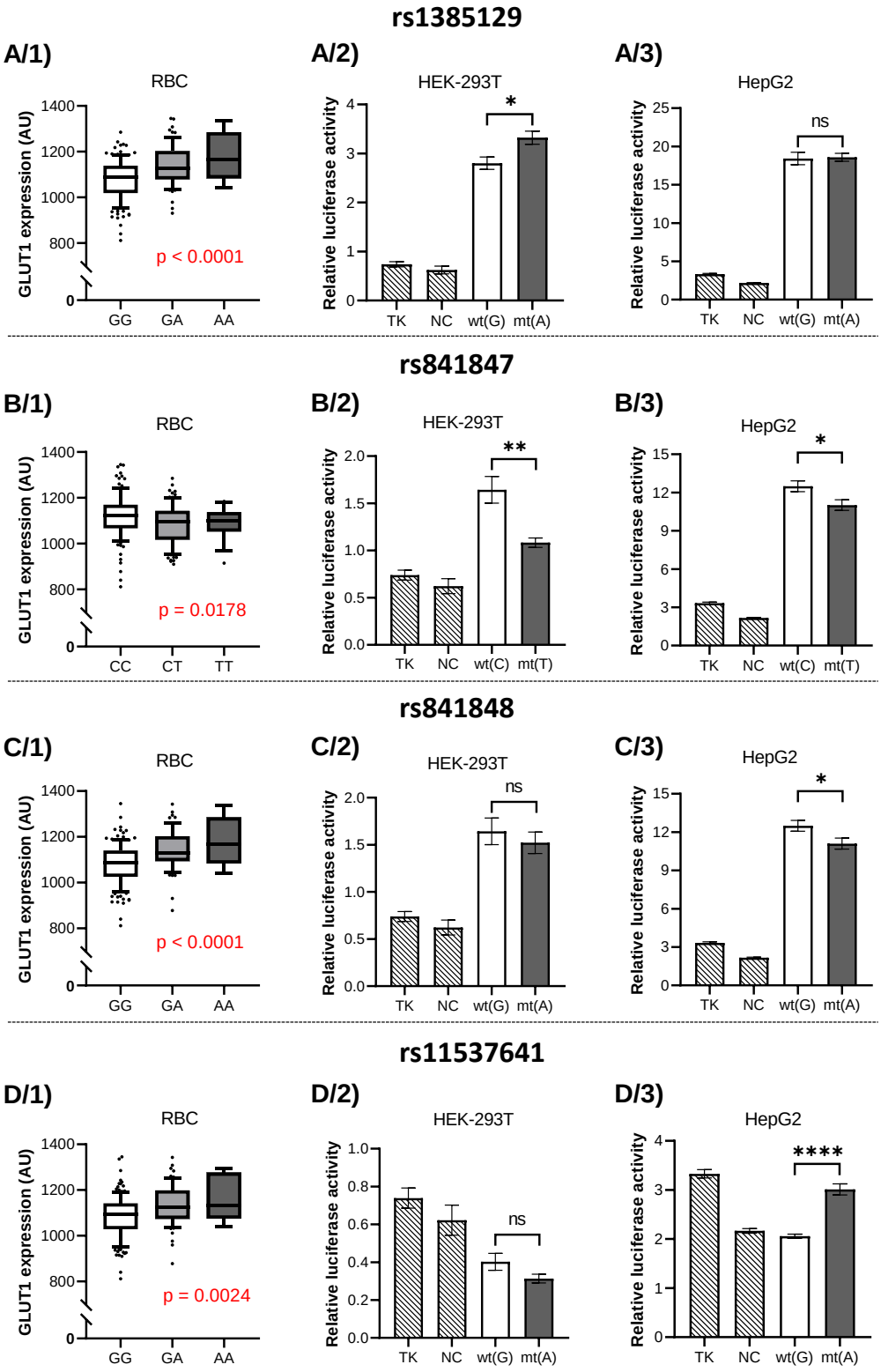

Figure 7. The effect of the 4 examined SNPs on GLUT1 expression and on luciferase activity. For rs1385129 the presence of the mutant allele showed significantly higher GLUT1 expression in RBCs, and luciferase activity in HEK-293T cells but not in HepG2 cells (A/1-3). For rs841847 the mutant allele had a decreasing effect both on GLUT1 expression and on luciferase activity (B/1-3). In case of rs841848

GLUT1 expression is higher in the presence of the mutant allele, but luciferase experiments do not confirm this result (C/1-3). Variant rs11537641 increases the GLUT1 expression level and the luciferase activity in HepG2 cells (D/1-3). Each boxplot represents samples with different genotypes, in the range of 10-90 percentile, while dots represent samples out of range. The stars represent significance at different levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Values are expressed as means ± SD.
wt: wild type allele; mt: mutant allele; TK: "empty" vector without insert; NC: negative control (with random sequence); ns: not significant; AU: arbitrary unit. Rearranged figure from Kulin et al. (45).

5.3. Regulatory background of GLUT1 expression

5.3.1. Metabolic factors

Since GLUT1 plays a significant role in glucose homeostasis, related metabolic factors can influence the expression level of GLUT1, which is supported by several previous studies (101–103). In the following we examined whether the effect of the SNPs change with different conditions, such as the reduced glucose and oxygen level, and the adding of insulin. The measurements were performed in the metabolically more relevant HepG2 cells.

In case of rs1385129 and rs841848, under low glucose concentrations a difference appears in the effect on luciferase activity between the wild-type and mutant alleles. In contrast, for rs841847 and rs11537641, the significant difference between the effects of the two alleles disappears (**Fig.8. A-D/1**). Interestingly, in case of rs841848, the presence of the FBS in the medium seems to have an influence on the outcome, as the removal of the FBS (for glucose and insulin treatments) results in an increased luciferase expression in the presence of the minor SNP, while for the hypoxia treatment, in FBS containing medium, decreased luciferase expression was observed. Insulin treatment (**Fig.8. A-D/2**) did not cause major changes, while under hypoxic conditions luciferase activity significantly increased regardless of the inserts or alleles used (**Fig.8. A-D/3**).

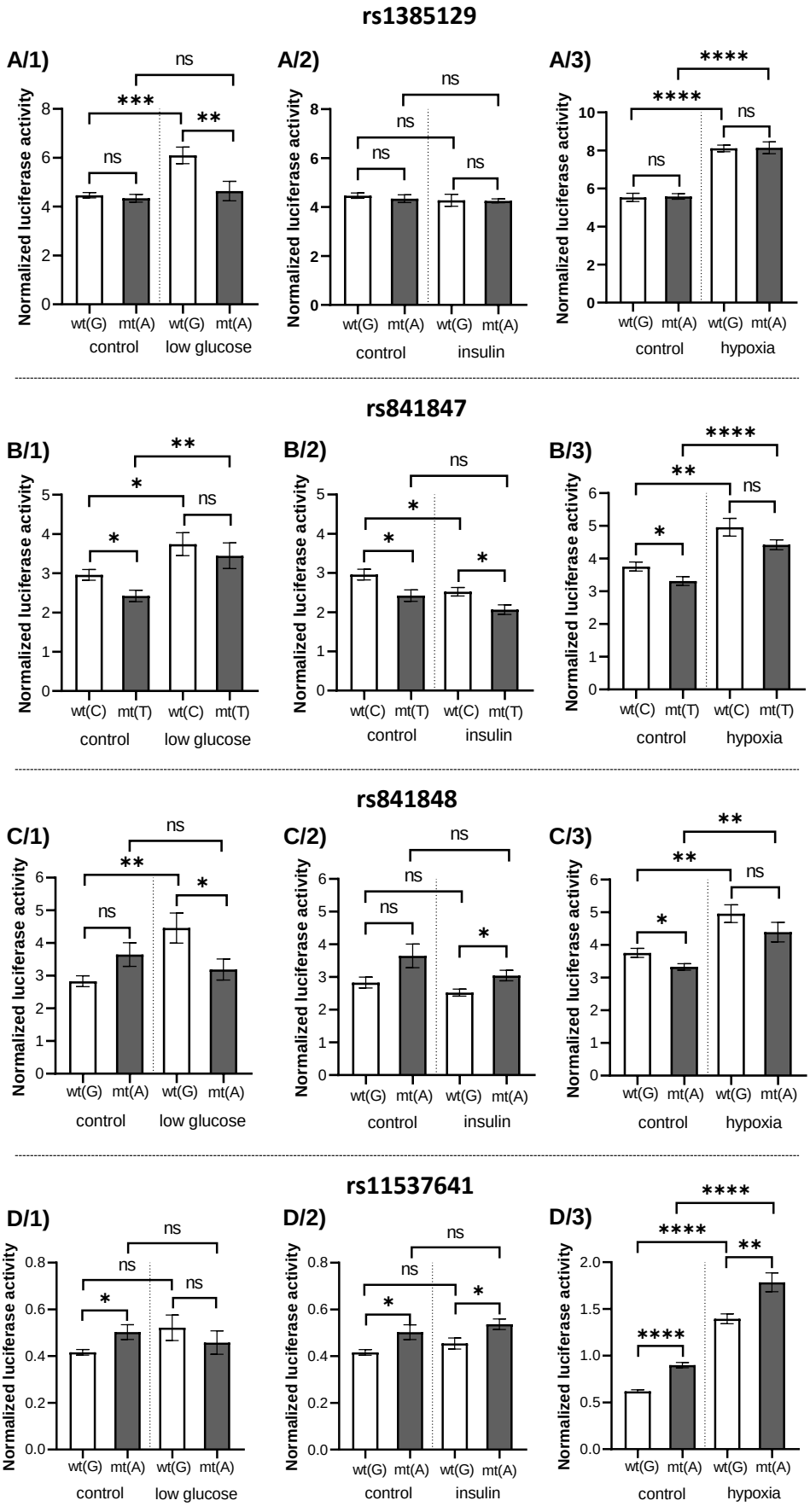
rs1385129
A/1)
A/2)
A/3)
Normalized luciferase activity
wt(G) mt(A) wt(G) mt(A)
control low glucose
control insulin
control hypoxia
rs841847
B/1)
B/2)
B/3)
wt(C) mt(T) wt(C) mt(T)
rs841848
C/1)
C/2)
C/3)
rs11537641
D/1)
D/2)
D/3)

Figure 8. The effect of the examined SNPs on luciferase activity under glucose, insulin, and hypoxia treatments. In case of rs1385129 and rs841848, under low glucose concentrations, a difference in the effect on luciferase activity between the wild-type and mutant alleles appears. In contrast, for rs841847 and rs11537641, the significant difference between the effects of the two alleles disappears. Insulin treatment did not cause major changes, while under hypoxic conditions luciferase activity significantly increased independent of the alleles. The stars represent significance at different levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Values are expressed as means ± SD. Data were normalized to the results of the "empty" vector. wt: wild type allele; mt: mutant allele; ns: not significant. Rearranged figure from Kulin et al. (45).

5.3.2. Transcription factors

According to our bioinformatics analysis, there are several predicted transcription factor (TF) binding sites that are altered in the presence of the tested SNPs (**see Table 9**). In the presence of the wild-type allele of the rs1385129 variant, we found binding sites for EGR1, CTCF and GCM1, while in the presence of the mutant allele, a new binding site appears for the ZNF281 transcriptional regulator. In case of the mutant allele of rs841847, we found that binding sites for ARNT2, BHLHE40, BHLHE41, ARNTL transcription factors disappeared, compared to the reference sequence. The wild type allele of the rs841848 variant affects the binding sites for two important transcriptional regulators (ZNF460 and ZNF528), while the sequence with the mutant allele introduces novel binding sites for STAT proteins (Stat1, Stat5a/b). In case of the variant rs11537641, the sequence with the wild type allele contains binding sites for the ZFP57 and Zfx transcriptional activators, while the mutant allele displays binding sites for ZBTB32, SREBF1, SREBF2 and FOXK1.

Table 9. Predicted transcription factor binding sites in the region of the examined SNPs. The table shows the binding sites (sequence) of the transcription factors (TF name) affected by one of the alleles of the SNPs. The concerned DNA strand and the T2DM-specific function of the TFs are also described. The p-value refers to the binding probability of the TFs. Strands labelled as 'D' (direct) and 'R' (reverse). The alleles of the examined SNPs are labelled with red color. Table from Kulin et al. (45).

SNP ID	Allele	TF Name	Strand	Sequence	P-value	Relevant function
rs1385129	wild	EGR1	D	CTCCTCCCACGGCC	3.2×10^{-4}	response to glucose, insulin, hypoxia
rs1385129	wild	CTCF	D	CGGCCAGCATGAGGCGACC	1.8×10^{-4}	-
rs1385129	wild	GCM1	R	CATGCTGGCCG	2.4×10^{-4}	-
rs1385129	mutant	Znf281	R	GCTGTGGGAGG	2.2×10^{-4}	-
rs841847	wild	ARNT2	R	GTCCCGTGCA	2.6×10^{-4}	response to hypoxia
rs841847	wild	BHLHE40	D	TGCACGGGAC	4.9×10^{-4}	-
rs841847	wild	BHLHE40	R	GTCCCGTGCA	2.5×10^{-4}	-
rs841847	wild	BHLHE41	R	GTCCCGTGCA	4.4×10^{-4}	-
rs841847	wild	ARNTL	R	TGTCCCGTGC	1.1×10^{-4}	-
rs841848	wild	ZNF528	R	CGGGAGGAAGGCTTTCC	2.5×10^{-4}	-
rs841848	wild	ZNF460	D	AAGCCTTCCTCCCGAG	2.5×10^{-4}	-
rs841848	mutant	STAT5a/ STAT5b	D	CCTCCCAAGAA	1.3×10^{-4}	response to insulin
rs841848	mutant	STAT1	R	GTTCTTGGGAG	4.9×10^{-4}	cellular response to insulin
rs841848	mutant	Stat5a	D	CTCCCAAGAACC	2.7×10^{-4}	response to insulin
rs11537641	wild	ZFP57	D	GTCAGGCCGCAGT	4.4×10^{-4}	-
rs11537641	wild	Zfx	R	TGTACTGCGGCCTG	1.1×10^{-4}	-
rs11537641	mutant	SREBF1	D	GTCAGGCCAC	2.8×10^{-4}	response to glucose, insulin
rs11537641	mutant	SREBF2	R	GTGGCCTGAC	1.2×10^{-4}	-
rs11537641	mutant	ZBTB32	R	TGTACTGTGG	1.6×10^{-4}	-
rs11537641	mutant	FOXK1	D	ACAGTACACACCGA	4.2×10^{-4}	cellular glucose homeostasis

5.4. *SLC2A1* SNPs and ABCG2-Q141K variant in type two diabetes

The final objective of my work was the observation of SNP frequency in control and type 2 diabetic samples. Based on previous studies, 3 SNPs (rs1385129, rs841847, rs841848) are associated with some complications of the disease, and in case of rs841847 even with T2DM (59). However, despite these previous results of the GLUT1 SNPs, no significant differences were observed among the examined groups in our examination. When studying the SNP rs2231142 (i.e., C421A) leading to the ABCG2-Q141K variant, there was also no significant difference between the control and the T2DM patients (**Fig.9.**). Due to the low sample size, we performed the analysis by combining the heterozygous and homozygous groups, but we did not find any significant differences between the groups. As the *SLC2A1* SNPs has been previously investigated in association with disease-related complications (diabetic, nephropathy, retinopathy) we also planned to analyze the allele frequencies in these samples, however with the total of 23 cases (albuminuria (n=16), neuropathy (n=11), nephropathy (n=7), retinopathy (n=4)) we were not able to get statistically relevant information.

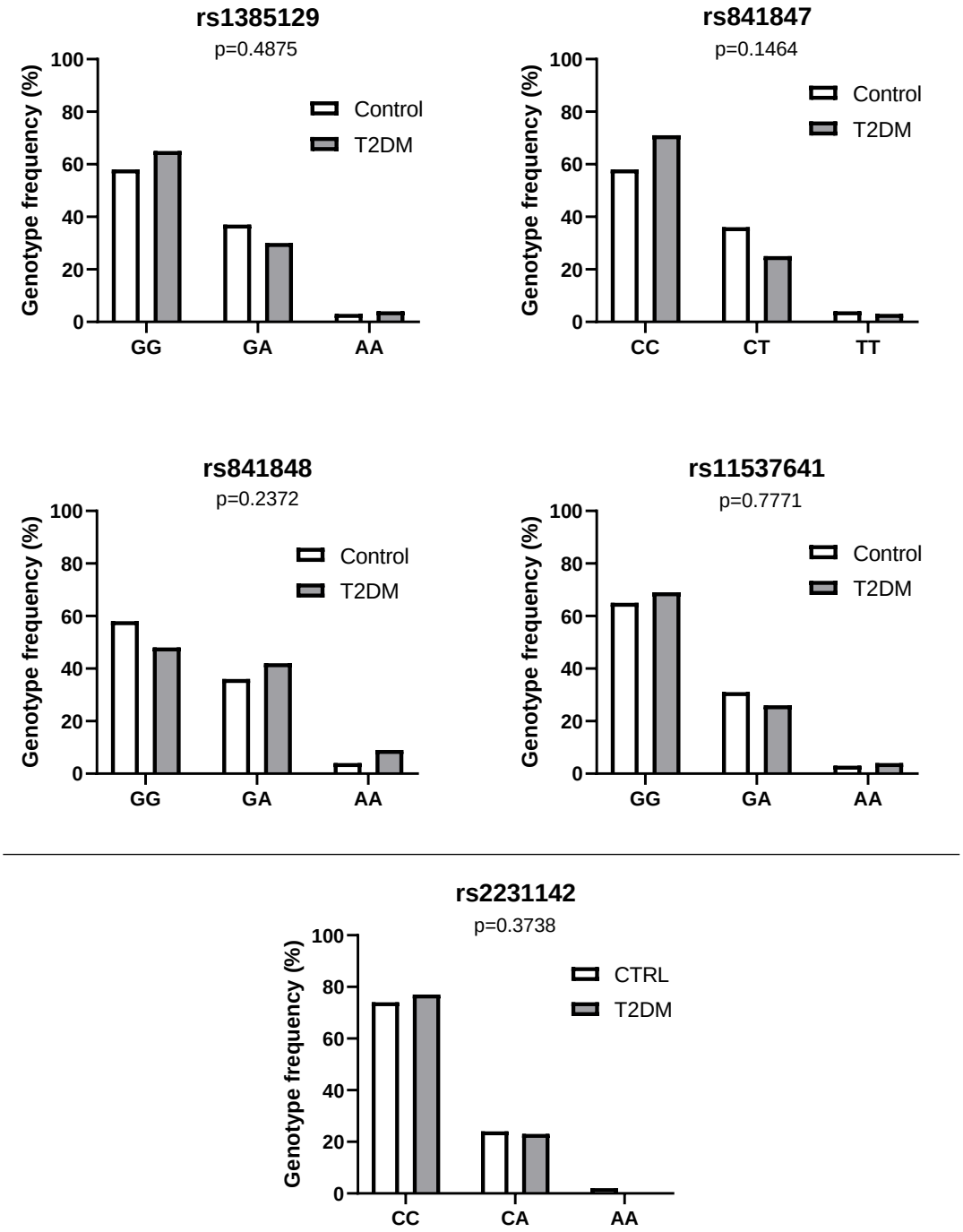

Figure 9. Genotype frequencies of the *SLC2A1* and *ABCG2* SNPs in control and T2DM groups. No significant differences were found between the groups, neither for the *SLC2A1* SNPs (rs1385129, rs841847, rs841848, rs11537641) nor for the *ABCG2* SNP (rs2231142/C421A; Q141K). Figures of the *SLC2A1* SNPs originate from Kulin et al. (45), while the figure of the *ABCG2* SNP was made based on Table 2 from Szabo et al. (93).

In addition to studying direct associations between the SNPs and the disease, we also investigated the indirect relationship based on disease-associated laboratory parameters. By analyzing the 4 examined *SLC2A1* SNPs in association with T2DM related laboratory parameters, there was no difference between the control and the T2DM groups. However, in case of the ABCG2-Q141K variant, we found significantly higher blood glucose and HbA1c levels in the T2DM patients carrying the ABCG2-Q141K variant (**Fig.10.**).

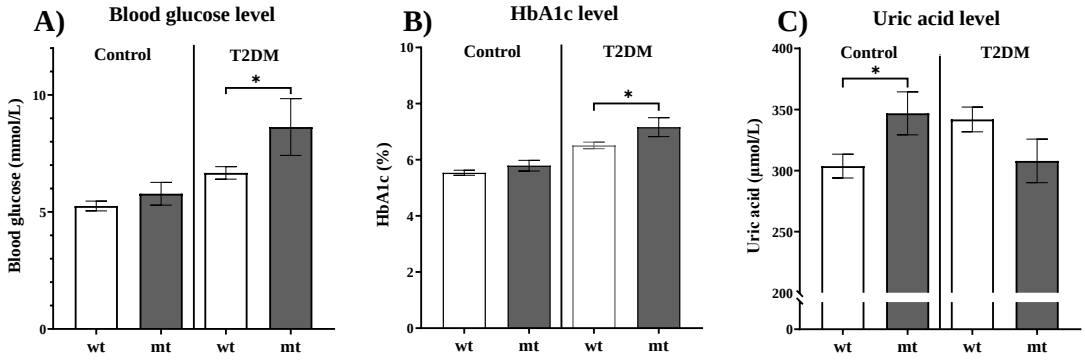

Figure 10. Blood glucose (Panel A), HbA1c (Panel B) and uric acid (Panel C) levels and the presence of the Q141K variant in the control and the T2DM groups. Values are expressed as means ± SE. wt: ABCG2 wild type; mt: hetero- and homozygotes for the C421A (ABCG2-Q141K) variant. Star (*) indicates a significant difference obtained in the individuals carrying the Q141K variant *$p<0.05$. The p values were calculated by Student's t test. Visually modified Figure from Szabo et al. (93).

6. Discussion

Membrane proteins play a key role in biological processes, and their quantitative changes are important under various pathological conditions. Therefore, several of these proteins are particularly important in drug development. Notably, members of the SLC and ABC transporter superfamily are of outstanding significance in this regard, as they influence the absorption, distribution, metabolism, excretion, and toxicity (ADME-Tox) of the drugs (4).

Type two diabetes mellitus is one of the most common metabolic diseases nowadays, and several membrane transporters play a crucial role in the development, progression, and treatment of the disease (90,91). In my thesis work I have investigated two membrane proteins in association with T2DM.

The primary focus of my thesis centered around the GLUT1 membrane protein. This transporter belongs to the SLC superfamily, specifically categorized within the SLC2A (GLUT) protein family. The GLUT1 membrane protein is a key player in the cellular glucose uptake from the blood, especially in tissues directly depending on glucose metabolism. These include the human RBCs, the brain and kidney vascular tissues, as well as numerous malignant tissues (29,50,51). Based on its function, alterations in GLUT1 expression can be linked to complex metabolic diseases, including type 2 diabetes and Alzheimer's disease (49). These changes usually have a regulatory background, driven by frequent genetic variations (polymorphism). In the GLUT1 study, I investigated the genetic and regulatory background of GLUT1 expression, as well as its possible association with T2DM.

The other branch of my work was about another important protein, ABCG2. In this study we have examined the potential relationship between the presence of the rs2231142 (C421A) SNP (results in the ABCG2-Q141K protein variant) and type two diabetes. We focused on the potential associations between the occurrence of this SNP variant and the basic metabolic parameters in T2DM (blood glucose, HbA1c, and uric acid levels).

The ABCG2-Q141K protein variant has a reduced stability and plasma membrane expression (104,105). It is also known that this variant significantly contributes to elevated serum uric acid levels and gout development (106–108). Wei et al. found that the diabetes indicator, HbA1c significantly correlated with serum uric acid level, which

raises the question if ABCG2-Q141K has a role in this association (79). Since ABCG2 function is also an important determinant of the ADME-Tox properties of numerous drugs, including those (e.g. metformin) used in the treatment of T2DM, this polymorphism may also affect the treatment efficacy of this disease (109–112).

6.1. GLUT1 expression and type two diabetes

According to my results, there is no association between T2DM and the expression level of GLUT1 membrane protein in RBCs. However, in a previous study significantly higher GLUT1 expression have been described in the placenta in patients with gestational diabetes, T1DM (54) and T2DM (55). This suggest that GLUT1 expression may have a cell-specific influence on the disease. Furthermore, the different expression level might have a role in the progression of T2DM. In a nephropathic rat model, it was found that hyperglycemia induces increased GLUT1 expression in the glomerular cells. The authors suggested that these cells fail to decrease GLUT1 expression as a respond to elevated extracellular glucose level, which was observed in other cell types, and the increased glucose uptake cause damage in the kidney cells, which may contribute to the development of nephropathy (53).

In our experiments, the expression levels of certain samples showed large deviation from the mean level. Behind it, we hypothesized regulatory changes, which can occur because of genetic variants. Therefore, my next step was to investigate the genetic (polymorphisms, mutations) and regulatory (metabolic factors, transcription factors) background of this phenomenon. Since there is no difference between the expression level of the control and case groups, in further experiments, I analyzed the combined values of the two groups. Therefore, I was able to obtain a relatively large number of samples and thus statistically more reliable results in genotyping analysis.

6.2. Genetic background of GLUT1 expression

In order to assess the relevance of genetic factors in complex diseases, GWAS and case-control studies are widely performed (113). However, the potential connection between direct protein expression levels and the presence of genetic variants is not specifically explored. To find such specific associations, in the present work we have performed a flow cytometry analysis to determine the expression level of GLUT1 in

RBC membrane and examined the role of the potentially connected SNPs by reporter expression studies.

Based on previous case-control studies (56–58) and our own sequencing experiments, we have selected four SNPs within the *SLC2A1* gene, to be examined in detail (rs1385129, rs841847, rs841848, rs11537641). Two of these variants (rs1385129, rs841848) are parts of relatively large haplotypes, thus, as lead SNPs, they provide information for relatively large regulatory segments of this gene.

As shown in the results chapter, the minor allele of three of the variants (rs1385129, rs841848, and rs11537641) significantly increased RBC GLUT1 expression, while the mutant allele of rs841847 had decreasing effect. To analyze the direct modulation effect of the SNPs, we used luciferase reporter expression constructs of the surrounding genetic areas.

Our detailed reporter assays demonstrated that the putative enhancer element within intron 2 of the *SLC2A1* gene, containing SNPs rs841847 and rs841848, is a functional enhancer in the human, similar to what was observed in mice (43). In addition, the insert containing the rs1385129, also shows a strong enhancer/promoter effect, while the region of rs11537641, had a suppressor effect in HEK cells.

Regarding the specific modulatory roles of these SNPs, the minor variant of rs1385129 slightly increased luciferase expression in the (renal derived) HEK-293T cells, while not in the (liver derived) HepG2 model cells. The difference in these results suggests that this SNP has a cell-specific effect. The human embryonic kidney derived (HEK) cells may reflect kidney-type regulatory features and rs1385129 was previously described as risk factor in diabetic nephropathy. Besides, as mentioned earlier, Heilig et al. found that GLUT1 expression increased in the renal glomerular cells of the nephropathic rat model (53). Taken together, this variant may contribute to diabetic nephropathy through the regulation of GLUT1 expression. The minor allele of rs841847 significantly decreased the luciferase expression in both cell types, and these results correlate with the outcome of the GLUT1 expression measurements, which means that this SNP has a general modulatory effect on the gene expression. The minor allele of rs841848 had no significant effect on luciferase expression in HEK-293T cells, but it showed an FBS-dependent effect in HepG2 cells. Here, there is a contradiction between the results of the RBC GLUT1 expression and the luciferase activity in HEK293T and

HepG2 cells. This might indicate an erythroid specific regulation, or it could mean that this is not a causal SNP, just a marker reflecting the modulating effect of another member from the haplotype. The minor allele of the rs11537641 variant significantly increased the luciferase expression in the HepG2 cells, which indicates a tissue-dependent effect of the variant. Taken together, these results suggest that three out of the four SNPs (rs1385129, rs841847, rs11537641) are eQTLs by directly influencing the expression of GLUT1, while the causality of the rs841848 SNP is questionable.

6.3. Regulatory background of GLUT1 expression

6.3.1. Metabolic factors

To investigate how metabolic factors modulate the effects of SNPs, we selected factors based on established literature. The regulatory effect of glucose (53), insulin (102) and hypoxia (103) on GLUT1 expression have been described in several previous studies.

In our experiments in HepG2 cells, condition-dependent and in some cases, SNP-dependent effects were observed. In case of rs1385129 and rs841848, under low glucose concentrations a difference appears in the effect on luciferase activity between the wild-type and mutant alleles. In contrast, for rs841847 and rs11537641, the significant difference between the effects of the two alleles disappears. These results indicate that the effect of the SNPs changes with the glucose level. The direct effect of insulin addition did not appreciably modify the reporter expression responses in the HepG2 cells. The effect of hypoxia, however increased the luciferase expression in case of all variants, corresponding to the observed regulation of the GLUT1 transporter in hypoxic conditions (103,114,115). These results highlight the dynamic nature of the interactions between genetic variants and metabolic factors in the HepG2 model cells, emphasizing the importance of considering environmental factors in understanding the functional consequences of genetic variations.

6.3.2. Transcription factors

The investigation of the transcription factor binding sites (TFBS) can be essential to understand the expression of the proteins, and the biological processes behind them. The bioinformatic analysis revealed several predicted transcription factor (TF) binding

sites that are altered in the presence of the tested SNPs. In case of rs1385129, we found three unique binding sites (EGR1, CTCF and GCM1) in the presence of the wild-type (wt) allele. Since EGR1 has an important role in the response to glucose (116), insulin (117) and hypoxia (118) this change may have major regulatory consequences. In the presence of the mutant allele, a new binding site appears for the ZNF281 transcriptional regulator. In the presence of the mutant allele of rs841847, five TF binding sites disappear, compared to the wt allele, including ARNT2, potentially related to hypoxia responses (119). The wild type allele of rs841848 affect binding sites for ZNF460 and ZNF528, while the sequence with the mutant allele has new binding sites for STAT proteins (Stat1, Stat5a, Stat5b), important in regulating cellular responses to insulin (120,121). In the case of the variant rs11537641 the sequence with the wild type allele contains binding sites for ZFP57 and Zfx, while the mutant allele affects four transcriptional factor binding sites (ZBTB32, SREBF1, SREBF2 and FOXK1). SREBF1 have a role in several cellular responses, including those related to insulin receptor (122), while FOXK1 affects cellular glucose homeostasis (123). Overall, this analysis provided insights into how transcription factor binding sites can be altered by the presence of an SNP and suggests that the effect of the SNPs on protein expression may manifest through these altered TF binding sites and the related regulation. However, experimental verification is needed to confirm this suggestion. For this, overexpression and knockout studies would be appropriate methods for observing the direct effect of the predicted transcription factors on GLUT1 expression. Besides, by using the same dual-luciferase system, we would be able to analyze if there is any change in the effect of the genetic variants.

6.4. *SLC2A1* SNPs and ABCG2-Q141K variant in type two diabetes

In addition to GLUT1, the other subject of my thesis is the Q141K variant of the ABCG2 protein. These independent studies can be connected through the common focus on type 2 diabetes.

Based on previous studies, in *SLC2A1*, three SNPs (rs1385129, rs841847, rs841848) are associated with some complications of the disease, and in case of rs841847 even with T2DM (59). Variant rs1385129 was found to increase the risk of diabetic nephropathy in Tunisian (56) and Kurdish (57) cohorts. The minor allele of the

rs841847 SNP has been described as a risk factor in diabetic retinopathy and nephropathy (58), but results for rs841848 have been controversial (56,60,61). Reasons for these conflicting results may be genetic heterogeneity in different populations or clinical heterogeneity in different studies. In my thesis, comparing control and T2DM groups, no significant differences were observed. One possible reason is that these SNPs have been described as risk factors in T2DM complications. Although we included samples with complications in our study, the limited sample size was insufficient for statistical analysis.

In case of *ABCG2*, when studying the SNP rs2231142 (i.e., C421A) leading to the ABCG2-Q141K variant, there was no difference in the frequency of this SNP between the control and the T2DM patients. Due to the low sample size, we performed the analysis by combining the heterozygous and homozygous groups, but even then, we did not find any significant differences between the groups. However, we found that T2DM patients carrying the SNP had significantly higher blood glucose levels than patients carrying only the wild-type alleles. In addition, the HbA1c levels, indicative of long-term blood glucose alterations, were also significantly higher in the T2DM patients carrying this SNP. In contrast, in the control group, the presence of the Q141K variant did not show any association with blood glucose or HbA1c levels. Interestingly, in a previous study they found that metformin, which drug is used as a treatment of T2DM, is transported by ABCG2 (111). These results suggest a potential role of the ABCG2-Q141K variant in T2DM. This role could either manifest through the combination with other genetic and environmental factors, or it could have an indirect role in T2DM through influencing treatment efficiency.

Consistent with its known role in uric acid transport, the ABCG2-Q141K variant resulted in significantly elevated plasma uric acid levels in the control population. However, this increased uric acid level was not observed in the type 2 diabetic patients. This may reflect the treatment of these patients, which included the xanthine oxidase inhibitor, allopurinol, in several cases. Allopurinol is commonly used in the treatment of conditions related to high levels of uric acid, such as gout. Xanthine oxidase is an enzyme involved in the conversion of hypoxanthine and xanthine to uric acid. By inhibiting this enzyme with allopurinol, the production of uric acid is reduced, which may explain the unexpected results.

7. Conclusions

7.1. GLUT1 expression and type 2 diabetes

- No direct association was observed between RBC GLUT1 expression levels and type 2 diabetes mellitus (T2DM).
- GLUT1 expression levels show a relatively wide range, in the background of which I found SNPs that, based on previous data, showed association with T2DM or its complications.

7.2. Genetic background of GLUT1 expression

- Four SNPs within the *SLC2A1* gene (rs1385129, rs841847, rs841848, rs11537641) were examined, revealing significant effects on RBC GLUT1 expression.
- Minor alleles of rs1385129, rs841848, and rs11537641 significantly increased, while rs841847 decreased the RBC GLUT1 expression.
- Reporter assays indicated the functional roles of these SNPs.
- The impact of rs841847 was consistent in both HEK293-T and HepG2 cell lines, while rs1385129 and rs11537641 exhibited cell-specific effects.
- Controversial results for rs841848 suggest a potential association with the GLUT1 expression pattern through another SNP in the haplotype.

7.3. Regulatory background of GLUT1 expression

- Metabolic factors such as glucose, insulin, and hypoxia showed variable effects on reporter expression responses.
- The effect of the SNPs is influenced by the glucose level. In case of rs1385129 and rs841848, under low glucose concentrations a difference appears in the effect between the wild-type and mutant alleles on luciferase activity. In contrast, for rs841847 and rs11537641, the significant difference between the effects of the two alleles disappears.
- Transcription factor binding site analysis revealed important alterations in the presence of the tested SNPs, suggesting their role in the regulatory network of GLUT1 expression.

7.4. *SLC2A1* SNPs and ABCG2-Q141K variant in type 2 diabetes

- The three SNPs (rs1385129, rs841847, rs841848) previously described as risk factors in T2DM or its complications were not associated with the disease in this cohort.
- The ABCG2-Q141K variant did not show direct association with T2DM but was associated with higher blood glucose and HbA1c levels in T2DM patients.
- The presence of the ABCG2-Q141K variant may affect the treatment response and contribute to long-term blood glucose alterations in T2DM patients.

8. Summary

Type 2 diabetes mellitus (T2DM) is a complex metabolic disease and various membrane transporters affect the development, progression, or treatments of the disease. In my work I have investigated two membrane proteins in association with T2DM. The GLUT1 membrane protein is a key glucose transporter in numerous cell types. The expression level of this protein has a role in several diseases, including cancer, Alzheimer's disease and T2DM. The ABCG2 protein is a multispecific xeno- and endobiotic transporter, affecting drug metabolism and playing a key role in uric acid extrusion. The ABCG2-Q141K variant, with reduced expression level has been shown to significantly affect gout development. Several other diseases, including T2DM have also been reported to be associated with high serum uric acid levels, suggesting that ABCG2 may also play a role in these conditions.

In this work GLUT1 expression level was measured in RBCs by flow cytometry, while the genetic background was analyzed by qPCR and luciferase assays. The C421A (Q141K) variant of *ABCG2* was determined by genotyping.

We found significant associations between RBC GLUT1 levels and four SNPs. In individuals with the minor alleles of rs841848, rs1385129, and rs11537641 had increased, while in those having the variant rs841847 had decreased erythrocyte GLUT1 levels. In the luciferase reporter studies performed in HEK-293T and HepG2 cells, a similar SNP-dependent modulation was observed in most cases. These associations were linked to potential regulatory factors, including reduced glucose or serum levels, hypoxic conditions, and alterations in transcription factor binding sites.

In the ABCG2-Q141K study we have compared cohorts of T2DM patients and healthy individuals regarding the major laboratory indicators of T2DM and determined the presence of the SNP rs2231142 (C421A), resulting in the ABCG2-Q141K protein variant. We found significantly higher blood glucose and HbA1c levels in the T2DM patients carrying the ABCG2-Q141K variant.

Our research on GLUT1 may contribute to a more detailed understanding of the genetic and regulatory background of GLUT1 expression and its potential role in associated diseases. The examination of the ABCG2-Q141K variant emphasizes the potential metabolic role of ABCG2 in the progression but also in the prevention and treatment of T2DM.

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10. Bibliography of the candidate's publications

10.1. Publications related to the Ph.D. dissertation

1. **Kulin Anna**, Kucsma Nóra, Bohár Balázs, Literáti-Nagy Botond, Korányi László, Cserepes Judit, Somogyi Anikó, Sarkadi Balázs, Szabó Edit, Várady György. Genetic Modulation of the GLUT1 Transporter Expression-Potential Relevance in Complex Diseases. Biology (Basel). 2022 Nov;11(11).
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10.2. Other publications

1. Szabó Edit, **Kulin Anna**, Korányi László, Literáti-Nagy Botond, Cserepes Judit, Somogyi Anikó, Sarkadi Balázs, Várady György. Alterations in erythrocyte membrane transporter expression levels in type 2 diabetic patients. Sci Rep [Internet]. 2021 Feb 2;11(1)2765. Available from: http://www.ncbi.nlm.nih.gov/pubmed/33531564
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