International Journal of Medical, Medicine and Health Sciences
ISSN: 2517-9969
Vol:13, No:6, 2019

Genotypic and Allelic Distribution of Polymorphic Variants of Gene *SLC47A1* Leu125Phe (rs77474263) and Gly64Asp (rs77630697) and Their Association to the Clinical Response to Metformin in Adult Pakistani T2DM Patients

Sadaf Moeez, Madiha Khalid, Zoya Khalid, Sania Shaheen, Sumbul Khalid

***Abstract*—**Background: Inter-individual variation in response to metformin, which has been considered as a first line therapy for T2DM treatment is considerable. In the current study, it was aimed to investigate the impact of two genetic variants Leu125Phe (rs77474263) and Gly64Asp (rs77630697) in gene *SLC47A1* on the clinical efficacy of metformin in T2DM Pakistani patients. Methods: The study included 800 T2DM patients (400 metformin responders and 400 metformin non-responders) along with 400 ethnically matched healthy individuals. The genotypes were determined by allele-specific polymerase chain reaction. *In-silico* analysis was done to confirm the effect of the two SNPs on the structure of genes. Association was statistically determined using SPSS software. Results: Minor allele frequency for rs77474263 and rs77630697 was 0.13 and 0.12. For *SLC47A1* rs77474263 the homozygotes of one mutant allele 'T' (CT) of rs77474263 variant were fewer in metformin responders than metformin non-responders (29.2% vs. 35.5 %). Likewise, the efficacy was further reduced (7.2% vs. 4.0 %) in homozygotes of two copies of 'T' allele (TT). Remarkably, T2DM cases with two copies of allele 'C' (CC) had 2.11 times more probability to respond towards metformin monotherapy. For *SLC47A1* rs77630697 the homozygotes of one mutant allele 'A' (GA) of rs77630697 variant were fewer in metformin responders than metformin non-responders (33.5% vs. 43.0 %). Likewise, the efficacy was further reduced (8.5% vs. 4.5%) in homozygotes of two copies of 'A' allele (AA). Remarkably, T2DM cases with two copies of allele 'G' (GG) had 2.41 times more probability to respond towards metformin monotherapy. *In-silico* analysis revealed that these two variants affect the structure and stability of their corresponding proteins. Conclusion: The present data suggest that *SLC47A1* Leu125Phe (rs77474263) and Gly64Asp (rs77630697) polymorphisms were associated with the therapeutic response of metformin in T2DM patients of Pakistan.

***Keywords*—**Diabetes, T2DM, *SLC47A1,* Pakistan, polymorphism.

Sadaf Moeez is with the International Islamic University, Islamabad, 44000 Pakistan (Corresponding Author; phone: +9233561555631; e-mail: sadaf_10oct@hotmail.com).

Madiha Khalid is with the PMAS Arid Agriculture University, Rawalpindi, 46000 Pakistan (phone: +923365734766; e-mail: mkmadiha87@gmail.com).

Zoya Khalid is with the FAST National University, Islamabad, 44000 Pakistan (phone: +92335716432; e-mail: zoyakhalid@nu.edu.pk).

Sania Shaheen is with the International Islamic University, Islamabad, 44000 Pakistan (phone: +923121854223; e-mail: saniaashaheen@gmail.com).

Sumbul Khalid is with the International Islamic University, Islamabad, 44000 Pakistan (phone: +923009549949; e-mail: sumbul.khalid@iiu.edu.pk).

I. Introduction

BESIDES the availability of 9 different classes of oral anti-diabetic drugs, metformin has been declared as the base for the treatment of type 2 diabetes mellitus (T2DM) all along with diet and exercise by European Association for the Study of Diabetes (EASD) and the American Diabetes Association (ADA) [1]. It has been considered as the best choice that leads to reduction of the HbA1c level without causing any hypoglycemia in individuals. At molecular level, metformin is considered as insulin sensitizer and is considered safely efficient. It is a hydrophilic molecule and the transportation of metformin in the intestine, liver and kidney is mediated by organic cation transporters (OCT). Its passive distribution is limited by its low lipid solubility across cell membranes [2].

A global observation is that in spite of the drug's proper usage, around 35% of T2DM individuals do not succeed to achieve initial optimum glycemic control by metformin monotherapy [3]-[5]. In this era of personalized medication it has been established that genetic factors is responsible for 64% to 94% of variations in any individual in renal clearance of a different drugs, including metformin [6]. Further, due to side effects of gastrointestinal, 5-10% of T2DM individuals are not able to bear metformin. Differences in the response of metformin may reveal phenotypic differences in the distribution and action of drug. Scientists have revealed different clinical effects of gender, age and BMI on clinical efficacy of metformin. This proposes that the genomic changes in the genes that encode their respective proteins play a crucial role in metformin pharmacodynamics and pharmacokinetics metformin at cellular level [4], [7], [8].

Multidrug and toxin extrusion proteins (MATEs) encoded by gene *SLC47A* are mammalian transporters and expressed predominately in the canalicular membrane of hepatocytes and brush-border membrane of proximal tubule epithelial cells in the kidney. Functionally, MATEs act as efflux transporters for different organic compounds, thus involve in the elimination process. So far, two isoforms of MATE's have been identified, MATE1 and MATE2K. Up till now, only few numbers of substrates are known including clinically used drugs such as metformin and cimetidine [9].

MATEs are secondary active transporters [10], [11]. The

vast majority of proteins that belong to the MATE family of transporters appear (by computer analysis) to have 12 transmembrane helices with intracellular amino and carboxyl terminal [12]. Human MATE genes are located in tandem on chromosome number 17 i.e., at 17p11.2. It is a region that is commonly deleted in Smith-Magenis syndrome, a genetic disorder with multiple congenital anomalies and mild mental retardation [13]. Its major function is to hinder the process of gluconeogenesis thus inhibiting the production of excessive hepatic glucose in liver [14].

The MATE family is also known as the *SLC47* family and the presence of any mutations, single nucleotide polymorphisms (SNPs), in the human MATE gene has been stated earlier in numerous populations [15], [12]. MATE1 and MATE2K are involved in the transportation of metformin. Metformin's excretion from the renal tubule cell to the lumen is carried out by MATE1 that is encoded by *SLC47A1* and MATE2K that is encoded by *SLC47A2* [16], [17]. Several SNPs that are involved in amino acid substitution are responsible for the reduced uptake of metformin therefore influence metformin's pharmacokinetics [18], [19].

MATE1 is extremely polymorphic in various inhabitants and variations in *SLC47A1* have been revealed to reduce uptake of metformin. Hence, *SLC47A1* plays an important role in triggering inter-ethnic and inter-patient changes in the clinical effectiveness of metformin. However, very limited number of studies has been performed around the globe so far and inconsistent results were documented when they correlate the genetic polymorphisms of gene *SLC47A1* to metformin therapeutic efficacy [12].

Till now, no studies have demonstrated the effect of SNPs of *SLC47A1* on metformin therapeutic efficacy in Pakistani T2DM individuals. Henceforward, we planned to assess the association between the genetic variations rs77630697 and rs77474263 of *SLC47A1* gene and the clinical response of metformin in Pakistani T2DM individuals.

In the current study, our main objective was to determine the genotypic and allelic frequencies of gene *SLC47A1* SNPs: rs77630697 and rs77474263 polymorphisms between T2DM metformin responder and metformin non-responder along with healthy individuals. The second objective was to link the *SLC47A1* rs77474263 and rs77630697 polymorphisms with the clinical pathological characteristics of metformin responder and metformin non-responder. Third objective was to know that whether these SNPs are affecting the structure and function of *SLC47A1* gene, thus changing the metformin's therapeutic efficacy.

The above selected drugs are the most common drugs for the treatment of T2DM as these are the cheapest and the most commonly available drugs in Pakistan. Metformin pharmacokinetics pathways involve different transporters including MATE1 encoded by *SLC47A1*. The selected SNPs are exonic so we hypothesized that these may affect the structure of MATE1 thus efficacy of metformin also gets effected. We selected SNPs in two ways: 1) SNPs that are present in high-likelihood candidate genes and 2) SNPs identified by ongoing GWASs for the metformin transporters encoded genes with respect to T2DM.

II. Methods

Study Population and Design

This was a case-control study. A total of 1200 unrelated individuals, including 800 clinically diagnosed T2DM individuals and 400 ethnically matched healthy individuals were enrolled into this study as per of their permission. 800 T2DM patients were further categorized in to 2 groups 400 were metformin responder (T2DM patients on monotherapy of metformin) and 400 were metformin non-responder (T2DM patients on combined therapy of metformin + sulfonylureas) Sample size was calculated by using online sample size calculator [20] by considering confidence level 95% and confidence interval 5 [21]. All included T2DM patients were clinically diagnosed by diabetologist of Pakistan Institute of Medical Sciences (PIMS) hospital, Pakistan-Islamabad.

Selection Criteria

Individuals who failed to match the drugs criteria were disqualified from the study. Individuals with T1DM, gestational diabetics, pregnant ladies and Mody were eradicated too. The whole research work was carried out by following the rules as per the statement of Helsinki and was properly permitted by the hospital. At the time of sample collection complete clinical data were collected from all T2DM patients and control individuals (Table I).

TABLE I
Basic Characteristic of Healthy Controls and T2DM Patients

Factors	Healthy controls	T2DM patients	p-value
Age (years)	49.55±14.002	50.04 ± 12.860	0.10
Gender			
Male	51%	53%	0.15
Female	49%	47%	
Height (m^2)	5.66±0.399	5.674±0.3801	0.2
Weight (kg)	67.28±.9.962	78.45±.11.898	**<0.001**
BMI (kg/m^2)	25.3370±.4.7304	29.4886±101.71	**<0.001**
Fasting Blood Glucose (mmol/L)	97.40±12.927	160.20 ±21.106	**<0.001**
Random Blood Glucose (mmol/L)	124.55 48.358	145.78±23.642	**<0.001**
HbA1c (%)	6.99±0.41	8.9±2.3	**<0.001**
BP Systolic	126.25±7.545	134.97±11.353	**<0.001**
BP Diastolic	81.93±3.345	85.26±4.343	**<0.001**
Total Cholesterol (mmol/L)	176.20±35.550	204.62 ± 32.615	**<0.001**
LDL (mmol/L)	104.68±23.081	138.56 ±26.224	**<0.001**
HDL (mmol/L)	56.30±13.711	43.55± 9.519	**<0.001**
Triglycerides (mmol/L)	139.11±2.738	176.19 ±34.780	**<0.001**

Blood Collection and DNA Extraction

Venous blood of all the involved patients and controls individuals were collected in 5 ml EDTA (ethylenediaminetetraacetic acid) vacutainers. Extraction of DNA from blood was done using a standard phenol-chloroform technique, and successively examined on 2% gel.

Genotyping

Allele specific PCR was performed. Primer sequence for **rs77474263** is **F1:** AGTGAGCTCGTACTGCTCC, **F2:** AGT

GAGCTCGTACTGCTC**T** and common reverse: TGCACCC AGACAGGATAATC with product size 169 bp. Primer sequence for **rs77630697** is **F1:** CATAAGCTCCGTGTTCTG TG**G**, **F2:** CATAAGCTCCGTGTTCTG TG**A** and common reverse: GGCCATGAAACCCACTTCAG with product size 221 bp. The reaction mixture was then processed in a thermocycler. Cycling conditions were 95 ˚C for 5 minutes for template denaturation followed by 35 cycles of PCR amplification. Further three temperatures were used for PCR: 94 ˚C (30 secs), 55˚C (30 secs), for both SNPs and 72 °C (1 minute). The gel was examined on gel documentation system relating the 100 bp DNA ladder (Fermentas, USA).

In-Silico Analysis

In-silico analysis was performed for SNPs (rs77474263 and rs77630697) of gene *SLC47A1*. Both sequence and structural properties were studied. Sequence properties depend on the physiochemical features (hydrophobicity, evolutionary conservation and volume and flexibility and rigidity) of amino acids. Structural properties involved the influence of variations on the protein structure and stability [22].

- SNP Functional Annotation

Functional annotation involves sequence of amino acids, functional prediction of a variant in coding and non-coding areas, regulatory elements of protein, miRNAs. SNPnexus and PROVEAN tool was used for filtering out the deleterious mutations with the silent mutations. The tools are accessible at [23], [24].

- Sequence Features

We have explored various sequence features including evolutionary conservation, flexibility-rigidity count and identification of disordered regions. Three different tools were utilized namely mutation assessor [25], FlexPred [26], and IUpred [27].

- 3D Structural Prediction of *SLC47A1*

The 3D structure of the protein is predicted by PHYRE2 which is a homology modelling server. The tool is available at [28].

- 3D Structure Prediction Validation and Energy Minimization

Further the quality of the model was analyzed by plotting Ramachandran plots using PROCHECK server. The model was refined by using YASARA which uses force field for energy minimization [29].

- Structural Feature of Solvent Accessibility

Solvent accessibility was measured by utilizing WESA available at [30]. It combines five methods; Bayesian statistics (BS), multiple linear regression (MLR), decision tree (DT), neural network (NN), and support vector machine (SVM). The residue is predicted either as buried or exposed.

- Structural Feature of Protein Stability

For computing protein stability changes we have utilized FoldX YASARA 3.0 beta version. YASARA is a molecular graphics, modeling and simulation program maintained by Center for Genomic Regulation, Barcelona, Spain. The mutations are classified as destabilizing if the free energy change between wild type and mutant type is greater than 5 kcal/mol.

- Structural Feature Molecular Mechanisms

The tool MutPred was utilized to determine the potential mechanisms affected by the missense mutations. This tool prioritizes the substitution that is causative of diseases and disrupts the structure and function of the protein. The structural and functional properties include the secondary structure, signal peptide and transmembrane topology, catalytic activity, macromolecular binding, post translational modifications, and metal-binding. The tool is accessible at link [31].

Statistical Analysis

Data were analyzed using IBM SPSS Statistics 20.0. Direct gene count method was used to calculate the genotypic and allelic frequencies. We used descriptive statistics with the mean ± SD in groups. To compare differences between continuous variables, student's *t* -test or the Mann-Whitney test was done. The chi-square test was done to evaluate the deviation of genotypes from Hardy-Weinberg Equilibrium (HWE). The difference in genotypic and allelic frequencies of the *SLC47A1* rs77474263 and rs77630697 polymorphisms was analysed between healthy controls and T2DM patients; metformin responder and non-responder individuals using Fisher's exact test. Multinomial logistic regression was used for calculating odds ratios (OR) and 95% confidence intervals (95% CI). To analyse differences in the level of HbA1c change between the genotypes, multivariate linear regression was used. $P < 0.05$ was considered as significant.

III. Results

Characteristics of Studied Subjects

A total of 975 T2DM cases were considered and after keeping in mind selection criteria 902 patients were selected. 102 patients were gone during follow up checkup. In the end present study was conducted on 800 T2DM individuals as they completed the follow up checkup. We categorized T2DM patients into 2 groups' metformin responders and metformin non-responders and 400 ethnically matched unrelated healthy controls were screened and clinically evaluated. Similar age distribution was observed between T2DM patients and healthy individuals though as expected, blood pressure, lipid profile, HbA1c, random and fasting glucose levels were higher in T2DM patients than in healthy individuals as shown in Table I.

Genotyping of SLC47A1 rs77474263 and rs77630697

All of the subjects were genotyped for two polymorphisms located in exon 2 and 4 of *SLC47A1* gene. In each group of study, both SNPs were following the HWE. The genotype and allele frequency distribution of *SLC47A1* rs77474263 and rs77630697 SNPs in T2DM individuals (metformin responder

and non-responder) along with healthy controls are summarized in Table II. Minor allele frequency for rs77474263 and rs77630697 was 0.13 and 0.12. As shown in Table II, statistically significant difference was observed between groups in the genotypic and allelic frequency ($p < 0.05$), representing that both studied SNPs have significant effect on the existence of T2DM in Pakistani population.

TABLE II
COMPARISONS OF GENOTYPIC AND ALLELIC FREQUENCIES OF *SLC47A1* POLYMORPHISMS IN T2DM PATIENTS (METFORMIN RESPONDERS AND NON-RESPONDERS) ALONG WITH HEALTHY CONTROLS

Genotype	Healthy Controls (n=400)	Metformin Responders (n=400)	Metformin Non-Responders (n=400)
***SLC47A1* rs77474263**			
CC	302 (75.5%)	267 (66.8%)	229 (57.2%)
CT	92 (23%)	117 (29.2%)	142 (35.5%)
TT	6 (1.5%)	16 (4%)	29 (7.2%)
Minor allele frequency			
T allele	0.13	0.19	0.25
Hardy– HWE P value	0.7	0.4	0.2
***SLC47A1* rs77630697**			
GG	314 (78.5%)	248 (62%)	194 (48.5%)
GA	78 (19.5%)	134 (33.5%)	172 (43%)
AA	8 (2%)	18 (4.5%)	34 (8.5%)
Minor allele frequency			
A allele	0.12	0.21	0.30
Hardy– HWE P value	0.2	0.9	0.6

Evaluation of Clinical Features between Metformin Responder and Non-Responder Groups (Baseline and after Treatment)

Table III presents the alterations from baseline in the clinical features of metformin responder and non-responder groups after metformin treatment successively for about 6 months. The mean (SD) of metformin daily dose that was required in responders and non-responders was 1000 mg and 1700 mg.

No significant change was reported in age, gender and height between responders and non-responders. In responders, the mean % variation of body weight (-2.58 ± -10.46 vs -0.90 ± -5.77, $p < 0.001$), BMI (-8.764 ± 2.3524 vs. -2.2735 ± 4.2688, $p < 0.001$), fasting blood glucose (-36.49 ± 4.337 vs -6.94 ± 46.86, $p < 0.001$), post- prandial blood glucose (-45.28 ± -11.73 vs -16.26 ± -5.75, $p < 0.001$), HbA1c (-18.22 ± 3.101 vs. 2.19 ± 59.85, $p < 0.001$), BP diastolic (-4.26 ± 10.95 vs. 6.48 ± 27.76, $p < 0.001$), total cholesterol (-13.11 ± 0.344 vs -1.81 ± -0.52, $p < 0.001$), was considerably low then non-responders. Conversely no noteworthy mean % change was observed in BP systolic, HDL, LDL and triglycerides as shown in Table III.

Influence of SLC47A1 (rs77474263 and rs77630697) Polymorphisms on Therapeutic Response to Metformin in T2DM Patients

A considerable difference was observed in the proportions of genotypic and allelic frequencies of *SLC47A1* rs77474263 (Table IV) and rs77630697 (Table V) gene polymorphism between metformin responder and non-responder groups.

TABLE III
CHARACTERISTICS OF T2DM PATIENTS BEFORE AND AFTER METFORMIN TREATMENT IN RESPONDERS AND NON-RESPONDERS

Factors	Metformin Responders	Metformin Non-Responders	p-value
Age	53.09± 12.390	49.88± 13.247	0.130
Gender			
Male	52%	50%	0.17
Female	48%	50%	
Height	5.66±0.4704	5.6706±0.39272	0.2
Weight			
Baseline	78.68±12.617	74.73±12.454	0.25
After Metformin 6 months therapy	76.65±11.297	74.05±11.735	0.27
Mean % Change	-2.58 ± -10.46	-0.90 ± -5.77	**<0.001**
BMI			
Baseline	26.47±4.57282	27.515±4.5310	0.263
After Metformin 6 months therapy	24.15±4.92093	27.528 ± 4.4518	**<0.001**
Mean % Change	-8.764±2.3524	0.0472± 4.2688	**<0.001**
Fasting Blood Glucose			
Baseline	192.64±18.536	198.27±35.065	0.22
After Metformin 6 months therapy	122.33±19.340	184.51±51.497	**<0.001**
Mean % Change	-36.49±4.337	-6.94± 46.86	**<0.001**
Random Blood Glucose			
Baseline	214.96±21.918	191.62±52.880	**<0.001**
After Metformin 6 months therapy	117.61±19.346	160.45±49.838	**<0.001**
Mean % Change	-45.28 ± -11.73	-16.26 ±-5.75	**<0.001**
HbA1c			
Baseline	8.89±0.474	8.9±0.553	0.26
After Metformin 6 months therapy	7.27±0.4887	9.1±0.884	**<0.001**
Mean % Change	-18.22± 3.101	2.19±59.85	**<0.001**
BP Systolic			
Baseline	134.02±12.226	132.06±11.035	0.30
After Metformin 6 months therapy	128.10±10.994	129.69±11.33	0.28
Mean % Change	-4.41±-10.076	-1.79 ± 2.67	0.25
BP Diastolic			
Baseline	85.58±4.261	84.15±4.331	0.08
After Metformin 6 months therapy	81.93±4.728	89.61±5.534	**<0.001**
Mean % Change	-4.26±10.95	6.48 ± 27.76	**<0.001**
Total Cholesterol			
Baseline	215.48±25.534	195.15±36.182	**<0.001**
After Metformin 6 months therapy	187.22±25.622	191.61±35.993	0.09
Mean % Change	-13.11±0.344	-1.81 ± -0.52	**<0.001**
HDL			
Baseline	43.05±10.994	47.80±12.613	0.21
After Metformin 6 months therapy	44.90±10.187	48.49±12.162	0.32
Mean % Change	4.29 ± -7.34	1.44 ± -3.57	0.29
LDL			
Baseline	134.48±31.613	136.46±29.924	0.35
After Metformin 6 months therapy	120.20±27.692	134.22±28.180	**<0.001**
Mean % Change	-0.22 ± -12.40	-1.64± -5.82	0.19
Triglycerides			
Baseline	186.92±18.953	189.82±35.424	0.07
After Metformin 6 months therapy	180.88±21.698	185.50±36.793	**<0.001**
Mean % Change	-3.23±14.48	-2.27 ± 3.864	0.06

TABLE IV
POLYMORPHISM RISK ANALYSIS AS FUNCTION OF THE INHERITANCE MODEL IN METFORMIN RESPONDERS AND NON-RESPONDERS CONSIDERING THE *SLC47A1* RS77474263 POLYMORPHISM

Model	Genotype	Metformin responders	Metformin Non-responders	OR (95% CI)	p-value
Codominant	C/C	267 (66.8%)	229 (57.2%)	1	
	C/T	117 (29.2%)	142 (35.5%)	1.41(1.0465 to 1.9135)	0.024
	T/T	16 (4%)	29 (7.2%)	2.11 (1.1194 to 3.9894)	0.021
Dominant	C/C	267 (66.8%)	229 (57.2%)	1	
	C/T-T/T	133 (33.2%)	171 (42.8%)	1.49 (1.1248 to 1.9979)	0.005
Recessive	C/C-C/T	384 (96%)	371 (92.8%)	1	
	T/T	16 (4%)	29 (7.2%)	1.87 (1.0023 to 3.5113)	0.04
Overdominant	C/C-T/T	283 (70.8%)	258 (64.5%)	1	
	C/T	117 (29.2%)	142 (35.5%)	1.33 (0.9890 to 1.7921)	0.05
Additive	C	651(81.4%)	600(75%)	1.45 (1.1464	
	T	149(18.6%)	200(25%)	to 1.8502)	0.002

TABLE V
POLYMORPHISM RISK ANALYSIS AS FUNCTION OF THE INHERITANCE MODEL IN METFORMIN RESPONDER AND NON-RESPONDER CONSIDERING THE *SLC47A1* RS77630697 POLYMORPHISM

Model	Genotype	Metformin responders	Metformin Non-responders	OR (95% CI)	p-value
Codominant	G/G	248 (62%)	194 (48.5%)	1	
	G/A	134 (33.5%)	172 (43%)	1.64(1.2232 to 2.2012)	0.001
	A/A	18 (4.5%)	34 (8.5%)	2.41(1.3233 to 4.4060)	0.001
Dominant	G/G	248 (62%)	194 (48.5%)	1	
	G/A-A/A	152 (38%)	206 (51.5%)	1.73(1.3075 to 2.2957)	0.0001
Recessive	G/G-G/A	382 (95.5%)	366 (91.5%)	1	
	A/A	18 (4.5%)	34 (8.5%)	1.97(1.0939 to 3.5531)	0.023
Overdominant	G/G-A/A	266 (66.5%)	228 (57%)	1	
	G/A	134 (33.5%)	172 (43%)	1.49(1.1240 to 1.9951)	0.005
Additive	G	630(78.8%)	560(70%)	1.58(1.2656	0.0001
	A	170(21.2%)	240(30%)	to 1.9931)	

For *SLC47A1* rs77474263 the carriers of one mutant allele 'T' (CT) of rs77474263 variant were fewer among metformin responders than those who were unsuccessful to respond (29.2% vs. 35.5%). Likewise, the response was further reduced (7.2% vs. 4.0%) in homozygotes 'T' allele (TT). Remarkably, T2DM individuals that were homozygous for allele 'C' (CC) had 2.11 times more probability to respond metformin monotherapy. Same pattern was detected when evaluated under several genetic models (42.8% vs. 33.2%, OR 1.49, 95% CI 1.1248 to 1.9979 for dominant; 92.8% vs. 96.0%, OR 1.87, 95% CI 1.0023 to 3.5113 for recessive; 64.5% vs. 70.8% and OR 1.45, 95% CI 1.1464 to 1.8502 for additive. No significant association was found for over-dominant OR 1.33, 95% CI 0.9890 to 1.7921 (Table IV).

For *SLC47A1* rs77630697, the homozygotes of one mutant allele 'A' (GA) of rs77630697 polymorphism were fewer in numbers among metformin responders than those who were unsuccessful to respond (33.5% vs. 43.0%). Likewise, the response was further reduced (8.5% vs. 4.5%) in homozygotes of two copies of 'A' allele (AA). Remarkably, T2DM individuals with two copies of allele 'G' (GG) had 2.41 times better chance to respond metformin monotherapy. Same pattern was detected when evaluated under several genetic models (51.5% vs. 38.0%, OR 1.73, 95% CI 1.3075 to 2.2957 for dominant; 91.5% vs. 95.5%, OR 1.97, 95% CI 1.0939 to 3.5531for recessive; 57% vs. 66.5%, OR 1.49, 95% CI 1.1240 to 1.9951 for over-dominant and OR 1.58, 95 % CI 1.2656 to 1.9931 for additive (Table V). Comparisons were also made between healthy controls and responders and non-responders for both rs77474263 and rs77630697 SNPs as shown in Tables VI-IX.

TABLE VI
POLYMORPHISM RISK ANALYSIS AS FUNCTION OF THE INHERITANCE MODEL IN HEALTHY CONTROLS AND METFORMIN RESPONDERS CONSIDERING THE *SLC47A1* RS77474263 POLYMORPHISM

Model	Genotype	Healthy Controls	Metformin Responders	OR (95% CI)	p-value
Codominant	C/C	302 (75.5%)	267 (66.8%)	1	
	C/T	92 (23%)	117 (29.2%)	1.43(1.0457 to 1.9788)	0.02
	T/T	6 (1.5%)	16 (4%)	3.01(1.1635 to 7.8195)	0.021
Dominant	C/C	302 (75.5%)	267 (66.8%)	1	0.006
	C/T-T/T	98 (24.5%)	133 (33.2%)	1.53(1.1275 to 2.0899)	
Recessive	C/C-C/T	394 (98.5%)	384 (96%)	1	0.03
	T/T	6 (1.5%)	16 (4%)	2.73(1.0595 to 7.0659)	
Overdominant	C/C-T/T	308 (77%)	283 (70.8%)	1	0.044
	C/T	92 (23%)	117 (29.2%)	1.38(1.0078 to 1.9008)	
Additive	C	696(87%)	651(81.4%)	1.53(1.1666	0.002
	T	104(13%)	149(18.6%)	to 2.0111)	

TABLE VII
POLYMORPHISM RISK ANALYSIS AS FUNCTION OF THE INHERITANCE MODEL IN HEALTHY CONTROLS AND NON-RESPONDER CONSIDERING THE *SLC47A1* RS77474263 POLYMORPHISM

Model	Genotype	Healthy Controls	Metformin Non-Responders	OR (95% CI)	p-value
Codominant	C/C	302 (75.5%)	229 (57.2%)	1	
	C/T	92 (23%)	142 (35.5%)	2.03(1.4877 to 2.7851)	<0.0001
	T/T	6 (1.5%)	29 (7.2%)	6.37(2.6027 to 15.6101)	0.0001
Dominant	C/C	302 (75.5%)	229 (57.2%)	1	
	C/T-T/T	98 (24.5%)	171 (42.8%)	2.30(1.7014 to 3.1122)	<0.0001
Recessive	C/C-C/T	394 (98.5%)	371 (92.8%)	1	
	T/T	6 (1.5%)	29 (7.2%)	5.13(2.1070 to 12.5047)	0.0003
Overdominant	C/C-T/T	308 (77%)	258 (64.5%)	1	
	C/T	92 (23%)	142 (35.5%)	1.84(1.3513 to 2.5125)	0.0001
Additive	C	696(87%)	600(75%)	2.23(1.7185	<0.0001
	T	104(13%)	200(25%)	to 2.8958)	

TABLE VIII
POLYMORPHISM RISK ANALYSIS AS FUNCTION OF THE INHERITANCE MODEL IN HEALTHY CONTROLS AND METFORMIN RESPONDERS CONSIDERING THE *SLC47A1* RS77630697 POLYMORPHISM

Model	Genotype	Healthy Controls	Metformin Responders	OR (95% CI)	p-value
Codominant	G/G	314 (78.5%)	248 (62%)	1	
	G/A	78 (19.5%)	134 (33.5%)	2.17(1.5716 to 3.0106)	<0.0001
	A/A	8 (2%)	18 (4.5%)	2.84(1.2184 to 6.6606)	0.015
Dominant	G/G	314 (78.5%)	248 (62%)	1	
	G/A-A/A	86 (21.5%)	152 (38%)	2.23(1.6372 to 3.0588)	<0.0001
Recessive	G/G-G/A	392 (98%)	382 (95.5%)	1	
	A/A	8 (2%)	18 (4.5%)	2.30(0.9921 to 5.3733)	0.05
Overdominant	G/G-A/A	322 (80.5%)	266 (66.5%)	1	
	G/A	78 (19.5%)	134 (33.5%)	2.07(1.5057 to 2.8723)	<0.0001
Additive	G	706(88.2%)	630(78.8%)	2.02(1.5412 to 2.6652)	<0.0001
	A	94(11.8%)	170(21.2%)		

TABLE IX
POLYMORPHISM RISK ANALYSIS AS FUNCTION OF THE INHERITANCE MODEL IN HEALTHY CONTROLS AND NON-RESPONDER CONSIDERING THE *SLC47A1* RS77630697 POLYMORPHISM

Model	Genotype	Healthy Controls	Metformin Non-Responders	OR (95% CI)	p-value
Codominant	G/G	314 (78.5%)	194 (48.5%)	1	
	G/A	78 (19.5%)	172 (43%)	3.56(2.5868 to 4.9245)	<0.0001
	A/A	8 (2%)	34 (8.5%)	6.87(3.1197 to 15.1677)	<0.0001
Dominant	G/G	314 (78.5%)	194 (48.5%)	1	
	G/A-A/A	86 (21.5%)	206 (51.5%)	3.87(2.8470 to 5.2796)	<0.0001
Recessive	G/G-G/A	392 (98%)	366 (91.5%)	1	
	A/A	8 (2%)	34 (8.5%)	4.55(2.0798 to 9.9622)	0.0001
Overdominant	G/G-A/A	322 (80.5%)	228 (57%)	1	
	G/A	78 (19.5%)	172 (43%)	3.11(2.26-4.27)	<0.0001
Additive	G	706(88.2%)	560(70%)	3.21(2.4744 to 4.1872)	<0.0001
	A	94(11.8%)	240(30%)		

Comparisons of differential values in metformin responders and non-responders with different *SLC47A1* variants rs77474263 and rs77630697 genotypes before and after metformin treatment is presented in Tables X-XIII. The average change in the level of HbA1c level per genotype is given in Tables XIV and XV. In metformin responder group, T2DM patients with CC and GG wild genotypes of SNPs rs77474263 and rs77630697 the average decrease in HbA1c level was largest (-0.123%). However, individuals with TT and AA mutant genotypes of SNPs rs77474263 and rs77630697 the HbA1c level was increased (0.91%) (Table XIV). Same pattern was observed in metformin non-responder group. T2DM patients with wild CC and GG genotypes of SNPs rs77474263 and rs77630697, the largest average decrease of HbA1c level was observed (0.72%). However, T2DM patients, with TT and AA genotypes of SNPs rs77474263 rs77630697 the levels of HbA1c increased (0.59%) (Table XV).

TABLE X
COMPARISONS OF DIFFERENTIAL VALUES IN METFORMIN RESPONDERS AND NON-RESPONDERS WITH *SLC47A1* VARIANT RS77474263 CC GENOTYPE BEFORE AND AFTER METFORMIN TREATMENT

Genotypes of *SLC47A1* rs77474263 (CC)	Metformin Responders	Metformin Non Responders	p-value
Age	53.±12.51	48.29±12.52	0.125
Gender			**0.015**
Male	51.7%	44.5%	
Female	48.3%	55.5%	
Height	5.67±0.322	5.66±0.4704	0.320
Weight			
Baseline	78.53±12.61	75.37±10.94	0.40
After Metformin 6 months therapy	76.61±11.41	75.48±10.768	0.30
Mean % Change	-2.444± 1.2	0.145±0.172	**<0.001**
BMI			
Baseline	27.03±4.981	26.4744±4.57282	0.263
After Metformin 6 months therapy	26.39± 4.656	27.1581±4.92093	0.326
Mean % Change	-2.36±0.325	2.60±0.348	0.110
Fasting blood sugar			
Baseline	172.10±19.001	140.51±7.158	**<0.001**
After Metformin 6 months therapy	125.90±61.16	140.48±6.616	**<0.001**
Mean % Change	-26.85±42.16	-0.021±0.542	**<0.001**
Random blood sugar			
Baseline	220.90±18.51	198.33±25.337	**<0.001**
After Metformin 6 months therapy	113.24±18.65	198.29±26.54	**<0.001**
Mean % Change	-48.74±0.75	-0.020±1.2	**<0.001**
HbA1c			
Baseline	8.75±0.556	8.82±0.4347	0.8
After Metformin 6 months therapy	7.20±0.596	7.98±0.4531	**<0.001**
Mean % Change	-17.7±0.04	-9.52±0.0184	**<0.001**
BP Systolic			
Baseline	133.69±10.61	136.707±10.44239	0.366
After Metformin 6 months therapy	127.33±11.83	133.79±10.91	**<0.001**
Mean % Change	-4.757±1.22	-2.13±0.47	**<0.001**
BP Diastolic			
Baseline	85.65±4.217	85.4327±4.45235	0.2
After Metformin 6 months therapy	81.54±5.239	84.4386±4.05122	**<0.001**
Mean % Change	-4.80±1.022	-1.158±0.401	**<0.001**
Total Cholesterol			
Baseline	198.01±25.653	226.7895±30.01729	**<0.001**
After Metformin 6 months therapy	186.58±25.645	224.397±30.48789	0.34
Mean % Change	-0.031±0.005	-1.06±0.48	**<0.001**
HDL			
Baseline	43.23±10.57	41.5848±6.71938	0.4
After Metformin 6 months therapy	45.32±9.898	41.6608±6.69782	**<0.001**
Mean % Change	0.198±0.672	0.192±0.022	**<0.001**
LDL			
Baseline	133.79±10.31	150.6433±12.3886	**<0.001**
After Metformin 6 months therapy	119.12±12.61	149.22±12.487	**<0.001**
Mean % Change	-10.96±22.30	-0.942±0.79	**<0.001**
Triglycerides			
Baseline	181.3473±3.191	180.01±25.62	0.336
After Metformin 6 months therapy	159.32±4.287	172.59±24.628	**<0.001**
Mean % Change	-12.146±34.34	-4.121±1	**<0.001**

TABLE XI
COMPARISONS OF DIFFERENTIAL VALUES IN METFORMIN RESPONDERS AND NON-RESPONDERS WITH *SLC47A1* VARIANT RS77474263 CT+TT GENOTYPE BEFORE AND AFTER METFORMIN TREATMENT

Genotypes of SLC47A1 rs77474263 (CT+TT)	Metformin Responders	Metformin Non Responders	p-value
Age	52.97±12.21	44.79±12.31	**<0.001**
Gender			
Male	30.8%	39.2 %	**<0.001**
Female	69.2%	60.8%	
Height	5.687±0.367	5.565±0.3292	0.454
Weight			
Baseline	78.98±12.658	81.69±10.524	0.073
After Metformin 6 months therapy	76.74±11.09	81.75±10.06	**<0.001**
Mean % Change	-2.84±1.568	0.073±0.464	**<0.001**
BMI			
Baseline	27.40±4.804	29.07±4.592	0.008
After Metformin 6 months therapy	26.65 ± 4.413	29.10±4.482	**<0.001**
Mean % Change	-2.73±0.391	0.103±0.11	**<0.001**
Fasting blood sugar			
Baseline	173.72±17.584	151.02±10.41	**<0.001**
After Metformin 6 months therapy	139.23±104.368	153.21±15.47	**<0.001**
Mean % Change	24.77±86.784	1.4501±5.06	**<0.001**
Random blood sugar			
Baseline	203.03±63.23	227.41±25.60	**<0.001**
After Metformin 6 months therapy	126.38±17.74	234.74±26.87	**<0.001**
Mean % Change	-37.75±45.49	3.223±1.27	**<0.001**
HbAc1			
Baseline	9.084±0.401	8.948±0.2664	**<0.001**
After Metformin 6 months therapy	8.103±0.336	8.7939±0.3064	**<0.001**
Mean % Change	-10.80±0.065	-1.722±0.04	**<0.001**
BP Systolic			
Baseline	134.70±14.979	136.50±10.334	0.219
After Metformin 6 months therapy	129.65±8.931	135.82±16.490	**<0.001**
Mean % Change	3.895±6.408	-0.498±6.156	**<0.001**
BP Diastolic			
Baseline	84.57±4.343	85.71±4.4316	0.616
After Metformin 6 months therapy	82.71±3.371	84.73±3.941	**<0.001**
Mean % Change	-2.19±0.972	-1.143±0.4906	**<0.001**
Total Cholesterol			
Baseline	203.14±37.206	225.11±30.921	**<0.001**
After Metformin 6 months therapy	188.51±25.625	222.95±31.332	**<0.001**
Mean % Change	-7.761±11.59	-0.959±0.412	**<0.001**
HDL			
Baseline	42.52±11.81	41.93±6.999	**0.667**
After Metformin 6 months therapy	44.06±10.73	41.98±6.698	**0.103**
Mean % Change	3.62±1.08	0.1192±0.301	**<0.001**
LDL			
Baseline	135.84±10.16	150.12±12.74	**<0.001**
After Metformin 6 months therapy	122.36±12.724	148.86±12.567	**<0.001**
Mean % Change	-9.92±25.23	-0.839±0.173	**<0.001**
Triglycerides			
Baseline	181.88±14.554	200.66±35.58	**<0.001**
After Metformin 6 months therapy	173.93±14.3008	198.39±37.82	**<0.001**
Mean % Change	-0.043±-1.73	-1.13±6.29	**<0.001**

TABLE XII
COMPARISONS OF DIFFERENTIAL VALUES IN METFORMIN RESPONDERS AND NON-RESPONDERS WITH *SLC47A1* VARIANT RS77630697 GG GENOTYPE BEFORE AND AFTER METFORMIN TREATMENT

Genotypes of SLC47A1rs77630697	Metformin Responders	Metformin Non Responders	p-value
Age	47.39±12.335	52.22±41	**<0.0001**
Gender			
Male	42.8%	52.4%	**<0.0001**
Female	57.2%	47.6%	
Height	5.667±0.3081	5.661±0.3263	0.853
Weight			
Baseline	75.08±11.30	78.93±12.85	0.002
After Metformin 6 months therapy	69.26±11.14	78.23±11.60	0.095
Mean % Change	-7.751±0.16	-0.886±1.25	**<0.0001**
BMI			
Baseline	26.48±4.487	27.29±4.840	0.081
After Metformin 6 months therapy	23.55±4.445	27.72±4.497	**<0.0001**
Mean % Change	-11.06±0.042	1.575±0.042	**<0.0001**
Fasting blood sugar			
Baseline	161.94±9.244	172.14±18.618	**<0.0001**
After Metformin 6 months therapy	140.84±8.867	166.76±17.703	**<0.0001**
Mean % Change	-13.029±0.377	-3.125±53.09	**<0.0001**
Random blood sugar			
Baseline	181.12±16.794	199.46±25.31	**<0.0001**
After Metformin 6 months therapy	127.12±15.831	162.59±26.199	**<0.0001**
Mean % Change	-29.699±-6.806	-18.484±0.889	**<0.0001**
HbAc1			**<0.0001**
Baseline	8.22±0.452	8.72±0.591	**<0.0001**
After Metformin 6 months therapy	7.69±0.593	7.97±0.336	**<0.0001**
Mean % Change	-6.44±31.194	-8.60±-43.147	**<0.0001**
BP Systolic			
Baseline	134.13±11.32	135.23±10.502	0.321
After Metformin 6 months therapy	127.37±10.859	134.72±11.584	**<0.0001**
Mean % Change	-5.04±0.461	-0.377±1.082	**<0.0001**
BP Diastolic			
Baseline	84.68±4.337	86.07±4.210	0.11
After Metformin 6 months therapy	83.58±3.666	85.51±6.005	0.78
Mean % Change	-1.299±0.671	-0.65±1.795	0.35
Total Cholesterol			
Baseline	202.40±24.873	204.26±37.340	0.583
After Metformin 6 months therapy	153.35±25.330	201.04±37.152	**<0.0001**
Mean % Change	-24.234±0.457	-1.576±0.19	**<0.0001**
HDL			
Baseline	41.30±11.45	41.89±7.313	0.614
After Metformin 6 months therapy	43.14±10.395	41.91±7.317	0.252
Mean % Change	4.45±1.055	0.047±0.004	**<0.0001**
LDL			
Baseline	137.42±28.72	137.71±20.757	0.909
After Metformin 6 months therapy	121.39±28.920	135.96±22.72	**<0.0001**
Mean % Change	-11.66±0.20	-1.27±1.963	**<0.0001**
Triglycerides			
Baseline	180.87±42.11	180.20±26.013	0.43
After Metformin 6 months therapy	157.67±46.78	173.26±25.012	**<0.0001**
Mean % Change	-12.82±4.67	-3.85±1.001	**<0.0001**

TABLE XIII
COMPARISONS OF DIFFERENTIAL VALUES IN METFORMIN RESPONDERS AND NON-RESPONDERS WITH *SLC47A1* VARIANT RS77630697 GG+GA GENOTYPE BEFORE AND AFTER METFORMIN TREATMENT

Genotypes of *SLC47A1* rs77630697 (GA+AA)	Metformin Responders	Metformin Non Responders	p-value
Age	45.41±12.51	52.85±13.33	**<0.0001**
Gender	41.7%	32.2%	
Male	58.3%	67.8%	**<0.0001**
Female			
Height	5.67±0.321	5.676±0.349	0.987
Weight			
Baseline	79.93± 12.45	82.13±10.65	0.114
After Metformin 6 months therapy	77.74±11.13	81.95±10.07	**<0.0001**
Mean % Change	-2.74±1.32	-0.219±0.58	**<0.0001**
BMI			
Baseline	27.67±5.04	29.05±4.527	0.018
After Metformin 6 months therapy	26.93±4.715	28.99±4.408	**<0.0001**
Mean % Change	-2.67±0.325	-0.206±0.119	**<0.0001**
Fasting blood sugar			
Baseline	169.87±10.51	171.56±17.74	0.092
After Metformin 6 months therapy	137.61±12.70	168.39±21.93	**<0.0001**
Mean % Change	-18.99±20.83	-1.847±23.61	**<0.0001**
Random blood sugar			
Baseline	214.41±60	228.20±25.82	0.011
After Metformin 6 months therapy	128.74±18.107	238.49±31.306	**<0.0001**
Mean % Change	-39.96±41.893	4.51±5.486	**<0.0001**
HbAc1			
Baseline	9.01±0.270	9.3±0.033	**<0.0001**
After Metformin 6 months therapy	8.5±0.495	9.0±0.021	**<0.0001**
Mean % Change	-5.66±0.225	-3.22±0.012	**<0.0001**
BP Systolic			
Baseline	134.99±14.10	135.45±10.33	0.740
After Metformin 6 months therapy	129.26±8.988	136.28±13.14	**<0.0001**
Mean % Change	-4.24±5.112	0.612±2.81	**<0.0001**
BP Diastolic			
Baseline	85.59±4.322	86.13±4.446	0.360
After Metformin 6 months therapy	82.41±3.155	88.61±681.23	0.302
Mean % Change	-3.715±1.167	2.879±676.78	**<0.0001**
Total Cholesterol			
Baseline	212.59±24.17	223.50±32.45	**<0.0001**
After Metformin 6 months therapy	196.85±26.542	221.42±32.56	**<0.0001**
Mean % Change	-7.403±2.372	-0.93±0.11	0.27
HDL			
Baseline	41.30±1.66	40.77±5.33	0.614
After Metformin 6 months therapy	42.46±1.98	40.61±5.31	0.252
Mean % Change	2.808±19.277	-0.392±-0.375	**<0.0001**
LDL			
Baseline	136.47±33.77	148.83±14.129	**<0.0001**
After Metformin 6 months therapy	122.82±26.867	147.28±14.67	**<0.0001**
Mean % Change	-10.002±6.903	-1.041±0.541	**<0.0001**
Triglycerides			
Baseline	185.08±21.677	185.42±14.89	**<0.0001**
After Metformin 6 months therapy	171.18±20.920	222.62±14.11	**<0.0001**
Mean % Change	-7.510±-6.398	20.06±-5.23	**<0.0001**

TABLE XIV
THE NUMBER OF METFORMIN RESPONDER AND THE AVERAGE CHANGE IN THE LEVEL OF HBA1C (%) PER *SLC47A1* GENOTYPE

Genotypes of *SLC47A1* rs77474263		Genotypes of *SLC47A1* rs77630697			
		GG	**GA**	**AA**	**Overall**
CC	**n**	183	47	0	230
	δ Hbac1 (95% CI)	-0.1232658 (-1.463674 to 0.1828574)	-0.3960088 (-1.424368 to 0 .6323509)	N/A	-3.48244 (-2.710886 to -4.253993)
	Odd Ratio	Ref	0.6730008	-	3.253
	P-value	0.0120	0.0450	-	**<0.001**
CT	**n**	17	108	17	142
	δ Hbac1	-0.584159	0.1071295	0.5909223	1.612242
	(95% CI)	(-2.187208 to 1.21889)	(-0.2598331 to 0 .4740922)	(-0.2312096 to 1.413054)	(0.9689337 to 2.25555)
	Odd Ratios	.6162152	1.113078	1.805653	1.612242
	P-values	0.0234	0.036	0.0159	**<0.001**
TT	**n**	0	11	17	28
	δ Hbac1 (95% CI)	N/A	0.2435378 (-0.5211497 to 1.008225)	0.914568 (-0.8764665 to 0 .10573306)	2.684756 (1.001994 to 4.367518)
	Odd Ratio	-	1.275755	0.6606256	14.65462
	P-values	-	0.0325	**<0.001**	0.002
Overall	**n**	200	166	34	400
	δ Hbac1 (95% CI)	-3.31244 (-4.632003 to -2.992876)	5.899263 (4.710858 to 7.087669)	3.59905 (1.869482 to 5.328617)	3.31244 (2.632003 to 3.992876)
	Odd Ratios	27.45202	34.7687	36.56347	27.45202
	P-values	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Results of in silico Analysis

Multiple studies have concluded that SNPs present in non-cancerous diseases more often appear in the non-coding regions of the genome [32]. In conjunction, for this current study SNPnexus showed that both the SNPs occur at the non-coding side as they are associated with diabetes mellitus type 2. Furthermore, SIFT and POLYPHEN presented that structure of the *SLC47A1* protein is damaged by the variations. Also, PROVEAN also predicted both mutations as deleterious as the predicted scores are below the cutoff.

Sequence Analysis

The evolutionary conservation of the mutated residues was analyzed by mutation accessor that showed that the residues at position 64 and 125 were highly conserved. Further, for the mutant G64D Glycine is predicted as the most flexible amino acid and its mutation to aspartic acid which is predicted as rigid by FlexPred will disrupt the protein function. For this residue torsion angles are uncommon. To make torsion angles, glycine is the only residue that is flexible enough. So when it changes into some other residue, it will force the local normal backbone into an improper conformation. As a result normal structure will be disrupted. For the mutant L125F FlexPred predicted both leucine and phenylalanine as rigid residues. Both the substitutions did not lie in the disordered regions as their value is predicted below the threshold as shown in Fig. 1.

TABLE XV
THE NUMBER OF METFORMIN NON-RESPONDERS AND THE AVERAGE CHANGE IN THE LEVEL OF HBA1C LEVEL (%) PER SLC47A1 GENOTYPE

Genotypes OF *SLC47A1* rs77474263		Genotypes of *SLC47A1* rs77630697			
		GG	**GA**	**AA**	**Overall**
CC	**n**	183	47	0	230
	δ Hbac1 (95% CI) Odd Ratio	-0.7225393 (-1.73876 to 0.3687975) Ref	.9416542 (-.2487684 to 2.132077)	N/A -	2.907998 (2.203518 to 3.612479) 18.32009
	P-value	0.0370	0.0450	-	**<0.001**
CT	**n**	17	108	17	142
	δ Hbac1 (95% CI) Odd Ratios	-1.741946 (-3.175655 to -.3082377) 0.1751791	0.3344946 (-0.0658474 to 0 .7348367) 1.31621	0.1160384 (-0.5534209 to 0 .7854977) 1.123039	1.216221 (0.6186892 to 1.813754) 3.374413
	P-values	0.017	0.056	0.0234	**<0.001**
TT	**n**	N/A	11	17	28
	δ Hbac1 (95% CI) Odd Ratio	-	0.4269204 (0.305358 to 0.4515177) 0.6525155	0.5909223 (-0.2312096 to 1.413054) 1.805653	3.278051 (0.7342355 to 5.821866) 26.52402
	P-values	-	0.0325	0.0159	0.012
Overall	**n**	200	166	34	400
	δ Hbac1 (95% CI) Odd Ratios	2.497617 (1.947774 to 3.04746) 12.1535	12.1535 (-.1199254 to .9641801) 1.525203	3.001402 (0.4577858 to 5.545018) 20.11372	1.53011 (1.316185 to 1.744035) 4.618685
	P-values	**<0.001**	**<0.001**	**<0.001**	**<0.001**

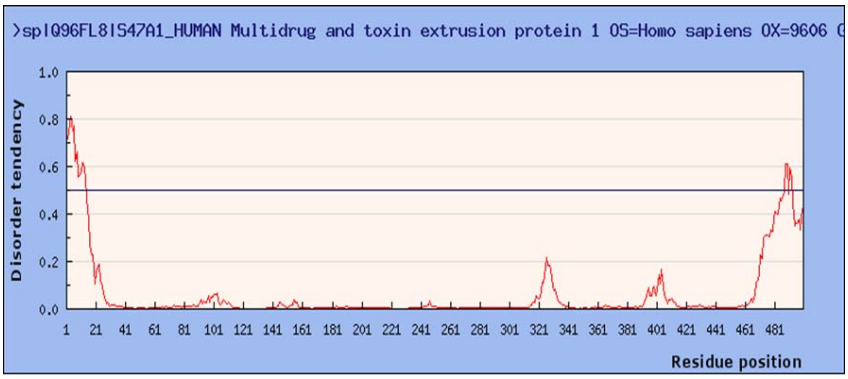

Fig. 1 Prediction of Disordered regions by using IUPred

Structural Analysis

For computing the structural impact of mutations first the 3D structure of *SLC47A1* was predicted by Phyre 2 which uses c5y50A as single highest scoring template. The 423 residues were modeled covering 78% of the sequence with 100 % confidence. The structure is shown in Fig. 2. The quality of the 3D structure generated from Phyre2 server was analyzed by plotting Ramachandran plot using PROCHECK. The plot shows the distribution of residues in the allowed and favored regions as shown in Fig. 3 and presented in Table XVI. Further the wild type and mutant structures were also minimized using YASARA energy minimization server. The change in total energy was observed along with RMSD values which indicate the deviation of mutants from the wild type. The results are given in Table XVII.

By using WESA tool we analyzed that whether the mutations are occurring at the surface or at the core of the protein. WESA showed that both mutations are significantly buried in the core of the protein. Consequently, the size difference in the wild type and mutant residues will affect the contacts with the nearby residues hence disrupting the structure of the protein. The protein stability changes determined by FoldX Yasara showed that both of the mutations are destabilizing the structure of the protein. The wild type had the protein stability value 88.93 kcal/mol. The results showed that mutation at G64D has protein stability value 42.2 ddG (kcal/mol) while mutation L125F has 51.3 ddG (kcal/mol). The folding free energy is important feature of protein stability. Hence these predicted values have shown that protein stability is affected by both mutations. Furthermore, the results of MutPred showed that mutations associated with the rs77630697 and rs77474263 SNPs of *SLC47A1* are highly damaging as the probability of them to be deleterious is more than 0.5. Results of *in-silco* analysis are presented in Table XVIII.

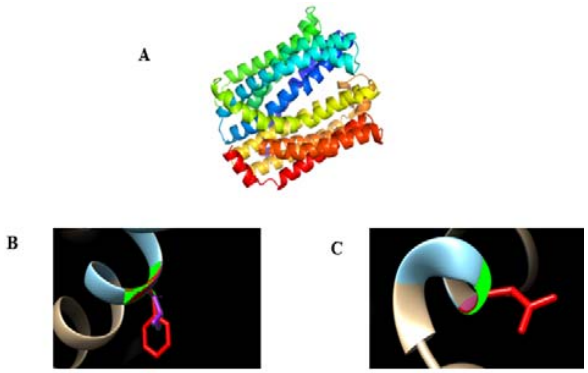

Fig. 2 (A) 3D structure of SLC47A1 predicted by Phyre2; (B) Superimposed mutant and wild type structure Leu125Phe; (C) Superimposed mutant and wild type structure Gly64Asp

TABLE XVI
DISTRIBUTION OF RESIDUES IN THE ALLOWED AND FAVORED REGIONS

Ramachandran Plot Statistics		
Residues in most favored regions [A,B,L]	373	94.9%
Residues in additional allowed regions [a,b,l,p]	19	4.8%
Residues in generously allowed regions [~a,~b,~l,~p]	1	0.3%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	393	100.0%
Number of end-residues (excl. Gly and Pro)	3	
Number of glycine residues	36	
Number of proline residues	10	
Total number of residues	442	

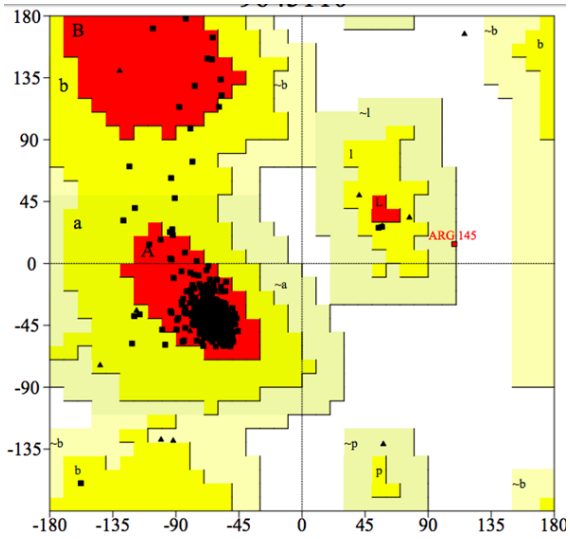

Fig. 3 Distribution of residues in the allowed and favored regions

TABLE XVII
WILD TYPE AND MUTANT STRUCTURES BY YSARA ENERGY MINIMIZATION SERVER

Models	RMSD	Total energy after minimization
Native structure	1.3265	-228278.9 KJ/mol
G64D	1.317	-228632.4 KJ/mol
L125F	1.3032	-227850.0 KJ/mol

TABLE XVII
RESULTS GENERATED FROM SNPNEXUS AND MUTPRED

SNPNexus	SNP	Allele	Gene	Predicted Function	Amino Acid	Details	SIFT Prediction
	rs77474263	C|T	SLC47A1	Non-coding	L125F	Non-synonymous	Highly damaging
	rs77630697	G|A	SLC47A1	Non-coding	G64D	Non-synonymous	Highly damaging
MutPred	**Mutation**		**Probability of deleterious mutation**			**Top 5 Features**	
	L125F		0.6			Gain of MoRF binding (P = 0.5272) Loss of stability (P = 0.5657) Gain of helix (P = 0.6868) Loss of glycosylation at P129 (P = 0.7545) Loss of catalytic residue at F128 (P = 0.8121)	
	G64D		0.8			Loss of catalytic residue at C63 (P = 0.0433) Loss of helix (P = 0.1299) Loss of ubiquitination at K68 (P = 0.1576) Gain of loop (P = 0.2045) Loss of stability (P = 0.4401)	

IV. DISCUSSION

Diabetes Mellitus (DM) is chronic disease developed when pancreas fails to produce insulin required or when human body is unable to utilize the produced insulin properly [33]. Regardless in the advancement of the treatment of T2DM, the growing frequency of T2DM has turn out to be a problem globally. Among the available numerous classes of agents for T2DM cure, metformin is one of the most commonly recommended drug globally including Pakistan, where the incidence of T2DM is increasing. There is a vast clinical difference in metformin response; hence the drug is commonly combined with extra drugs like sulfonylureas to treat T2DM which has been considered as second line of therapy.

Clinical trial studies have shown that more than one third of individuals getting metformin monotherapy fail to attain satisfactory control on levels of fasting glucose [34]. The key cause for the lack of response in the behavior of metformin in T2DM individuals may be due to changes in genes that are involved in drugs pharmacokinetics and pharmacodynamics [35]. In our present study, we established a noteworthy association with *SLC47A1* rs77474263 and *SLC47A1* rs77630697 gene polymorphism and metformin clinical efficacy. T2DM patients with carriers of CC and GG genotypes had 2.11 and 2.41 times more probability to response towards metformin use when linked to T2DM individuals with TT and AA genotypes. In addition, metformin gives strong beneficial effects on BMI, blood pressure and lipid profile. To the best of our knowledge, this

was the first study from Pakistani population and there are very few studies on the genotyping of *SLC47A1* Leu125Phe (rs77474263) and Gly64Asp (rs77630697) from different world populations. Previous studies conducted globally, showed that orally administered drugs successfully reduce HbA1c levels by 0.5–1.5% [36].

This was a case-control study. Polymorphisms in the gene *SLC47A1* could result in either 'loss-of-function' or 'gain-of-function' mutations resulting in altered function of the efflux transporters. Polymorphisms including c.983A > C (p.D328A, ss104806857), c.373C > T 69 (p.L125F, rs77474263) and c.191G > A (p.G64D, rs77630697) were studied in the medical trials but presented no influence on metformin pharmacokinetics [37]. Studies of [38] and [39] are the key studies which did not find any association between the above studied SNPs and metformin pharmacokinetics. It has been demonstrated [40] that individuals with minor allele A have shown twofold reduction in HbA1c level than those with G allele in case of SLC47A1 rs2289669 polymorphism.

The MAF (%) of the SNP rs77474263 in *SLC47A1* gene in other populations is described: Europeans (0.00105%), Americans (T=0.1494%), Asians (0.0022), European Americans (0.001%), Africans (0.0004%) and East Asians (0.002). The variant rs77630697 in *SLC47A1* gene had a MAF of 0.007%, 0.0027% in East Asians and Asians whereas 0.0000%, 0.0000% and 0.0000% 48.5%, in Americans, European and Africans respectively [41].

Our *in-silico* studies have shown that both the mutations found in the protein of *SLC47A1* affects the 3D structure. For rs77474263, amino acid substitution occurs at position 125 where leucine is substituted to phenylalanine. Though both are non-polar amino acids, still this substitution is disrupting the 3D structure of a protein. Leucine is non-polar because of the presence of isobutyl side chain, whereas in the case of phenylalanine, it is hydrophobic due to the inert and hydrophobic nature of the benzyl side chain. For rs77630697, amino acid substitution occurs at position 64 where glycine is substituted to aspartic acid and as a well-established fact, we know it plays a crucial role in the helix formation due to its small size and it occupies a specific internal position in the helix so it was assumed that any amino acid substitution for glycine causes delay/disturbance in the helix propagation. Therefore, the difference in the shape of these two amino acids may be one of the reasons in the change of structure of this protein. These alterations in the 3D environment *in vivo* cause loss of the normal function of *SLC47A1* in different diseases depending upon the nature of the substituted amino acid and its position.

Results of the current study might have a practical implication in future personalized treatment of T2DM patients. However, small sample size of the current study can be considered as a limitation of the study, therefore more research in different ethnic groups with a larger sample size is required to elucidate the role of *SLC47A1* Leu125Phe (rs77474263) and Gly64Asp (rs77630697) variants in metformin response. In serum, level of insulin was not measured in T2DM patients. Level of metformin in the T2DM patient's serum was not measured. mRNA based study was not done due to limitation of funds which can help to check the effects of these exonic SNPs on the expression of gene. Moreover studying the more SNPs of SLC47A1gene may add more information regarding to efficacy of metformin in T2DM patients.

In conclusion, we summarized that the rs77474263 and rs77630697 genetic polymorphisms of *SLC47A1* seem to be an important factor in metformin therapeutic response in Pakistani T2DM patients. Though, it needs to be validated in larger sample size.

Acknowledgments

Entire group is thankful to all the participants who agreed to be a part of this study. We thank International Islamic University for providing lab facilities. We also thank PIMS hospital for permitting us to collect blood from T2DM patients.

Conflict of Interest: None

References

[1] Inzucchi SE, Bergenstal RM, Buse JB, et al. American Diabetes Association (ADA) European Association for the Study of Diabetes (EASD) Management of hyperglycemia in type 2 diabetes: a patient-centered approach: position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). *Diabetes Care*. 2012; 35:1364–1379.

[2] Nathan DM, Buse JB, Davidson MB, et al. American Diabetes Association. European Association for Study of Diabetes Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care*. 2009; 32:193–203.

[3] Graham GG, Punt J, Arora M, et al. Clinical pharmacokinetics of metformin. *Clinical Pharmacokinetics*. 2011; 50: 81–98.

[4] Cook MN, Girman CJ, Stein PP, Alexander CM. Initial monotherapy with either metformin or sulphonylureas often fails to achieve or maintain current glycaemic goals in patients with type 2 diabetes in UK primary care. *Diabetic Medicine*. 2007; 24: 350–358.

[5] Kahn SE, Haffner SM, Heise MA, et al. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. *New England Journal of Medicine*. 2006; 355: 2427-2443.

[6] Bailey CJ and Turner RC. Metformin. *New England Journal of Medicine*. 1996; 334: 574–579.

[7] Leabman MK, Huang CC, Kawamoto M, et al. Pharmacogenetics of Membrane Transporters Investigators. Polymorphisms in a human kidney xenobiotic transporter, OCT2, exhibit altered function. *Pharmacogenetics*. 2002; 12: 395–405.

[8] Hermann LS, Schersten B, Melander A. Antihyperglycaemic efficacy, response prediction and dose-response relations of treatment with metformin and sulphonylurea, alone and in primary combination. Diabet Med. 1994;11:953–60.

[9] Reitman ML, Schadt EE. Pharmacogenetics of metformin response: a step in the path toward personalized medicine. J Clin Invest. 2007;117:1226–9.

[10] Damme K, Nies AT, Schaeffeler E, Schwab M. Mammalian MATE (SLC47A) transport proteins: impact on efflux of endogenous substrates and xenobiotics. *Drug Metabolism & Review*. 2011; 43: 499-523.

[11] Giacomini KM, Sugiyama Y. Membrane transporters and drug response. In: Goodman LS, Brunton LL, Chabner B, Knollmann BC, eds. Goodman & Gilman's Pharmacological Basis of Therapeutics. 12th ed. New York: McGraw-Hill Education; 2011.

[12] Omote H, Hiasa M, Matsumoto T, Otsuka M, Moriyama Y. The MATE proteins as fundamental transporters of metabolic and xenobiotic organic cations. Trends Pharmacol Sci. 2006;27:587–93. doi: 10.1016/j.tips.2006.09.001.

[13] Takane H, Shikata E, Otsubo K, Higuchi S, Ieiri I. Polymorphism in

human organic cation transporters and metformin action. *Pharmacogenomics.* 2008; 9: 415–422.
[14] Bi W, Yan J, Stankiewicz P, et al. Genes in a refined Smith-Magenis syndrome critical deletion interval on chromosome 17p11.2 and the syntenic region of the mouse. *Genome Research.* 2002; 12: 713–728.
[15] Hundal RS, Krssak M, Dufour S, et al. Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes.* 2000; 49: 2063–2069.
[16] Aier MH Jr, Paulsen IT. Phylogeny of multidrug transporters. *Seminars in Cell & Developmental Biology*. 2001; 12: 205–213.
[17] Tsuda M, Terada T, Mizuno T, Katsura T, Shimakura J, Inui K. Targeted disruption of the multidrug and toxin extrusion 1 (mate1) gene in mice reduces renal secretion of metformin. *Molecular Pharmacology*. 2009; 75: 1280–1286.
[18] Chen Y, Li S, Brown C, et al. Effect of genetic variation in the organic cation transporter 2 on the renal elimination of metformin. *Pharmacogenetics and Genomics*. 2009; 19: 497-504.
[19] Zolk O. Disposition of metformin: variability due to polymorphisms of organic cation transporters. Annals of Medicine. 2012; 44:119-129. *Pharmacology Therapy*. 2014; 96: 370–9.
[20] Available at www.surveysystem.com. Accessed on 1/11/2018.
[21] Kirby A, Gebski V, Keech AC. Determining the sample size in a clinical trial. The *Medical Journal of Australia*. 2002; 177: 256-7.
[22] Khalid Z and Sezerman OU. Prediction of HIV Drug Resistance by combining Sequence and Structural Properties. IEEE/ACM transactions on computational biology and bioinformatics. 2018; 15: 966-973.
[23] Available at http://snp-nexus.org/. Accessed on 13/12/2018.
[24] Available at http://provean.jcvi.org/seq_submit.php. Accessed on 13/12/2018.
[25] Available at http://mutationassessor.org/r3/. Accessed on 13/12/2018.
[26] Available at http://flexpred.rit.albany.edu/. Accessed on 14/12/2018.
[27] Available at http://iupred.enzim.hu/. Accessed on 14/12/2018.
[28] Available at http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index. Accessed on 14/12/2018.
[29] Krieger E, Joo K, Lee J, Lee J, Raman S, Thompson J, Tyka M, Baker D, Karplus K. Improving physical realism, stereochemistry, and side-chain accuracy in homology modeling: Four approaches that performed well in CASP8. Proteins. 2009; 77 Suppl 9:114-22.
[30] Available at http://pipe.sc.fsu.edu/wesa.html. Accessed on 15/12/2018.
[31] Available at http://mutpred.mutdb.org/. Accessed on 15/12/2018.
[32] Khalid Z and Ugur. Interpreting the prevalence of regulatory SNPs in cancers and protein-coding SNPs among non-cancer diseases using GWAS association studies. 2014: 95-98.
[33] Mahrooz A, Parsanasab H, Hashemi-Soteh MB, et al. The role of clinical response to metformin in patients newly diagnosed with type 2 diabetes: a monotherapy study. Clin Exp Med. 2015;15(2):159-65.
[34] Shu Y, Sheardown SA, Brown C, et al. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *Journal of Clinical Investigation.* 2007; 117: 1422–1431.
[35] Sissung TM, Troutman SM, Campbell TJ, et al. Transporter pharmacogenetics: transporter polymorphisms affect normal physiology, diseases, and pharmacotherapy. *Discovery medicine*. 2012; 13: 19-34.
[36] Lozano E, Herraez E, Briz O, et al. Role of the plasma membrane transporter of organic cations OCT1 and its genetic variants in modern liver pharmacology. *Bio Medical research international*. 2013; 692-701.
[37] Topic E. The role of pharmacogenetics in the treatment of diabetes mellitus. *Journal of Medical Biochemistry.* 2014; 33: 58–70.
[38] Toyama K, Yonezawa A, Tsuda M, et al. Heterozygous variants of multidrug and toxin extrusions (MATE1 and MATE2-K) have little influence on the disposition of metformin in diabetic patients. *Pharmacogenetics Genomics.* 2010; 20: 135-138.
[39] Tzvetkov MV, Vormfelde SV, Balen D, et al. The effects of genetic polymorphisms in the organic cation transporters OCT1, OCT2, and OCT3 on the renal clearance of metformin. *Clinical Pharmacology & Therapeutics.* 2009; 86: 299-306
[40] Tkac I, Klimcakova L, Javorsky M, et al. Pharmacogenomic association between a variant in SLC47A1 gene and therapeutic response to metformin in type 2 diabetes. *Diabetes Obesity and Metabolism.* 2013; 15: 189–91.
[41] Retrieved from: https://www.ncbi.nlm.nih.gov/projects/SNP/. Accessed on 18/12/2018.