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Genetic Polymorphism in the Organic Cation Transporters 1 (OCT1) Gene and its Effect on Therapeutic Efficacy and Gastrointestinal Side Effects of Metformin in Patients with Type 2 Diabetes Mellitus in Basrah/ Southern Iraq.

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This study aims to detect the association of the OCT1 genetic polymorphism with the efficacy and gastrointestinal side effects of metformin in newly diagnosed type 2 diabetes and drug naïve patients in Basrah/Southern Iraq. This was a prospective cohort population-based study of (102) newly diagnosed type 2 diabetics from February 2022 to December 2022. Newly diagnosed type 2 diabetes, drug naïve patients with an HbA1c range of (6.5-9.9) were included in the study. All the participants received immediate-release metformin. Metformin responders were patients whose HbA1c levels decreased by =1% after three months of treatment. Patients were genotyped for one of the most common SNPs in the OCT1 gene (SLC22A1): M420del (rs72552763) of axon 7, using ARMS- PCR genotyping assays. Gastrointestinal side effects were observed in 15% of the patients. Out of the total of 102 participants, 69 were responders and 33 were non-responders. The homozygous genotype (AA) "reference type" of the SLC22A1 (rs72552763) gene polymorphism was significantly found in the responders' group; p-value = 0.0001. The homozygous genotypes (deletion/deletion) of the SLC22A1 (rs72552763) gene were more common among the non-responders' group; p-value = 0.0001. About 87% of those with gastrointestinal side effects carried the AA genotype. All the patients without gastrointestinal side effects carried the homozygous del/del genotype; P-value 0.005. There was a significant association between the rs72552763 gene polymorphism and metformin efficacy and GI side effects.

Keywords: GI side effects; OCT1 polymorphism; Metformin; Type-2 Diabetes Mellitus.

Type 2 diabetes mellitus (T2DM) is a chronic, progressive disease that requires strict supervision from the time of diagnosis through all stages of the disease [1, 2]. Metformin is the first choice of oral glucose-lowering drugs for treatingT2DM [3]. Metformin decreases the blood glucose level by inhibiting hepatic gluconeogenesis, and increasing peripheral glucose uptake [4]. It may also increase gut glucose utilization [5].

Metformin is not metabolized inside the body [6]. As Metformin is cationic drug at physiological pH. Its transportation through cell membranes is mediated by many transporters. One of these transporters is the organic cation transporter 1 (OCT1) [7]. The OCT1 is a protein transporter that belongs to the Solute Carrier family *(SLC22A), and* is located in the sinusoidal membrane of the hepatocytes and is considered the major transporter of metformin into hepatocytes, the target of metformin action [8, 9]. Some studies reported that human OCT1 is also located in the lateral membrane of the intestinal epithelial cells [9]. The OCT1 gene (*SLC22A1*) is highly polymorphic [10]. One of the most prevalent function variants was the methionine 420 deletion (Met420del) which was recognized among 1,079 individuals from 53 populations analyzed (minor allele frequency around the words is 14.1 %). There were other loss of function variants including Arg61Cys (3.2 %), Gly465Arg (1 %), Ser14Phe (0.8 %), and Gly401Ser (0.7 %) [11].

Metformin is frequently associated with gastrointestinal (GI) side effects in about 30 % of the patients. These side effects include nausea, bloating, abdominal pain, and diarrhea. Around 5–10% of these patients cannot continue metformin treatment due to severe GI side effects[12].

Several studies have shown the association of OCT1 polymorphism with response to metformin [13, 14]. A study in South India included 122 T2DM patients concluded that carriers of two copies of allele (AA) of rs622342 had 5.6 times greater probability of responding to metformin treatment [15].The current study has three aims. First, to investigate the frequency of SNP [Met420del/ **rs72552763]** of OCT1 (SLC22A1 gene). Second, to assess the association between this SNP and response and GI side effects of metformin therapy in T2DM patients. Third, to evaluate the association between response and GI side effects of metformin in T2DM patients from Basrah/Southern Iraq.

MATERIALS AND METHODS

In this prospective cohort study, we interviewed 984 T2DM patients from two main tertiary care centers in Basrah/Southern Iraq; 450 patients from Almawani Specialized Endocrine and Diabetes Center from (February 2021 to May 2022) and 534 patients from Faiha Specialized Diabetes, Endocrine, and Metabolism Center (FDEMC) from (May 2022 to December 2022).

Eight hundred thirty-two patients were excluded from a total of 984 interviewed patients for the following reasons: seven patients were type 1 diabetes, 541 patients were not newly diagnosed, 265 patients were treated with other glucose-lowering drug before enrollment, two patients were pregnant, one cancer patient, eight patients took OCT1 inhibitors (three patients took cimetidine and five patients took clopidogrel), and eight patients refused to enter into the study. Consecutively, 152 patients were enrolled. A signed informed consent was obtained from all the patients. During the follow-up period, 22 patients failed to follow up, two patients added another glucose-lowering drug to metformin monotherapy, three patients did not have blood samples for genetic and biochemical analysis, two patients stopped metformin due to intolerance, and 21patients had a false genetic test. Ultimately, 102 T2DM patients were included in the study and their data were analyzed.

All the participants received immediate-release metformin tablets supplied by the centers in which the study was conducted. The regimen was given by slow titration to ensure patient compliance and limit side effects as follows: in the first week, the participants were given 500mg tablets once daily. In the second week, the participants were given 500mg tablets twice daily and then 1g twice daily for three months.

The inclusion criteria involved: Newly diagnosed T2DM (drug naïve) patients with HbA1C range (6.5-9.9 %), with an age ranged from 25 and 75 years old (male and female).

On the other hand, exclusion criteria involved: Those with GI diseases such as gastro duodenal ulcers, malignancies, hormonal therapy, and patients currently on glucose-lowering drugs. Patients were monitored during the first three months of metformin monotherapy.

In the absence of any acute GI disease, the GI symptoms of metformin therapy included: bloating, abdominal pain, diarrhea, nausea, vomiting, and anorexia. [12].

Response to metformin is defined as the reduction of HbA1c levels of e”1% from baseline value after three months of metformin treatment [16].

Data were directly obtained from the

participants during interviews, using a questionnaire designed to record the patient's information (Appendix 1) which included name, age (years), sex, family history of DM, drugs history, GI side effect, micro vascular complications and blood pressure.

Five ml of venous blood was collected from all the patients. Three ml of whole blood was placed in an anticoagulant tube for HbA1c analysis at baseline and after three months of metformin monotherapy. The residual (2ml) was put in EDTA-tube for genetic testing that was done once only.

Genetic Analysis

Patients were genotyped for one of the most common SNPs in the OCT1 gene (*SLC22A1*): M420del (rs72552763) of axon 7 by using ARMS-PCR genotyping assays. Polymerase chain reaction (PCR) was carried out by using a specific pair of primers designed for the (SLC22A1) gene using the NCBI database, and primer 3software.

The sequences of the primers that were used for the amplification analysis of the OCT1 (SLC22A1) gene for SNP identifications include two forwards

M420del-F (A): GGGCAGCCTGCCTCGCCA,

M420del-F (Del): GGGCAGCCTGCCTCGCCT, and one Reverse

M420-R: GCCTGAGGGAGGCTTTGGAG, using product size 645bp.

Laboratory measurements

The following instruments and materials were used to accomplish the study: Geneaid DNA extraction, Agarose, Go Tag Green Master Mix, Nuclease Free Water TAE 40X, Ethidium Bromide Solution (10mg/ml) Quantiflor dsDNA System (Promega, USA). In addition, the following Instruments were used for Genetic Analysis: Germany-dry-bath DB-005Dry block heater (Taiwan), QuantusFlorometer (Promega, USA), Vortex Mixer in Shakers(BIOTEC- FISHER SCO, Germany), ComboMini Gel Electrophoresis system (Avans Biotechnology electrophoresis, Breda), Micro spin Centrifuge (My Fugene, China), Digital Magnetic Hotplate-Stirrer (MS-H-pro Dragon LAB, China), Centrifuge -13300 rpm (Hattich-Mikro 185, Germany), The high performance thermal cycler for DNA amplification by PCR (Analytic jena –BiometraTAdvanced, Germany).

Extraction and quantification of DNA

The DNA Genome was extracted from the blood samples based on the protocol of Geneaid DNA extraction. The procedure of DNA extraction is carried out in 4 steps: lysis, binding, washing, and elution.

The concentration of extracted DNA was detected by using Quantus Fluorometer and was measured by using (a QuantiFluor® ONEdsDNA System kit). The QuantiFluor®ONEdsDNA System consists of a fluorescent DNA binding dye that allows sensitive quantitation of small amounts of double-stranded DNA (ds DNA).

Optimization of polymerase chain reaction was carried out after many trials, using Master mix 12.5 ìl, water 7.5 ìl, Forward primer 1 ìl, Reverse primer 1 ìl and DNA sample 3 ìl, with a total volume of up to 25 ml.

Polymerase chain reaction (PCR)

Thermocycler Program for (Met420del)/ (SLC22A1) gene involves initial Denaturation at 95 C° for 5 minutes (one cycle), Denaturation at 95 C° for 30 seconds (30 cycles), Annealing at 52 C° for30 seconds (30 cycles), extension at 72 °C for 30 seconds (30 cycles), Final extension at 72 °C for 5 minutes (one cycle) hold at 4 °C for ".

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to ensure the existence of PCR amplification. The solutions used were: TBE Buffer (Tris-borate-EDTA) (10X), DNA ladder marker, and Ethidium bromide (10 mg/ml).

Statistical analysis

Data were analyzed by using SPSS software version 26. The qualitative data were presented as absolute numbers, frequencies, and percentages. The quantitative data were presented as mean± standard deviation. To evaluate the association among the quantitative variables, we used independent student t., Mann Whitney U, and ANOVA tests. For qualitative data, Chi-Square or Fisher's Exact test have been used. A P-value of less than 0.05 was considered as significant.

RESULTS

One hundred and two patients completed the study and their data were analyzed; 54 men and 48 women. The age ranged between 33 to 70 years; with a mean age of 52.87±10.91 for women and 51.70±10.63 for men. The mean HbA1c was 8.188± 1.066%.About one-sixth of the patients

had hypertension, and more than 50% of the participants had a family history of DM. The demographic and clinical characteristics of the patients are summarized in Table 1.

Gastrointestinal (GI) side effects of metformin

Metformin was well tolerated with fewer adverse effects. After three months of treatment, the side effects were observed in 15% of the patients as follows: abdominal discomfort (7%), nausea (4%), diarrhea (2%), and metallic taste (2 %). There was no observed evidence of hypoglycemia or lactic acidosis during the study.

After three months of metformin treatment, the enrolled patients were divided into two groups; those with GI side effects and those without GI side effects. There were no significant differences between the two groups regarding baseline characteristics (age, sex). No significant differences between groups in HbA1c both in baseline and after 3 months values (p-value>0.05), but the reduction "% in HbA1c (0.90%) was significant between groups (p-value 0.002), and this means that the group with GI side effect had more reduction in HbA1c than the group without GI side effects after metformin treatment. As shown in Table 2 Figure 1.

Molecular Assays

In gel electrophoresis, the PCR products were stained with ethidinium bromide, and we detected three genotypes AA, A>del, and del>del. the fragment length was 621bp. As shown in Figure 2.

Characteristics of single-nucleotide polymorphisms of SLC22A1/OCT1 gene in the present study

Gene: SLC22A1, SNP code: rs72552763, Position: CHR6:160139849 (GRCH38.P13), Nucleotide position and variation: 1258A > del, AMINO ACID CHANGE AND POSITION: Met420del, Met '! del, Axon 7, Reference allele: A, Reference allele frequency: 0.74, Minor allele (alternative allele): Del, Minor allele frequency: 0.26.

Demographic and baseline clinical characteristics of the rs72552763

The present study showed no significant differences in sex, age, hypertension, and baseline HbA1c% were observed between different genotypes of the rs72552763, but the patients with the A/A genotype of rs72552763polymorphism

Table 1. Baseline characteristics of the study participants

Parameters	Mean ± SD
Age (year)	52.24 ± 10.746
Sex	
Men%	(54) 52.9%
Women %	(48) 47.1%
HbA1c%	8.188± 1.066
Hypertension	
Yes	(18) 17.6%
No	(84) 82.4%
Family history of DM	
Yes	56.90%
No	43.10%
Social level	
Low	(71) 69.6%
Medium	(31) 30.4%
High	(0) 0.00%

Table 2. Characteristics of patients with and without gastrointestinal side effects induced by metformin treatment

Parameters	Group without gastrointestinal side effects (n =87), 85%	Group with gastrointestinal side effects (*n* = 15), 15%	P-value
Age (years)	51.82±11.079	54.67±8.466	0.345*
Sex			
Men n (%)	(46) 52.9%	(8) 53.3%	0.974**
Women n (%)	(41) 47.1%	(7) 46.7%	
HbA_{1c} (%) baseline	8.136± 1.069	8.486± 1.037	0.243*
HbA1c(%) after 3 months	7.854± 1.455	7.580± 1.033	0.487*
"HbA1c (%)	0.282±1.324	0.906±0.475	0.002*

* Mann Whitney U Test, ** Chi-Square Test

exhibited significantly greater reductions in their HbA1c level (p-value 0.000) than patients carrying (A/del) or (del/del) genotypes of rs72552763 polymorphism as shown in Table 3.

Effect of SNP (rs72552763) on metformin efficacy and side effects in T2DM patients

In the present study, we divided the recruited patients according to response to metformin monotherapy into two groups, the responders and the non-responders group, and response to metformin therapy depends on HbA1c level. The responders´ group involved patients whose HbA1C levels decreased by e"1% after three months of treatment, while the non-responders´ group involved patients whose HbA1c decreased by less than 1% after treatment [16]. after treatment, we had 69 responders and 33 non-responders. After genotyping both groups of participants, we found a significant difference in the alleles and genotype frequencies between the responders and the non-responders groups (P-value 0.0001). In which Homozygous genotypes (AA) "reference type" of the SLC22A1 (rs72552763) gene polymorphism was associated with the responder´s group (p-value 0.0001), while homozygous genotypes (deletion/deletion) of the SLC22A1 (rs72552763) gene were more common among the non-responders group (p-value 0.0001). About 81% of the responder´s group had reference alleles "A". (P-value 0.000). All data are shown in Table 4, and Figure 3.

In the present study, about 87% of the patients with GI side effects carried the homozygous AA genotype, while all the patients without GI side effects carried the homozygous del/del genotype (P-value 0.005). A similar thing was observed with alleles, in which all the patients with GI side effects carried the reference allele "A", while all the patients without GI side effects carried the minor (alternative) allele "del" (p-value 0.019). As shown in Table 4and Figure 4.

Table 3. Comparison of demographic and clinical variables based on OCT1 genetic variant

Variables rs72552763	Genotypes AA	A/ deletion	Deletion	p-value
Sex				
Men	40 (54.1%)	1 (33.3%)	13 (52.0%)	0.859*
Women	34 (45.9%)	2 (66.7%)	12 (48.0%)	
Age (y)	52.34± 10.194	46.33±15.177	52.64± 12.086	0.627**
Hypertension				
Yes	12 (16.2%)	0 (0.0%)	6 (24.0%)	0.570*
No	62 (83.8%)	3 (100.0%)	19 (76.0%)	
HbA1c baseline %	8.17± 1.08	7.90± 1.49	8.26± 1.01	0.845**
HbA1c after 3 months %	7.49± 1.04	7.60±1.70	8.77±1.84	0.000**
" HbA1c %	0.67± 1.00	0.30± 0.72	-0.51± 1.55	0.000**

* Fisher's Exact Test, ** ANOVA test, "= 1st measure minus 2nd measure of HbA1c%

Table 4. Association of genotypes of (rs72552763)/ Met420del polymorphism with efficacy and side effects of metformin in T2DM

Genotypes		AA	Del>delDeletion	A>delDeletion	P *value
Response	Responders	60 (81.1%)	7 (28%)	2 (66.7%)	0.000
	Non-responders	14 (18.9%)	1 (33.3%)	18 (72%)	
Side effects	Group without GI side effects (n=87)85%	6170.1%	2528.7%	11.1%	0.005
	Group with GI side effects(n=15), 15%	1386.7%	00.0%	213.3%	

*Fisher exact test

Table 4 Association of genotypes of (rs72552763)/ Met420del polymorphism with **efficacy** and side effects of metformin in **T2DM**

DISCUSSION

Metformin is considered the drug of the first line for the treatment of T2DM [17], one of the essential issues about metformin use is a high variation in response to metformin [18], in addition to its high prevalence of metformin-induced gastrointestinal side effects, and many people suffer from these adverse effects during metformin therapy. These symptoms may result in poor patient adherence or even discontinuation of treatment [17].

Association of the rs72552763 polymorphisms with metformin efficacy

There is a very high variation in response to metformin, and more than 36% of T2DM patients treated with metformin monotherapy have poor glycemic control. This may be related to genetic and non-genetic factors [18, 19]. Several

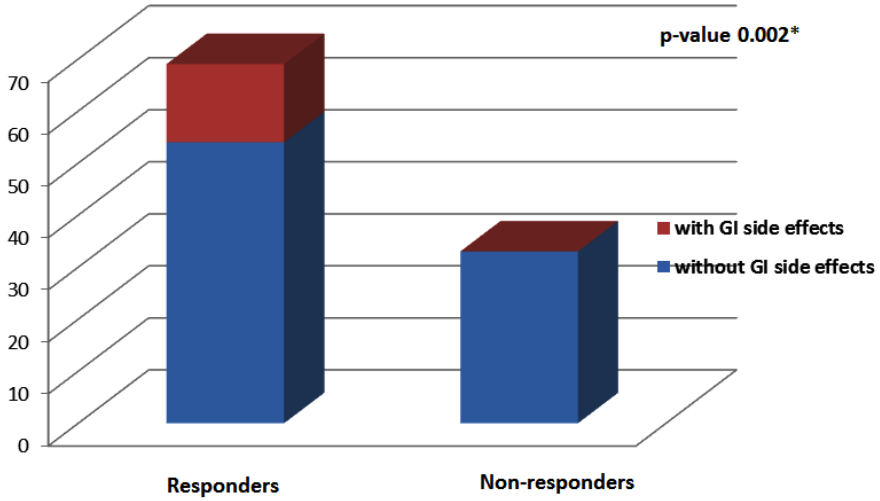

Fig. 1. The relationship between response and GI side effects of metformin in patients after three months of treatment. *Fisher exact test

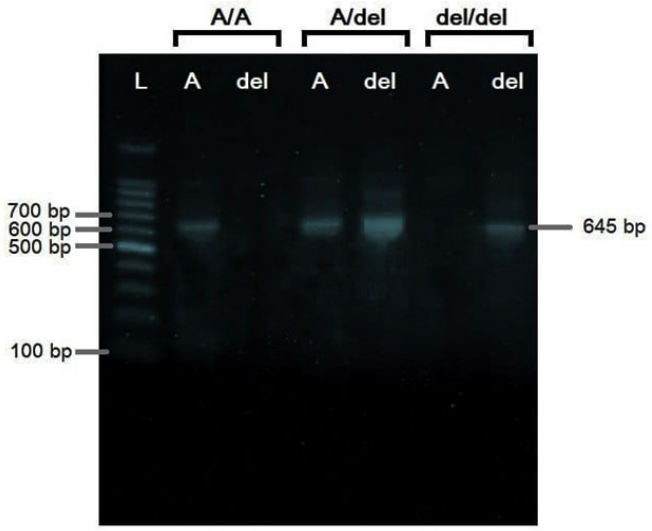

Fig. 2. Gel electrophoresis of (rs72552763) gene polymorphism

studies have shown that the efficacy of metformin is affected by genetic polymorphism in the OCT1 transporters OCT1 (*SLC22A1*) [20].

In the current study, there was a significant association between the SLC22A1 rs72552763/ (Met420del) gene polymorphism and metformin efficacy. We observed this SNP is related to changes in HbA1c levels, in which individuals carrying the homozygous AA-rs72552763 genotype had a significant decrease in HbA1c levels after three months of treatment compared to individuals carrying other genotypes of rs72552763, the "% HbA1c for rs72552763 AA-genotype was 0.67 while for A/del (rs72552763) and del/del (rs72552763) genotypes were 0.30, and -0.51 respectively. This may be due to less metformin

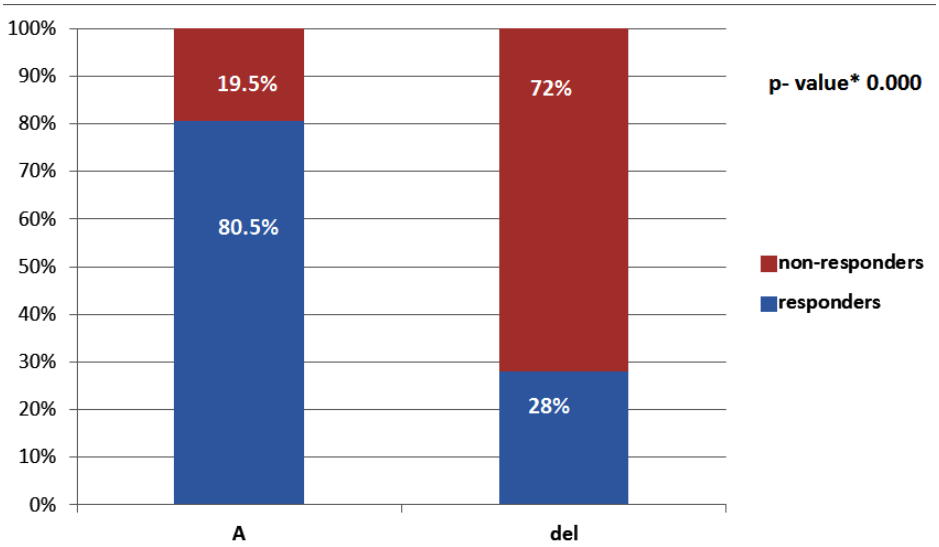

Fig. 3. Effect of alleles of (rs72552763) gene polymorphism on the efficacy of metformin in T2DM. *Fisher exact test

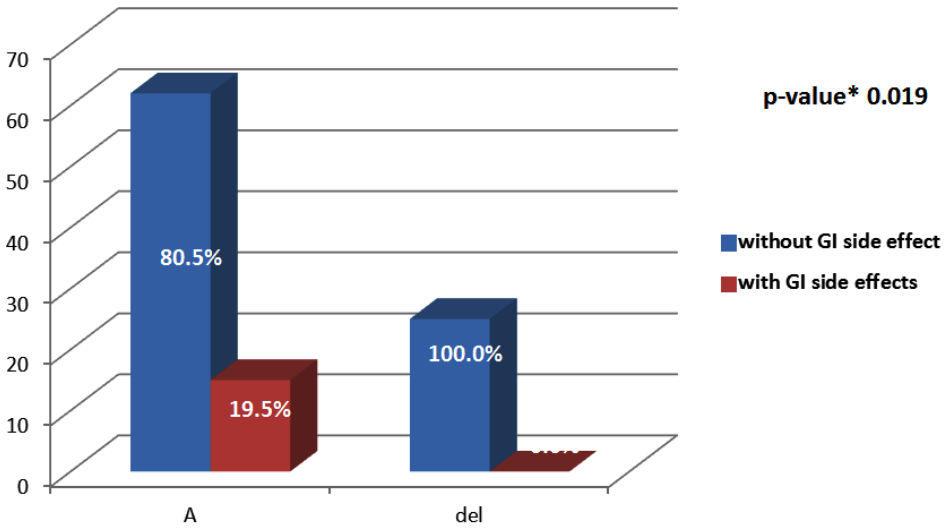

Fig. 4. Association of alleles of (rs72552763)/ Met420del polymorphism with metformin side effects in type 2 diabetes patients. *Fisher exact test

transported into the hepatocytes in individuals with OCT1 reduced-function alleles. The assumption is that the cellular uptake of metformin appeared to be the initial step in its activation of AMPK. And the liver is considered the major site of metformin action [5]. We supposed in the present study that the patients who carried the reduced function variants required a higher dose of metformin to achieve good glycemic control.

Similar findings were observed by Becker *et al.,* who detected a significant increase of 0.02% in the HbA1c levels among patients carrying the CC-rs622342 genotype [21].

A study done by Shu *et al*. in 2007 on twelve healthy volunteers given a glucose load by OGTT in two subsequent metformin doses (1000) mg in the evening and 850 mg in the morning showed decreased acute response to metformin in subjects with defective variants [14]. Becker *et al.* found that polymorphism of rs622342 of OCT1 has been associated with the glucose-lowering effect of metformin [21].

On the other hand, Zhou et al. investigate the effect of 156 T > C on the glycemic response to metformin in Han Chinese and Indian populations. They found no significant effect of the minor allele C, while the T/T genotype showed a greater reduction in HbA1c levels ($P = 0.020$) [22].

Association of rs72552763 polymorphisms with GI side effects of metformin

The most common GI side effects of metformin include (nausea, vomiting, and diarrhea) which occur in about 25% of patients [23]; these effects cause early discontinuation of metformin in (4-10%) of the patients [24]. The pathophysiology of metformin-induced GI side effects is still unknown; despite various hypotheses that have been suggested, including stimulation of intestinal serotonin secretion, alterations in glucose and incretin metabolism, and bile-salt malabsorption [25]. Some studies showed that GI side effects of metformin may be due to a local increase in the metformin concentration in the intestinal tissue after oral administration of the drug. [26, 27].

In the current study, we observed after three months of metformin treatment, about 15% of the patients suffered from GI side effects. We showed a significant association between response and GI side effects of metformin, the patients with GI side effects were responders to metformin treatment, and all the non-responders had no GI side effects. In addition group with GI side effects had more reduction in HbA1c level than the group without GI side effects after metformin treatment. The probable explanation for the relationship between side effects and metformin efficacy may present in the form of connected pathways or similar targets for the side effects and therapeutic response. Dujic et al. concluded in their study the relation between OCT1 variants and common GI side effects induced by metformin [28].

Opposite results were observed by Rashid et al., who showed that GI side effects were not associated with the response to metformin [21]. A study by Laura and her colleagues also found no association between metformin therapy and GI side effects [29]. A study was done on populations from North Caucasia, North Africa, and Sub-Sahara African ancestry. They found a significant effect of metformin therapy in producing GI side effects [30].

In the present study, the frequency of the rs72552763 (Met420del) polymorphism among the Iraqi population in Basra city/southern Iraq was 0.26 for the alternative allele and 0.74 for the reference allele, as compared with different populations, we found that the alternative allele frequency of the present study is higher than European alternative allele 0.13, African 0.05, Asian 0.007, East Asian 0.01. (NCBI database).

The current study investigates the association of one of the most common SNPs, rs72552763/ met420del of the SLC22A1/OCT1 gene and the presence of common GI side effects of metformin therapy in T2DM patients.

There was a significant association between GI side effects and genotypes of rs72552763, in which most of the patients with GI side effects had AA genotypes, while all the patients without GI side effects had del/del genotypes.

Regarding the pharmacokinetics of metformin, the major role of OCT1 in hepatocytes has been attributed to the hepatic uptake of this drug [8]. It is unlikely that OCT1 reduced function in the liver may affect the GI side effects even if genetic variations are considered to exert functional effects.

Tarasova et al. investigate two of the loss-of-function OCT1 variants, rs12208357 (R61C)

and rs34059508 (G465R), and they found no significant association with GI side effects. But in the same study, Tarasova et al. screened the effect of other OCT1 variants, rs628031 (M408V) and rs36056065 (8 bp insertion) with GI side effects of metformin in 53 tolerant and 193 intolerant patients. They found a significant association with the presence of GI side effects [24].

Dujic et al., in a study conducted on 2,166 (251 severely intolerant and 1,915 tolerant) T2DM patients, showed reduced activity of OCT1 variants (rs12208357 [R61C], rs55918055 [C88R], rs34130495 [G401S], rs72552763 [M420del], and rs34059508 [G465R]) is a significant determinant of metformin associated with GI side effects in which carriers of two reduced function alleles had 2.4 times higher chance of developing GI side effects. Odds (95% CI =1.48–3.93, P=0.001) [22]. Another study done by Dujic et al., with 92 participants, from an unspecified population, also found that the rs122083571 and rs72552763 polymorphisms were significantly associated with GI side effects (OR = 2.31, 95% CI [1.07–5.01], P = .034) [28].

The detection of specific SNPs influencing the glycemic response to metformin monotherapy can provide new information about the underlying molecular mechanisms of the T2DM patients' response to therapy. Finally, this would permit development in the treatment of T2DM.

Despite the great efforts that have been made in two large tertiary care centers, there is a limitation in this study related to the small sample size of patients involved.

In conclusion, minority of the participants suffered from GI side effects. In addition, we found response to metformin therapy depends on HbA1c level. Interestingly, we found a significant association between SLC22A1 rs72552763/ (Met420del) gene polymorphism and metformin efficacy and GI side effects. The patients with GI side effects were significantly associated with the responders' ´group, and all the non-responders had no GI side effects. This relationship between GI side effects and metformin efficacy may be due to connected pathways or similar targets for the side effects and therapeutic response.

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Conflict of Interest

Authors state no conflict of interest.

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