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Testing the Association Between TRPM6 rs2274924 Gene, Metabolic Syndrome and Staphylococcus Aureus in Patients With Type 2 Diabetes Mellitus

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Abstract: The TRPM6 gene is associated with multifactorial diseases: diabetes mellitus, obesity, hypertension, components of the metabolic syndrome. The aim of this study is to test the association between TRPM6 rs2274924 gene, metabolic syndrome and S. aureus based on clinical data and biochemical, haematological, microbiological and genetic laboratory investigations in patients with type 2 diabetes mellitus. The TRPM6 gene sequencing was performed by the Advanced NGx assay based on clinical and laboratory data, on 152 subjects from Giurgiu County Emergency Hospital. The results were processed by the graph Pad prism 7 program, VMD. In conclusion, the TRPM6 rs2274924 gene is associated with metabolic syndrome and S.aureus.

Keywords: TRPM6 gene, metabolic syndrome, hypertension, diabetes, S.aureus.

1. Introduction

The TRPM6 (Transient Receptor Potential Cation Channel Subfamily M Member 6) gene is mainly expressed in the kidney and colon, and it encodes a protein containing an ion channel domain and a protein kinase domain. Mutations of this gene are associated with hypomagnesaemia and, secondarily, with hypocalcaemia. TRPM6 is a gene that encodes a protein called TRPM6. This gene includes functions of transferase activity, transferring phosphorus-containing groups, ion channel activity. What is essential is the ion channel and the serine/threonine-protein kinase. Isoforms of the type M6-kinase lack the ion channel region. TRPM6 belongs to the melastatin (TRPM1)-related transient receptor (TRPM) channel family. TRPMs are Ca(2+)-permeable cation channels mostly located to the plasma membrane.

The structural mechanism of TRPM channels includes intracellular N and C termini, 6 transmembrane segments and a pore region between segments 5 and 6. The N-terminal domain has a conserved region and the C-terminal domain contains a TRP motif, a coiled-coil region, and, in TRPM6, an alpha protein kinase domain. TRPM6 may be downregulated in the distal convoluted tubule in response to cyclosporine, with magnesium loss as a result. The TRPM6 protein contains 2022 amino acids and has a calculated molecular mass of around 234 kD. The TRPM6 gene consists of 39 exons spanning over a region of 166 kb [1, 2]. The cytogenetic location of TRPM6 gene is 9q21.13, on the long arm (q) of chromosome 9, at position 21 (Fig. 1).

The TRPM6 gene plays an important role in epithelial magnesium transport and in the active magnesium absorption in the intestine and kidney. The normal functioning of TRPM6 gene provides information for obtaining a protein which acts as a channel allowing ions of magnesium (Mg^{2+}) to flow into cells; the channel also allows small amounts of calcium ions (Ca^{2+}) to pass through cells.

Magnesium is involved in various cell processes, including the production of cellular energy,

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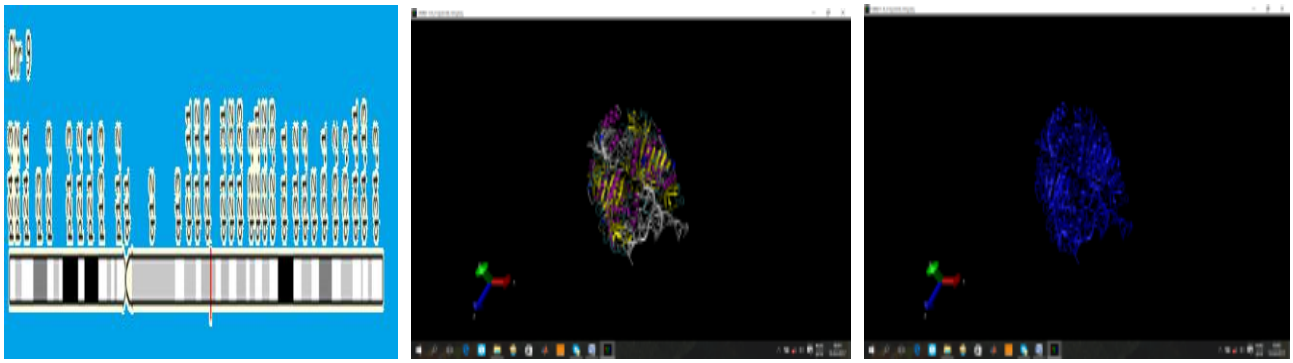


(a) (b) (c)

Fig. 1 (a) Location of the gene in TRPM6 on chromosome 9 (by [36]**), (b) Secondary structure, (c) Tertiary structure of the correpsonding protein: 3D structure of gene TRPM6(5CD1.pdb, processed by VMD version 1.9.1 Newcartoon). TRPM6: helix A (red), helix B (blue).**

maintenance of the DNA structure (nucleotides), protein production, cell growth and death. Additionally, Mg^{2+} is needed for the production of a substance called parathyroid hormone, which regulates blood calcium levels. Magnesium and calcium are also necessary for the normal functioning of the nerve cells that control muscle movement (motor neurons). The TRPM6 channel is embedded in the membrane of epithelial cells in the large intestine, distal convoluted tubules, the lungs and the testes. When the body needs additional Mg^{2+}, the TRPM6 channel allows it to be absorbed in the intestine and filtered from the fluids that pass through the kidneys by the distal convoluted tubules. When the body has sufficient or too much Mg^{2+}, the TRPM6 channel does not filter out the Mg^{2+} from fluids but allows the ion to be released from the kidney cells into the urine. The channel also helps to regulate Ca^{2+}, but to a lesser extent. At least 38 mutations in the TRPM6 gene have been proven to cause hypomagnesemia. This condition is characterized by low magnesium levels (hypomagnesemia) and low calcium levels (hypocalcemia) in the body, leading to neurologic problems which start in childhood, including muscle spasms and convulsions. TRPM6 gene mutations result in a lack of functional proteins. Magnesium deficiency has been correlated with a group of metabolic disorders and associated chronic diseases such as oxidative stress, systemic inflammation, endothelial dysfunction, insulin resistance, hypertension, T2DM and coronary heart disease [3]. Obesity, type 2 diabetes and metabolic syndrome are related conditions characterized by chronic inflammation, partly attributable to Mg^{2+} deficiency. In metabolic diseases, a low Mg^{2+} status due mainly to unhealthy diets contributes to the generation of a pro-inflammatory environment that exacerbates the metabolic disorder. Mg^{2+} supplementation appears to encourage the correction of this condition, but at this point it is difficult to interpret whether the beneficial effects of Mg^{2+} occur through a direct effect on the metabolic pathways or an indirect action on inflammation, or both, of type 2 diabetes and metabolic syndrome. The correction of poor dietary habits and, eventually, the supplementation of Mg^{2+} might represent a cheap but valuable tool to contain the onset and the progression of these conditions [4]. The TRPM6(SNPs:p.Lys1584Glu) polymorphism was associated with an increased risk of DM [5]. Haplotype analyses suggested a significant risk of type 2 DM among carriers of rare non-synonymous SNP alleles in TRPM6 (Lys1584Glu in exon 27[rs2274924]), when magnesium intake was less than 250 mg per day. Compared to non-carriers, women who were carriers of haplotype 1393Ile-1584Glu had an increased risk of DM2 (OR, 4.92, 95%CI, 1.05-23.0) only when they had a low magnesium intake (< 250 mg/day). Results provide

suggestive evidence that two common non-synonymous TRPM6 coding region variants, Ile1393Val and Lys1584Glu polymorphisms, might confer susceptibility to type 2 diabetes in women with low magnesium intake [6]. Hypomagnesemia affects insulin resistance and is a risk factor for type 2 diabetes mellitus (DM2) and gestational diabetes mellitus (GDM). A single nucleotide polymorphism (SNP) was identified in the epithelial magnesium channel TRPM6(1584)E) to confer susceptibility for DM2. By measuring total glycosylated hemoglobin (TGH) in pregnant women as a measure of glucose control, it was demonstrated that the variants of TRPM6(K(1584)E) gene are associated with higher values of TGH and provide a higher probability of developing GDM. The impaired response of TRPM6(K(1584)E) to insulin represents a unique molecular pathway leading to GDM where the defect is located in TRPM6 [7].

The aim of the study is to test the association between TRPM6 rs2274924 gene, metabolic syndrome and S. aureus based on clinical data and biochemical, haematological, microbiological and genetic laboratory investigations in patients with type 2 diabetes.

2. Materials and Methods

The 152 subjects were selected for this study, of whom: MS (n = 20, women = 10, men = 10), DM2 (n = 60, women = 30, men = 30), and clinically healthy controls (n = 72). The clinical data (body mass index (BMI), high blood pressure (HBP)), the biochemical laboratory data (glucose, triglycerides, cholesterol, HDL, LDL, calcium, magnesium, HbA1c, uric acid, vitamin C, Vitamin D, vitamin B12, folic acid, homocisteina, zinc) and the hematological laboratory data (RBC, HCT, HGB, WBC) were recorded for these subjects [8]. The subjects were selected at Giurgiu County Emergency Hospital and they expressed their informal consent to participate in the study.

The sequencing of TRPM6 gene was performed by Advanced NGx test. This is a genetic test presenting a combination of nutrigenetics tests. It uses DNA, it is performed on a Ion Torrent machine and it includes 400 genetic variations with a number of 99 genes [9].

The results were statistically processed by Hardy-Weinberg equilibrium for genotype distribution and frequency. The values $p < 0.05$ were considered statistically significant. Calculation and interpretation of Odds ratio (calculation of the disease risk conferred by genotypes). OR = 1 risk; OR > 1 protection [10].

For the data processing the following software was used: Visual Molecular Dynamics (VMD) version 1.9.1 — for 3D visualization of molecules [11].

The Hardy-Weinberg equilibrium was tested for each SNP in the control and patient groups using the One Way ANOVA statistical method. The data were statistically analyzed by One-Way ANOVA (One-Way analysis of variance) using Graph Pad Prism7 software. A statistical significance level of $p < 0.0001$ was used. The statistical significance between the control and patient groups was examined by One-Way ANOVA (One-Way analysis of variance), which is a linear regression used for the analysis of the TRPM6 rs2274924 gene. Statistical analysis used Graph Pad Prism software version 7.0.3 for Windows 7 [12]. S. aureus strains isolated from different types of infections (blood cultures, tracheobronchial secretions, pharyngeal exudates, urinary tract infections) were also included in this study. Strain identification was performed using API 20 STAPH microassay galleries (BioMerieux, Lyon, France) and, respectively, the VITEK 1 automated system. The strains are maintained on preservation media (WHO) and are in the microorganism collection of the Microbiology Laboratory of the Faculty of Biology. Qualitative techniques were used to determine the spectrum of sensitivity to antibiotics — the standardized diffusimetric method (Kirby-Bauer) [13, 14]. Determining the adherence and invasion capacity of the cell substrate by assessing the number of viable

cells. The three main patterns of adherence of enterobacteria to an in vitro cell substrate described in the literature were highlighted, namely: localized adherence; aggregative adherence, diffuse adherence (with cells diffusely adhered to the host cell membrane surface). The adherence pattern indirectly indicates the pathogenicity and virulence mechanisms (pathotype) of the respective strain [15].

3. Results and Discussion

In the case of the TRPM6 rs2274924 gene processed by the Graph Pad Prism 7 program, statistically significant differences were obtained for: Cholesterol processed by ANOVA test ($p = 0.0001$) is statistically significant ($p < 0.05$)(yes), it results that the gene is highly associated with MS; Triglycerides processed by ANOVA test ($p = 0.0877$) are not statistically significant ($p < 0.05$)(ns), it results that the gene is not highly associated with MS; HDL processed by ANOVA test ($p = 0.0053$) is statistically significant ($p < 0.05$) (yes), it results that the gene is highly associated with MS; Glucose processed by ANOVA test ($p = 0.0001$) is statistically significant ($p < 0.05$) (yes), it results that the gene is highly associated with MS; LDL processed by ANOVA method ($p = 0.0013$) is statistically significant ($p < 0.05$) (yes), it results that the gene is highly associated with MS; Uric acid processed by ANOVA test ($p = 0.0030$) is statistically significant ($p < 0.05$) (yes), it results that the gene is highly associated with MS; HbA1c processed by ANOVA test ($p = 0.0001$) is statistically significant ($p < 0.05$) (yes), it results that the gene is highly associated with MS; Calcium processed by ANOVA test ($p = 0.0053$) is statistically significant ($p < 0.05$)(yes), it results that the gene is highly associated with MS; Magnesium processed by ANOVA test ($p = 0.0053$) is statistically significant ($p < 0.05$) (yes), it results that the gene is highly associated with MS; Sex processed by ANOVA test ($p = 0.0053$) is statistically significant ($p < 0.05$) (yes), it results that the gene is associated with MS; Age processed by ANOVA test ($p = 0.0053$) is statistically significant ($p < 0.05$) (yes), it results that the gene is highly associated with MS; HBP processed by ANOVA test ($p < 0.0001$) is statistically significant ($p < 0.05$) (yes), it results that the gene is highly associated with MS; BMI processed by ANOVA ($p < 0.0001$) is statistically significant ($p < 0.05$) (yes), it results that TRPM6 rs2274924 gene is highly associated with metabolic syndrome; Vitamin D processed by ANOVA ($p < 0.0001$) is statistically significant ($p < 0.05$) (yes), it results that the gene is highly associated with MS; Vitamin C processed by ANOVA ($p < 0.0001$) is statistically significant ($p < 0.05$) (yes), it results that TRPM6 rs2274924 gene is highly associated with MS; Vitamin B12 processed by ANOVA ($p < 0.0001$) is statistically significant ($p < 0.05$) (yes), it results that TRPM6 rs2274924 gene is highly associated with MS; Folic acid processed by ANOVA test ($p = 0.0053$) is statistically significant ($p < 0.05$) (yes), it results that the gene is highly associated with MS; Homocysteine processed by ANOVA ($p < 0.0001$) is statistically significant ($p < 0.05$) (yes), it results that the gene is highly associated with MS; Zinc processed by ANOVA ($p < 0.0001$) is statistically significant ($p < 0.05$) (yes), it results that TRPM6 rs2274924 gene is highly associated with metabolic syndrome; RBC processed by ANOVA ($p = 0.0053$) is statistically significant ($p < 0.05$)(yes), it results that the gene is highly associated with MS; HCT processed by ANOVA ($p < 0.0001$) is statistically significant ($p < 0.05$) (yes), it results that the gene is highly associated with MS; HGB processed by ANOVA ($p < 0.0001$) is statistically significant ($p < 0.05$) (yes), it results that TRPM6 rs2274924 gene is highly associated with metabolic syndrome, and WBC processed by ANOVA ($p < 0.0001$) is statistically significant ($p < 0.05$) (yes), it results that TRPM6 rs2274924 gene is highly associated with metabolic syndrome (Fig. 2 and Fig. 3).

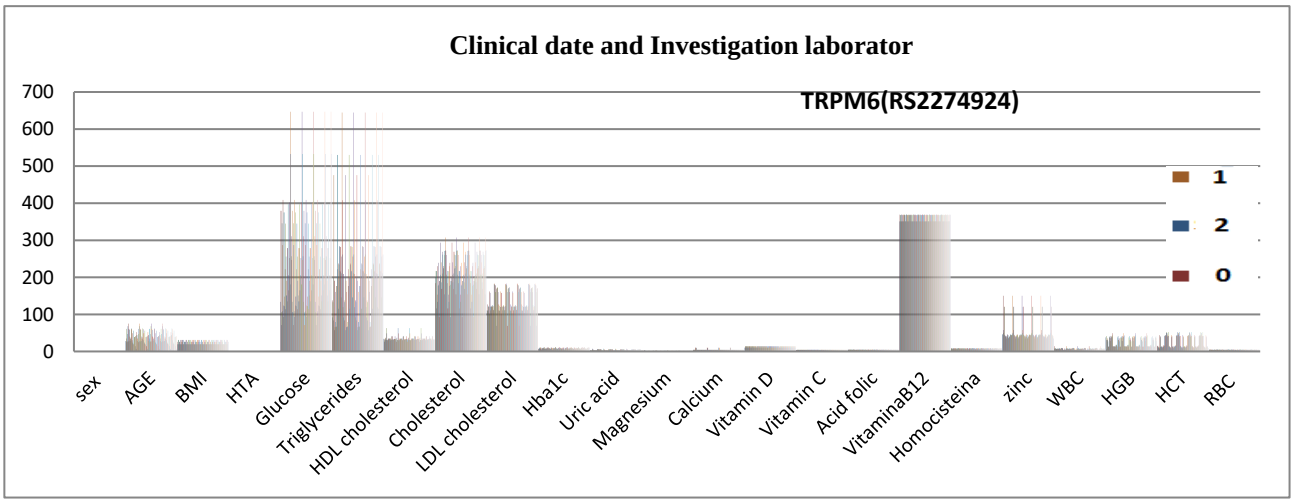


Fig. 2 Processing of clinical data and biochemical and haematological laboratory investigations.

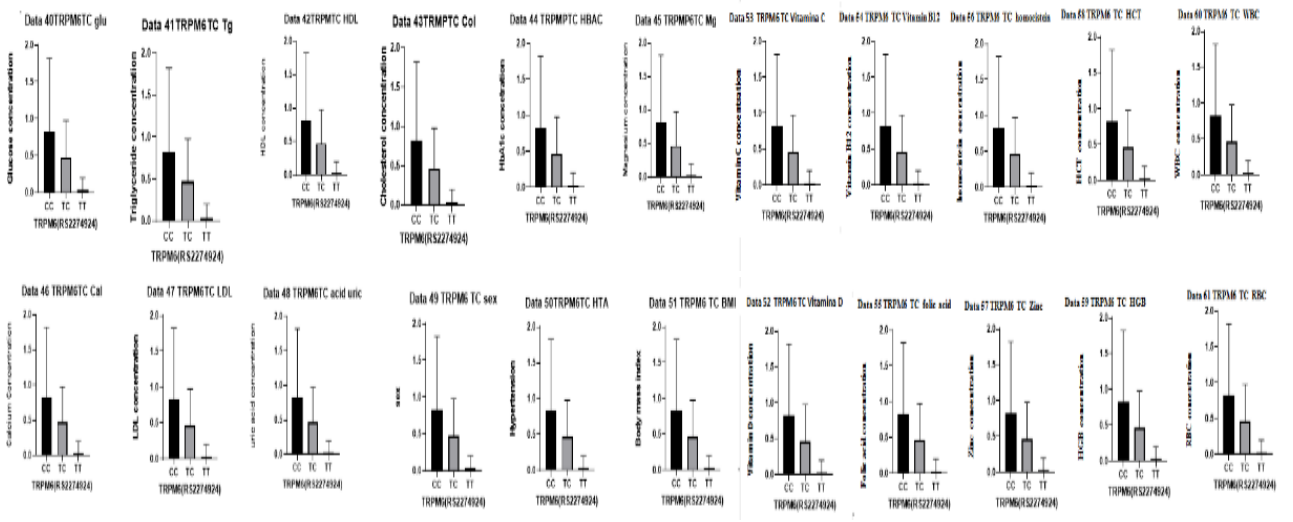


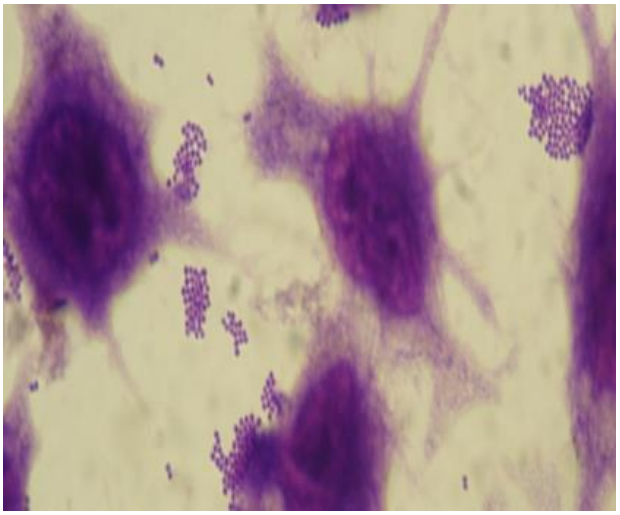
Fig. 3 Processing through the Graph Pad Prism 7.0.3 program based on clinical data and laboratory investigations of the TRPM6 rs2274924 gene.

Following the statistical processing by One Way ANOVA linear regression method using Graph Pad Prism 7.0.3 software, for the patient group, the clinical parameters and laboratory investigations are statistically significant: age, sex, BMI, HTA, cholesterol, HDL, triglycerides, LDL, HbA1c, calcium, magnesium, glucose, uric acid, vitamin C, vitamin D, vitamin B12, folic acid, homocysteine, zinc, RBC, HCT, HGB, WBC ($p < 0.0001$) OR = 2. It showed that the TRPM6 rs 2274924 gene is associated with metabolic syndrome in patients with DM2. The Advanced NGx assay shows that the TRPM6 gene (rs2274924T/C) controls intestinal absorption of magnesium as well as its reabsorption in the kidneys. Through an incompletely elucidated mechanism, women carrying the TC haplotype have an increased risk of type 2 diabetes if their daily dietary intake of magnesium is below 250 milligrams.

In patients with type 2 diabetes mellitus, in whom the TRPM6 gene associated with the metabolic syndrome has been identified, S. aureus strains have been isolated from: blood cultures, tracheobronchial secretions, pharyngeal exudates, urinary tract infections, based on clinical and laboratory data. In the case of S.aureus, in the MRSA phenotype-strains are resistant to all beta-lactams except the newer

cephalosporins with anti-MRSA activity. The analysis of S. aureus strains shows some heterogeneity in virulence and resistance, depending on the source of isolation. The S. aureus strains investigated were generally susceptible to antibiotics, but resistance phenotypes (MRSA and MLSb i) were also detected. The adherence capacity test showed that the selected strains exhibit a high degree of adherence, being found in the diffuse, aggregative pattern that is predominant. Diffuse-aggregative shows adhered cells and diffusely-adhered cells on the HeLa cell membrane surface, but also between cells (Fig. 4).

(a)



(b)

Fig. 4 a) Penicillin resistance phenotype of *S. aureus* strains, b) Microscopy image with adherence patterns — diffuse-aggregative in *S.aureus* Col. Giemsa (OI, 100x).

TRPM6 was found to be highly expressed in the gut and kidneys, but also in the lungs and testes. Recent studies indicate a regulation of TRPM6 expression by nutritional magnesium. Magnesium homeostasis in humans depends primarily on the balance between intestinal absorption and renal excretion. Magnesium deficiency can result from reduced dietary intake, intestinal malabsorption or renal wasting. The control of magnesium homeostasis in the body lies mainly in the renal tubules [16].

Zinc deficiency is associated with alterations in taste and smell [17]. Zinc deficiency is associated with increased severity of Covid 19 and degenerative disease. Patients with Covid 19 have very high levels of chromium, calcium, copper and low levels of zinc, magnesium, manganese, iron, lead, arsenic and thallium [18]. The use of Zn against viruses has been studied from 1970 to the present and Zn has been shown to affect viral replication, protein synthesis and processing, membrane fusion and RNA polymerase activity [19]. Studies conducted on a 2-month-old infant in Japan suffering from refractory seizures, born to non-consanguineous parents, with a birth weight and height of 3342 g and 50. 5 cm, and who did not have epileptic and electrolyte disorders in the family, found hypocalcaemia (3.16mg/dl (4.60-5.17)) and hypomagnesaemia (0.6 mg/dl range = 1.82-2.4 mg/dl), although 1.25-dihydroxyvitamin D3 of 85 pg/ml (normal range 20-70) was insufficient (4.1 ng/ml). Treatment with intravenous infusion of calcium gluconate and magnesium sulphate was performed. Then, after the age of 1 year treatment with oral magnesium succeeded in maintaining serum 25-hydroxyvitamin D3 levels. In vitamin D3 metabolism, magnesium plays an essential role in activating 25-hydroxylase, which converts vitamin D3 into 25-hydroxyvitamin D3. Magnesium deficiency could lead to secondary dysregulation of vitamin D3 metabolism. Hypomagnesemia 1 (HOMG1) is an extremely rare disease with autosomal recessive inheritance caused by mutations in the TRPM6 gene.

It turns out that the 2-month-old patient with HOMG1 is associated with heterozygous mutations in the TRPM6 gene due to hypomagnesemia with secondary hypocalcemia [28]. Emerging evidence indicates that one of the main physiological functions of TRPM6 is to maintain cellular metabolism of Mg^{2+} and probably Ca^{2+} and Zn^{2+}. Recent experiments with genetic animal models have shown that TRPM6 are essential for epithelial Mg^{2+} transport in the placenta and gut. Homologs of the TRPM6 gene have been found in all vertebrate species. Several alternatively spliced mRNA isoforms are derived from the human gene. Three alternative 5′ exons (exons 1A, 1B and 1C) of the gene can be spliced with a second common exon, resulting in three full-length mRNA variants, TRPM6a, TRPM6b and TRPM6c. To date, the TRPM6a cDNA variant has been widely used for heterologous expression studies, while the functional properties of the other TRPM6 gene products remain unknown. Until recently, the functional evaluation of the TRPM6 channel has been limited to the heterologously expressed human TRPM6a isoform. Hypomagnesaemia is improved after high-dose Mg2+ administration. Despite this treatment, serum Mg2+ levels remain in the subnormal range, and it has been suggested that Mg2+ deficiency is associated with an increased risk of insulin resistance and type 2 diabetes. A recent study for genome-wide associations showed that two SNPs in TRPM6, rs3750425 and rs2274924, could confer susceptibility to type 2 diabetes in women with low Mg2+ intake. Moreover, these SNPs may be associated with a higher probability of developing gestational diabetes. Genome-wide association studies to correlate the intake of Mg2+ with a jeun glucose and insulin levels were performed in ~50,000 healthy Europeans. The authors reported that a higher Mg2+ intake was associated with lower glucose and insulin concentrations, while rs2274924 in TRPM6 was associated with higher glucose levels. Recently, SNPs in human TRPM6 have been shown to be associated with a neural tube closure defect, i.e., meningomyelocele. Therefore, SNP profiling in TRPM6 may prove to be a useful new diagnostic indicator of insulin resistance and fetal developmental abnormalities. Recent experiments with cultured cells, TRPM6 gene-modified mouse models and clinical data from patients with mutations in the TRPM6 gene strongly support the notion that TRPM6 plays a critical role in maintaining the Mg2+ balance in the body [20]. Measurement of HbA1c in 997 pregnant women as a measure of glucose control showed that the TRPM6(K1584E) gene is associated with increased HbA1c conferring increased risk of developing gestational diabetes [21]. The TRPM6 gene was identified by RT-PCR by signaling mRNA expression and T cell activation in peripheral human blood lymphocytes and mouse spleen T cells. TRPM6 gene protein was detected in two types of WT and KD mice. Associations between the two types of mice and the following haematological parameters were identified: HCT (WT1:42.1, WT2:41.8, WT3:40.3, KD1:42.6, KD2:41.7, KD3:36.8), RBC (WT1 = 9.49, WT2 = 9.31, WT3 = 9.42, KD1 = 9.87, KD2 = 9.56, KD3 = 8.71), HBG (WT1 = 12.9, WT2 = 12.8, WT3 = 12.3, KD1 = 13.6, KD2 = 12.8, KD3 = 11.4) [21]. Protein content, WBC and the percentage of polymorphonuclear neutrophils (%PMN) in the blood were measured. TRPM6 and TRPM7 gene mRNA expressions were measured by qRT-PCR, showing that in the control group PaO_2 and PaO_2/FiO_2 values are low, while in the other group $PaCO_2$, protein content, WBC, PMN%, TNF-a, IL-6 and the lung coefficient values are increased. It results that the values of the two genes TRPM6 and TRPM7 were significantly increased [22].

Low levels of folic acid and vitamin B12 have been found in mothers with offspring with neural tube defects. Elevated homocysteine levels denote vitamin B12 and folic acid deficiency during pregnancy. The relationship between red cell folic acid and serum folic acid and homocysteine may be useful for detecting folate deficiency during pregnancy and

indicate pregnancies at risk of neural tube defects [23]. Results show that vitamin B12 supplementation and foods enriched with folic acid reduce homocysteine and may prevent vascular and neural tube diseases [24].

Staphylococcus aureus is a coagulase-positive species, a versatile opportunistic pathogen, capable of causing infections at variable sites, ranging from skin infections to systemic infections. CNNM are divalent metal cation transport mediators of cyclin and CBS. In humans one finds the cyclin M(CNNM) family comprising 4 members, CNNM1-4, and in mammals, four proteins are also found, which are members of the CNNM family, CNNM1-CNNM4. These proteins contain a domain of unknown function 21 (DUF21) that spans the plasma membrane three and a half times, followed by cystathionine-β-synthase (CBS) domains in the cytoplasmic region. Such proteins with a tandem set of DUF21 and CBS domains can exist in eukaryotes, from yeasts to mammals and in prokaryotes, Salmonella typhimurium (S. typhimuriu) and Staphylococcus aureus (S. aureus). These proteins of the CNNM family are highly conserved in evolutionary terms, which is suggestive of their crucial biological function. In 2002, it was discovered that genomic analyses of hereditary hypomagnesemia showed that patients commonly carried mutations in Trpm6, which encodes the cation channel. The transient receptor cation channel of subfamily M member 6 (TRPM6) is permeable to Mg^{2+} and can mediate its influx into gut and kidney cells. The analysis of another type of hereditary hypomagnesemia revealed mutations in Cnnm2, which suggested the possibility that the protein produced, cyclin M2 (CNNM2) and its family proteins are also involved in Mg^{2+} transport across the plasma membrane [25]. Based on clinical data, BMI, age, gender, ethnicity, cardiovascular disease, hypertension, diabetes, liver disease, renal disease, it results that S. aureus presents increased mortality risk in patients hospitalized with COVID-19 [26].

4. Conclusions and Recommendations

In conclusion, the TRPM6 rs2274924 gene ($P < 0.0001$, $OR = 2$) is associated with metabolic syndrome and S. aureus in patients with type 2 diabetes mellitus, based on clinical data and biochemical, haematological, microbiological and genetic laboratory investigations: biochemical, haematological, microbiological and genetic.

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This study was conducted from 20.01.2014-19.01.2015 and 20.01.2015-19.01.2016 as part of a volunteer activity at Giurgiu County Emergency Hospital (Volunteer Contract No. 2/20.01.2014 and contract No. 2/20.01.2015 subject to art. 13 (5) of Law No. 677/2001).

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