

## ASSOCIATION OF THE GENETIC POLYMORPHISM RS11640851 MT1A 80 C/A AND TYPE 2 DIABETES MELLITUS IN THE CENTRAL BALKAN POPULATION

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Type 2 diabetes mellitus (T2DM) is the most common type of diabetes and is becoming an increasingly prevalent global health issue. Polymorphisms in genes coding metallothioneins, a group of small zinc-binding proteins that participate in antioxidative protection, are believed to be involved in T2DM pathogenesis. This study aimed to investigate the potential association of the single nucleotide polymorphism (SNP) rs11640851 MT1A 80 C/A and the T2DM risk and to determine the impact of the genotype and allelic distribution on the diabetes-related biochemical parameters. The study included 298 subjects, 112 with T2DM and 186 healthy, non-diabetic controls. The participants' fasting glycemia and HbA1c levels were measured, while the SNP in the MT1A gene was determined using the PCR-RFLP method. There were no significant differences in the genetic distribution and allele frequency between control subjects and diabetic patients ( $p > 0.05$ ). There was likewise no association between the SNP and diabetes-associated laboratory parameters, fasting serum glucose and HbA1c levels. However, 79.6% of allele C carriers had fasting glucose levels above 7 mmol/L, versus 53.3% of subjects homozygous for allele A ( $p = 0.005$ ). Although our study did not find a direct association between the MT1A genetic variants and the occurrence of T2DM, we observed an effect of the allele C on glycemic control in the patients. Further research in a larger population is needed to expand these findings and to improve the understanding of metallothionein genes and their impact on the development of T2DM.

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**Key words:** type 2 diabetes, metallothionein, zinc, single nucleotide polymorphism

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### Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by hyperglycemia caused by a relative insulin deficiency (1). It is the most common type of diabetes by far, accounting for roughly 95% of all cases, affecting over 400 million people worldwide (2). Overall incidence of DM has doubled between 1990 and 2017, with the vast majority of T2DM cases (3). The chronic hyperglycemia of diabetes is associated with long-

term damage, dysfunction, and failure of different organs and systems, especially cardiovascular and peripheral nervous systems, as well as the eyes and kidneys (1).

Alterations in the metabolism of essential trace elements, including zinc, have been consistently observed in patients with T2DM (4). Reduced levels of serum zinc in diabetic patients have been reported in multiple studies (5), and some linked hypozincemia to the severity of diabetes and associated complications, such as diabetic retinopathy, diabetic nephropathy and diabetic peripheral neuropathy (6 – 9). Earlier studies have also found that Zn ions are involved in insulin synthesis, storage, secretion, and as insulin signaling, leading some authors to propose the inclusion of impaired zinc metabolism to the list of metabolic disorders in diabetes (10).

Moreover, studies have demonstrated that a family of small, cysteine-rich proteins called metallothioneins (MTs), may have an important role in Zn ion buffering and signaling, as well as in nitric oxide (NO) signaling and oxidative stress defense. Previous studies have suggested that MTs have important roles in essential metals homeostasis, defense against heavy metal poison-

ing and as anti-oxidative protection (11) due to their ability to scavenge reactive oxygen and nitrogen species (ROS/RNS) (12 – 14).

There are four main MTs isoforms in humans (15, 16), two of which, MT1 and MT2, are expressed in the majority of tissues, most abundantly in the liver, pancreas, intestine and kidney (17).

Interest in MTs has grown over the years, as they were found to be implicated in a variety of pathological processes, from accumulation and toxicity of heavy metals (Cd, Hg, Pb), to association with multiple types of cancer, cardiovascular diseases and diabetes mellitus, and psychiatric disorders such as autism (18).

The expression levels and function of MTs are highly variable and dependent on polymorphisms present in the MT coding genes, which may contribute to the occurrence and development of various pathologies. To date, only a handful of studies have investigated the association between MT1 and MT2 gene single nucleotide polymorphisms (SNPs) and their contribution to the development of T2DM (18 – 20).

One of the SNPs of particular interest in T2DM is rs11640851 MT1A 80 C/A, also known as MT1A Thr27Asn or MT1A +647 A/C, a polymorphism located in the coding region of MT1A gene that leads to an amino acid substitution. Association of this polymorphism and T2DM was suspected (21), but it was never reported before in the Central Balkan population.

This study aimed to investigate the potential association of SNP rs11640851 MT1A 80 C/A and the T2DM risk, and to determine the impact of the genotype and allelic distribution on the diabetes-related biochemical parameters, specifically patients' glycemia and glycosylated hemoglobin levels.

## Materials and Methods

### Patients

In the study, a total of 112 patients with T2DM were recruited from the Endocrinology, Diabetes and Metabolic Diseases Clinic, University Clinical Center Niš. The patients were recruited while attending the clinic for their routine check-ups. Additionally, 186 healthy subjects were recruited as non-diabetic controls.

### Biochemical analyses

Fasting serum glucose and glycosylated hemoglobin (HbA1c) levels were measured in all of the study participants using standard methods on an automated clinical chemistry analyzer Beckman-Coulter AU680 at the Medical and Clinical Biochemistry Center, University Clinical Center Niš. Genotyping was performed in the Laboratory for Functional Genomics and Proteomics of the Scientific Research Center for Biomedicine of the Medical Faculty in Niš.

MT1A 80 C/A rs11640851 genotyping

The subject's DNA was extracted from 200  $\mu$ L of whole blood, which was sampled in 3 mL EDTA tubes. A commercial DNA purification kit (Genomic DNA Purification Kit, Thermo Scientific, Lithuania) was used for DNA extraction according to the manufacturer's instructions. Genotyping of the SNP in MT1A gene 80 C/A rs11640851 was performed using the PCR-RFLP method adapted from Cipriano et al. 2006 (22). The primers used were forward, 5'-CACTCAGCTGGCAGCATTG-3' and reverse 5'-ACTTGGCTCAGCCCCAGATT-3'. The reaction mixture consisted of 0.1  $\mu$ L HotStart DNA polymerase (FIREPol DNA polymerase, Solis Bio-Dyne, Tartu, Estonia), 2  $\mu$ L FIREPol Buffer B 10x, 1.2  $\mu$ L MgCl<sub>2</sub>, 0.2  $\mu$ L dNTP mix (20 mM of each), 0.4  $\mu$ M of each primer, 1 ng/ $\mu$ L of DNA template, and PCR grade water was added up to a total volume of 20  $\mu$ L. Amplification was performed using the following program: initial denaturation at 95 °C for 5 minutes, 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 61 °C for 45 s, elongation at 72 °C for 60 s, and final elongation at 72 °C for 7 minutes. The PCR product (187 bp) was digested using the MnlI restriction enzyme (NEB, Ipswich, MA, USA) at 37 °C for 50 minutes, and the resulting fragments were resolved using vertical electrophoresis on an 8% polyacrylamide gel. The gel was then stained in ethidium bromide solution and observed under UV light. The presence of the allele A was identified by the undigested 187 bp band, while the allele C was identified by two bands of 140 and 47 bp. Heterozygous samples displayed all three bands.

### Statistical analysis

The distribution of the genotypes for the polymorphism was assessed for deviation from the Hardy-Weinberg equilibrium (HWE). The characteristics of the study group were expressed as the median and interquartile range, or the mean and standard deviation or frequency (with or without percentages). Student's t-test (for normally distributed data) or the Mann-Whitney U test (non-normally distributed data) was employed for the comparison of two independent samples. ANOVA (for normally distributed data) with Tukey as Post Hoc Test and Kruskal Wallis (non-normally distributed data) with Mann-Whitney U test as Post Hoc were used to compare more than two defined groups. Chi-square ( $\chi^2$ ) test was used to compare data between groups when data were defined as categorical. All analyses were performed using IBM SPSS Statistics for Windows, v.24.0 (IBM Corp, Armonk, NY, USA) with the significance level set at  $p < 0.05$ .

## Results

General characteristics and biochemical parameters of the subjects are displayed in Table 1. Subjects with diabetes were on average of older

**Table 1.** Characteristics of the study population

	Non-Diabetics (n = 186)	Diabetics (n = 112)	Test (t/Z, $\chi^2$ ) and significance
Sex (male/female)	30.65%/69.35%	50.00%/50.00%	$\chi^2 = 11.124$ ; p = 0.001
Age (years)	60.31 $\pm$ 9.70 60 (13)	65.12 $\pm$ 9.19 65 (12)	T = -4.229; p < 0.001
Weight (kg)	80.52 $\pm$ 15.33 78 (20)	88.63 $\pm$ 15.40 88 (22)	Z = -4.393; p < 0.001
Body mass index (kg/m <sup>2</sup> )	28.09 $\pm$ 4.11 27.92 (5.68)	30.63 $\pm$ 4.44 31.00 (5.83)	T = -4.972; p < 0.001
Serum glucose level (mmol/L)	5.57 $\pm$ 0.53 5.50 (0.70)	8.34 $\pm$ 2.65 8.00 (2.70)	Z = -11.820; p < 0.001
HbA1c (%)	5.43 $\pm$ 0.40 5.40 (0.50)	7.60 $\pm$ 1.71 7.00 (2.40)	Z = -13.257; p < 0.001

t- Data are presented as mean  $\pm$  standard deviation and media (interquartile range) or frequency (%). Student – t Test; Z-Mann Whitney U Test; Chi-Square ( $\chi^2$ ) Test

**Table 2.** Genotypes and alleles of MT1A in relation to diabetes presence

	MT1A AA	MT1A AC	MT1A CC	Allele A frequency	Allele C frequency
Non-diabetics (n = 180)	75	78	27	0.63	0.37
Diabetics (n = 99)	45	41	13	0.66	0.34
Test and significance	$\chi^2 = 0.424$ ; p = 0.809			$\chi^2 = 0.4453$ ; p = 0.505	

**Table 3.** Genetic dominant model of MT1A genotypes in relation to diabetes presence

	MT1A AA genotype	MT1A C allele (AC+CC genotype)	Test and significance
Non-diabetics (n = 180)	75	105	$\chi^2 = 0.374$ ; p = 0.541
Diabetics (n = 99)	45	54	

age, had greater overall body weight, and had increased BMI (p < 0.001). Diabetic patients had significantly increased fasting glucose levels compared to the control subjects (p < 0.001), as well as significantly increased levels of HbA1c (p < 0.001).

Of the total number of 298 subjects enrolled in the study, 279 were genotyped for MT1A 80 C/A polymorphism. Of these, 180 were control subjects and 99 had diabetes.

The distribution of genotypes of MT1A 80 C/A in subjects is summarized in Table 2, and was in Hardy-Weinberg equilibrium in both the control and diabetic groups (p > 0.05). No significant differences in genotypes were found between the diabetes patients and controls. Minor allele frequency (allele C) was 0.37 in the control group and 0.34 in the diabetes group, which was not a significant difference (p > 0.05).

To assess the potential impact of the C allele on diabetes risk, a genetic dominant model that classified AA genotypes differently from AC and CC genotypes was used (Table 3). However, no significant difference in C allele frequency was found between the control and diabetes groups.

The association of MT1A 80 C/A and the laboratory parameters of diabetes, fasting glucose and HbA1c levels, was also explored in the diabetes group (Table 4). Average glycemia in the allele C carriers was 8.68  $\pm$  2.82 mmol/L vs. 8.15  $\pm$  2.60 mmol/L in the genotype AA patients, which was not a significant difference (p > 0.05). Also, HbA1c levels were similar between allele C carriers and non-carriers (7.67  $\pm$  1.89% vs. 7.59  $\pm$  1.67%, p > 0.05).

As the subjects in the diabetes group were all receiving anti-diabetic medication, a proportion of them did not show serum glucose and HbA1c values characteristic of diabetes, which were  $\geq$  7.0 mmol/L for glycemia and  $\geq$  6.5% for HbA1c, i.e. medication was masking the true diabetes phenotype. For this reason patients were stratified based on the aforementioned cutoff values for glycemia and HbA1c, both individually and together (Table 5). After the exclusion of subjects with regulated biochemical parameters, it was revealed that 79.6% of allele C carriers had fasting glucose levels above 7 mmol/L, versus 53.3% of subjects homozygous for allele A (p = 0.005). On the other hand, patients with values of

**Table 4.** Serum glucose level and HbA1c with respect to MT1A genotype

	MT1A AA genotype	MT1A C allele (AC+CC genotype)	Test and significance
Fasting glucose (mmol/L)	8.15 ± 2.60 7.20 (3.80)	8.68 ± 2.82 8.30 (2.20)	Z = -1.409; p = 0.159
HbA1c (%)	7.67 ± 1.89 6.80 (2.90)	7.59 ± 1.67 7.45 (2.50)	Z = -0.197; p = 0.844

**Table 5.** Genotype frequencies of MT1A with stratification by laboratory parameters

	MT1A AA genotype (n = 45)	MT1A AC+CC genotype (n = 54)	Test and significance
Fasting glucose (> 7 mmol/L)	24 (53.3%)	43 (79.6%)	$\chi^2 = 7.759$ ; p = 0.005
HbA1c (> 6.5 %)	32 (71.1%)	39 (72.2%)	$\chi^2 = 0.015$ ; p = 0.903
Fasting glucose (> 7 mmol/L) and HbA1c (> 6.5 %)	20 (44.4%)	36 (66.7%)	$\chi^2 = 4.934$ ; p = 0.026

HbA1c  $\geq$  6.5% revealed no differences regarding the presence or absence of allele C (72.2% vs. 71.1%, respectively,  $p > 0.05$ ). In addition to that, among patients that displayed both glycemia and HbA1c levels above the cutoff values, 66.7% were allele C carriers, versus 44.4% allele A homozygotes ( $p = 0.026$ ).

### Discussion

Type 2 diabetes mellitus is a metabolic disorder characterized by hyperglycemia dominantly caused by insulin resistance. It manifests with a complex pathology involving dyslipidemia and hyperlipidemia, an increase in weight due to accumulation of adipose tissue, as well as increased oxidative stress, proinflammatory state and endothelial dysfunction (1, 23, 24). Elevated glucose causes non-enzymatic glycation of proteins and lipids, leading to the accumulation of advanced glycation end products (AGEs), which further exacerbate oxidative stress, LDL oxidation and activation of immune cells (25, 26).

Oxidative stress is a crucial mechanism of pathogenesis in diabetes. Some of the typical findings in both diabetic patient and experimental animal models are impaired functionality of antioxidative protection enzymes, such as superoxide dismutase (SOD), glutathione peroxidase, catalase and paraoxonase (PON), as well as increased production of ROS/RNS and levels of oxidative stress markers, such as malondialdehyde (27). Excessive production of ROS/RNS worsens insulin resistance and accelerates the development of the microvascular and macrovascular complications of diabetes (28). Some authors even suggested that diabetes mellitus itself should be considered an oxidative stress disease (29).

Metallothioneins provide a substantial contribution to antioxidative defenses. Thanks to their high thiol (-SH) group content, metallothioneins react directly with ROS/RNS, neutralizing them similar to glutathione (GSH). In the case of •OH radicals, MTs neutralize them at a rate of approximately 340 times higher than that of GSH (13). Metallothioneins also sequester dangerous Fenton metal ions, such as Cu<sup>+</sup> and Fe<sup>2+</sup> (30). Additionally, MTs provide a stable availability of Zn ions, which are crucial cofactors of numerous enzymes, including SOD (31). Given the involvement of MTs in antioxidative defenses, a number of researchers have explored the possible associations between MT alterations and various pathological conditions (32).

In this study, we investigated the potential association of the genetic polymorphism MT1A 80 C/A rs11640851, which causes amino acid substitution asparagine (allele A) to threonine (allele C) at position 27 of the MT1A polypeptide chain, where the Asn variant is the wild type. We found that there were no significant differences in the genotype distribution and allelic frequency of MT1A 80 C/A SNP between control subjects and patients with T2DM. Previous studies conducted in the central Italian population found a significant association of rs11640851 allele C with longevity in elderly women, as well as an increased T2DM occurrence with a higher risk of diabetic cardiovascular complications in the general population (21, 22). With this in mind, a genetic dominant model was used to assess whether allele C was a potential risk allele in our study, but no significant differences were found between the control group and the group of patients with diabetes.

There was likewise no association between MT1A 80 C/A SNP and diabetes-related laboratory parameters, fasting serum glucose and HbA1c levels. Interestingly, the Italian group found an

association between the allele C and elevated fasting glucose and HbA1c levels in the group of diabetic patients with cardiovascular complications (21). Another, surprising, finding in the same study was that peripheral blood mononuclear cells of allele C carriers exhibited increased intracellular MT content, as well as reduced intracellular release of Zn ions upon NO stimulation. A more recent study in India did not find any association between MT1A 80 C/A and laboratory parameters of diabetes, but it did find an increased frequency of allele C in the group of diabetic patients (33).

Since the subjects in the diabetes group were receiving various types and doses of anti-diabetic medication, there was a possibility that this variation in medication, along with its varying effectiveness, could have influenced the laboratory measurements of fasting serum glucose and glycated hemoglobin A1c levels. To address this issue, we have stratified the patient group based on their glycemia and HbA1c levels by implementing cutoff values of 7 mmol/L for glycemia, and 6.5% for HbA1c. When stratified by HbA1c values alone, no difference was observed in the genotype and allelic representation. However, there was a clear difference in serum glucose levels between the AA genotype patients and the patients carrying allele C, where 79.6% of allele C carriers had glycemia above 7 mmol/L, compared to 53.3% of the AA homozygotes. When cutoff values for both glycemia and HbA1c were implemented, the significance remained. This finding suggests that MT1A 80 C/A does have an impact on the level of glycemia control in diabetic patients.

The group of researchers led by Mocchegiani provided an explanation for how the presence of the MT1A 80 C allele influences the risk of diabetes including glycemic control in patients with type 2 diabetes. Under conditions of increased oxidative stress and pro-inflammatory signaling the expression of MT1 and MT2 genes is continuously upregulated, as these genes are induced by IL-6 signaling and the presence of ROS/RNS. However, under these altered conditions, increased total Zn binding capacity, without the corresponding increase in zinc pool leads to Zn ion sequestration. Reduction in inducible Zn ion availability inside cells not only impairs the function of antioxidative protection enzymes that require Zn

as a cofactor but also disrupts zinc signaling and causes NF- $\kappa$ B over activation (34 – 37).

However, the majority of studies have found that MTs exhibit both antioxidant and anti-apoptotic properties, improving cell survival and functionality (14). Overexpression of MT protects cardiomyocytes from oxidative damage, loss of contractility and apoptosis induced by high-fat diet, while the absence of MT exacerbates diabetic cardiomyopathy in a rodent model (38 – 42). Other studies have found that MTs have neuro-protective properties in the retinal and brain tissues against oxidative stress damage (31, 43 – 46). Additionally, MTs have been found to help in the prevention of diabetes-induced tissue ischemia, by activation of angiogenesis through induction of HIF-1/SDF-1/VEGF pathway (47, 48). It should be noted that experimental animal models are unable to accurately predict physiological and pathological processes in humans reliably, indicating that the mechanisms related to metallothioneins and their function in metabolism are still not fully understood.

### Conclusion

This study did not find a direct association between genetic polymorphism in MT1A 80 C/A and the occurrence of type 2 diabetes mellitus. However, there was evidence of poorer glycemic control in diabetic patients with respect to the MT1A alleles they are carrying. A possible implication of the study is in the implementation of personalized medicine, by the inclusion of genetic testing in the diagnostics, for better optimization of treatment. Further research in a larger population is needed to expand these findings and to improve the understanding of metallothionein gene polymorphisms and their impact on the occurrence and progression of diabetes.

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MT1A 80 C/A SA DIJABETESOM MELITUSOM TIPA 2 U  
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Tip 2 dijabetesa melitusa (T2DM) najčešći je tip dijabetesa, koji postaje sve zastupljeniji globalni zdravstveni problem. Veruje se da polimorfizmi u genima koji kodiraju metalotioneine, grupu malih proteina koji vezuju cink i uključeni su u antioksidativnu zaštitu, učestvuju u patogenezi T2DM-a. Cilj ovog istraživanja bilo je ispitivanje potencijalne povezanosti pojedinačnog polimorfizma nukleotida rs11640851 MT1A 80 C/A sa rizikom od T2DM-a, kao i utvrđivanje uticaja genotipa i raspodele alela na biohemijske parametre povezane sa dijabetesom. U istraživanju je učestvovalo 298 ispitanika – njih 112 imalo je T2DM, a preostalih 186 zdravih ispitanika činilo je kontrolnu grupu. Ispitanicima su mereni glikemija natašte i nivo HbA1c. Polimorfizam u MT1A genu utvrđivan je pomoću PCR-RFLP metode. Nisu primećene značajne razlike u distribuciji genotipova i frekvenciji alela između kontrolne grupe i bolesnika sa dijabetesom ( $p > 0,05$ ). Takođe, nije bilo povezanosti između polimorfizma i laboratorijskih parametara povezanih sa dijabetesom, glikemije natašte i nivoa HbA1c. Ipak, nivo glukoze iznad 7 mmol/L zabeležen je kod 79,6% nosilaca alela C, odnosno kod 53,3% ispitanika koji su homozigoti za alel A ( $p = 0,005$ ). Iako u našem istraživanju nije pronađena direktna povezanost između genetskih varijanti MT1A gena i pojave T2DM-a, uočen je uticaj alela C na kontrolu glikemije kod bolesnika. Kako bi se proširila navedena saznanja i unapredilo razumevanje uticaja gena za metalotioneine na razvoj T2DM-a, neophodna su dalja istraživanja sa većim brojem ispitanika.

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