Reactive Oxygen Species Modulator-1 (ROMO-1) Polymorphism Rs6060566 and The Risk of Myocardial Infarction in Slovenian Subjects With Type 2 Diabetes Mellitus

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Abstract

Background

We aimed to examine the role of the rs6060566 polymorphism of the reactive oxygen species modulator-1 (*ROMO-1*) gene in the development of myocardial infarction (MI) in Caucasians with type 2 diabetes mellitus (T2DM).

Methods

A total of 1072 subjects with T2DM were enrolled in cross-sectional case-control study: 335 subjects with MI and 737 subjects without clinical signs of coronary artery disease (CAD). Genetic analysis of the rs6060566 polymorphism was performed in all subjects. To assess the degree of coronary artery obstruction, a subpopulation of 128 subjects with T2DM underwent coronary computed tomography (CT) angiography. Next, endarterectomy samples were obtained during myocardial revascularization from diffusely diseased coronary arteries in 40 cases, which were analysed for ROMO-1 expression according to their genotype.

Results

There were no statistically significant associations between different genotypes or alleles of the rs6060566 polymorphism and MI in subjects with T2DM. The carriers of the C allele of the *ROMO-1* rs6060566 had a threefold increased likelihood of having coronary artery stenosis (AOR = 3.27, 95% CI 1.16–9.20). Furthermore, the carriers of the C allele showed higher number of positive cells for ROMO-1 expression in endarterectomy samples of coronary arteries.

Conclusions

In accordance to our study, the rs6060566 polymorphism of the *ROMO-1* gene is not the risk factor for MI in Caucasians with T2DM. However, we found that subjects carrying the C allele were at a 3.27-fold increased risk of developing severe CAD compared with those who had nonobstructive CAD. Moreover, The C allele carriers showed statistically higher number of cells positive for ROMO-1 compared with T allele carriers in coronary endarterectomy samples.

Background

Type 2 diabetes mellitus (T2DM) is a heterogeneous group of metabolic disorders that affects around 8% of world population and was thought to be responsible for 5 million deaths in 2015 worldwide [1]. In addition, is one of the major risk factors for coronary artery disease (CAD) and more than 40% of patients with acute coronary syndrome (ACS) have diabetes mellitus (DM) [2]. Coronary artery disease is

characterized by atherosclerosis in coronary arteries and can be asymptomatic, whereas ACS usually presents with a symptom, such as unstable angina, and is frequently associated with myocardial infarction (MI) regardless of the presence of CAD [3]. Because of the proatherosclerotic, proinflammatory, and prothrombotic states associated with diabetes, diabetic patients with ACS are at high risk of subsequent cardiovascular events [4]. The 7-year risk of developing MI in diabetic patients was comparable to the risk of MI in nondiabetic patients who had a prior MI, which suggests that diabetes contributes significantly to the development of MI and can possibly be considered as a coronary heart disease (CHD) risk equivalent [5].

In a physiological system, the imbalance between antioxidant defence mechanism and reactive oxygen species (ROS) production leads to oxidative stress and subsequent pathological conditions [6]. Coronary vascular disease (CVD) occurs as a consequence of accelerated atherosclerosis, mainly driven by oxidative stress [7]. Numerous defence genes are involved in maintaining the balance between oxidant production and their removal by ROS-scavenger enzyme systems.

Reactive oxygen species modulator − 1 (*ROMO-1*) gene is located in chromosome 20q11.22 [8] and produces a small transmembrane protein located in the inner mitochondrial membrane. It is a unique nonselective cation channel that is suggested to be regulated in response to fluctuation in free iron concentration and of its redox state [9]. Nevertheless, ROMO-1 is vital for normal mitochondrial morphology and function [10] and its activation induces ROS production in mitochondrial respiratory chain leading to oxidative stress and cell death [11]. Moreover, ROMO-1 is involved in cell proliferation [12], cell apoptosis [13], it is thought to have a role in replicative senescence [14] and in carcinogenesis and tumour progression tumour cells [15].

Up to now, however, only one study examined relationship between *ROMO-1* polymorphism and diabetes vascular complication [16]. The aim of the present study was to assess the potential role of the *ROMO-1* polymorphism rs6060566 in the development of MI in Slovenian subjects with T2DM. Furthermore, we also explored ROMO-1 expression in coronary endarterectomy specimens with immunohistochemically staining.

Methods

Subjects

In this retrospective cross-sectional case-control study, 1072 unrelated Caucasians with T2DM lasting at least 10 years were enrolled. Participants were divided into two study groups: 335 subjects with MI and 737 subjects with no history of CAD, no signs of ischemic changes on electrocardiogram and no ischemic changes during submaximal stress testing. Subjects were classified as having T2DM according to the current American Diabetes Association criteria [17]. The diagnosis of MI was made according to the established universal criteria [18]. Subjects with MI were included in the study 1 to 9 months after the acute event.

All subjects enrolled in the study belonged to Caucasian ethnicity. After an informed consent for the participation in the study was obtained, a detailed interview was made including active smoking status and blood was drawn for biochemical analyses and genotyping. Body mass index (BMI) was calculated as weight in kilograms divided by the height in meters square.

Coronary computed tomography angiography

Further, to assess the degree of coronary artery obstruction, a subpopulation of 128 asymptomatic subjects with T2DM underwent coronary computed tomography (CT) angiography for diagnostic purposes at International Centre for Cardiovascular Diseases MC Medicor, Izola, Slovenia. Non-invasive visualization of epicardial coronary artery tree and detection of stenosis was performed on dual source Dual energy CT scanner (Siemens, Germany). The acquisition and reading of the coronary CT angiograms was assessed by BC, a senior expert cardiac radiologist. Normal coronary arteries were defined by the absence of obstructive or nonobstructive atherosclerotic plaque. Nonobstructive CAD was defined by the presence of plaque occupying a cross-sectional area stenosis <50%. The severity of CAD was classified by the degree of stenosis of the cross-sectional area (<50%, ≥50%≤75% and >75%) and by the number of diseased vessels (score from 0 to 3; as 0 for no vessel disease (VD), 1 for single VD, 2 for double VD and 3 for triple VD). Angiographically diagnosed diseased left main coronary artery (LMCA) was scored as 1 with disregarding stenosis in any of two major branches: left anterior descending (LAD) or left circumflex (LCx). In addition, if LMCA was not affected by atherosclerosis, we assigned score 1 for each LAD or/and LCx, respectively. At last, diseased right coronary artery (RCA) was scored as 1.

Biochemical analyses

Glucose, glycated haemoglobin A1c (HbA1c), high sensitivity C-reactive protein (hsCRP), total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, and triglycerides were determined by standard biochemical methods.

Genotyping

Genomic DNA was extracted from 100 µl of whole blood using a Qiagen isolation kit. The rs6060566 polymorphism of the *ROMO-1* gene was genotyped by KBioscience Ltd using their own novel fluorescence-based competitive allele-specific polymerase chain reaction (KASP) assay. Details of the method used can be found at http://www.kbioscience.co.uk/.

Immunohistochemistry

In the second part of the study, we included 40 subjects with T2DM with angina pectoris that had surgical myocardial revascularization. Coronary endarterectomy tissue samples were obtained during myocardial revascularization from diffusely diseased coronary arteries. With respect to different rs6060566 genotypes, relative expression for ROMO-1 in resected tissue samples was analysed by immunohistochemistry.

Sections from formalin-fixed, paraffin-embedded tissue blocks of 40 coronary endarterectomy specimens were cut at a thickness of 5 μm. Immunohistochemistry was carried out using a VENTANA BenchMark Ultra Slide staining System (Roche Diagnostics). Endarterectomy specimens were stained with antibody against ROMO-1 (1:100, rabbit polyclonal, HPA012782, Sigma Prestige Antibodies, St. Louis U.S.A.). For the detection of primary rabbit immunoglobulins the NovoLink Max Polymer Detection System (Leica Biosystems Newcastle Ltd, United Kingdom) was used according to the manufacturer's instructions. The sections were further incubated with chromogen diaminobenzidine (DAB). Subsequently, reaction with the peroxidase produced a brown precipitate at the ROMO-1 sites. Using a light microscope (Axio Scope 2, Zeiss Group, Germany), two researchers PN and DP independently evaluated the slides and manually counted ROMO-1 positive cells at 400 × magnification. Numerical areal density of cells which were immunoreactive for ROMO-1 was calculated (the number of positive cells per mm^2) as described before [19].

Statistical analyses

Normally distributed continuous variables were expressed as means ± standard deviations, and as median (interquartile range) when asymmetrically distributed. Normality of the continuous variables was examined by the Kolmogorov-Smirnov test. Normally distributed continuous variables were tested using an unpaired Student's t test and the Mann-Whitney U-test when asymmetrically distributed. Discrete variables were compared with Pearson χ^2 test, which was also used to test whether the genotypes distribution departures form Hardy-Weinberg equilibrium (HWE). However, when in the contingency table cells with expected frequencies <5 were identified, the Fischer's Exact test was used to determine if there is a significant relationship between two categorical variables.

Additionally, all variables that showed significant differences by univariate analysis were put into a stepwise multiple logistic regression. A *p*-value of <0.05 was considered to be statistically significant. Statistical analysis was performed using the SPSS program version 19 (SPSS Inc. Chicago, IL).

Results

Clinical characteristics and biochemical analysis

The clinical characteristics and biochemical analysis of the Slovenian subjects with T2DM are listed in Table 1. Cases (335 subjects with MI) had lower BMI, lesser waist circumference and better-controlled hypertension. Additionally, they had a higher total and LDL cholesterol, triglycerides, and lower HDL cholesterol. Moreover, cases had longer duration of T2DM. The two groups of subjects were well matched with regard to age, gender, fasting glucose, HbA1c, hsCRP level and concomitant history of cerebrovascular insult (CVI) or transitory ischaemic attack (TIA).

Table 1
Demographic and clinical characteristics of cases and controls in Slovenian subjects with T2DM

	Cases	**Controls**	
	(Myocardial infarction)	(Without CAD)	*p*-value
Number	335	737	
Age (years)	64.33 ± 9.79	64.12 ± 9.08	0.75
Male gender (%)	200 (59.7)	399 (54.1)	0.09
BMI (kg/m^2)	29.64 ± 4.14	30.69 ± 4.59	**< 0.001**
Waist circumference (cm)	104.99 ± 11.42	107.86 ± 12.73	**0.02**
Systolic blood pressure (mm Hg)	148.06 ± 19.75	150.78 ± 19.74	0.05
Diastolic blood pressure (mm Hg)	82.11 ± 10.59	84.63 ± 11.52	**< 0.001**
DM duration (years)	15 (10–23)	13 (9–18)	**< 0.001**
Fasting glucose (mmol/l)	8.87 ± 2.,90	8.60 ± 2.54	0.25
HbA1c (%)	7.88 ± 1.34	7.50 ± 1.9	0.64
Total cholesterol (mmol/l)	5.15 ± 1.45	4.64 ± 1.12	**< 0.001**
HDL cholesterol (mmol/l)	1.14 ± 0.30	1.24 ± 0.35	**< 0.001**
LDL cholesterol (mmol/l)	2.94 (2.22–3.76)	2.50 (2.02–3.10)	**< 0.001**
Triglycerides (mmol/l)	1.90 (1.34–2.70)	1.60 (1.10–2.43)	**< 0.001**
Smoking prevalence (%)	43 (22.8)	66 (9.0)	0.05
CVI (%)	27 (8.1)	44 (6.0)	0.20
TIA (%)	17 (5.1)	21 (2.8)	0.07
hsCRP (mg/l)	2.40 (1.28–4.80)	2.40 (1.30–3.90)	0.15

Abbreviations: BMI: Body mass index; DM: Diabetes mellitus; HbA1c: Glycated haemoglobin A1c; CVI: Cerebrovascular insult; TIA: Transitory ischaemic attack; hsCRP: high-sensitivity C-reactive protein. Values in bold indicate statistically significance.

Genotyping analysis

The genotype and allele frequencies of the *ROMO-1* rs6060566 polymorphism are shown in Table 2. Genotype distributions for both cases (subjects with MI) and controls (subjects without CAD) did not miss HWE (cases: $p = 0.70$; controls: $p = 0.83$, Pearson χ^2 test; respectively). Moreover, in each of the studied group genotype (cases: $p = 0.40$ and controls: $p = 0.21$, Pearson χ^2 test) and allele (cases: $p = 0.24$

and controls: $p = 0.1$, Pearson χ^2 test) frequencies were not significantly different from those reported for the datasets in the 1000 Genomes Project Phase 3 European population.

Table 2
Genotype and allele frequencies distribution of the rs6060566

		Cases (%) (N = 335)	Controls (%) (N = 737)	*p*-value
Genotypes	CC	10 (3.0%)	21 (2.8%)	0.98
	CT	90 (26.9%)	202 (27.4%)	
	TT	235 (70.1%)	514 (69.7%)	
Alleles	C allele (%)*	110 (16.4%)	244 (16.6%)	0.94
	T allele (%)	569 (83.6%)	1230 (83.4%)	
p (HWE)		0.70	0.83	
Abbreviations: HWE: Hardy-Weinberg equilibrium; * MAF: Minor allele frequency.				

Association analyses

Further, binary logistic regression analyses for different genetic models found no significant associations between different genotypes or alleles of the rs6060566 polymorphism and the risk of MI in Slovenian subjects with T2DM. Estimates of odds ratio (OR)s were adjusted (AOR)s (Table 3) for the variables (BMI, waist circumference, diastolic blood pressure, total cholesterol, HDL and LDL cholesterols, triglycerides, duration of DM in years) that were significant in the univariate analyses (Table 1).

Table 3
Binary logistic regression analyses for the association between rs6060566 of the *ROMO-1* and MI in Slovenian subjects with T2DM

Genetic Model	Cases/Controls	AOR (95% CI)	*p*-value
Co-dominant			
CC vs. TT*	10/21 vs. 235/514	1.12 (0.12–10.83)	0.92
CT vs. TT*	90/202 vs. 235/514	1.68 (0.84–3.36)	0.14
Dominant			
[CC + CT] vs. TT*	100/223 vs. 235/514	1.64 (0.83–3.22)	0.15
Recessive			
CC vs. [CT + TT]*	10/21 vs. 325/716	0.96 (0.10–9.17)	0.97
* The reference; OR: Odds ratio; AOR: Adjusted OR for BMI, waist circumference, diastolic blood pressure, total cholesterol, HDL and LDL cholesterols, triglycerides, duration of DM in years; CI: Confidence interval.			

No significant differences in genotype and allele frequency distribution for the rs6060566 polymorphism were observed among subjects with different distribution and extent of CAD, which was defined by coronary CT angiography (Table 4).

Table 4
Genotype and allele frequency distributions of the rs6060566 polymorphism in 128 subjects with T2DM who underwent coronary CT angiography

	Number of diseased vessels					Percentage of the cross-sectional area stenosis			
	None	1 VD	2 VD	3 VD	*p*-value	< 50%	≥ 50% ≤ 75%	> 75%	*p*-value
	N (%)	N (%)	N (%)	N (%)		N (%)	N (%)	N (%)	
CC	2 (4.2)	0	0	0	0.895*	2 (2.1)	1 (3.6)	0	0.262*
CT	14 (29.2)	4 (18.2)	10 (25)	4 (25)		25 (25.8)	12 (42.9)	1 (33.3)	
TT	33 (66.7)	18 (81.8)	31 (75)	12 (75)		70 (72.2)	15 (53.6)	2 (66.7)	
C	18 (18.4)	4 (9.1)	10 (12.2)	4 (12.5)	0.44†	29 (14.9)	14 (15)	1 (16.7)	0.213†
T	80 (81.6)	40 (90.9)	72 (87.8)	28 (87.5)		165 (85.1)	42 (75)	5 (83.3)	
* The *p*-value was obtained with Fischer s Exact test									
† The *p*-value was obtained with Pearson χ^2 test									
Abbreviations: VD: Vessel disease.									

Finally, we performed multinomial logistic regression analysis to evaluate the association of the rs6060566 polymorphism with CAD. Because of the low frequency of the minor C allele (Table 4) the analyses were performed assuming the dominant genetic model ([CC + CT] vs. TT). The final model is shown in Table 5. The dependent variables describing the severity of CAD were the number of diseased and extent of stenosis (none diseased vessel and stenosis < 50% were used as references, respectively). Independent variables included in the model were dominant genetic model (TT genotype was used as reference), age, gender, lipid parameters and duration of T2DM in years. We did not observe any interactions between dominant genetic model and CAD without adjustment for the possible confounders (Table 5). Nevertheless, when well-known CAD risk factors (age, gender, lipid parameters and duration of T2DM in years) were fixed in the model the association between carriers of the [CC + CT] genotypes and ≥ 50%≤75% cross-sectional area stenosis became statistically significant ($p = 0.025$, multinomial logistic regression). The carriers of the C allele of the *ROMO-1* rs6060566 had a threefold increased likelihood of having coronary artery stenosis (AOR = 3.27, 95% CI 1.16–9.20, Table 5).

Table 5
Multinomial logistic regression analyses for the association between dominant genetic model ([CC + CT] vs. TT*) of the rs6060566 polymorphism and CAD

	OR (95% CI)	*p*-value	AOR (95% CI)	*p*-value
≥ 50% ≤ 75%	2.25 (0.95–5.34)	0.067	3.27 (1.16–9.20)	**0.025**
> 75%	1.3 (0.11–14.89)	0.835	0.94 (0.03–25.8)	0.969
1 VD	0.44 (0.08–2.56)	0.364	0.25 (0.02–2.58)	0.245
2 VD	0.67 (0.18–2.5)	0.547	0.88 (0.12–6.39)	0.899
3 VD	0.66 (0.11–4.08)	0.661	0.85 (0.03–2.78)	0.928
Abbreviations: OR: Odds ratio; AOR: Adjusted ORs. ORs were adjusted for age, gender, lipid parameters and duration of T2DM in years. Statistically significant result (*p*-value < 0.05) is highlighted in bold.				
* The reference				

Coronary computed tomography angiography

A total of 128 subjects with T2DM underwent coronary angiography (Fig. 1). A single vessel disease (1 VD) was observed in 22 (17%) subjects, two-vessel disease (2 VD) in 41 (32%) and three-vessel disease (3 VD) in 16 (13%) subjects. Further, 49 (38%) subjects had normal all major epicardial coronary arteries (LMCA, LAD, LCx and RCA) on CT angiograms (Fig. 1a). Moreover, 97 (76%) subjects had nonobstructive CAD (cross-sectional area stenosis of < 50%), in 28 (22%) subjects a cross-sectional area stenosis of ≥ 50%≤75% was detected while only 3 subjects (2%) had stenosis of > 75% (Fig. 1a). As can be seen in

Fig. 1a, subjects with 2 VD (14/41, 34.1%) and nonobstructive CAD (11/97, 11.3%) suffered from nonfatal MI more often than other subjects in both comparative groups (number of diseased vessels and percentage of the cross-sectional area stenosis). Of note, there was a statistically significant difference ($p = 0.0096$, Pearson χ^2 test) in frequency distribution between subgroups with and without MI with regard to the extent of the CAD (Fig. 1a). In contrast, no difference ($p = 0.283$; Fischer s Exact test) was observed between subgroups with regard to the coronary cross-sectional area stenosis (Fig. 1a). Regarding the number of the involved vessels, a significantly higher frequency ($p = 0.013$; Pearson χ^2 test) of MI was found in subjects with 2 VD. Interestingly, subjects with two affected coronary arteries showed a 3.72 fold risk for MI (OR = 3.72, 95% CI 1.27–10.84, Fig. 1a).

Coronary angiography revealed that of the 128 subjects more than 50% of them had developed CAD in LAD (Fig. 1b), while the remainder of the coronary arteries were spared of atherosclerotic disease more frequently. Atherosclerotic changes were noticed in LMCA in 39 subjects (30.5%), while a slightly higher percentage of atherosclerotic disease was seen in LCx and RCA (41.4%) (Fig. 1b). Furthermore, the relationship between presence or absence of CAD and coronary arteries was statistically significant ($p = 0.0055$, Pearson χ^2 test; Fig. 1b).

As depicted in Fig. 1c, subjects with CAD (CAD+) in LMCA or LAD had about 3.5-fold higher risk of experiencing MI ($p = 0.007$ for LMCA and $p = 0.01$ for LAD, Pearson χ^2 test; respectively) compared with subjects without CAD (CAD-). However, in subjects with diseased LCx and RCA MI occurred more frequently than in subjects with disease-free coronary arteries, although the difference was not statistically significant ($p = 1.0$ for LCx and $p = 0.9$ for RCA, Pearson χ^2 test; respectively).

Immunohistochemistry

At the end of this study, the coronary artery segments, which were obtained by endarterectomy from subjects with advanced atherosclerosis, were examined with immunohistochemical staining. A statistically significantly higher numerical areal density of ROMO-1 positive cells was found in 17 subjects with the C allele (Fig. 2) in comparison with 23 subjects with ROMO-1 TT genotype (wild type) ($835 \pm 215/mm^2$ versus $412 \pm 153/mm^2$; $p < 0.001$, Student s t test).

Discussion

Our study investigated the role of oxidative stress *ROMO-1* gene polymorphism rs6060566 in Slovenian subjects with T2DM who experienced MI. We found no association of the rs6060566 polymorphism with MI. In contrast, we have identified a significant relationship between carriers of the [CC + CT] genotypes (under assumption of the dominant genetic model) and ≥ 50%≤75% cross-sectional area stenosis. Thus, we found that subjects carrying the C allele were at a 3.27-fold increased risk of developing severe CAD compared with those who had nonobstructive CAD.

Moreover, a significantly higher frequency of MI was found in subjects with 2 VD as compared to the subjects in whom CAD was not detected by CTA. At first glance, it seems that the frequency of subjects with MI increased with the number of involved vessels. Our results are partly supported by in-depth study conducted on 12.594 diabetes patients by Gyldenkerne et al. [20] who found that the extent of CAD is a major risk factor for MI and death in patients with DM. We could not confirm this increased risk for MI in

a group of subjects with 3 VD, most likely because of small sample size. Although it is known that diabetic patients suffer from more extensive CAD and hence higher incidence of multi-vessel CAD than non-diabetic subjects [21], in our research, 3 VD was diagnosed only in 16 out of 128 subjects who underwent coronary angiography. On the other hand, it is important to note, that all of them were diagnosed without atherosclerotic lesions in LMCA and none of them have suffered a MI. Furthermore, all 16 subjects underwent either percutaneous coronary intervention (PCI) or coronary artery bypass grafting surgery (CABG). We can recognize that appropriate myocardial revascularization strategy with optimal medical therapy appear to led to reduced incidence of MI in this small group of Slovenian subjects with 3 VD. Recently, a large-scale meta-analysis based on 18.224 patients with DM receiving PCI and CABG has demonstrated the superiority of CABG in reducing mortality, MI and need for repeat revascularization in patients with DM and complex CAD (including LMCA and/or multivessel disease) [22]. However, in our study we did not examine clinical outcomes of PCI or CABG in subjects with complex CAD.

In our study, subjects with CAD localized in LMCA and LAD had significantly higher occurrence of nonfatal MI compared to subjects without CAD in respective coronary arteries. They had about a 3.5-fold higher risk of MI. Our observations parallel with well-known facts that diabetes approximately doubles the risk of MI and death among patients with known CAD [23, 24]. On the other hand, the current study shows that MI is less frequent in subjects with disease-free coronary arteries. Thus, in the absence of angiographically significant CAD, patients with diabetes treated with contemporary prophylactic therapy have the same risk of cardiovascular events as patients without diabetes [25]. Therefore, optimal medical therapy and appropriate selection of myocardial revascularization strategy is critical for patients with DM [26].

T2DM patients are strongly prone to atherothrombotic complications in coronary arteries as well as in other macro- and microvasculature. One of major contributors to susceptibility for atherothrombotic complications is genetic background [27]. Many studies [28, 29] have shown association of different genetic loci and their polymorphisms with higher risk of atherosclerosis, CAD and MI. Numerous identified risk polymorphisms have no known pathophysiological function, but others are involved in inflammatory response, lipid function, transportation, endothelial dysfunction or oxidative stress regulation.

As oxidative stress is recognized as central pathogenic process in accelerated atherosclerosis in T2DM, various studies [7] investigated associations of oxidative stress genes (e.g. Nicotinamide Adenine Dinucleotide Phosphate (NADPH1) oxidase, Myeloperoxidase, Glutathione peroxidase 1, Glutathione S-transferase [30], NAD(P)H1: Quinone oxidoreductase (NQO1), Superoxide dismutase 1 and 2 [31], Thioredoxin reductase 2 [32], Uncoupling protein 2 [33], etc.) with micro- and macrovascular complications in T2DM. In general, studies yielded conflicting results, some of this discrepancies could be attributed to different study populations, races, small sample sizes, study types (i.e. retrospective) and design. It should be noted that many association studies were conducted on one single common polymorphism and hence have not scanned for the gene-gene or gene-environment influences on the risk for MI.

Oxidative stress occurs when ROS production exceeds the elimination capacity of antioxidant system. Most ROS are generated in mitochondrial respiratory chain [34] and although by-products, they are vital for normal processes, such as the maintenance of the vascular tone, cell adhesion, immune responses and cellular growth [35]. Increased expression of ROMO-1, firstly identified in tumour cells, increases ROS production [34], leading to oxidative stress and cell death [11]. Product of *ROMO-1* is small transmembrane protein located in inner mitochondrial membrane that has been recently found to be a unique nonselective cation channel. Its function is suggested to be regulated in response to fluctuation of free iron concentration and redox state of iron [9]. It is proposed that ROMO-1 activation causes recruitment of B-cell lymphoma-extra-large (Bcl-xL) protein to the outer mitochondrial membrane that in turn reduces its membrane potential, resulting in ROS production [13].

ROMO-1 is essential protein involved in several cell functions. It is vital for maintaining mitochondrial cristae shape [10, 36] and its loss causes mitochondrial cytochrome c leakage, which is one of a key molecule involved in the intrinsic apoptotic pathway [36]. Its role in TNF-alpha-induced apoptosis was furthermore confirmed by Kim et al. [13].

Hwang et al. [11] showed that enforced ROMO-1 expression leads to massive cell death - necrosis - by excessive ROS production. It was shown that in physiological states ROMO-1 derived ROS were indispensable for the proliferation of both normal and cancer cells, respectively [12]. Moreover, Chung el al. [36] showed that ROMO-1 might be principal factor in deregulation of nuclear factor-κB and related pathways that contribute to tumour cell proliferation and invasion. It is also thought to have a role in replicative senescence [14]. Lung, colorectal cancer and gliomas [38] have been linked to ROMO-1. In non-small cell lung cancer ROMO-1 can serve as disease biomarker and is predictor of poor survival and malignant effusions in these patients [39, 40]. In colorectal cancer, ROMO-1 expression predicts poor survival and higher invasiveness [15]. Its role is also being investigated concerning pulmonary and renal fibrosis [41], obstructive sleep apnoea syndrome [42] and Fanconi anaemia [43].

Further, according to our knowledge, this is the first study investigating the expression of the ROMO-1 in coronary endarterectomy samples. The C allele carriers showed statistically higher number of cells positive for ROMO-1 compared with T allele carriers. This result corroborates the findings by Petrovič et al. [16], in which greater ROMO-1 expression was found in fibrovascular membranes of subjects with microvascular complication of T2DM, namely proliferative diabetic retinopathy. We also assumed that ROMO-1 rs6060566 polymorphism might be associated with MI. However, we failed to show the presumable association in Slovenian subjects with T2DM. One possible explanation for our results might be that some of the controls had clinically silent CAD that we did not detect with our inclusion criteria, or had other macro- or microvascular complications that could also be induced by oxidative stress. Otherwise, subjects with MI had longer duration of DM and higher levels of HbA1c. It is generally believed that the relative risk for MI increases with any increase in glycaemia above the normal range [44]. On the other hand, cases had better managed blood pressure, which is probably a consequence of recent MI, thus recently optimized medical therapy with presumed better compliance.

Conclusion

In conclusion, in this study we did not observe any association between rs6060566 polymorphism of the *ROMO-1* gene and the risk of MI in Caucasians with T2DM. However, we found that subjects carrying the C allele were at a 3.27-fold increased risk of developing severe CAD compared with those who had nonobstructive CAD. Moreover, The C allele carriers showed statistically higher number of cells positive for ROMO-1 compared with T allele carriers in coronary endarterectomy samples. Furthermore, it seems likely that the extent of CAD was a risk factor for MI in a subgroup of subjects in whom two-vessel disease was diagnosed with CT coronary angiography.

New opportunities for research activity should open up to explore the contribution of oxidative stress on perpetuating the atherosclerotic process in coronary artery system in subjects with T2DM.

Abbrevations

ROMO-1: Reactive oxygen species modulator-1; MI: Myocardial infarction; T2DM: Type 2 diabetes mellitus; CAD: Coronary artery disease; CT: Computed tomography; AOR: Adjusted odds ratio; CI: confidential interval; ACS: Acute coronary syndrome; DM: Diabetes mellitus; CHD: Coronary heart disease; ROS: Reactive oxygen species; CVD: Coronary vascular disease; VD: Vessel disease; LMCA: Left main coronary artery; LAD: Left anterior descending; LCx: Left circumflex; RCA: Right coronary artery; BMI: Body mass index; HbA1c: Glycated haemoglobin A1c; hsCRP: High sensitivity C-reactive protein; LDL: Low density lipoproteins; HDL: High density lipoproteins; PCR: Polymerase chain reaction; DAB : Diaminobenzidine; KASP: Competitive Allele-Specific Polymerase chain reaction; HWE: Hardy-Weinberg equilibrium; SPSS: Statistical Package for the Social Sciences; CVI: Cerebrovascular insult; TIA: Transitory ischaemic attack; MAF: Minor allele frequency; OR: Odds ratio; PCI: Percutaneous coronary intervention; CABG: Coronary artery bypass grafting; NADPH: Nicotinamide Adenine Dinucleotide Phosphate; NQO1: NAD(P)H1 Quinone oxidoreductase; Bcl-xL: B-cell lymphoma-extra-large.

Declarations

Ethics approval and consent to participate

The research (number 0120-372/2017) was approved by the Republic of Slovenia National Medical Ethics Committee, Ministry of Health and was performed in compliance with the Helsinki Declaration. The following members: Chairman dr. Božidar Voljč, MD; Prof. dr. Matej Cimerman, MD; dr. Urh Grošelj, MD; Prof. dr. Matjaž Jereb, MD; dr. Dušica Pleterski-Rigler, MD and Mr. Tone Žaklelj constituted the quorum for the ethical review of research. None of the members had any conflict of interest in the matter on which they voted. All study participants were fully informed of the research objectives, and those who agreed to participate signed an informed consent form.

Availability of data and materials

The datasets of the current study are available from the co-author DP upon reasonable request.

Consent for publication

Not applicable.

Competing interests

The authors affirm that they have no conflicts of interest related to the content of this article.

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Authors contribution

MT wrote the manuscript, SMR carried out the statistical analyses, interpretation of the data and participated in writing and correcting the manuscript. DP revised the manuscript. All authors approved the final version of the manuscript.

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Figures

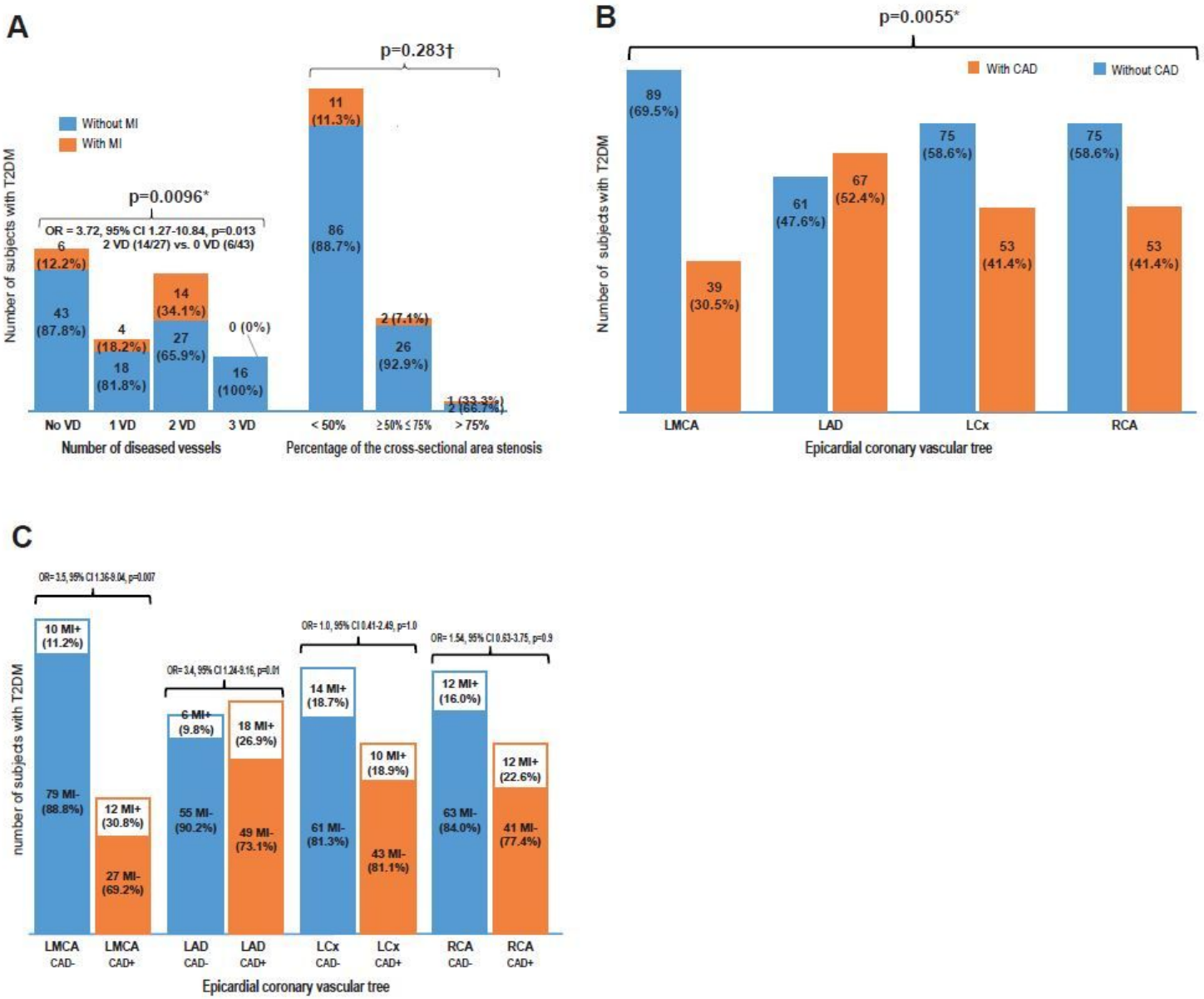

Figure 1

1 Findings at coronary CT angiography. a Distribution of Slovenian subjects with T2DM according to the extent and severity of CAD. b Atherosclerotic burden in major coronary arteries. c Relation between specific coronary artery and the risk of MI in subjects with CAD (CAD+) in comparison with subjects without CAD (CAD-). * The p-value was obtained with Pearson χ2 test † The p-value was obtained with Fischer s Exact test

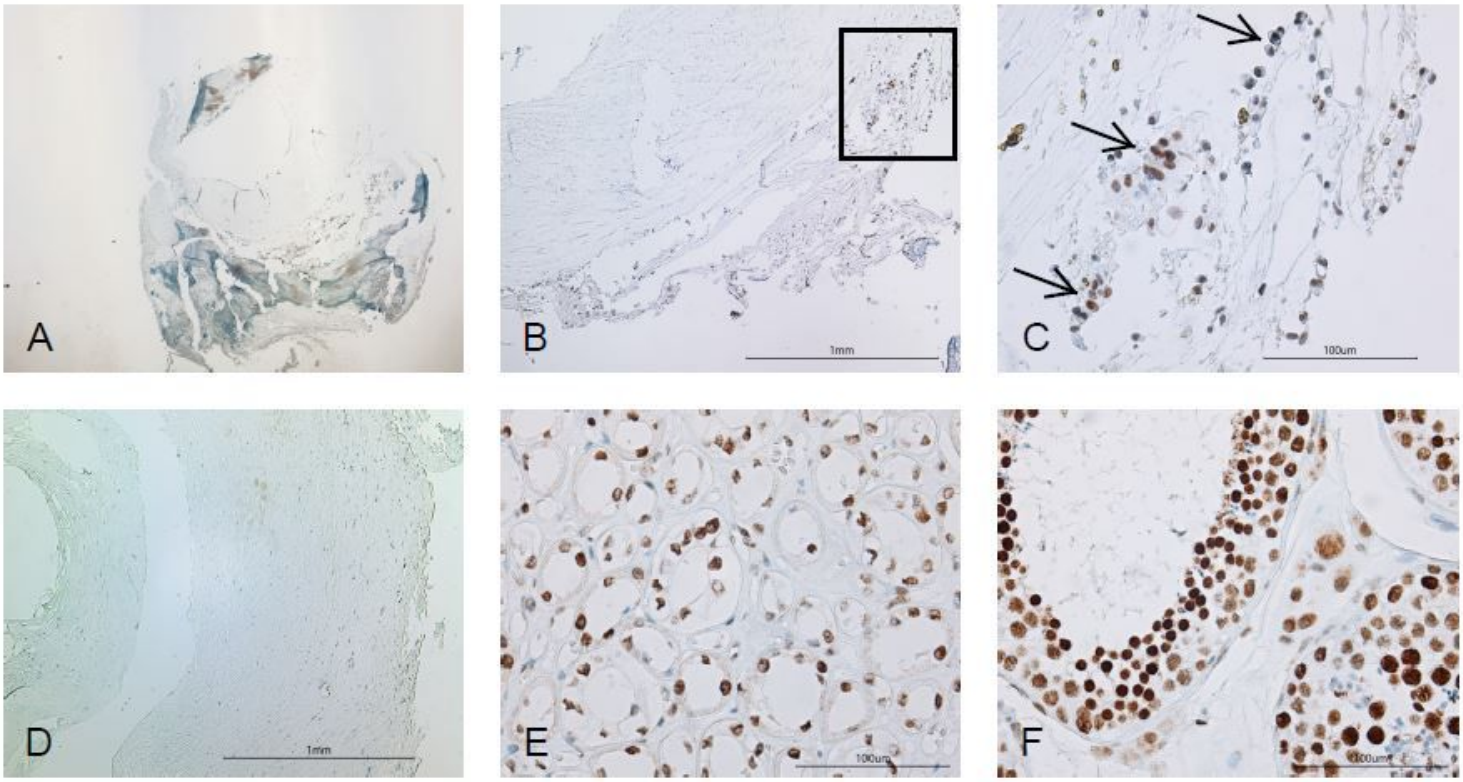

Figure 2

Histology and immunohistochemistry for ROMO-1 in endarterectomized tissue samples of coronary arteries (a-d) and positive controls (e-f): ROMO-1 positive reaction in human kidney medulla (e) and testis (f). (a-c) ROMO-1 expression in representative tissue section in the C allele carrier of the rs6060566 polymorphism, (c) is a higher magnification of the square in (b). A portion of the coronary artery (c) in which immunohistochemistry for ROMO-1 reveals positive brown cells (arrows). No staining of cells in the coronary artery of the T allele carrier (d). Original magnification ×25 (a), ×100 (b, d, e) and ×400 (c, f).