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**Assessment and Role of Certain Markers of Endothelial Dysfunction
and Oxidative Stress in Outpatients with Metabolic Syndrome**

DISSERTATION SUMMARY

for the acquisition of the educational and scientific degree

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Списък на използваните съкращения

ADMA	- Asymmetric dimethylarginine
BMI	- Body mass index
CRI-I	-Castelli risk index I
CO	-Carbon monoxide
CRP	-C - reactive protein
DDAH1	-Dimethylarginine dimethylaminohydrolase 1
eNOS-	- Endothelial nitric oxide synthase
ED	- Endothelial dysfunction
GGT	- γ -glutamin transferase
GPx	-Glutathione peroxidase
GSH	-Glutathione
GST	- Glutathione S трансфераза
HO-1	-Heme-oxygenase-1
iNOS	-Inducible nitric oxide synthase
ICAM-1	- Intercellular adhesion molecule 1
I κ B	-Inhibitor of NF- κ B
IL-6	- Interleukin-6
IL-8	- Interleukin-8
IL-10	- Interleukin-10
MDA	-Malondialdehyde
MetS	-Metabolic syndrome
NO	-Nitric oxide
NF- κ B	-Nuclear factor kappa B
Nrf2	- factor (erythroid – derived-2) –like-2)
ONOO-	- Peroxynitrite
OS	-Oxidative stress
PBMC	- Peripheral blood mononuclear cells
PRMT-I	- Protein methyltransferase-1

ROS	-Reactive reactive species
CIMT	- Carotid intima media thickness
TNF α	- Tumor necrosis factor alpha
TBA	-Thiobarbituric acid
TC	- Total cholesterol
VCAM-1	-Vascular cell adhesion molecule 1
WC	-Waist circumference

1. INTRODUCTION

Metabolic syndrome (MetS) is a global problem that affects an increasing number of people of working age. Metabolic syndrome (also known as syndrome X or insulin resistance syndrome) is a cluster of metabolic and cardiovascular disorders whose common denominator is insulin resistance (Arcaro G, 2002; Hill MA et al., 2021).

The complications of metabolic syndrome are numerous, but the most important are cardiovascular and cerebrovascular complications (heart attacks and strokes), as these often lead to disability or death (Tziomalos K, et al., 2010; Fornoni A, Raij L, 2021; Hill MA et al 2021). They are associated with an increased impact of risk factors: untreated hypertension, diabetes mellitus with poor blood sugar control, hyperlipidemia, dyslipidemia, high uric acid levels, abdominal obesity, reduced physical activity, and an unhealthy diet (Lundman et al., 2003; Kanbay et al., 2013; Chan NN, Chan JC, 2002; Konukoglu D, Uzun H., 2017; Brook RD et al., 2001; Perticone F et al., 2001)

The development of endothelial dysfunction is considered the primary pathogenic factor linking MetS to the influence of risk factors. The components of MetS and associated vascular risk can impair vascular endothelial function and the structure of the vascular wall and may lead to subclinical atherosclerosis and vascular disease (Kim JA et al., 2006; Tziomalos K et al, 2010; Jamwal S, Sharma S, 2018; Shayo SC et al, 2019; Hill MA et al, 2021; Fornoni A, Raij L, 2021).According to current understanding of endothelial dysfunction as a universal and key factor in the pathogenesis of vascular damage, it precedes the pathomorphological changes in the vascular wall associated with cardiometabolic diseases

Therefore, endothelial dysfunction is considered a triggering pathogenic mechanism for the development and progression of CVD, which is the leading cause of disability for thousands of people worldwide. The social significance of disabling complications of CVD underscores the need to seek new approaches for early prevention. The search for ways to reduce vascular wall damage is one of the most promising areas in the prevention of cardiometabolic diseases.

The literature on endothelial dysfunction in MetS is scarce and contradictory. Markers of endothelial dysfunction remain poorly studied. Elevated levels of total cholesterol, triglycerides, and glucose, as well as certain pro-inflammatory markers (C-reactive protein,

interleukin-6, and tumor necrosis factor- α), are not sufficient criteria for endothelial dysfunction in cardiometabolic diseases and cardiovascular risk.

The molecular mechanisms underlying endothelial dysfunction have not been sufficiently studied, but its development is believed to be closely linked to the inflammatory response due to an imbalance between oxidants and antioxidant defenses in the body and the development of oxidative stress in MetS (Perticone F et al., 2001; Mertens I et al., 2006; Devaraj S et al., 2011). Disruption of the redox balance plays a key role in endothelial damage by reducing endothelial NO-dependent vasodilation, stimulating vascular inflammation, and activating redox-sensitive cellular and intercellular signaling pathways, thereby pathogenetically linking oxidative stress, chronic inflammation, and endothelial dysfunction (Shayo SC et al., 2019; Hill MA et al., 2021; Fornoni A, Raij L, 2021). This has led to increased research interest and the conduct of new targeted studies aimed at investigating the complex mechanisms of endothelial damage and protection in MetS, as well as opportunities for therapeutic intervention.

2. AIM AND OBJECTIVE

AIM: To investigate and evaluate the role of certain markers of endothelial activation and oxidative stress in outpatients with metabolic syndrome

To achieve this aim, the following research objectives were defined:

1. To measure plasma levels of ADMA, a marker of endothelial dysfunction
2. To examine the markers of oxidative stress malondialdehyde (MDA)) in individuals with metabolic syndrome
3. To examine lipid markers, glucose levels, uric acid levels, WC and body mass index (BMI)
4. To investigate the correlation between ADMA and MDA and atherogenic lipid indexes
5. To investigate the expression of the transcription factors Nrf2, NF- κ B, and NO-1 in PBMCs in individuals with metabolic syndrome
6. To investigate the relationship between ADMA, NO-1, and Nrf2, NF- κ B in individuals with metabolic syndrome
7. To investigate and evaluate age-related changes in CIMT in individuals with metabolic syndrome

8. To evaluate the relationship between CIMT, ADMA, NO-1, NF- κ B, and Nrf2 in metabolic syndrome

3. MATERIAL AND METHODS

3.1 MATERIAL

Participants

The investigation study was approved by the local Ethics Committee at the Medical University of Varna (Protocol. 86/26 September 2019, Varna, Bulgaria). Written informed consent was obtained from all participants. A total of 84 people (69 females, 5 males) were included in this case-control study. They were divided into two groups: those newly diagnosed with MetS (n = 63) and nonmetabolic, clinically healthy individuals (a control group) (n = 20). According to CEP-ATP III criteria (the presence of three or more of the following: waist circumference ≥ 102 cm (men) or ≥ 88 cm (women); blood pressure $\geq 130/85$ mmHg or treatment for hypertension; triglycerides ≥ 1.7 mmol/L (≥ 150 mg/dL); high-density lipoprotein (HDL) cholesterol < 1.03 mmol/L (< 40 mg/dL) for men or < 1.29 mmol/L (< 50 mg/dL) for women), 63 individuals were selected for the MetS group. The participants were newly diagnosed and indicated as having MetS based on the abovementioned criteria and were not under treatment for the condition. Inclusion and exclusion criteria of the study were as follows: inclusion criteria—individuals with elevated blood pressure, blood sugar, obesity, dyslipidaemias, and high levels of triglycerides, cholesterol, and uric acid in the blood, as well as those who have relatives (mother, father) with diabetes mellitus, arterial hypertension, and obesity; exclusion criteria-vascular accident, autoimmune, psychiatric, and malignant diseases.

Table 1. Baseline characteristics of the study groups

Groups	Control N=20	MetS N=63	P values
BMI kg/m ²	23.2±0.72	29.3± 1,27	0.0001
WC	74.6±2.53	93±1,31	0,0001
SBP mmHg	123±3.01	144±1.07	0.0001
DBP mmHg	79.5±1.89	90.1±0.99	0.0001
TG mmolL	0.70±0.071	1.72±0.122	0,0001
HDL mmolL	2.28±0.27	1.38±0.068	0.0001

Data expressed as mean $\pm$ SEM. MetS indicates metabolic syndrome; BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; TG, triglycerides

3.2 Methods

The necessary tests were conducted in the Clinical Laboratory and Doppler Sonography Room at the St. Marina Hospital in Varna and the Department of Biochemistry, Molecular Medicine and Nutrigenomics, Medical University of Varna,

3. 2. 1 Survey method

A questionnaire developed for the purposes of the study was used to collect information on demographic data (gender, age, place of residence), educational level, presence of components of the metabolic syndrome and other cardiovascular risk factors (arterial hypertension, type 2 diabetes mellitus and obesity), family history, genetic predisposition to vascular diseases, presence of chronic CVDs, dyslipidemia, lifestyle, data on physical activity

3. 2. 2 Biochemical and clinical-laboratory methods

The following clinical and laboratory tests are performed on the participants:

- height, waist circumference (WC), body mass index (BMI)

- venous measurement of blood sugar and serum levels of TC, (total cholesterol) - total cholesterol; HDL-C (High-density Lipoprotein Cholesterol) - high-density cholesterol; LDL-C (Low density Lipoprotein Cholesterol) - low-density cholesterol; TRGL (triglycerides)- triglycerides with Biochemical Analyzer ADVIA 1800
- atherogenic indices Castelli I risk index (Castelli risk index I, CRI-I) = $\text{TC}/\text{HDL-cholesterol}$ • Castelli II risk index (Castelli risk index II, CRI-II) = $\text{LDL-cholesterol}/\text{HDL-cholesterol}$ •
- Plasma concentrations of asymmetric dimethylarginine Asymmetric dimethylarginine (ADMA) were determined using an ELISA assay kit (DLD Diagnostica GMBH, Germany), after it was validated locally. The method was validated in accordance with the international standard for quality and competence of medical laboratories (BDS/EN/ISO 15189). Measurements were performed in duplicate. Data are presented in mM/L.
- Lipid peroxidation was analyzed with the level of malondialdehyde (MDA) measured by its reactivity to thiobarbituric acid (TBA) in plasma using the method of Porter et al.²⁸ Data are presented in mM/L.

3.2. 3 Genetic methods

Peripheral Blood Mononuclear Cell (PBMC) Collection

Peripheral blood mononuclear cells were separated from whole blood (6 mL) and collected in lithium heparin vacutainers. The separation of PBMCs was carried out using a Ficoll separation medium with a density of 1.077 g/L and 50 mL LeucoSep™ centrifuge separation tubes (GreinerBioOne, Kremsmunster, Austria) with a separation barrier for PBMC separation based on density gradient centrifugation and purification, following the manufacturer's instructions.

Gene Expression Analysis

TRI reagent (AmbionR, Life Technologies, Waltham, MA, USA) was used for total RNA extraction from PBMCs. cDNA was synthesized from 0.1 µg of total RNA with a Revert Aid First Strand cDNA Synthesis Kit (ThermoScientific, Waltham, MA, USA). Relative gene expression analysis was performed with two-step real-time qPCR. SYBR Green qPCR MasterMix with ROX (KAPA SYBR FAST qPCR Kit, KAPA BIOSYSTEMS, Wilmington, MA, USA) was used for the real-time PCR reaction and primer sets were

as follows: U6 F: GCTTCGGCAGCACATATACTAAAAT; R: CGCTTCACGAATTTGCGTGTTCAT;
HMOX1 F: TCAGGCAGAGGGTGATAGAAG; R: TTGGTGTCATGGGTCAGC;
NRF2 F: TCCAGTCAGAAACCAGTGGAT; R: GAATGTCTGCGCCAAAAGCTG; NF-kB F: AGAGGCTTCCGATTTTCGATATGG; R: GGATAGGTCTTTTCGGCCCTTC. Real-time PCR was carried out on an Applied BiosystemsR 7500 Real-Time qPCR instrument (Waltham, MA, USA). Gene expression levels of the genes of interest were calculated according to the 2- $\Delta\Delta C_t$ method, where U6 served as endogenous control

3.2.4 Carotid Ultrasonography

Carotid intima-media thickness (CIMT), which is a surrogate marker for the presence and progression of atherosclerosis, was determined by B-mode ultrasound [23]. In the study, we examined the carotid arteries of patients using an Aloka Alpha 6 device (ProSound Digital Color System) by B-mode ultrasound and a linear transducer (38 mm) with a frequency of 4–13 MHz (16 MHz BBH-Rx). Determining in advance the depth, the course of the artery, and the level of its division into external and internal branches with Doppler sonography, we followed the blood flow and visualized the changes in the vessel wall.

The main indicator that could be calculated in this study was CIMT, which is considered by many authors as a surrogate marker for the presence and progression of atherosclerosis. We made three standard measurements for the CIMT indicator on each side, resp. at 0.5, 1, and 2 cm below the bifurcation of the carotid artery, taking as reliable the mean value of the intima-media thickness (IMT). We measured arterial intima thickness and intimal thickening as an indicator of early atherosclerosis. Using the ESC/ESH criteria for arterial hypertension (AH), we defined normal CIMT (<0.9 mm) and increased CIMT (>0.9 mm).

3.2.5. Statistical Analysis

Data were presented as mean $\pm$ SEM or percentage (%), depending on what was deemed more appropriate for the purpose. Data analysis was performed on GraphPad Prism v. 8.3. and SPSS v. 23. Standard statistical methods, such as descriptive statistics, unpaired Student's t-tests for normally distributed parameters, and one-way ANOVA, GraphPad Prism v. 8.3., with Bonferroni correction, were used.

4. RESULTS AND DISCUSSION

4. 1.INVESTIGATION OF THE RELATIONSHIP BETWEEN PLASMA LEVES OF ADMA, MDA AND METABOLIC MARKERS IN INDIVIDUALS WITH METHABILIC SYNDROME

1.1. Study of the level of ADMA, MDA and lipid profile, glucose in plasma in individuals with metabolic syndrome and control group and the correlation between them

1.1.1 Comparative analysis of the values of ADMA in plasma in individuals with metabolic syndrome (MetS) and the control group (C)

The level of ADMA in plasma was significantly increased in the metabolic group compared to the control by 20% (0.42 ± 0.02 to 0.52 ± 0.01)

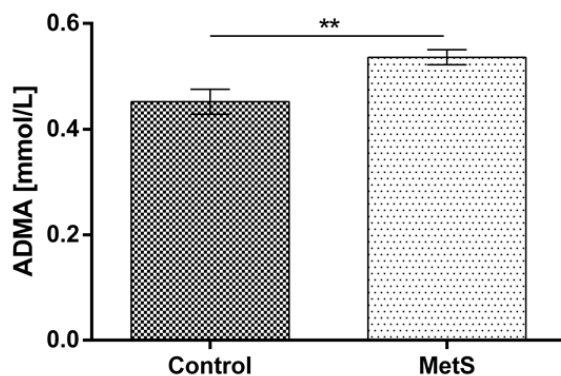


Fig 7 Plasma ADMA levels in subjects with metabolic syndrome (MetS) and controls (C). Data are presented as mean±SEM statistical significance was presented as $p<0.05$, $**p<0.0001$

Таблица1

1.2 Comparative analysis of lipid profile indicators in individuals with metabolic syndrome (Mets) group and the control group (C

Table1

Lipid Markers	C	MetS	P -Value
TG	0.699±0.063	1.65±0.131	P=0.0001
LDL	2.34±0.113	3.28±0.21	P=0.0005
HDL	2.28± 0.140	0.65±0.060	P=0.0020
TC	4.25±0.13	5.69±0.81	P=0.0001

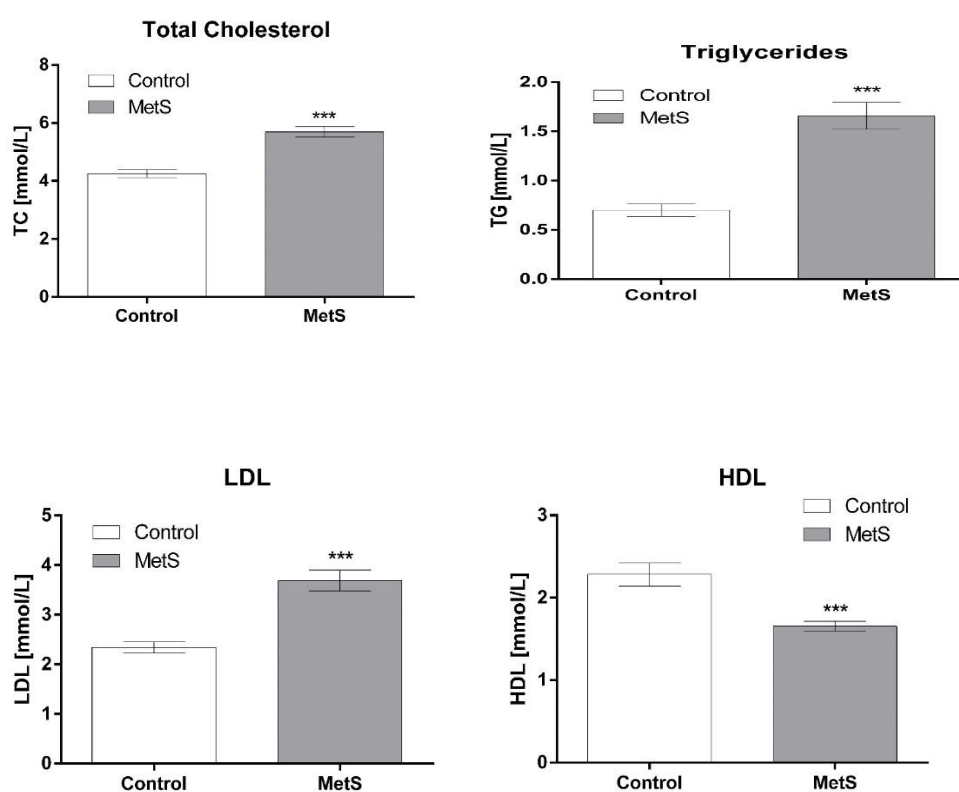


Figure 8 Changes in lipid profile (HDL, LDL, TG TC) in subjects with metabolic syndrome (MetS) and control (C). Data are presented as mean±SEM statistical significance was presented as $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$

1.1.3 Comparative analysis of some lipid indexes in subjects with metabolic syndrome (MetS) group and the control group) група и контролната група (C)

Table 2

Atherogenic indexes	C	MetS	P Value
TC/HDL	1.977±0.364	3.059±0.985	0.0002
LDL/HDL	1.148±0.375	1.868±0.918	0.0061

Table 3 Correlations between AMA levels and lipid profile indicators and lipid indices

		TG	TC	LDL	HDL
ADMA	Person correlation	.414	.084	.147	-489
	P	0.001	0.657	0.437	0.004

		TC/HDL	LDL/HDL	
ADMA	Pearson correlation	.524	.424	
	p	0.0031	0.0193	

1.1.4 Comparative analysis of glucose levels in subjects with metabolic syndrome (MetS) group and the control group (C)

Glucose values were significantly increased in the metabolic group compared to the control by 25% (3.98 +0.39 to 4.98+0.73 (Fig.10)

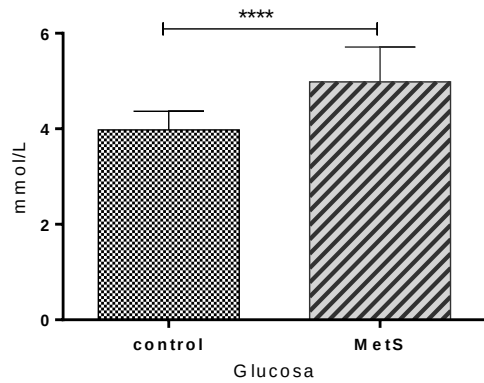


Figure 10 Plasma glucose levels in subjects with metabolic syndrome (MetS) and controls (C). Data are presented as mean $\pm$ SEM statistical significance was presented as $p<0.05$, ** $p<0.0001$

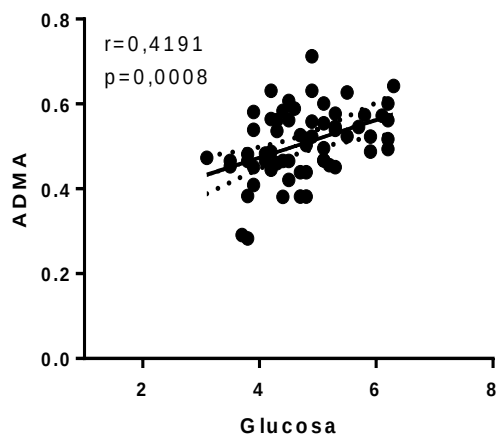


Fig 11 Correlation between plasma levels of ADMA and glucose in individuals with metabolic syndrome

1.1.5 Comparative analysis of BMI in subjects with metabolic syndrome (MetS) group and the control group (C)

BMI values were significantly increased in the metabolic group compared to the control by 24% (22.9 ± 1.788 to 28.31 ± 3.45) (Fig.12)

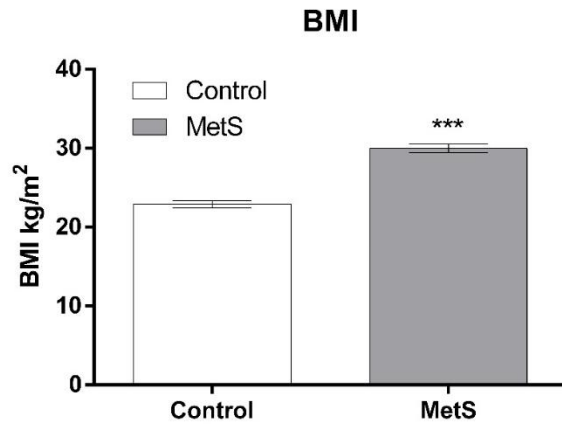


Figure 12 Changes in BMI in subjects with metabolic syndrome (MetS) and controls (C). Data are presented as mean±SEM statistical significance was presented as $p<0.05$, ** $p<0.0001$

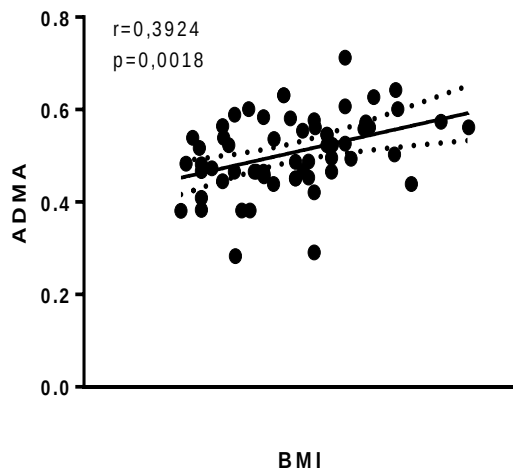


Figure 13 Correlation between plasma ADMA levels and BMI in individuals with metabolic syndrome

1.1.6 Comparative analysis of WC values in subjects with metabolic syndrome (MetS) group and the control group (C)

WC values were significantly increased in the metabolic group compared to the control by 22% (74.64± 7.85 to 90.81± 8.27) (Fig.14)

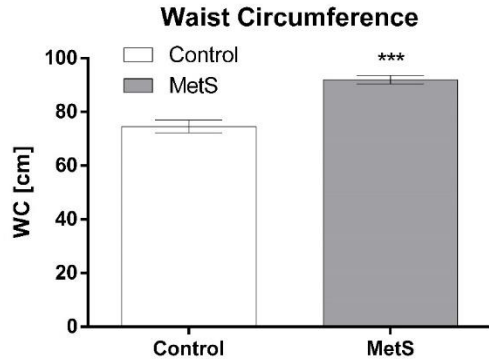


Figure 14 Changes in WC in subjects with metabolic syndrome (MetS) and controls (C). Data are presented as mean±SEM statistical significance was presented as $p < 0.05$, ** $p < 0.0001$

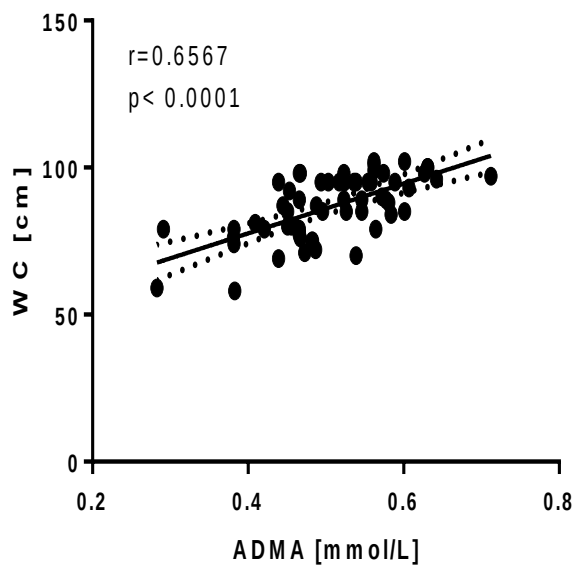


Figure 15 Correlation between plasma ADMA levels and WC in individuals with metabolic syndrome

Comparative analysis of uric acid (UA) values in subjects with metabolic syndrome (MetS) group and the control group (C)

Uric acid values were non-significantly increased in the metabolic group compared to the control (Fig.16)

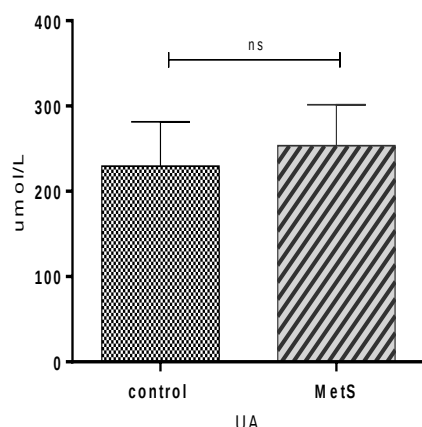


Figure 16 Plasma levels of UA in subjects with metabolic syndrome (MetS) and controls (C). Data are presented as mean $\pm$ SEM statistical significance was presented as $p<0.05$), ** $p<0.0001$

1.1.8 Comparative analysis of MDA values in subjects from the metabolic and control groups

The level of MDA in the plasma was significantly increased in subjects from the metabolic syndrome group compared to the control by 78% (1.05 ± 0.13 to 1.87 ± 0.11) (Fig. 17).

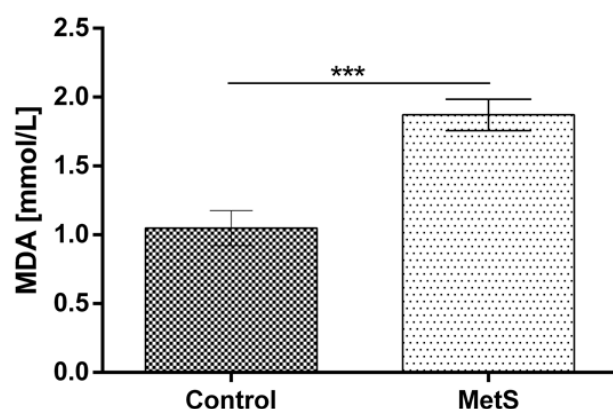


Figure 17 Plasma MDA levels in subjects with metabolic syndrome (MetS) and controls (C). Data are presented as mean $\pm$ SEM statistical significance was presented as $p<0.05$), *** $p<0.001$.

Table 4 Correlations between MDA levels and lipid profile indicators and lipid indices

		TG	TCI	LDL	HDL
MDA	Pearson correlation	.467	.163	.303	.270
	p	0.018	0.386	0.020	0.113

Таблица

		CHol/HDL	LDL/HDL	
MDA	Pearson correlation	.284	.364	
	p	0.128	0.048	

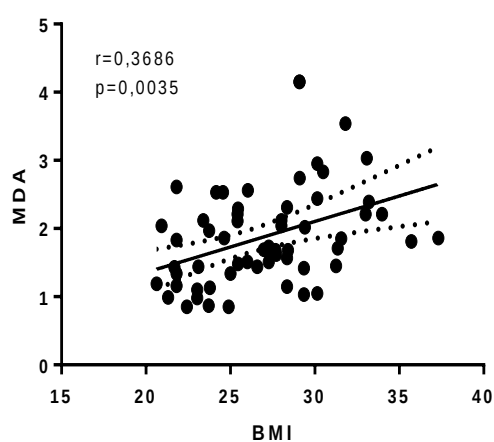


Figure 18 Correlation between BMI and MDA levels in the plasma of individuals with metabolic syndrome

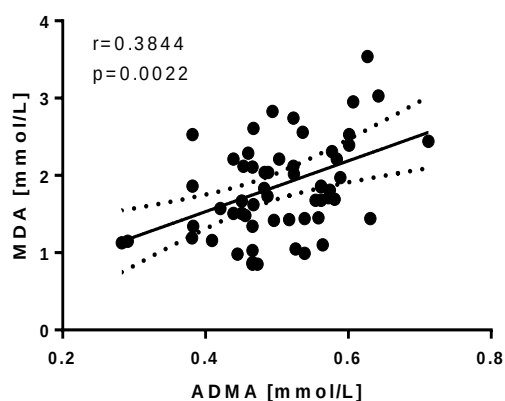


Figure 19 Correlation between MDA and ADMA levels in the plasma of individuals with metabolic syndrome

1.1.9 Discussion

Endothelial dysfunction, characterized by reduced nitric oxide (NO) bioavailability, is one of the key factors in the pathogenesis of MetS (Fornoni A, Raij L, 2002; Tziomalos K et al., 2010; Palomo I et al., 2011). In recent years, there has been a significant increase in interest in asymmetric dimethylarginine (ADMA), which acts as a physiological regulator of NO biosynthesis (Palomo J et al., 2011).

Endothelial NO, which is synthesized from L-arginine with the help of NO synthase (eNOS), is an important regulator of vascular homeostasis. In addition to being a potent vasodilator, NO also acts as an inhibitor of the adhesion of various inflammatory cells to the vascular wall. It suppresses platelet aggregation and smooth muscle cell proliferation. Inactivation and/or impaired synthesis of NO is common in patients with risk factors for cardiovascular disease (CVD) and can lead to the development of endothelial dysfunction, hypertension, thrombosis, and atherogenesis (Desjardins F, Balligand JL, 2006)

ADMA is an inhibitor of eNOS and is considered one of the key factors in endothelial dysfunction (Tan B et al., 2007). It is believed that elevated serum levels of ADMA and reduced NO synthesis lead to the development of endothelial dysfunction and CVD, a triggering factor in the development of atherosclerosis (Kawashima S, 2004)

The results of our studies show significantly higher plasma levels of ADMA in individuals with MetS compared to the control group (Fig. 7). Elevated plasma ADMA levels have been demonstrated in patients with type 2 diabetes (Abbasi, 2001), hypercholesterolemia (Bode-Boëger SM et al., 1996, Böger RH et al., 2003), hypertriglyceridemia (Lundman P et al., 2001), hypertension, and obesity (Perticone F et al., 2010), as components of metabolic syndrome.

Elevated plasma levels of glucose and proatherogenic lipids such as TG, OX, and LDL were observed, while HDL levels were reduced in the metabolic group. (Figs 8,9, 10) WC and BMI were also elevated in the metabolic group Figs. 12, 14 Furthermore, our data show a positive correlation between ADMA and TG, ADMA and TG/HDL, ADMA and LDL/HDL, ADMA and WC, and ADMA and BM (Figs 11,13,15)

The evidence linking hypercholesterolemia, as well as other risk factors such as obesity, hyperuricemia, and endothelial dysfunction, is conflicting. A number of studies report a significant association between elevated ADMA levels, hypercholesterolemia, and endothelial

dysfunction in individuals with MetS (Dayal S, 2005; Ferlazzo N et al., 2021; Jamwal S, Sharma S., 2018; Kwiecien S et al., 2012).

It is known that ADMA is metabolized by dimethylarginine dimethylamine hydrolase (DDAH1) to dimethylamine and citrulline (Leiper J et al 2007, Pope AJ et al 2009). On the other hand, ADMA is produced by the degradation of methylated proteins under the action of enzymes called protein arginine methyltransferases type I (PRMT-I) (Vallance N et al., 2004, Ferlazzo N et al., 2021, Leiper J et al., 2007). The expression of PRMT-I in endothelial cells is regulated by the presence of native or oxidized LDL cholesterol, both of which are elevated in patients with MetS (Pope AJ et al., 2009),

It has been demonstrated that hypercholesterolemia can impair the function or regulation of DDAH1 and lead to intracellular accumulation of ADMA and increased oxidative stress (Bode-Böger, 1996). Increased gene expression of PRMT-I is another mechanism by which ADMA levels rise in hypercholesterolemia (Böger RH et al., 2000).

Risk factors such as hypercholesterolemia and hyperglycemia stimulate the production of free radicals in metabolic syndrome (Bekyarova, 2007). The results of the present study show an increase in plasma MDA levels, a key factor in lipid peroxidation and oxidative stress in individuals with MetS (Fig. 17). We also established a correlation between MDA and lipid markers, as well as between MDA and BMI in the metabolic group (Fig.18

In the present study, we found a significant positive correlation between ADMA, a marker of endothelial dysfunction, and plasma MDA, a marker of oxidative stress and oxidative damage (Fig.19)

It is known that oxidative stress reduces the activity of DDAH1, which degrades ADMA, and increases its synthesis by activating PRMT, leading to elevated plasma ADMA levels, inhibition of eNOS, and reduced NO bioavailability (Ito et al., 1999) Pope AJ et al., 2009; Böger RH et al., 2000; Jamwal S, Sharma S, 2018) (Fig.20)

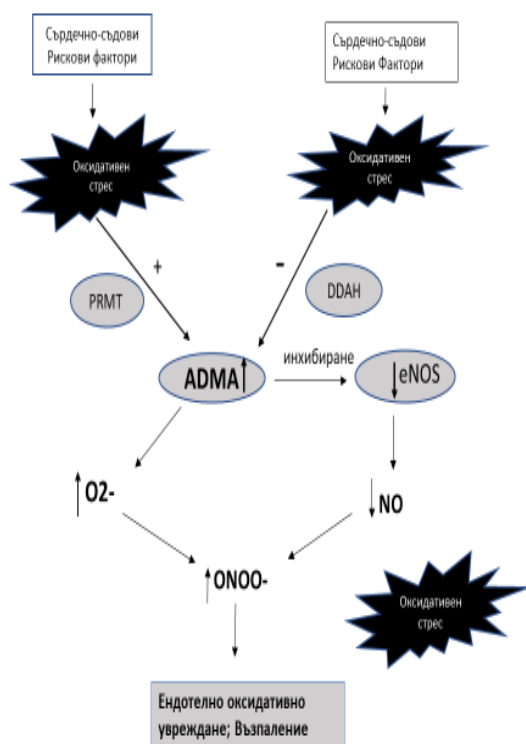


Figure 20 A diagram illustrating the mechanisms underlying the increased formation of asymmetric dimethylarginine (ADMA). Oxidative stress due to an increase in cardiovascular risk factors such as diabetes, hypercholesterolemia, hyperglycemia, and hypertension can stimulate ADMA production and/or inhibit ADMA degradation, leading to reduced inhibition of eNOS and decreased NO availability. Furthermore, NO bioavailability may be reduced by ADMA due to superoxide-induced NO degradation and the formation of peroxynitrite (Pope AJ et al., 2009)

Chronic administration of ADMA reduces the activity of superoxide dismutase (SOD), glutathione peroxidase, and catalase, and increases oxidative stress and endothelial dysfunction (Kwiecien S, 2012). This is accompanied by elevated circulating levels of MDA, a marker of lipid peroxidation and oxidative stress (Leiva E et al., 2003; Kwiecien S et al., 2012).

ADMA can directly inhibit eNOS, leading to the generation of less NO and more superoxide (O_2^-), an increase in lipid peroxidation, and direct damage to endothelial cells (Ito A et al., 1999). Furthermore, O_2^- can inactivate NO to form peroxynitrite ($ONOO^-$), which lacks the protective properties of NO but has a damaging effect on endothelial cells and exacerbates

endothelial dysfunction (Förstermann U & Li H, 2011). Furthermore, superoxide radicals induce the oxidation of LDL, which plays an important role in atherogenesis. (Förstermann & Li, 2011). A possible mechanism for reducing eNOS activity and NO bioavailability is also the inhibitory effect of free radicals and pro-inflammatory cytokines on the expression of this enzyme in the vascular endothelium (Pope AJ et al., 2009).

It is possible to speculate that hypercholesterolemia, elevated concentrations of ADMA and OX lead to a reduction in endothelial NO (Leiper J et al, 2007). The reduced bioavailability of NO due to decreased eNOS activity and/or NO degradation leads to impaired endothelium-dependent vasodilation, a tendency toward thrombus formation, and vascular wall remodeling (Chan JP et al, 2000).

Furthermore, OX can also, by stimulating an inflammatory response, increase the expression of pro-inflammatory cytokines and adhesion molecules and contribute to the progressive development of endothelial dysfunction in MS (Sydow K, Münzel T, 2003)

It has been established that hypercholesterolemia and elevated levels of ADMA and MDA lead to endothelial dysfunction, a key factor in the development of proatherogenic changes in endothelial cells and vascular events, including stroke and myocardial infarction (Vladimirova-Kitova LG et al., 2008; Petrova JJ et al., 2015)

Therefore, OS represents an important biochemical link between the risk factors stimulating ED and early proatherogenic changes in the endothelium. The relationship between ADMA and OS in the pathogenesis of endothelial dysfunction in MetS has been poorly studied.

Breaking this vicious cycle is crucial for limiting the development of endothelial dysfunction. Evidence suggests that the administration of antioxidants restores DDAH1 activity and reduces intracellular ADMA levels. Melatonin reduces plasma ADMA levels by preventing the oxidative stress-induced decrease in DDAH activity in a fructose-induced model of metabolic syndrome in rats (Tain, 2010). Resveratrol, known as a scavenger of OH/O₂ and peroxide radicals, is capable of inducing antioxidant defense mechanisms, limiting lipid peroxidation, and restoring the activity of DDAH1, an enzyme that degrades ADMA in endothelial cells under high glucose-mediated oxidative stress (Bonnefont-Rousselot, 2016). No data have yet been published on the effects of antioxidants on blood ADMA-MDA-NO parameters in humans (Maas, 2005).

This study showed that levels of ADMA, a key factor in endothelial dysfunction, and MDA, a marker of oxidative stress and lipid peroxidation, and proatherogenic lipids are elevated in individuals with metabolic syndrome. It is possible that factors other than hyperlipidemia may lead to elevated plasma levels of ADMA and MDA.

Based on the findings of the present study, we suggest a potential role for ADMA and MDA as predictive biomarkers for endothelial dysfunction and cardiometabolic risk.

ADMA and MDA may serve as useful diagnostic markers for detecting early changes in cardiovascular damage when traditional screening tests are still normal.

Our future research will focus on the cellular and molecular mechanisms underlying changes in ADMA and MDA, with an emphasis on the transcription factors Nrf2 and NO-1, which serve as markers of cellular antioxidant defense.

4. 2. RELATIONSHIP BETWEEN Nrf2, HO-1, NF-KB EXPRESSION, PLASMA ADMA LEVEL AND OXIDATIVE STRESS IN INDIVIDUAL WITH METABOLIC SYNDROME

2.1 Investigation of Nrf2, NF- κ B, and HO-1 expression in peripheral blood mononuclear cells (PBMCs) in metabolic syndrome

Our studies show that Nrf2 mRNA (over 130%), HO-1 mRNA expression (over 185%) and NF- κ B mRNA (over 53%), in peripheral blood mononuclear cells (PBMCs) in the metabolic syndrome group were significantly elevated compared to control subjects.

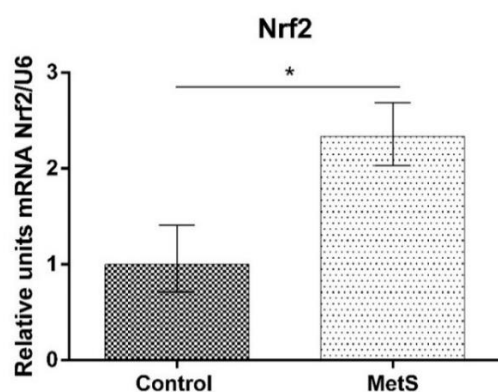


Figure 21 Nrf2 gene expression in PBMCs from individuals with metabolic syndrome (MetS) and controls (C). Data are presented as mean $\pm$ SEM; statistical significance is indicated as $p < 0.05$), * $p < 0.05$

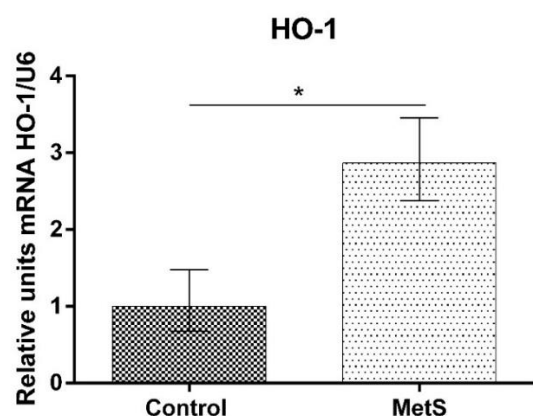


Figure 22 HO-1 gene expression in PBMCs in individuals with metabolic syndrome (MetS) and controls (C). Data are presented as mean $\pm$ SEM; statistical significance is indicated by $p < 0.05$), * $p < 0.05$

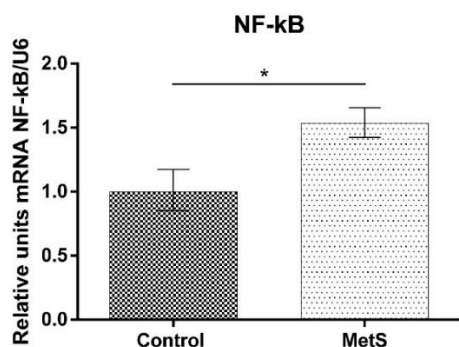


Figure23. *NF-κB* gene expression in PBMCs from individuals with metabolic syndrome (MetS) and controls (C). Data are presented as mean $\pm$ SEM; statistical significance was set at $p < 0.05$.

2.2 Изследване на промяната в плазмените нива на ADMA MDA при лица с метаболитен синдром

The plasma MDA level was significantly increased in subjects with metabolic syndrome group compared to controls by 78% (1.05 ± 0.13 to 1.87 ± 0.11)

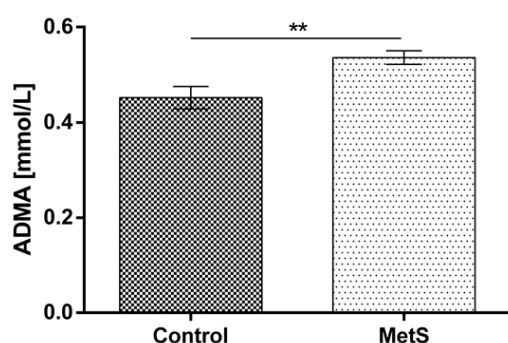


Figure 24 Plasma ADMA levels in subjects with metabolic syndrome (MetS) and controls (C). Data are presented as mean $\pm$ SD statistical significance was presented as $p < 0.05$, $**p < 0.01$

The plasma MDA level was significantly increased in subjects with metabolic syndrome group compared to controls by 78% (1.05 ± 0.13 to 1.87 ± 0.11).

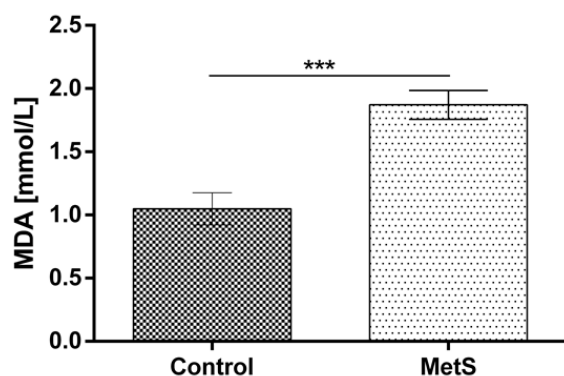


Figure 25 Plasma MDA levels in subjects with metabolic syndrome (MetS) and controls (C). Data are presented as mean $\pm$ SEM statistical significance was presented as $p < 0.05$, *** $p < 0.001$

2.3 Discussion

In the present study, we investigated the expression of NF- κ B, a transcription factor mediating the inflammatory response, and Nrf2, a critical regulator of protection against oxidative stress, as well as HO-1, an endothelial protector and antioxidant, and the relationship between them and plasma ADMA in individuals with MetS.

Our data show that plasma ADMA levels, as a marker of endothelial dysfunction, and MDA, as a marker of oxidative stress and lipid peroxidation, are elevated in MetS (Figs. 24 25). Some studies report a significant association between elevated plasma ADMA levels, hypercholesterolemia, and endothelial dysfunction in individuals with metabolic syndrome (Palomo I. et al., 2011; Dayal S, Lentz SR, 2005; Ferlazzo N et al, 2021). It has been established that hypercholesterolemia and elevated plasma levels of ADMA and MDA cause endothelial dysfunction, a key factor in the development of proatherogenic changes in endothelial cells and vascular events such as stroke and myocardial infarction. Hypercholesterolemia may disrupt the function or regulation of dimethylamine hydrolase, thereby leading to intracellular accumulation of ADMA and increased oxidative stress (Ito A, et al., 1999).

In our study, we found a significant positive correlation between plasma MDA, a marker of oxidative stress, and plasma ADMA, a marker of endothelial dysfunction. It is known that ADMA is metabolized by dimethylarginine dimethylamine hydrolase (DDAH1) to dimethylamine and citrulline (Leiper J et al. 2007; Pope AJ et al. 2009). Oxidative stress, TNF- α , homocysteine, and nitrosative stress have been shown to reduce DDAH activity.

Elevated plasma concentrations of ADMA have been found to increase superoxide (O_2^-) production by NADPH oxidase, thereby increasing oxidative stress and reducing NO bioavailability (Sydow K, Munzel, 2003). ROS and lipid peroxidation products are a decisive factor in the activation of the transcription factors Nrf2 and NF- κ B (Kensler TW et al. 2007; Chen ZH, Niki E 2006).

The expression of the nuclear factor Nrf2 is increased in PBMCs (Fig. 21). Nrf2 is located in the cytoplasm, and its translocation to the nucleus is regulated by various stimuli that have the potential to disrupt the redox balance (Chen B et al., 2015; Zhang M et al, 2013; Zhou et al., 2014). Consequently, in a stressful environment, such as oxidative/inflammatory insults, Nrf2 moves to the nucleus and binds to its ARE sites to induce the transcription of enzymes to counteract the effect of increased ROS generation.

It has been established that Nrf2 can activate DDAH-1, which metabolizes ADMA, an endogenous inhibitor of the nitric oxide synthase eNOS (Pope AJ et al., 2009). Increased DDAH1 leads to lower plasma ADMA levels, which would improve endothelial function through adequate NO production (Nair N, Gongora E, 2017). Therefore, Nrf2 may improve endothelial NO production and endothelial function by regulating the DDAH/ADMA/eNOS pathway (Fig. 3.2)

HO-1 is an Nrf2-dependent gene that exerts beneficial effects by protecting against oxidative damage (Kim YM et al., 2011). HO-1 plays an important role in reducing the production of reactive oxygen species (ROS) through its ability to break down heme and produce carbon monoxide, biliverdin/bilirubin, and release free iron, with the latter being stored as ferritin (Abraham N, Kappas A, 2005; Farombi EO & Surh YJ, 2006; Peterson SJ et al, 2019). It has been demonstrated in clinical studies that HO-1 deficiency in humans causes oxidative stress and damage to endothelial cells (Yachie A et al, 1999).

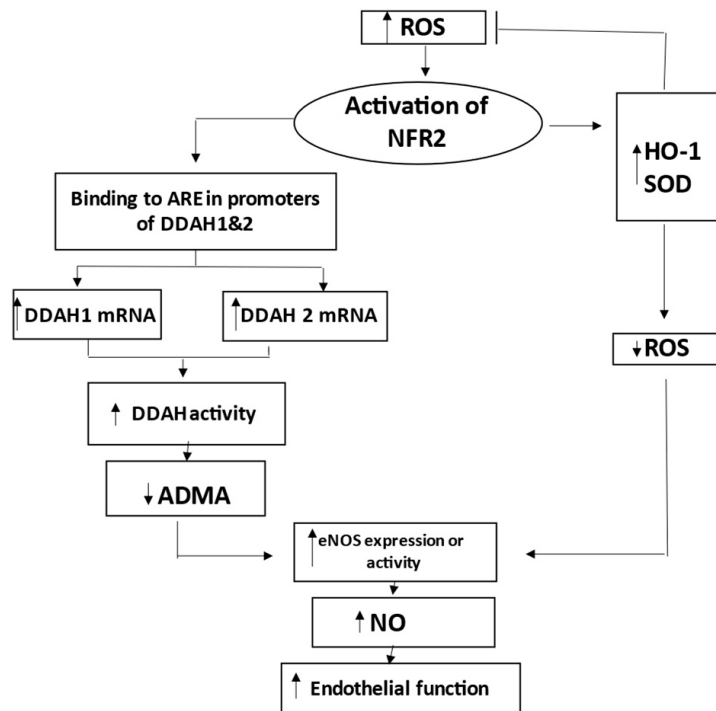


Figure 26 Schematic representation of the role of nuclear factor erythropoietic 2-related factor 2 (Nrf2) in preserving endothelial function during activation by reactive oxygen species (ROS). Nrf2 increases the expression of HO-1 and other genes to reduce ROS. Nrf2 also increases DDAH-1 and DDAH-2, leading to the degradation and reduction of ADMA, which is an inhibitor of eNOS. Reduced ADMA levels lead to improved NO production and associated endothelial function. Thus, Nrf2 coordinates HO-1/DDAH/eNOS pathways that enhance endothelial NO generation and endothelial function. ADMA stands for asymmetric dimethylarginine; ARE, antioxidant response element; DDAH, dimethylarginine dimethylamino hydrolase; eNOS, endothelial nitric oxide synthase; HO-1, heme oxygenase-1; and SOD, superoxide dismutase (Bekyarova G et al 2023).

Conversely, increased production of the HO-1 protein represents an attempt to minimize cellular damage (Schipper HM 2004). The enzyme heme oxygenase-1 is the first line of defense for cells against oxidative stress. It has been demonstrated that HO-1 participates in the regulation of many physiological as well as pathophysiological processes. HO-1 is expressed at low levels in endothelial cells and leukocytes under physiological conditions, but it is highly inducible in response to various pathophysiological stresses/stimuli. (Kim YM et al., 2011) Activation of the cytoprotective Keap1-Nrf2 signaling pathway regulates a large number of genes encoding enzymes that enhance the antioxidant capacity of cells, such as heme oxygenase (HO-1), superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, and others

(Osburn WO et al., 2006). In this way, the induction of antioxidant defense factors increases resistance to subsequent, more severe ROS attacks on cells (Jaiswal AK, 2004).

The present study demonstrated increased expression of HO-1 in PBMCs (Fig. 22). The activation of HO-1, also known as HSP32, is considered an adaptive and protective response against oxidative stress induced by various stressors such as oxidized lipoproteins, cytokines, hemodynamic changes, angiotensin II, and nitric oxide (NO) in the vascular wall. (Abraham NG, Kappas A, 2008. Naito Y et al 2014).

The protective mechanism of HO-1 is associated with the generation of heme metabolites—CO, bilirubin, and ferritin (Paine A et al, 2010; Wunder C & Potter RF, 2003, Kawashima S, Yokoyama M. 2004) It has been established that biliverdin and CO mediate most of the anti-inflammatory, antioxidant, and anti-apoptotic effects of HO-1.

Bilirubin scavenges peroxy radicals, which are among the most potent stimulators of lipid peroxidation (Yesilkaya AR et al., 2000). In addition to its antioxidant properties, CO also has anti-inflammatory effects and enhances the cytoprotective effects of bilirubin. Like NO, CO has a vasodilatory effect (Durante W, 2002). In addition to its antioxidant properties, CO also has anti-inflammatory effects (Otterbein L E, Choi AMK, 2000) and anti-proliferative effects (Song R et al., 2003). The anti-apoptotic action of HO-1 is mediated by CO (Brouard S et al, 2000). These properties of HO-1 suggest an anti-atherogenic role for this enzyme (Ishikawa K, 2003).

The present study demonstrated increased expression of HO-1 in PBMCs (Fig.22). The enzyme HO-1, as an Nrf2-dependent gene, exerts beneficial effects through endothelial protection against oxidative damage (Yachie A et al., 1999). Elevated plasma levels of HO-1 have been reported in diabetes (Bao W, 2010) and in other chronic diseases (Sato T et al, 2006; Mateo I et al, 2010; Miyazaki et al, 2010). Induction of the HO-1 enzyme has a cytoprotective effect in diabetes (Abraham VG, Kappas A, 2008), hypertension, ischemia/reperfusion (Yet SF et al., 2001), and atherosclerosis (Juan SH et al., 2001). Conversely, inhibition of HO-1, for example, in diabetic mice, enhances oxidative stress, causes damage to endothelial cells, and accelerates the formation of atherosclerotic lesions, which demonstrates the protective role of HO-1 against the development of atherosclerosis (Kishimoto Y et al., 2019)

We hypothesize that HO-1 induction is an adaptive response against oxidative stress-mediated ADMA accumulation, NO loss, and the development of ED in individuals with MetS. Hem oxygenase-1 plays an important role in the adaptive and protective response of cells to increased

free radical generation and is a marker of oxidative stress (Bauer M & Bauer I, 2002; Valko M et al., 2007). Through its antioxidant activity, HO-1 can counteract the harmful effects of ROS in obesity, hypertension, diabetes, metabolic syndrome, and CVD (Abraham VG, Kappas A, 2008)

Activation of HO-1 inhibits ROS production, supports endothelium-dependent relaxation and NO production, and improves insulin sensitivity while preventing vascular complications in an experimental model of MetS (El-Bassossy HM et al., 2014).

It has been reported that HO-1 induction inhibits ROS production in the aorta of animals with fructose-induced MetS (El-Bassossy, HM et al, 2014). It is hypothesized that HO-1 induction is compensatory in nature, and elevated levels support the view that it serves as a marker of oxidative stress in the vascular wall.

Patients with higher HO-1 levels have higher levels of atherogenic lipids, glucose, and other risk factors compared to other patients. It has been established that HO-1 induction reduces lipid peroxidation in plasma and the arterial wall (Ishikawa K, 2003). Conversely, oxidative stress in the absence of HO-1 causes increased damage to endothelial cells (Yachie A et al., 2021).

Elevated levels of HO-1 in circulating plasma have been observed in individuals with impaired glucose tolerance and type 2 diabetes (Bao et al. 2010). It is believed that increased HO-1 expression is an adaptive and compensatory response to protect against oxidative stress caused by high glucose levels in diabetes mellitus (Bao W et al., 2010). Morsi WG et al. (2006) report increased expression and activity of HO-1 in endothelial cells in atherosclerosis. HO-1 expression is a factor that promotes the stabilization of atherosclerotic plaques

In the present study, we found that plasma levels of ADMA (an endogenous eNOS inhibitor) and MDA are elevated in humans, and that a functional relationship exists between them.

HO-1 induction may restore reduced eNOS activity and compensate for the loss of NO bioavailability not only by inhibiting the generation of NADPH oxidase activity and superoxide radicals, but also by increasing SOD and catalase activity and the antioxidant capacity of ECs in MetS (Pae, HO et al 2010; El-Bassossy, HM et al, 2014). CO, possessing antioxidant properties, may prevent the decoupling of eNOS.

According to Pae HO et al. (2010), there are three possible mechanisms for the preservation of vascular NO via HO-1 and heme degradation products: (1) modulation of eNOS expression and

activity, (2) prevention of vascular NO inactivation, and (3) compensation for the loss of vascular NO.

Moderate activation of the HO-1 enzyme demonstrates antioxidant, anti-inflammatory, anti-apoptotic, anti-proliferative, and anti-atherogenic properties (Abraham NG, Kappas, A 2008). Despite the immense interest in this enzyme, the role of HO-1 in the pathogenesis of MetS remains unclear, which necessitates further studies on the subject.

It has been demonstrated in clinical studies that a deficiency of HO-1 in humans causes oxidative stress and damage to endothelial cells (Yachie A et al., 1999). Conversely, increased production of the HO-1 protein represents an attempt to minimize cellular damage (Schipper HM 2004).

Activation of the Nrf2 transcription factor, by increasing HO-1 expression, can inhibit oxidative stress and increase eNOS activity and NO production (Pae HO et al, 2010). HO-1, by modulating Nrf2 expression, induces adaptive reprogramming and enhances antioxidant defense (Biswas C et al., 2014). It has been reported that increased expression of HO-1 and heme degradation products may improve vascular function, at least in part, by compensating for the loss of NO (Pae HO et al., 2010).

According to a number of authors, the study of HO-1 provides an opportunity to assess the body's adaptive mechanisms under conditions of oxidative stress and the potential for therapeutic intervention. Investigating the relationship between ADMA, HO-1, and oxidative stress would help clarify the complex pathophysiological mechanisms of endothelial dysfunction in MetS, which is important for the prevention, early diagnosis, and treatment strategy of MetS.

By increasing the expression of HO-1 and other antioxidant genes, Nrf2 can reduce ROS, DDAH-1, and DDAH-2, as well as ADMA in plasma, and increase eNOS activity and NO bioavailability (Pope AJ et al, 2009). Therefore, by coordinating the HO-1/DDAH/eNOS pathways, Nrf2 can improve endothelial NO production and endothelial function.

Data from a previous study indicate that ADMA, depending on its concentration, can increase the production of ROS, which act as secondary mediators and activate NF- κ B in endothelial cells (Abraham NG, Kappas A, 2008). Data from the present study show increased NF- κ B expression in PBMCs (Fig. 3.4), which corresponds to higher plasma levels of MDA and ADMA in MetS. ADMA can activate NF- κ B, cytokine expression, soluble ICAM-1 and

VCAM-1 expression, and monocyte adhesion, and may mediate their pro-inflammatory actions in the endothelium (Mangoni AA et al, 2021). It has been demonstrated that reduced eNOS/NO activity upon ADMA accumulation activates the transcription factor NF- κ B, which is responsible for the expression of endothelin-1 and other pro-inflammatory mediators, including TNF α , IL-6, free radicals, chemokines, and adhesion molecules (ICAM-1, ELAM-1), as well as iNOS, contributing to endothelial dysfunction.

In conclusion, the results of the present study demonstrate the importance of the transcription factor Nrf2, which plays a key role in endothelial protection, and of NF- κ B, a transcription factor mediating the inflammatory response in the pathogenesis of endothelial dysfunction in MetS. Investigating the relationship between ADMA, HO-1, and the transcription factors Nrf2 and NF- κ B would contribute to elucidating the complex pathophysiological mechanisms of endothelial damage in MetS, which is important for the prevention, early diagnosis, and treatment strategy of CVD.

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4. 3. CHANGE IN INTIMA-MEDIA THICKNESS (CMT) ASSOCIATED WITH REDOX BALANCE AND AGE IN INDIVIDUALS WITH METABOLIC SYNDROME

3.1 Comparative analysis of CMT values in individuals with metabolic syndrome and the control group

CMT values were significantly higher in the metabolic syndrome group compared to the control group by 20% (0.703 ± 0.021 to 0.922 ± 0.061) (Fig. 27)

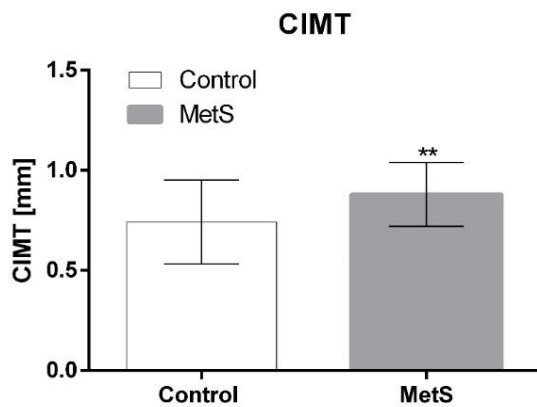


Figure 27 CMT in individuals with metabolic syndrome (MetS) and the control group (C). Data are presented as mean $\pm$ SEM (statistical significance is indicated at $p < 0.05$).

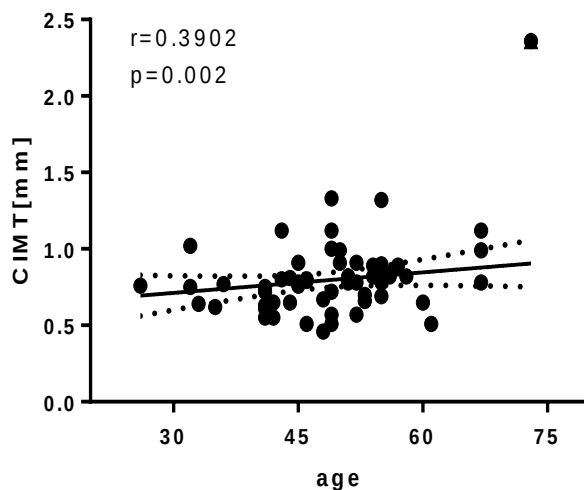
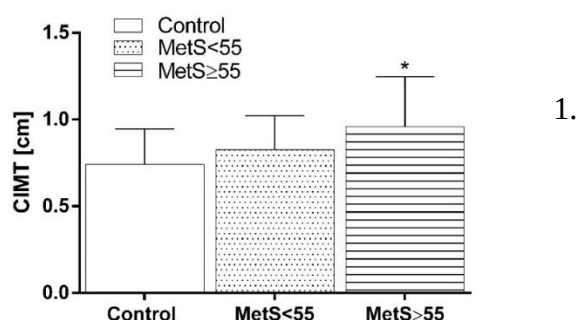


Figure 28 Correlation between CMT and age in individuals with metabolic syndrome (MetS) and controls (C). Data are presented as mean $\pm$ SEM (statistical significance is indicated at $p < 0.05$).

3.2 Comparative analysis of CIMT values according to age in individuals with MetS

CIMT values were non-significantly elevated in the MetS<55 group compared to the control group by 7% (0.703 ± 0.021 to 0.755 ± 0.051) and in the MetS $\geq$ 55 group by 31% (0.703 ± 0.021 to 0.922 ± 0.061) (Fig. 29)



Фигура 29 Changes in CIMT according to age in individuals with MetS. Data are presented as mean $\pm$ SEM. Control group: CIMT < 0.9 mm, age < 55. The MetS group was divided into two subgroups: MetS with age < 55 and MetS with age $\geq$ 55.

3.3 Expression of NF- κ B, Nrf2, and HO-1 in PBMCs by age

As shown in Figure 30, significantly increased expression of the three genes under study was observed in the MetS group, age <55. Here, the magnitude of the increased mRNA levels is as follows: 83% for NF- κ B ($p < 0.05$); 251% for Nrf2 ($p < 0.05$); and 337% for HO-1 ($p < 0.05$).

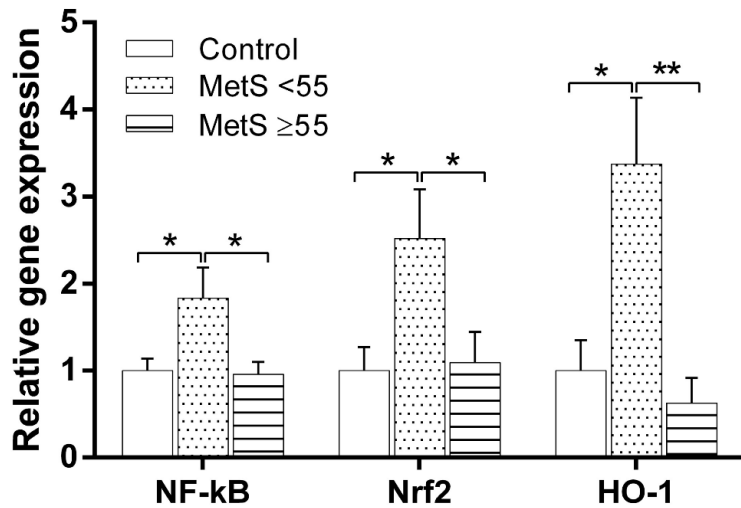


Figure 30 Expression of NF- κ B, NRF2, and HO-1 in PBMCs, according to age in MetS. Control group: age < 55. The MetS group is divided into two subgroups: MetS with age < 55 and MetS with age $\geq$ 55. * $p < 0.05$, ** $p < 0.01$,

3.4. NF- κ B, Nrf2, and HO-1 Expression in PBMC Based on CIMT

The NF- κ B, NRF2, and HO-1 expression, depending on CIMT, followed the same pattern as the dependence on age. The MetS group was subdivided according to the values of their CIMT: MetS with CIMT < 0.90 mm and MetS with CIMT $\geq$ 0.90 mm. A CIMT of 0.90 mm was considered a cut-off value

A significantly increased expression of the three studied genes in the MetS group with CIMT < 0.90 mm was detected as follows: 80% for NF- κ B ($p < 0.01$); 260% for Nrf2 ($p < 0.05$), and 303% for HO-1 ($p < 0.05$). Again, a trend was observed with NF- κ B, NRF2, and HO-1 expression levels in PBMC changing with age and HO-1 being the most affected among the three studied genes. Similar to the results about the dependence of NF- κ B, NRF2, and HO-1 expression on age, here, data show that in the MetS subgroup with individuals with CIMT $\geq$ 0.90 mm, these genes were under-regulated, achieving the levels of the control group or even lower, with a statistically significant difference between the MetS subgroups with different CIMT being established

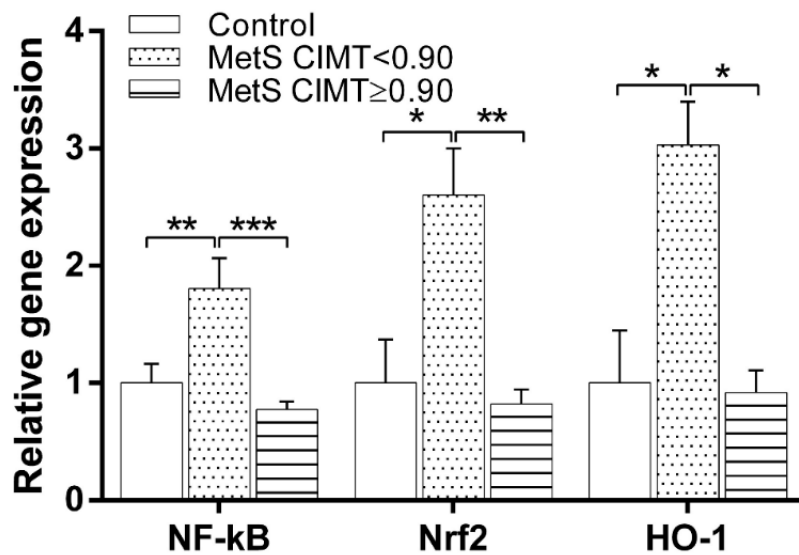


Figure 31. Relative expression levels of *NF-kB*, *Nrf2*, and *HO-1* in PBMC, depending on CIMT. Control group: IMT < 0.90 mm. The MetS group was divided into two subgroups: MetS with CIMT < 0.90 mm and MetS with CIMT ≥ 0.90 mm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5 Plasma levels of MDA, ADMA, and HO-1 by age

Plasma levels of MDA, ADMA, and the HO-1 enzyme change with age. The data show significantly higher plasma levels of MDA and ADMA in both the MetS <55 and MetS ≥55 subgroups. HO-1 is higher only in the MetS <55 subgroup. The difference between the MetS subgroups is not significant (Fig. 32)

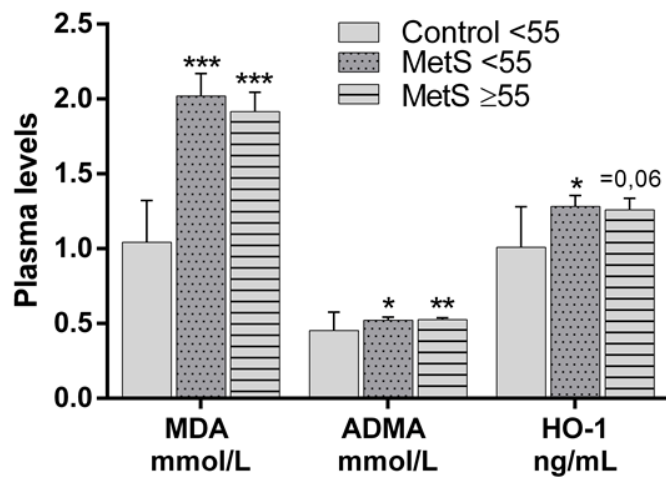


Figure 32 Plasma levels of MDA, ADMA, and HO-1 in the study subjects, by age. The control group consisted of individuals aged <55, while the MetS group was divided into two subgroups: MetS aged <55 and MetS aged ≥55.

3.6. MDA, ADMA, and HO-1 Plasma Levels Based on CIMT

Data about plasma levels of MDA, ADMA, and HO-1 based on CIMT (mm) are shown in Figure 33. There were significantly higher plasma levels of the studied parameters in both MetS subgroups, based on CIMT, as compared to the control group. A difference between the two MetS subgroups based on CIMT was not established.

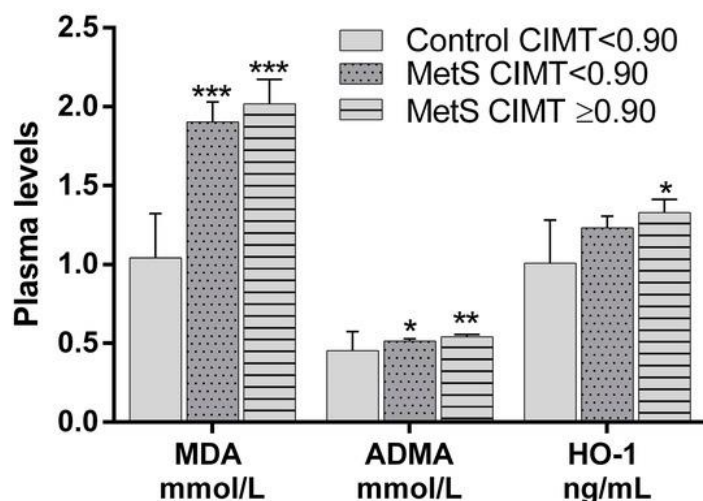


Figure 33 Plasma levels of MDA, ADMA, and HO-1 in study subjects based on CIMT.

Control group: CIMT < 0.90 mm. The MetS group was divided into two subgroups: MetS with CIMT < 0.90 mm and MetS with CIMT ≥ 0.90 mm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.7 Discussion.

Metabolic syndrome is defined by the WHO as a pathological condition characterized by abdominal obesity, insulin resistance, hypertension, and hyperlipidemia (Saklayen MG, 2018). The components of MetS and the associated cardiovascular risk can impair vascular endothelial function and the structure of the vascular wall and may lead to atherosclerosis and vascular diseases (Mathieu P et al, 2006).

CIMT is a widely used marker of endothelial dysfunction and increased risk of CVD and atherosclerosis (Kawada T et al., 2016, Jung JM et al., 2014; Scuteri A et al., 2004). CIMT is used as a structural marker of subclinical atherosclerosis and is associated with ED and age (Pezzano A et al., 2006). CIMT is a marker for assessing the subclinical manifestations of cardiovascular and metabolic diseases and is a strong predictor of future cerebrovascular and cardiovascular events (Aydin M et al., 2013).

The data from our study show an increase in CIMT in individuals with MetS (Fig. 16). Other authors have also reported an increase in CIMT in cases of diabetes mellitus, hypertension, obesity, and components of MetS (Rubio-Guerra AF et al., 2010; Ungvari Z et al., 2011; Dawson JD et al., 2009). An increase in CIMT, as measured by ultrasound, is directly associated

with an increased risk of myocardial infarction and stroke even in older adults without a history of cardiovascular disease (Robertson CM et al., 2012).

We found a positive correlation between CIMT and age (Fig. 27). Our data show a gradual increase in CIMT with age. Fig. 28 However, a statistically significant higher mean CIMT was found only in the MetS >55 subgroup compared to the control group (0.96 ± 0.29 vs. 0.74 ± 0.21 , $p < 0.05$).

There is evidence that the presence and severity of MetS associated with CIMT in various populations is a phenomenon conceptualized as early vascular aging (Koç AS et al., 2019). The mechanisms underlying these changes are not fully understood, but it is believed that oxidative stress and inflammation play a very important role. It has been demonstrated that the consequences of oxidative stress and low-grade inflammation, which cause ED and endothelial damage, become more pronounced with advancing age (Jia G et al., 2019). There is strong evidence suggesting that Nrf2 protects against the induction of cellular aging. Vascular oxidative stress during aging is associated with Nrf-2 dysfunction (Kloska D et al., 2019).

Nrf2, a redox-sensitive transcription factor, upregulates the expression of heme oxygenase 1 (HO-1) and other genes (Ungvari Z et al., 2011) and plays an important role in endothelial protection against oxidative stress and in regulating cellular redox balance (Chen B et al., 2015). Reactive oxygen species (ROS), products of lipid peroxidation, and low-grade inflammation are key factors in the activation of the transcription factors Nrf2 and NF- κ B (Motohashi, H, Yamamoto, M. 2004). The relationship between CIMT and biomarkers of endothelial function associated with oxidative stress and inflammation in individuals with MetS remains unclear. We found no data in the literature regarding changes in CIMT and age-related and redox balance in MetS.

We studied how expression levels of NF- κ B, Nrf2, and HO-1 in PBMCs of MetS individuals varied according to age and CIMT. The control group was designed to contain healthy individuals aged < 55 years. The MetS group included individuals both under and over 55 years of age. As shown in Figure 30, a significantly increased expression of the three studied genes in the age < 55 MetS subgroup was detected. The increase in the mRNA levels in the age < 50 MetS subgroup was as follows: 83% for NF- κ B ($p < 0.05$), 251% for Nrf2 ($p < 0.05$), and 337% for HO-1 ($p < 0.05$). It is obvious that the expression level of these genes in PBMC changes with age and that HO-1 appears to be the most affected among the studied genes. In contrast, in the MetS subgroup with individuals aged over 55 years, it is visible that these genes

are under-regulated, achieving the levels of the control group. Thus, the difference between the age < 55 MetS and age $\geq$ 55 MetS subgroups was determined to be statistically significant (Figure 30).

As shown in Figure 31, a significantly increased expression of the three studied genes in the MetS group with IMT < 0.9 mm was detected. The increase in the mRNA levels in the MetS with CIMT < 0.90 mm subgroup was as follows: 80% for NF- κ B ($p < 0.01$), 260% for Nrf2 ($p < 0.05$), and 303% for HO-1 ($p < 0.05$). Again, a trend was detected with NF- κ B, Nrf2, and HO-1 expression levels in PBMC changing with age and HO-1 being the most affected among the studied genes. Similar to the results about the dependence of NF- κ B, Nrf2, and HO-1 expressions on age, here, data show that in the MetS subgroup with individuals with IMT $\geq$ 0.90 mm, these genes were under-regulated, achieving the levels of the control group or even lower, leading to the establishment of statistical significance between the MetS subgroups with different CIMT (Figure 31)

We can speculate that protective mechanisms involving Nrf2 and HO-1 decline with age (Figure 30). This may be a cause of impaired endothelial function and accelerated endothelial damage to blood vessels, as evidenced by increased CIMT and an increased risk of cardiovascular complications and events.

Our results show that the expression of these genes in PBMCs changes with age, and HO-1 appears to be the most affected of the genes studied (Fig. 30). Nrf2, a redox-sensitive transcription factor, increases the expression of heme oxygenase 1 (HO-1) and other genes (Ungvari Z et al, 2011) and plays an important role in endothelial protection against oxidative stress and regulates cellular redox balance. HO-1 is expressed at low levels in endothelial cells and white blood cells under physiological conditions; it is highly inducible in response to stress (Abraham, NG, Kappas A 2005). HO-1 has been described as a key regulator of adaptive cellular responses to oxidative stress and low-grade inflammation and plays a role in cellular homeostasis through the potent antioxidant activity of bilirubin and the anti-inflammatory effect of carbon monoxide (Ferrándiz ML, Devesa I. 2008). The increased expression of Nrf2 and HO-1 in individuals with MetS represents an attempt to minimize endothelial damage (Abraham NG, Kappas A 2008).

Our research data show that the expression of Nrf2, HO-1, and NF- κ B is most significant in the metabolic group of individuals under 55 years of age (Fig.30). In contrast, in the MetS subgroup of individuals over 55 years of age, the expression of these genes is lower, reaching the levels

of the control group, and thus the difference between MetS <55 and MetS ≥55 is statistically significant)

The reduced expression of Nrf2 in individuals with MetS ≥55 may increase vulnerability to risk factors and accelerate endothelial damage associated with metabolic disorders and other pathophysiological conditions in aging. We hypothesize that these changes in the expression of Nrf2 and HO-1 also account for the higher CIMT values in MetS ≥ 55.

In this study, we investigated the relationship between plasma levels of ADMA, an endogenous inhibitor of nitric oxide synthase, is a marker of endothelial dysfunction, and MDA and HO-1 as markers of lipid peroxidation and oxidative stress, and CIMT as a marker of endothelial damage, an independent predictor of cardiovascular risk in individuals with MetS.

We found that redox balance parameters, e.g., MDA, ADMA, and plasma HO-1 levels, vary by age (Fig. 32). The data show significantly higher plasma levels of MDA and ADMA in both the MetS <55 and MetS ≥ 55 subgroups. HO-1 has higher plasma levels only in the MetS <55 subgroup. The difference between the MetS subgroups is not significant, indicating that these parameters are not age-related (Fig 33).

It has been demonstrated that elevated levels of MDA, as a marker of oxidative stress and lipid peroxidation, and ADMA, as an endogenous competitive inhibitor of nitric oxide synthase, can lead to reduced eNOS activity and NO levels, which contributes to ED in diabetes mellitus (Said, M.A. 2022).

ADMA can activate NF-κB, which is responsible for the expression of endothelin-1 and other pro-inflammatory mediators, such as TNF-alpha, interleukin-6 (IL-6), free radicals, chemokines, and adhesion molecules, as well as inducible NO synthase (iNOS), contributing to endothelial damage (Mangoni AA et al, 2021). We found that increased NF-κB expression correlates with elevated plasma levels of ADMA and MDA. Furthermore, it was found that elevated plasma concentrations of ADMA cause an increase in superoxide (O₂⁻) production. Superoxide can combine with NO to form peroxynitrite, a radical (ONOO⁻) with potentially harmful effects on the vascular wall, which further contributes to the exacerbation of endothelial damage and an increase in IMT (Cooke JP, 2005; Zsuga J et al., 2007)

Activation of the Nrf2/HO-1 signaling pathway plays a critical role in the antioxidant protection of endothelial cells in response to various inflammatory and oxidative stimuli [Peterson SJ,

2019]. Elevated plasma levels of HO-1 can inhibit oxidative stress and low-grade inflammation, thereby improving endothelial function by increasing NO levels (Pae HO, 2019).

Our data showing elevated plasma levels of ADMA, MDA, markers of oxidative stress, and CIMT in individuals with MetS are consistent with other studies (Scuteri, A et al., 2004; Jung, JM et al., 2014; Kawada, T et al., 2016; Nair N. Gongora E, 2017). Our data show that plasma HO-1 and CIMT are elevated in individuals with MetS, which is consistent with the findings of other authors (Idriss NK et al., 2010). According to Pae et al (2010), HO-1 activation may improve endothelial function, at least in part, by compensating for the loss of NO bioavailability at elevated plasma ADMA levels.

Redox imbalance with advancing age is likely responsible for the earlier onset of morphological changes in the endothelium, as evidenced by increased CIMT, and for the risk of late clinical complications and events (Kishimoto, Y et al., 2019).

Nrf2 reduces oxidative stress by regulating the expression of NO-1 and other antioxidant genes, while ADMA, MDA, and NF- κ B increase oxidative stress and low-grade inflammation, contributing to ED by inhibiting the eNOS pathway) (Poredos P et al., 2004).

Endothelial dysfunction caused by atherosclerosis risk factors occurs well before measurable changes in the vascular wall, such as CIMT (Aydin M et al., 2013). Since age-dependent CIMT and redox balance are interrelated and indicative of different aspects of the atherosclerotic process, their early assessment may be important for cardiovascular prevention.

Based on current and previous findings, we propose measuring ADMA levels in conjunction with markers of oxidative stress (MDA, HO-1, and Nrf2), may be a promising step in developing an effective model for monitoring the severity of CIMT and early vascular damage in individuals with MetS. CIMT is an important tool for early screening to assess subclinical manifestations of cardiovascular and metabolic diseases and a strong predictor of future cerebral and cardiovascular complications (Aydin M et al, 2013).

In conclusion, our study investigates the relationship between biochemical markers of endothelial dysfunction and markers reflecting morphological changes in blood vessels, taking into account the role of the balance between endothelial damage factors and endothelial protection factors. Individuals with unbalanced ROS production and/or impaired antioxidant defense systems and age-related redox imbalance are at high risk for increased CIMT and cardiovascular risk.

5. CONCLUSIONS

1. In individuals with metabolic syndrome, levels of ADMA a key factor in endothelial dysfunction and MDA, marker of oxidative stress—are significantly elevated in plasma, and a positive correlation exists between them.
2. Compared to healthy controls, individuals with MetS have significantly elevated levels of proatherogenic lipids (TG, TG/HDL, LDL/HDL), plasma glucose, BMI, and WC.
3. ADMA and MDA may be useful diagnostic markers for detecting early changes in cardiovascular damage when traditional screening tests are still normal.
4. Elevated expression of the transcription factors Nrf2 and NF-kB has been observed in peripheral blood mononuclear cells (PBMCs): Nrf2 plays a key role in endothelial protection against oxidative stress, and NF-kB regulates the inflammatory response in individuals with MetS.
5. Nrf2, a redox-sensitive transcription factor, increases HO-1 expression, regulates cellular redox balance, and plays an important role in endothelial protection against oxidative stress.
6. Compared to healthy controls, individuals with metabolic syndrome demonstrate significantly higher CIMT values with advancing age.
7. The relationship between elevated CIMT values and age-related changes in Nrf2 and HO-1 antioxidant defense and redox imbalance (ADMA and MDA) in individuals with metabolic syndrome was analyzed.
8. Individuals with reduced antioxidant defense against oxidative stress and/or age-related redox imbalance are at high risk for increased CIMT and cardiovascular risk.

6. CONTRIBUTIONS

Original Contributions

1. For the first time in Bulgaria, the relationship between ADMA and HO-1, an antioxidant and marker of oxidative stress, has been investigated in individuals with MetS. The study of HO-1 allows for the assessment of the body's adaptive mechanisms under conditions of oxidative stress and the potential for therapeutic intervention.
2. For the first time in Bulgaria, the relationship between ADMA, HO-1, and the transcription factors Nrf2 and NF- κ B is being investigated, which would help clarify the complex mechanisms of endothelial damage and has practical significance for the prevention, early diagnosis, and treatment strategy for individuals with MetS.
3. Links have been identified between indicators such as ADMA and markers of oxidative stress (MDA, HO-1, and Nrf2) as well as the lipid profile (total cholesterol, triglycerides, HDL), BMI, and glucose, which has practical implications for monitoring and preventing cardiovascular complications in individuals
4. The importance of a comprehensive assessment, including ADMA as a marker of endothelial dysfunction, along with markers of oxidative stress (MDA, HO-1, and Nrf2) and CIMT as a marker of subclinical atherosclerosis and cardiovascular risk, is emphasized
5. Comprehensive testing of the ADMA marker of endothelial activation and markers of oxidative stress (MDA, HO-1, and Nrf2) may be a promising step toward developing an effective model for monitoring CIMT and the early detection of vascular damage in individuals with MetS.

Applied Contributions

1. The study of the transcription factors Nrf2 and NF- κ B provides a more reliable and comprehensive understanding of the mechanisms of endothelial damage compared to studies focusing solely on individual markers of oxidative stress and inflammation.

2. This study points the way toward future therapeutic approaches aimed primarily at the transcription factors Nrf2 and NF- κ B, which regulate the expression of genes encoding vascular damage and protective factors in individuals with MetS
3. The set of methods presented in the study can be effectively applied as an innovative approach in clinical practice for diagnosing endothelial damage in individuals with MetS.

7. PUBLICATIONS RELATED TO THE DISSERTATION

1. Bekyarova GY, **Bekyarov NA**, Madjova VH, Madjova CR, Kalevska ED. Age-Associated Changes in Carotid Intima–Media Thickness in Relation to Redox Balance Indices in Metabolic Syndrome *Appl.Sci* 2024 14 (16), 7218 doi.org/10.3390/app14167218, IF= 2.5
2. Bekyarova GY, Vankova DG, Madjova VH, **Bekyarov NA**, Salim AS, Ivanova DG, Stoeva SM, Gerova DI, Kiselova-Kaneva YD. Association between Nfr2, HO-1, NF-kB Expression, Plasma ADMA, and Oxidative Stress in Metabolic Syndrome. *Int J Mol Sci.* 2023 Dec 2;24(23):17067. doi: 10.3390/ijms 24231706, IF= 4.9
3. Bekyarova G, Kiselova-Kaneva Y, Madjova V, Gerova D, **Bekyarov N**, Todorova M, Stoeva S. The predictable role of ADMA and oxidative stress in endothelial dysfunction - a pilot study. *Journal of IMAB, Annual Proceeding (Scientific Papers) Suppl 12; 2022; vol. 28, 37-40.*

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