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Increased levels of anti-heat-shock protein 60 (anti-Hsp60) indicate endothelial dysfunction, atherosclerosis and cardiovascular diseases in patients with mixed connective tissue disease

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Abstract Heat-shock protein 60 (Hsp60) has been shown to provoke inflammation, and anti-Hsp60 may facilitate the development of atherosclerosis. In this study, we have investigated 30 patients with mixed connective tissue disease (MCTD) and assessed anti-Hsp60 and their relationship to cardiovascular diseases (CVD). Out of 30 patients with MCTD, 15 had CVDs. Anti-Hsp60 antibody was determined by enzyme-linked immunosorbent assay. Since endothelial dysfunction and accelerated atherosclerosis are characteristic to MCTD, a wide array of MCTD-, endothelial dysfunction- and CVD-associated parameters was investigated: serum lipid levels, paraoxonase activity (PON1), rich nuclear ribonucleoprotein U1 (anti-U1RNP), anti-endothelial cell antibodies, anti-cardiolipin and anti-β2-glycoprotein I antibody isotypes (anti-CL and anti-β2GPI), endothelin-1 (ET-1) levels, also intima–media thickness (IMT), a quantitative indicator of atherosclerosis. In MCTD, anti-Hsp60 antibody levels were significantly higher than in healthy individuals ($p < 0.02$). MCTD patients with CVD had significantly higher levels of anti-Hsp60 compared to MCTD without CVD ($p = 0.001$). Patients with MCTD had significantly lower high-density lipoprotein cholesterol ($p = 0.02$) and PON activity ($p < 0.001$), and significantly increased systolic ($p < 0.0002$) and diastolic ($p < 0.001$) blood pressure compared to healthy individuals. Anti-U1RNP levels ($p < 0.002$) and IMT were higher in patients compared to controls ($p = 0.002$). The CVD-positive MCTD patients had increased anti-Hsp60 ($p < 0.0013$), anti-CL IgG ($p = 0.0005$), ET-1 serum concentration ($p < 0.05$) and IMT levels ($p < 0.001$) compared to MCTD patients without CVD. Anti-Hsp60 showed a strong correlation with anti-ox-LDL ($r = 0.36$, $p = 0.01$) and serum ET-1 ($r = 0.62$, $p < 0.001$) and negative correlation with PON activity ($r = -0.47$, $p = 0.01$). Anti-Hsp60 indicates endothelial injury, CVD, and can function as a novel atherosclerotic risk factor, also a valuable diagnostic marker in patients with MCTD.

Keywords Mixed connective tissue disease · Anti-Hsp60 antibody · Dyslipidemia · Endothelial dysfunction · Atherosclerosis · Cardiovascular disease

Abbreviations

AECA	Anti-endothelial cell antibodies
ANA	Antinuclear antibodies

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Apo(A1,B)	Apolipoprotein
β2GPI	β2-Glycoprotein I
BMI	Body mass index
BP	Blood pressure
CL	Cardiolipin
CS	Corticosteroid
CVD	Cardiovascular disease
dsDNA	Double-stranded deoxyribonucleic acid
ECG	Electrocardiography
ELISA	Enzyme-linked immunosorbent assay
EMG	Electromyography
ENG	Electroneurography
ET-1	Endothelin-1
GN	Glomerulonephritis
HDL	High-density lipoprotein
hsCRP	High-sensitivity C-reactive protein
Hsp	Heat-shock protein
HRCT	High-resolution CT scanning
IMT	Carotid artery intima–media thickness
LDL	Low-density lipoprotein
MCTD	Mixed connective tissue disease
NSAID	Nonsteroidal anti-inflammatory drugs
oxLDL	Oxidized low-density lipoprotein
PAH	Pulmonary arterial hypertension
PON	Paraoxonase activity
U1-RNP	Uridine-rich ribonucleoprotein

Introduction

Heat-shock proteins (Hsp) are intracellular proteins conserved during the evolution. Hsps have pivotal function in innate immunity and can be found in all species from prokaryotes to vertebrates, indicating a high degree of structural similarity between various species. Hsps are traditionally classified based on their molecular weight, including low-molecular-weight Hsps, such as Hsp10, Hsp40, Hsp60, Hsp70 and Hsp110. The basic functions of Hsps are intracellular molecular transport management, the functional restoration of damaged proteins or their elimination by programmed cell death [1]. The Hsps are typically localized within the cell, but may appear on the surface of activated endothelial or mononuclear cells [2, 3]. Endothelial cells can be activated following environmental stress, oxidized LDL (oxLDL), infection, cytokine stimulation and produce large amounts of Hsps. Due to antigenic homology between certain species, Hsps and the antibodies produced against them provoke inflammation, moreover as primary antigens, may play a role in the development of atherosclerosis [4–9]. In terms of cardiovascular diseases (CVD), the most important role is attributed to the Hsp60 protein, which shows approximately 60 % sequence homology with the Mycobacterial Hsp65, the Chlamydia Hsp60 and *E. coli* GroEL [10]. The Hsp60-producing endothelial cells are the primary targets of inflammatory processes, and via molecular mimicry between bacterial and human Hsp60 antigens, pathogenic antibodies are produced.

The pathogenic role of Hsp60 and its epitopes has been previously described in autoimmune diseases, such as Crohn's disease, type 1 diabetes mellitus, multiple sclerosis or psoriasis [5, 10].

Mixed connective tissue disease (MCTD) is a systemic autoimmune, inflammatory disorder, characterized by the simultaneous damage of multiple organs [11–14]. The most common symptoms of MCTD are polyarthritis, the swelling of the hands and fingers, Raynaud's phenomenon, myositis, esophageal dysmotility, pulmonary arterial hypertension (PAH) and the presence of interstitial lung disease [15–17]. Serologically, autoantibodies against the uridine-rich nuclear ribonucleoprotein U1 (anti-U1RNP) are characteristic to the disease. Over the past two decades, it became evident that the sera of MCTD patients contain higher titers of anti-endothelial cell antibodies (AECA) and antibodies against phospholipid structures, such as cardiolipin (anti-CL) and β2-glycoprotein I (anti-β2GPI). These autoantibodies may alter the disease phenotype and exacerbate the disease course [18–21].

The disease starts at the age of 30–40 with a 90 % female dominance. In MCTD, similar to other systemic autoimmune diseases, even from the early stages of the disease, signs of atherosclerosis are evident: lipid abnormalities, hypertension, endothelial dysfunction and vascular damage [22]. Until the mid-1990s, PAH was the primary cause of death in MCTD. However, in our cohort of 280 MCTD patients, cardiovascular mortality is in the first place, indicating that CVD-related diagnostic procedures and CVD monitoring are pivotal in the appropriate follow-up of these patients.

In addition to classical risk factors for atherosclerosis, the key pathogenic role of inflammatory processes, indicated by increased high-sensitivity C-reactive protein (hsCRP) levels and the presence of vascular endothelium-damaging antibodies, is now evident in MCTD. However, the primary antigen that provokes vessel-wall injury, resulting in early-onset atherosclerosis is unknown.

Increasing number of studies indicates that antigens initiating atherosclerotic process belong to the family of Hsps, and anti-Hsp60 has a clear atherosclerosis provoking effect.

Symptoms of cardiovascular damage are apparent from the very early disease phase of patients with MCTD. Therefore, the primary aims of our study were to investigate whether MCTD patients had antibodies against Hsp60

in their sera, moreover to assess whether there was a relationship between these antibodies and endothelial dysfunction, atherosclerosis and CVD. We also investigated the correlation among anti-Hsp60 antibodies, traditional cardiovascular risk factors, and other pathogenic antibodies which could lead to endothelial damage and atherosclerosis in MCTD.

Materials and methods

Patients

The patients were recruited from The Institute of Internal Medicine, Department of Clinical Immunology Outpatient Clinic between December 2011 and June 2012, where they received treatment and followed-up regularly. Thirty female patients with MCTD were enrolled in this study; all met the criteria set by Alarcon-Segovia [23] and Villarreal, which are as follows: the presence of at least four clinical symptoms, such as synovitis, Raynaud's phenomenon, myositis, swollen hands, fingers, sclerodactyly, along with positive serum test for anti-U1RNP antibodies. Of the 30 patients, 10 patients received corticosteroid (CS) treatment, 6 were administered with CS and methotrexate combination and five received CS and cyclophosphamide combination, while nine patients were taking nonsteroidal anti-inflammatory drugs (NSAIDs). Twenty sex- and age-matched healthy individuals were enrolled, as controls.

The subjects' written consent was obtained according to the Declaration of Helsinki (most recently at the General Assembly in October 2008). The study has been approved by local ethics committee/Institutional Review Board of the University of Debrecen.

Clinical diagnostic procedures

All patients have undergone chest X-ray; in case of alveolitis suspected, high-resolution CT scanning (HRCT) and pulmonary function tests were performed. Patients with lower extremity numbness or pain have undergone electroneurography (ENG). In patients with suspected myositis, serum creatine kinase and myositis-specific autoantibody assessment, muscle biopsy and/or electromyography (EMG) was performed. Early detection of PAH was carried out by Doppler echocardiography. In case of echocardiography raised the suspicion of PAH, right heart catheterization was carried out. The diagnosis of CVD was established if (1) the patient has had a myocardial infarction, or ECG signs of myocardial infarction were found, (2) clinical symptoms of angina were verified by coronarography, (3) known ischemic heart disease diagnosed by noninvasive tests or tissue Doppler method or (4) the patient had dilated cardiomyopathy.

Determination of anti-Hsp60

Anti-human Hsp60 IgG autoantibodies were measured by enzyme-linked immunosorbent assay (ELISA) (Lionex Diagnostics and Therapeutics, Braunschweig, Germany) as described previously [24, 25]. Briefly, multi-well plates were coated with human Hsp60 or M. bovis Hsp65 (0.1 μg/well). The plates were blocked and incubated with 100 μl 1:200 diluted serum samples. Specific antibody binding was detected with peroxidase-conjugated rabbit antihuman IgG antibodies (Dako North America Inc., Carpinteria, CA, USA). All patient and control samples were measured simultaneously on the same plate. Results were expressed as OD values.

Measurement of serum lipids

Total cholesterol levels were determined by spectrophotometry. Triglyceride and high-density lipoprotein C (HDL-C) were assessed by immune turbidimetric method. The determination of serum lipoprotein was carried out with agarose gel electrophoresis, measured on Cliniscan densitometer. The level of low-density cholesterol (LDL-C) was measured enzymatically and with a colorimetric assay (Roche LDL-C plus 2nd gen., Roche Inc., Basel, Switzerland). Apolipoprotein A1 (ApoA1) and apolipoprotein B (ApoB) concentrations were assessed by immunonephelometric method (ORIG Diagnostica, Espoo, Finland).

Serum paraoxonase (PON1) assessment

Serum paraoxonase activity (PON) measurement was carried out using spectrophotometry as previously described, using paraoxon, as substrate [22].

Immunological tests

Determination of antinuclear antibodies (ANA) was performed on human epithelial cell lines (HEp-2) by indirect immunofluorescence method. Serum levels of ANA at 1:64 dilution were considered positive.

The following autoantibodies were assessed by ELISA method: anti-U1RNP (Pharmacia and Upjohn, Freiburg, Germany), anti-SSA, anti-SSB, anti-Jo1 anti-Scl70 (Hycor Biomedical, Indianapolis, IN, USA), anti-double-stranded (ds) DNA, anti-cardiolipin (anti-CL IgG, IgM, IgA), anti-beta2-glycoprotein I (anti-beta2-GPI IgG, IgM, IgA) (Orgentec, Mainz, Germany). Cutoff values were as

follows: anti-U1-RNP: 5 U/mL; anti-SSA, anti-SSB, anti-Jo1: 10 U/mL; anti-Scl70, anti-DNA: 20 U/mL; anti-CL IgG, IgA: 7 U/mL; anti-CL IgM: 10 U/mL; anti-beta2-GPI IgG, IgM, IgA: 5 U/mL.

Anti-oxLDL determination was performed by ELISA in accordance with the manufacturer's instructions (ImmuLisa oxLDL, IMMCO Diagnostics, Buffalo, New York, NY, USA).

Plasma level of anti-endothelial cell antibodies (AECA) was determined by a cellular ELISA with cultured human umbilical vein endothelial cells according to the method previously described in details [21]. The calibration curve values were given in units/mL with the cutoff value of 35 U/mL.

The level of endothelin-1 (ET-1) was measured by ELISA method according to the manufacturer's instructions (Biomedica Gruppe, Vienna, Austria). The lowest measurable level of ET-1 was from 0.1 fmol/mL. Intra-assay and inter-assay coefficient were <5 and <10 %, respectively.

Evaluation of the carotid artery intima–media thickness (IMT)

Carotid artery IMT was determined by high-resolution Duplex ultrasonography using a HP Sonos 5500 instrument, equipped with 5–10 MHz linear transducer, as described and reviewed previously in details [26, 27]. Briefly, longitudinal and transverse section images were taken of the carotid system. If no plaques were detected from a mediolateral transducer position, longitudinal images were captured of the common carotid arteries, 10 mm proximally from the carotid bulb. Images were captured in the end-diastolic phase, and IMT data were evaluated offline using the AVITA software (Hi-Tech Systems Ltd., Basingstoke, UK). IMT was recorded as the distance between the first (lumen–intima border) and the second (media–adventitia border) echogenic lines. On both sides, ten measurements were performed; the mean of the individual values was calculated, and results were presented in millimeters.

Statistical analysis

Normality of continuous variables was evaluated by Shapiro–Wilk test. On variables, which showed not-normal distribution, Mann–Whitney test was used to compare controls and MCTD patients. Correlations were determined using Spearman correlation coefficient. Statistical significance was assumed when $p < 0.05$. Statistica for Windows (StatSoft, Tulsa, OK, USA) was used for data analysis.

Table 1 Demographic data of MCTD patients and controls

	Healthy controls $n = 20$	MCTD patients $n = 30$	p value
Gender (male/female)	0/20	0/30	
Age (years) mean ± SD	47.9 ± 8.4	53.0 ± 12.1	0.1
Disease duration (years) mean ± SD		15.0 ± 7.57	
Cardiovascular diseases in the family	0/20	4/30	0.1
Menopause	11/20	19/30	0.6

Table 2 Clinical manifestations of patients with MCTD

Affected organs	Patients with MCTD n (%)
Polyarthritis	25 (85.3)
Raynaud's phenomenon	22 (73.0)
Swollen hands and fingers	16 (53.3)
Pulmonary arterial hypertension	14 (46.6)
Interstitial lung disease	18 (60.0)
Myositis	13 (43.3)
Serositis	20 (66.6)
Neuropathy	10 (33.3)
Esophageal dysmotility	9 (30.0)
Skin vasculitis	9 (30.0)
Glomerulonephritis	3(10.0)
Antiphospholipid syndrome	17 (56.6)
Venous thrombosis	12/17
Arterial thrombosis	5/17
Cardiovascular disease	15 (50.0)

Results

Patient characteristics and clinical manifestations

The average age of patients with MCTD at the time of the study was 53.0 ± 12.1 years; the disease duration was 15.0 ± 7.6 years (Table 1). When patients and the control group were compared, regarding family history or CVD risk factors, there were no differences, including smoking habits or time of menopause.

Organ involvement in MCTD patients is summarized in Table 2. The most common clinical symptoms were the metacarpophalangeal, interphalangeal joints swelling of the hands, Raynaud's phenomenon, interstitial lung disease and serositis. Fourteen patients had PAH during the course of the disease. Three patients had glomerulonephritis (GN) with a histological diagnosis of membranous GN in one patient, while in two cases diffuse proliferative GN.

Table 3 Anti-Hsp60, body mass index (BMI), BP, lipid parameters and PON of MCTD patients

Parameters	Control $n = 20$	MCTD $n = 30$	p value
Anti-Hsp60 (OD)	104.5 (69.0–173.0)	173.0 (125.0–265.0)	0.02
BMI(kg/m2)	22.0 ± 3.2	28.9 ± 4.8	<0.0001
Systolic BP (mmHg)	125.5 ± 14.6	151.7 ± 26.3	0.0002
Diastolic BP (mmHg)	78.0 ± 8.9	94.5 ± 13.3	<0.001
Total cholesterol (mmol/L)	4.7 ± 1.0	6.2 ± 1.4	0.007
LDL (mmol/L)	2.5 ± 0.7	3.8 ± 1.2	<0.0001
HDL (mmol/L)	2.1 ± 0.5	1.7 ± 0.4	0.02
ApoA1 (g/L)	1.4 ± 0.4	0.9 ± 0.3	<0.0001
ApoB (g/L)	0.9 ± 0.1	1.0 ± 0.3	0.2
PON (U/mL)	88.4 (68.0–130.1)	173.0 (125.0–265.0)	<0.0001

Apo(A1,B) apolipoprotein, *BMI* body mass index, *BP* blood pressure, *HDL* high-density lipoprotein, *Hsp* heat-shock protein, *LDL* low-density lipoprotein, *PON* paraoxonase activity

Table 4 Serum autoantibody levels and IMT in MCTD patients and controls

	Control $n = 20$	MCTD $n = 30$	p value
Anti U1-RNP (U/mL)	4.9 (2.3–6.7)	11.9 (5.6–21.3)	0.002
AECA (U/mL)	17.3 ± 9.0	48.0 ± 27.5	<0.0001
Anti-oxLDL (U/mL)	10.6 ± 5.6	27.7 ± 18.6	0.0003
Anti-CL IgG (U/mL)	5.0 (3.5–6.9)	11.5 (4.3–23.8)	0.02
Anti-CL IgA (U/mL)	2.65 (1.5–5.1)	3.3 (2.3–4.3)	0.4
Anti-CL IgM (U/mL)	2.1 (1.1–3.3)	2.9 (1.4–4.3)	0.3
Anti-β2GPI IgG (U/mL)	4.0 (3.0–4.4)	4.0 (3.4–6.5)	0.1
Anti-β2GPI IgA (U/mL)	4.0 (3.1–4.6)	3.8 (2.8–5.0)	0.06
Anti-β2GPI IgM (U/mL)	1.6 (0.7–3.0)	2.5 (1.0–3.4)	0.3
hsCRP (g/L)	1.45 ± 1.14	30.3 ± 19.1	<0.0001
IMT (mm)	0.6 ± 0.08	0.7 ± 0.1	0.002

AECA anti-endothelial cell antibodies, *β2GPI* β2 glycoprotein I, *CL* cardiolipin, *hsCRP* high-sensitivity C-reactive protein, *IMT* carotid artery intima–media thickness, *oxLDL* oxidized low-density lipoprotein, *U1-RNP* uridine-rich ribonucleoprotein

Antiphospholipid syndrome was association with MCTD in 17 patients; 12 patients had venous thrombosis, while five patients had arterial thrombosis. Regarding general vascular and cerebrovascular diseases, two patients had transient ischemic attack, and one patient required surgery due to obliterative vascular disease of the lower extremity.

Altogether, 15 out of 30 patients had CVDs. Two patients suffered from myocardial infarction with corresponding ECG abnormalities. Unstable angina was detected in 3 patients, and 8 patients had ischemic heart disease, while 2 MCTD patients had dilated cardiomyopathy and required regular cardiac monitoring.

Anti-Hsp60 and serological abnormalities in association with CVD

The anti-Hsp60 values were significantly higher in the MCTD patients than in the control group [control: 104 (69–173) vs. 173 (125–265); $p < 0.02$] (Table 3). The patients' systolic and diastolic blood pressure (BP) were both higher in MCTD than in controls [systolic BP control: 125.5 ± 14.6 mmHg, MCTD: 151.7 ± 26.3, $p = 0.0002$; diastolic BP control: 78 ± 8.9 vs. MCTD: 95.4 ± 13.3, $p < 0.0001$]. In patients, the total cholesterol and LDL fraction increased compared to the control group. HDL cholesterol and ApoA1 cholesterol levels were significantly lower in MCTD than in controls (HDL cholesterol MCTD: 1.7 ± 0.4 vs. controls: 2.1 ± 0.5 mmol/L, $p = 0.024$; ApoA1: MCTD: 0.9 ± 0.3 g/L, control: 1.4 ± 0.4 g/L, $p < 0.0001$). In patients with MCTD and PON1, the PON was reduced compared to the control subjects' values [MCTD 88.4 (68–150 U/mL) vs. control: 173 (125–265 U/mL), $p < 0.001$].

In patients with MCTD, along with the increase of anti-Hsp60 serum levels, we found significantly elevated anti-U1RNP, anti-oxLDL, AECA and anti-CL IgG values compared to healthy subjects (Table 4). The serum concentration of the inflammatory parameters, ET-1, anti-oxLDL and hsCRP, was higher in the patients than in the control population [ET-1: control vs. MCTD: 1 (0.4–2.9 fmol/L) vs. 2.6 (1.1–3.1 fmol/L), $p < 0.05$; anti-oxLDL: control vs. MCTD: 10.6 ± 5.6 vs. 27.7 ± 18.6 U/mL, $p = 0.0003$; hsCRP: control vs. MCTD: 1.45 ± 1.14 vs. 30.3 ± 19.1 g/L, $p < 0.0001$]. The carotid IMT was significantly higher in MCTD than in the age- and sex-matched control group, indicating increased atherosclerotic tendency in these patients (0.6 ± 0.08 vs. 0.7 ± 0.1 mm, $p = 0.002$).

Correlation of anti-Hsp60 levels with anti-oxLDL, ET-1 and PON1

We correlated various investigated parameters with anti-Hsp60 levels in patients with MCTD. We found negative correlation between anti-Hsp60 antibodies and the PON1 PON ($r = -0.4743$, $p = 0.01$). Moreover, there was a close positive correlation between anti-Hsp60 antibody levels and anti-oxLDL ($r = 0.36$, $p < 0.01$) and between anti-Hsp60 and the serum concentrations of ET-1 ($r = 0.62$, $p < 0.0001$) (Fig. 1a–c).

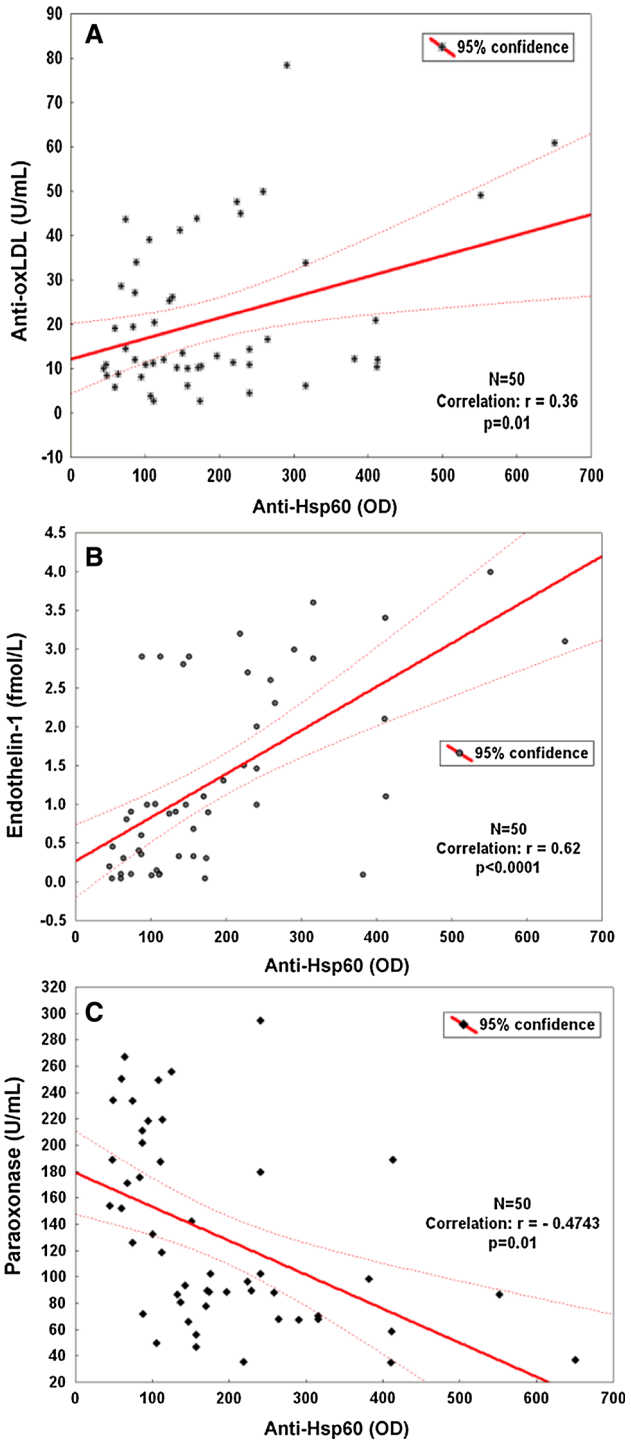

Fig. 1 Correlation between antibodies against Hsp60 and various parameters associated with endothelial damage and atherosclerosis. **a** Anti-Hsp60 and anti-oxLDL. **b** Anti-Hsp60 and endothelin-1 (*ET-1*). **c** Anti-Hsp60 and paraoxonase activity (*PON1*)

Comparison between MCTD patients with and without CVDs

Finally, we compared various serological markers and clinical parameters between MCTD patients with or without CVD complications (Table 5). We found that anti-Hsp60 antibody levels were significantly higher in MCTD patients having CVD complications, compared to MCTD without CVD: 259 (176–316) vs. 137 (84–157), respectively; $p < 0.001$). The MCTD CVD-positive group had significantly higher systolic and diastolic BP (Table 5). Patients with CVD had a clearly reduced paraoxonase (PON1) activity; paraoxonase functions as an antioxidant and prevents the oxidation of LDL; therefore, its activity is strongly associated with anti-atherosclerotic processes. PON1 activity: MCTD CVD-positive patients: 68.9 ± 22.7 U/mL versus MCTD CVD-negative patients: 124 ± 60.2 U/mL; $p = 0.005$. Other serological markers of inflammation, such as hsCRP, as well as ET-1, a potent vasoconstrictor, involved in the development of atherosclerosis were also increased in MCTD patients with CVD, compared to patients without CVD complications: hsCRP: 44 (26.6–48) versus 22.4 (9.4–43) g/L, respectively; $p < 0.04$; ET-1: 2.6 (1.1–3.1) versus 1 (0.4–2.9) fmol/L, respectively; $p = 0.05$. The assessment of the important ultrasound imaging marker of atherosclerosis, the carotid artery IMT indicated that the CVD-positive MCTD group had increased values compared to the CVD-negative patient subset: 0.8 ± 0.1 vs. 0.6 ± 0.1 mm, respectively; $p < 0.0003$).

Table 5 Anti-Hsp60 and cardiovascular risk factors in patients with or without CVD

	MCTD patients without CVD ($n = 15$)	MCTD patients with CVD ($n = 15$)	p value
Anti-Hsp60 (OD)	137 (84–157)	259 (176–316)	0.001
Systolic BP (mmHg)	138.0 ± 25.1	165.3 ± 19.9	0.003
Diastolic BP (mmHg)	89.7 ± 10.4	99.3 ± 14.4	0.04
PON (U/mL)	124.7 ± 60.2	68.9 ± 22.7	0.005
Endothelin-1 (fmol/L)	1.0 (0.4–2.9)	2.6 (1.1–3.1)	0.05
hsCRP (g/L)	22.4(9.4–43.0)	44.0 (26.6–48.0)	0.04
IMT (mm)	0.6 ± 0.1	0.8 ± 0.1	0.0003

BP blood pressure, *CVD* cardiovascular disease, *hsCRP* high-sensitivity C-reactive protein, *Hsp* heat-shock protein, *IMT* carotid artery intima–media thickness, *PON* paraoxonase activity

Discussion

In the work presented here, we investigated anti-Hsp60 antibody levels and its relationship to atherosclerosis and cardiovascular events in patients with MCTD. Primarily, we found that patients with MCTD have higher serum levels than that of healthy subjects of the same age group. Moreover, within MCTD patients, the level of anti-Hsp60

antibody levels further increased in those with accompanying cardiovascular events. Fifteen MCTD patients were diagnosed with CVD, suffering from unstable angina, chronic ischemic heart disease or dilated cardiomyopathy; all presented with significantly higher anti-Hsp60 antibody levels, compared to the 'MCTD-only' group, showing no evidence of past, or ongoing cardiovascular events.

Our findings confirmed previous observations that MCTD patients have lipid abnormalities, compared to healthy individuals. The total cholesterol, LDL cholesterol levels were increased in MCTD compared to values from healthy subjects, while the levels of HDL, ApoA1 and importantly, the natural antioxidant PON clearly decreased. In patients, both systolic and diastolic BP were more pronounced when compared to healthy individuals.

Previously, we have shown that MCTD is characterized by increased lipid oxidation (oxLDL), decreased activity of the PON1 paraoxonase enzyme and reduced levels of ApoA1, which has a key role in stabilizing the paraoxonase enzyme [22]. Decreased PON activity in the arterial wall is insufficient to protect LDL cholesterol from oxidation [28, 29]. These findings indicate that accelerated atherosclerosis is characteristic to MCTD.

Recent findings support the idea that Hsp60 is not only localized in the intracellular mitochondria, but appears in the endothelial cell surface, moreover can be released by injured and dead cells [30, 31]. Fluid shear stress of the arterial wall has been shown to induce Hsp60 expression in endothelial cells in vitro and in vivo, accelerates the intracellular production of endothelial cell adhesive proteins and enhances monocyte adhesion to the endothelial cell surface [32]. In humans, large concentrations of Hsp60/65 have been identified in macrophages, residing in atherosclerotic plaques of the carotid artery, and also these Hsps have been detected in endothelial cells and in minor amounts in smooth muscle cells, as well [33, 34].

In our investigated MCTD patient cohort, the presence of antibodies to Hsp60 was strongly associated with atherosclerotic events, IMT increase, raised systolic and diastolic pressure values, high anti-oxLDL levels, along with decreases PON. Generally, in MCTD patients with cardiovascular abnormalities, increased hsCRP indicates ongoing inflammatory processes. The investigated endothelial cell damaging factors anti-CL IgG and ET-1 showed strong correlation with anti-Hsp60 antibodies.

In MCTD, no evidence has been gathered that endothelial cell injury, triggered by bacterial or chlamydial infection would be a primary etiological factor, which could provoke increased production of Hsp60, or via molecular cross-reaction/mimicry would lead to the production of anti-Hsp60 autoantibodies. However, previous findings confirmed that one of the members of the Hsp family, human Hsp60 showed homology to the Mycobacterial Hsp65 and Chlamydia Hsp60, which contributed to the production of anti-Hsp60 antibodies [35]. Moreover, it has been demonstrated that the presence of antibodies against Hsp60 in the early pathogenic events is the earliest serological marker of atherosclerosis [35]. A large prospective study found that levels of Hsp60 were significantly elevated in subjects with prevalent/incident carotid atherosclerosis and that these levels were correlated with IMT [36]. Hsp60 was also correlated with anti-lipopolysaccharide, anti-Chlamydia and anti-Hsp60 antibodies, various markers of inflammation, as well as the presence of chronic infections. The increased levels of antibodies were independent of gender and investigated risk factors. Moreover, the risk of atherosclerosis associated with high Hsp60 levels was amplified when subjects had clinical and/or laboratory evidence of chronic infections [36]. The 5-year follow-up of these patients showed that the antibody levels remained persistently elevated, especially in subjects in whom carotid IMT progression was apparent [36].

Anti-Hsp60 is increased in ca. 70 % of the general population in addition the anti-Hsp60 antibody-positive subjects have significantly higher susceptibility to atherosclerosis and coronary artery disease, compared to the anti-Hsp60-negative population. The severity of the coronary artery disease was associated with increased antibody titers [37, 38].

In our work along with the assessment of anti-Hsp60, for the first time, we investigated the pathological changes in anti-oxLDL levels, particularly in association with CVD complications in MCTD. The presence of anti-Hsp60 closely correlated with the increase of anti-oxLDL levels. Previously, we depicted that in patients with acute coronary syndrome, anti-oxLDL levels were significantly higher than in healthy individuals [39]. Antibodies against ox-LDL directly damage vascular synovium, or the damage is caused by circulating ox-LDL–immune complexes and their deposition [40]. The modified LDL is taken up by macrophages by the scavenger receptors, which transforms them into foam cells, and they accumulate in the endothelial cell layer. The modification of LDL affects chemotaxis, and these particles become cytotoxic at the end of the oxidative process. The level of antibodies against ox-LDL indicates the degree of oxidative stress is related to the extent of atherosclerosis and predisposes to myocardial infarction. In patients with myocardial infarction, the serum level of anti-ox-LDL was found to be substantially higher than in controls. Other factors such as age, smoking, elevated BP and high total cholesterol levels contribute to a 2.5-fold increase in the probability to develop myocardial infarction [41, 42].

Regarding another systemic autoimmune disease, in patients with systemic sclerosis, the presence of anti-Hsp60 antibodies did not show association with the duration of

disease, the lung function tests or IMT [43]. These patients were not characterized by increased circulating Hsp60, and Hsp65 levels, or anti-oxLDL values [43].

Circulating soluble anti-Hsp60 antibodies may be used as prognostic biomarker, indicators of CVDs. A prospective follow-up study, including 195 healthy subjects, described that anti-human Hsp60 antibody titers were significantly higher in those patients who subsequently developed cardiovascular complications compared to those subjects who did not have CVD events [44]. Studies on a large patient cohort indicated that soluble Hsp60 levels were high in subjects with carotid artery atherosclerosis and Hsp levels correlated with IMT values [45]. Borderline hypertensive patients had elevated serum levels of soluble Hsps, while in patients who have already developed hypertension had clearly high heat-shock protein serum concentrations. Increased serum Hsps and inflammatory markers showed clear correlation in patients who had very early cardiac and microvascular damage, although coronary angiography identified normal arteries [46].

Our patient cohort with MCTD had antibodies with endothelial activating/damaging properties, as well as various inflammatory factors that increased endothelial and tissue damage. AECA in the presence of elevated ET-1 levels were characteristic to patients with MCTD, associated with PAH. Endothelial cell damage is indicated by the increase in serum ET-1 levels in patients with PAH. Moreover, endothelial damage is reflected by the results of a 30-year follow-up study on MCTD patients, where antibodies against β2GPI and other antiphospholipid antibodies lead to secondary antiphospholipid syndrome, in 30 % of patients. Altogether, these findings support the idea that endothelial damage and endothelial activation are a pivotal feature of MCTD patients [46–49].

In our MCTD patients with CVD, besides myocardial infarction, unstable angina and ischemic heart disease, two patients had dilated cardiomyopathy. It has been previously demonstrated that Hsp60 shows protein sequence homology with the cardiac myosin heavy chain, acetylcholine receptor, DNA-binding proteins and thyreoglobulin [50]. In a study on dilated cardiomyopathy, 10 % of the patients had antibodies against Hsp60 without CMV or enterovirus infection [51]. These data indicate that atherosclerosis, autoimmune processes of the myocardial damage without prior bacterial infection, is important in the pathogenesis. Further studies and observations are necessary to assess the role of infectious factors contributing to exacerbations or acceleration of the cardiovascular damage.

Altogether, our findings support the idea that the immune-mediated endothelial dysfunction and damage, as well as accelerated atherosclerosis, leading to CVDs present in patients with MCTD. Antibodies against Hsp60 clearly indicate endothelial injury, CVD and can function as a novel atherosclerotic risk factor, as well as a valuable diagnostic marker in patients with MCTD. Increased levels of anti-Hsp60 in patients with MCTD can provide a sufficient background for the early introduction of endothelial protection in the future management of the disease, to prevent serious cardiovascular conditions in these patients.

Besides traditional risk factors for atherosclerosis, novel inflammatory mediators, autoantibodies play a pivotal role in early, accelerated atherosclerosis in patients with MCTD. Herein, we introduce a novel marker, antibodies against Hsp60, which clearly indicate endothelial injury, atherosclerotic processes and concomitant CVD in patients with MCTD. Generally, MCTD patients and MCTD patients with CVDs were characterized by increased anti-Hsp60 antibody levels. Besides anti-Hsp60, we identified that of general indicators of inflammation and atherosclerosis, patients with MCTD had lipid abnormalities, indicated by lower HDL cholesterol and PON levels and increased anti-U1RNP, anti-oxLDL, IMT and ET-1 levels. Anti-Hsp60 correlated with anti-oxLDL, ET-1 and with decreased PON activity.

Our findings support the idea that immune-mediated endothelial dysfunction, accelerated atherosclerosis present in patients with MCTD, signified by anti-Hsp60. Anti-Hsp60 clearly indicates endothelial injury, CVD and can function as a novel atherosclerotic risk factor, as well as a valuable diagnostic marker in patients with MCTD. Increased levels of anti-Hsp60 in patients with MCTD can provide a sufficient background for the early introduction of endothelial protection and anti-platelet therapy (e.g., aspirin, statins, clopidogrel or abciximab) in the future management of the disease, to prevent serious cardiovascular conditions in these patients.

Conflict of interest The authors declare that they have no conflict of interest.

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