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Myosteatosi in NAFLD patients correlates with plasma Cathepsin D

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Abstract: Previously, we have shown that hepatic lipid accumulation induces the secretion of cathepsin D (CTSD), and that plasma CTSD levels are associated with increased inflammation and disease severity in nonalcoholic fatty liver disease (NAFLD). Although it is clear that the liver is a major source of plasma CTSD, it is unknown whether other metabolically active organs such as the muscle, also associate with plasma CTSD levels in NAFLD patients. Therefore, the aim of this study was to explore the relation between lipid accumulation in the muscle (myosteatosi) and plasma CTSD levels in forty-five NAFLD patients. We observed that hepatic steatosis positively associated with plasma CTSD levels, confirming the previously established link between plasma CTSD and the liver. Furthermore, a positive association between myosteatosi and plasma CTSD levels was observed, which was independent of sex, age, BMI, waist circumference and hepatic steatosis. By establishing a positive association between myosteatosi and plasma CTSD levels, our findings suggest that, in

addition to the liver, the muscle is also linked to plasma CTSD levels in NAFLD patients. The observed link between myosteatosi and plasma CTSD levels supports the concept of a significant role of the skeletal muscle in metabolic disturbances in metabolic syndrome-related disorders.

Keywords: Plasma CTSD levels; myosteatosi; steatosis; ectopic lipid accumulation; NAFLD.

Introduction

Due to the growing prevalence of metabolic syndrome (MetS), nonalcoholic fatty liver disease (NAFLD) is an increasing worldwide health problem. Being the most common cause of chronic liver diseases in the Western world [1], NAFLD is a progressive disease ranging from liver steatosis to nonalcoholic steatohepatitis (NASH), hepatic fibrosis, cirrhosis, liver failure and ultimately hepatocellular carcinoma [2, 3]. Given the disease burden associated with NAFLD, more research is needed to elucidate the underlying disease mechanisms as a basis to develop novel therapies.

Previously, our group has shown that, following hepatic steatosis, increased secretion of cathepsin D (CTSD, a lysosomal enzyme) is associated with higher levels of inflammation, disturbed lipid metabolism and disease severity in *in vitro* and *in vivo* models for NAFLD, suggesting a pathophysiological role of CTSD in NAFLD [4, 5]. In addition, we observed that plasma CTSD levels are also elevated in NASH patients [6], further emphasizing the association between plasma CTSD levels and NAFLD progression. Of note, studies have shown increased plasma CTSD levels in type 2 diabetes [7], Alzheimer's disease [8] and inflammatory bowel disease [9], suggesting that extra-hepatic organs are also linked to plasma CTSD under pro-inflammatory conditions. Importantly, extra-hepatic organs such as the gut, adipose tissue and muscle also induce metabolic disturbances in MetS and NAFLD,

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raising the possibility that next to the liver, also other extra-hepatic organs are linked to plasma CTSD in MetS and NAFLD patients [10-12]. Among the aforementioned extra-hepatic organs, the muscle is increasingly being implicated in the progression of MetS. Accumulating evidence has shown that ectopic fat infiltration in skeletal muscle, referred to as myosteatorsis, includes intramuscular and intermuscular lipid accumulation, and is strongly associated with obesity, diabetes and MetS [13, 14]. For instance, intermuscular fat has been shown to be a predictor of insulin sensitivity [15, 16], highlighting the link between myosteatorsis and systemic metabolism. In addition, intramuscular fat is a metabolically active component of the muscle, which contributes to the secretion of inflammatory cytokines that induce systemic inflammation [14]. Whether the muscle is also linked to plasma CTSD in NAFLD patients has yet to be explored.

Therefore, our study aim was to investigate whether, besides the liver, also the muscle correlates with plasma CTSD levels in NAFLD patients. For this reason, forty-five NAFLD patients were enrolled in this study, in which muscle signal intensity loss on in-phase and opposed-phase imaging (chemical shift MRI) was assessed to determine myosteatorsis based on the formula as previously described [17]. Furthermore, fasting blood samples were analyzed for the measurement of glucose- and lipid-related parameters, liver enzymes as well as plasma CTSD levels. Our findings demonstrate a positive association between the degree of myosteatorsis and plasma CTSD levels independent of sex, age, BMI, waist and hepatic steatorsis in NAFLD patients. Therefore, our data suggest that in addition to the liver, also the muscle is linked to plasma CTSD levels. Taking into account that plasma CTSD aggravates metabolic disturbances, the observed link between myosteatorsis and plasma CTSD levels supports the concept of a significant role of the skeletal muscle in metabolic disturbances in metabolic syndrome-related disorders.

Methods and materials

Subject characteristics

Forty-five subjects were included in the present study as previously described [17]. All participants had proven NAFLD via liver biopsy or chemical shift magnetic resonance imaging (MRI). Metabolic syndrome was diagnosed based on the International Diabetes Federation (IDF) definition [18]. Exclusion criteria were excessive ethanol consumption (male > 14 and female >

7 standard beverages per week), causes for secondary hepatic fat accumulation (medication, Wilson's disease, viral infections, starvation or parenteral nutrition and microvesicular steatorsis on liver biopsy), pregnancy and breastfeeding, a history of bariatric surgery, liver cirrhosis and/or hepatocellular carcinoma, malignancy(s) within the last 5 years and individuals about to undergo or recovering from a surgical or otherwise medical procedure. All subjects were recruited from Maastricht University Medical Center (MUMC+) and CO-EUR (a second line eating disorder clinic) between September 2015 and October 2018.

Informed consent: Informed consent has been obtained from all individuals included in this study.

Ethical approval: The study was approved by the Medical-Ethical Committee of Maastricht University (ClinicalTrials.gov Identifier: NCT02422238), and was performed in accordance with the principles of the Declaration of Helsinki, as revised in 2008.

Biochemical analyses

After overnight fasting, venous blood was collected into EDTA tubes that were put on ice after blood collection at Maastricht University Medical Center (MUMC+). After centrifuging (1000 x g; 10 min; 4°C), plasma was snap-frozen in liquid nitrogen and then stored at -80°C until analyses. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyl transpeptidase (GGT), bilirubin, Alkaline Phosphatase, total cholesterol, low density lipoprotein cholesterol (LDL-cholesterol), high density lipoprotein cholesterol (HDL-cholesterol), triglyceride, fasting plasma glucose and hemoglobin A1c (HbA1c) were determined using routine analyses at the clinical chemistry department of the Maastricht UMC+ hospital. Plasma CTSD levels were determined using the CTSD enzyme-linked immunosorbent assay according to the manufacturer's protocol (Uscn Life Science, Wuhan, China). The absorbance for CTSD levels was measured on a Benchmark 550 microplate reader (Bio-Rad, Hercules, CA). Skeletal muscle mass index (SMI) (appendicular skeletal mass/height²) were calculated with dual-energy X-ray absorptiometry (DXA).

Magnetic resonance imaging (MRI) – hepatic fat fraction

Hepatic fat fraction (hepatic steatorsis) was assessed as previously described [17] [19]. In brief, four circular regions of interest (ROI) of 5 cm² from each MRI section (in total

3 MRI sections) were drawn in the liver, where artefact, vascular and biliary structures were avoided. Then, the ROI was copied from the in-phase (IP) image to the opposed-phase (OP) image. The mean signal intensity (SI) loss of all 12 ROIs was calculated as previously described [17, 20].

Magnetic Resonance Imaging (MRI) – muscle fat fraction

The MRI technique (chemical shift MRI) was performed to determine muscle fat fraction (myosteatorsis) in all participants after a minimal fasting period of 3 hours within one month of the other measurements. MRI images were obtained with a 1.5T or 3T Achieva MRI system (Philips, Best, The Netherlands). The muscle fat fraction was measured with the simultaneously obtained 3D fast gradient echo mDixon images as previously described [17]. Parameters employed for 3D fast gradient echo mDixon sequence were for 1.5T and 3T respectively: flip angle = 15° and 10°, TR = 5.8 and 3.4 ms, TE1/TE2 = 2.37/4.75 and 1.19/2.37ms, field-of-view (FOV) = 320 x 320 and 384 x 384 mm, acquisition matrix = 288 x 227 and 252 x 209, slice thickness 5 and 3 mm. The multifidus and erector spinae muscle were segmented bilaterally at the lumbar 1 (L1) level using Weasis software and muscle fat fraction (chemical shift MRI) was calculated with the formula: $(SI_{IP} - SI_{OP}) / SI_{IP} \times 100\%$ as previously described [17].

Transient elastography

Liver stiffness measurements (LSM) were performed in all participants using the FibroScan® (touch 502, Echosens, Paris, France). Both the M probe (3.5 MHz) and the XL probe (2.5 MHz) were available for this study. At least 10 valid measurements, a 60% success rate and an interquartile range of less than 30% of the median elasticity were accepted for further analysis. The final result of LSM is the median of the valid (at least 10) individual measurements. The cut-off value for significant fibrosis (histological grade 2 fibrosis (F2)) and advanced fibrosis (F3) were 7.0 kPa and 8.7 kPa, respectively [21]. In the database, we divided fibrosis into no fibrosis (<7.0 kPa) and significant fibrosis (≥7.0 kPa) groups for further analysis according to the cut-off value of 7.0 kPa.

Data statistics

Statistical analyses were performed using SPSS 25.0 (IBM, Armonk, NY, USA) and Graphpad Prism 6.0 for Microsoft Windows. All data were expressed as means ± SEM. The differences of myosteatorsis between males and females were tested using unpaired *t*-test in Graphpad Prism 6.0. Pearson's correlations were performed to determine simple correlations between plasma CTSD levels and other parameters. Subsequently, multiple linear regression analyses were performed to analyze the association between plasma CTSD levels and hepatic fat fraction/fibrosis/myosteatorsis, in which plasma CTSD levels were added as dependent variable and either hepatic fat fraction, fibrosis or myosteatorsis as independent variables, resulting in model 1 (simple regression), model 2 (model 1 + adjustment for sex), model 3 (model 2 + adjustment for age), model 4 (model 3 + adjustment for BMI) and model 5 (model 4 + adjustment for waist circumference). Additionally, the association between plasma CTSD levels and myosteatorsis was also investigated in a multiple linear regression analysis adjusted for sex, age, BMI and waist circumference, using myosteatorsis as dependent variable and plasma CTSD levels as independent variable. Only in the multiple linear regression analyses of plasma CTSD levels and myosteatorsis, hepatic fat fraction was adjusted in the model 6 (model 5+hepatic fat fraction). A *p*-value <0.05 was considered statistically significant.

Results

Anthropometric and clinical characteristics of the study participants

The baseline characteristics of participants are summarized in Table 1. As previously described [17], forty-five NAFLD patients participated in our study, of which 24 subjects were males and 21 individuals were females, with an average age of 51.1 ± 1.7 years old and a mean BMI of 32.9 ± 0.8 Kg/m². The average of myosteatorsis was $35.57 \pm 1.65\%$, ranging from 8.08% to 54.31%. Finally, plasma CTSD levels ranged from 40150.00 pg/mL to 1364000.00 pg/mL, with a mean of 440776.82 ± 43143.42 pg/mL.

Table 1: Baseline clinical characteristics of the study participants.

	Mean $\pm$ SEM	Ranges
Age, yrs	51.1 $\pm$ 1.7	20~65
Sex (M/F)	24/21	-
BMI (kg/m ²)	32.9 $\pm$ 0.8	24.6~46.3
Waist circumference (cm)	108.9 $\pm$ 1.8	85.8~146.9
Hip (cm)	114.0 $\pm$ 1.8	96.0~154.3
WHR	0.96 $\pm$ 0.01	0.73~1.10
SBP (mmHg)	134.5 $\pm$ 2.2	107~177
DBP (mmHg)	81.0 $\pm$ 1.4	62~115
Heart rate (bpm)	66.2 $\pm$ 1.4	49~97
HbA1c (%)	5.87 $\pm$ 0.14	4.50~9.10
Fasting glucose (mmol/L)	6.3 $\pm$ 0.3	4.8~13.0
Total Cholesterol (mmol/L)	5.2 $\pm$ 0.1	3.6~7.7
Triglycerides (mmol/L)	2.15 $\pm$ 0.34	0.65~15.58
HDL Cholesterol (mmol/L)	1.26 $\pm$ 0.06	0.50~2.20
LDL Cholesterol (mmol/L)	3.2 $\pm$ 0.1	1.8~5.4
GGT (U/L)	54.9 $\pm$ 7.6	8~256
ALT (U/L)	46.2 $\pm$ 6.8	7~272
AST (U/L)	32.0 $\pm$ 3.0	11~118
Bilirubin (umol/L)	8.5 $\pm$ 0.6	3.7~23.1
Alkaline phosphatase (IU/L)	95.4 $\pm$ 4.7	52~186
Hepatic fat fraction (%)	19.51 $\pm$ 1.61	6.62~45.49
Fibroscan liver stiffness (Kpa)	6.9 $\pm$ 0.4	3.6~12.2
Fibrosis (Yes/No)	18/25	-
Myosteatosis (%)	35.57 $\pm$ 1.65	8.08~54.31
Skeletal muscle mass index (kg/m ²)	8.64 $\pm$ 0.19	5.69~11.20
CTSD levels (pg/mL)	440776.82 $\pm$ 43143.42	40150.00~1364000.00

Data are mean $\pm$ SEM. BMI, body mass index; WHR, waist/hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, hemoglobin; HDL, high density lipid protein; LDL, low density lipid protein; GGT, gamma-glutamyl transpeptidase; ALT, alanine transaminase; AST, aspartate aminotransferase.

Hepatic steatosis rather than fibrosis is positively associated with plasma CTSD levels

To validate our previous finding that the liver contributes to plasma CTSD levels, we assessed whether hepatic steatosis and fibrosis associated with plasma CTSD levels in our current NAFLD patients. As shown in Table 2, linear regression analyses were performed by adjusting for sex, age, BMI and waist circumference. Our data showed that hepatic fat fraction positively associated with plasma

CTSD levels (standardized coefficient $\beta=0.300$, 95% CI: 0.003~0.595, $p=0.048$) in model 1. Though the association was nearly significant after adjustment for sex (model 2: standardized coefficient $\beta=0.288$, 95% CI: -0.012~0.586, $p=0.059$), and age (model 3: standardized coefficient $\beta=0.308$, 95% CI: -0.002~0.617, $p=0.051$), hepatic fat fraction was still significantly associated with plasma CTSD levels after correcting for BMI (model 4: standardized coefficient $\beta=0.316$, 95% CI: 0.019~0.610, $p=0.037$) and further adjustment for waist circumference (model 5: standardized coefficient $\beta=0.325$, 95% CI: 0.024~0.623,

Table 2: Hepatic fat fraction (steatosis) is positively associated with plasma CTSD levels.

Dependent variable: plasma CTSD levels						
Models	Hepatic fat fraction			Fibrosis		
	Adjusted R square	Standardized coefficient β (95% CI)	p value	Adjusted R square	Standardized coefficient β (95% CI)	p value
Model 1	0.068	0.300 (0.003~0.595)	0.048	0.009	0.182 (-0.135~0.504)	0.250
Model 2 (Model 1+Sex)	0.062	0.288 (-0.012~0.586)	0.059	0.019	0.189 (-0.126~0.510)	0.229
Model 3 (Model 2+Age)	0.047	0.308 (-0.002~0.617)	0.051	-0.005	0.187 (-0.133~0.513)	0.242
Model 4 (Model 3+BMI)	0.134	0.316 (0.019~0.610)	0.037	0.050	0.073 (-0.267~0.414)	0.663
Model 5 (Model 4+Waist circumference)	0.120	0.325 (0.024~0.623)	0.035	0.026	0.094 (-0.274~0.466)	0.603

Data were analyzed by multiple linear regression models: Model 1, simple regression; Model 2, model 1 + adjustment for sex; Model 3, model 2 + adjustment for age; Model 4, model 3 + adjustment for BMI; Model 5, model 4 + adjustment for waist circumference. $p<0.05$ is statistically significant.

Figure 1: Myosteatosi s is gender-dependent and positively correlates with plasma CTSD levels in NAFLD patients. Pearson’s correlation was performed to analyse correlation. $p<0.05$ is statistically significant. $**p<0.01$.

$p=0.035$) (Table 2). These data demonstrate that hepatic steatosis is positively associated with plasma CTSD levels independent of sex, age, BMI and waist circumference. As displayed in Supplementary Figure 1, plasma CTSD levels are not significantly different between ‘no fibrosis’ and ‘significant fibrosis’ groups. Similarly, we did not observe associations between plasma CTSD levels and fibrosis (as shown in table 2). Altogether, these data demonstrate that hepatic steatosis, but not fibrosis, is independently associated with plasma CTSD levels, confirming the link of the liver to plasma CTSD levels.

The positive association between myosteatosi s and plasma CTSD levels independent of sex, age, BMI, waist circumference and hepatic fat fraction

To investigate whether the muscle also links to plasma CTSD levels, the association between myosteatosi s and plasma CTSD levels was analyzed by Pearson’s correlation and multiple linear regression analyses. As shown in Figure 1A, we observed that plasma CTSD levels positively

Table 3: Myosteatorosis is positively associated with plasma CTSD levels independent of sex, age, BMI, waist circumference and hepatic fat fraction (steatosis).

Dependent variable: plasma CTSD levels			
Myosteatorosis			
Models	Adjusted R square	Standardized coefficient β (95% CI)	p value
Model 1	0.108	0.358 (0.067~0.644)	0.017
Model 2 (Model 1+Sex)	0.210	0.525 (0.219~0.822)	0.001
Model 3 (Model 2+Age)	0.194	0.537 (0.223~0.843)	0.001
Model 4 (Model 3+BMI)	0.198	0.471 (0.136~0.799)	0.007
Model 5 (Model 4+Waist circumference)	0.177	0.474 (0.129~0.812)	0.008
Model 6 (Model 5+Hepatic fat fraction)	0.280	0.488 (0.165~0.805)	0.004

Data were analyzed by multiple linear regression models: Model 1, simple regression; Model 2, model 1 + adjustment for sex; Model 3, model 2 + adjustment for age; Model 4, model 3 + adjustment for BMI; Model 5, model 4 + adjustment for waist circumference; Model 6, model 5 + adjustment for hepatic fat fraction (steatosis). $p < 0.05$ is statistically significant.

correlated with myosteatorosis ($r=0.358$, $p=0.017$). Moreover, in Figure 1B, we also found that myosteatorosis was gender-dependent, showing higher values for myosteatorosis in females than males ($p < 0.01$). To further evaluate whether myosteatorosis was dependently or independently associated with plasma CTSD levels, multiple linear regression analysis was performed by adjusting for sex, age, BMI, waist circumference and hepatic steatosis. As displayed in table 3, myosteatorosis positively associated with plasma CTSD levels (model 1: standardized coefficient $\beta=0.358$, 95% CI: 0.067~0.644, $p=0.017$), even after adjustment for sex (model 2: standardized coefficient $\beta=0.525$, 95% CI: 0.219~0.822, $p=0.001$), age (model 3: standardized coefficient $\beta=0.537$, 95% CI: 0.223~0.843, $p=0.001$), BMI (model 4: standardized coefficient $\beta=0.471$, 95% CI: 0.136~0.799, $p=0.007$), waist circumference (model 5: standardized coefficient $\beta=0.474$, 95% CI: 0.129~0.812, $p=0.008$) and further correcting for hepatic fat fraction (model 6: standardized coefficient $\beta=0.488$, 95% CI: 0.165~0.805, $p=0.004$). Vice versa, and shown in Supplementary table 1, we also observed that plasma CTSD levels independently associated with myosteatorosis independent of sex, age, BMI, waist circumference and hepatic steatosis. As such, these data indicate an independent positive association of myosteatorosis with plasma CTSD levels in NAFLD patients, suggesting that, in

addition to the liver, also the muscle interacts with plasma CTSD levels.

Discussion

The exact mechanisms underlying NAFLD development and progression are not yet fully elucidated. Previously, our studies have demonstrated that plasma CTSD levels are associated with the development of hepatic inflammation and dyslipidemia, suggesting that plasma CTSD is a key player in metabolic disturbances in NAFLD [4, 6]. In the current study, our data shows the positive and independent association between myosteatorosis and plasma CTSD levels in NAFLD patients, identifying a link between plasma CTSD levels and the muscle. This observed link supports the concept of a significant role of the skeletal muscle in metabolic disturbances in metabolic syndrome-related disorders.

The consumption of highly caloric, lipid-rich diets is one of the major propellers of MetS and the development of metabolic syndrome associated disorders [22, 23]. Previously, we established lysosomal lipid accumulation as a link between increased cellular uptake of lipids, metabolic disturbances and hepatic inflammation in mouse models for NASH [4]. In addition to interfering

with proper lysosomal function and thus triggering pro-inflammatory pathways within cells, lysosomal lipid accumulation causes the leaking of lysosomal contents, culminating in increased secretion of CTSD into the plasma [24] [25, 26]. In line, we have previously shown that NAFLD progression is positively associated with plasma CTSD levels [6], indicating that increased metabolic dysfunction and inflammation are accompanied by increased plasma CTSD levels. Furthermore, our studies show that enhanced plasma CTSD levels in adult NASH patients are likely derived from Kupffer cells [6], thus pinpointing the liver as a source of plasma CTSD following hepatic steatosis and inflammation. In the current study, the observation that myosteatorosis in NAFLD patients independently associates with plasma CTSD levels raises the possibility that the muscle, as a metabolic organ, also contributes to plasma CTSD levels. Mechanistically, the increased secretion of CTSD induced by lipid overload in the muscle can be explained by lysosomal exocytosis, a process that is regulated by intracellular Ca^{2+} channels [25]. Moreover, lipid accumulation in cells is able to induce chronic intracellular Ca^{2+} overload, subsequently stimulating lysosomes to fuse with the plasma membrane and thus releasing lysosomal enzymes (i.e., CTSD) into the circulation via exocytosis [27, 28]. Vice versa, and potentially even more interesting, our observed link also raises the possibility that plasma CTSD levels increase myosteatorosis, considering plasma CTSD as a mediator inducing ectopic lipid accumulation. Whether myosteatorosis leads to plasma CTSD levels, vice versa, or both is a matter of future debate. Nevertheless, our data show a link between myosteatorosis and plasma CTSD in NAFLD patients, implying an involvement of the skeletal muscle in MetS-associated disorders.

Previously, myosteatorosis has been strongly associated with obesity, diabetes and MetS, suggesting that myosteatorosis plays an important role in metabolic disturbances in the context of metabolic syndrome-related disorders [13]. Of note, ample evidence has demonstrated that myosteatorosis induces the secretion of local and systemic pro-inflammatory cytokines [14], which are key mediators in the pathogenesis of NAFLD [29]. In addition to pro-inflammatory cytokines, our current study suggests that myosteatorosis may further contribute to systemic metabolic dysfunction potentially via increased secretion of CTSD. How plasma CTSD further fuels this metabolic dysfunction is a topic for future research, though interactions with insulin and lipid metabolism appear to be involved players [4, 30].

Furthermore, myosteatorosis has been shown to contribute to the aetiology of insulin resistance through the impairment of insulin signalling [31, 32], modulation of adipokine (e.g., adiponectin) secretion [33] and inhibiting nutritive muscle blood flow [34]. Importantly, insulin resistance is recognized as a critical pathophysiological factor in NAFLD [35]. As such, the aforementioned findings suggest that myosteatorosis is potentially involved in the pathophysiology of NAFLD by modulating insulin resistance. In line, as previously shown in this cohort, myosteatorosis is positively associated with insulin resistance in NAFLD patients [17]. Indeed, our previous studies have demonstrated that inhibiting circulating CTSD reduces plasma insulin levels and improves insulin resistance, suggesting that plasma CTSD aggravates insulin resistance [5, 36]. As such, these findings provide support for the possibility that myosteatorosis contributes to insulin resistance in NAFLD via increased CTSD secretion.

Although the cohort that we used is relatively small, we demonstrate for the first time a positive and independent association between myosteatorosis and plasma CTSD levels in NAFLD patients. On one hand, our findings indicate that the muscle, in parallel with the liver, associates with plasma CTSD levels, which may further trigger and exacerbate metabolic dysfunction in NAFLD patients. In addition, whether other metabolically active organs, for instance, adipose tissue also link to plasma CTSD levels in NAFLD should be further addressed in the future and studies on larger cohorts should be conducted to validate our findings. Furthermore, more research is necessary to better understand the underlying mechanism between the interaction of myosteatorosis and plasma CTSD levels in the context of MetS.

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Conflict of interest: Ronit Shiri-Sverdlov is a member of Biomolecular Concept's Editorial Advisory Board.

Data Availability Statement: The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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