İSTANBUL ÜNİVERSİTESİ YAYINEVİ

RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.2021.896230
İst Tıp Fak Derg 2021 / J Ist Faculty Med 2021

THE RELATIONSHIP BETWEEN THE EXPRESSION LEVELS OF TISSUE INHIBITOR OF METALLOPROTEINASES-3 (TIMP3) AND SEVERITY OF ATHEROSCLEROSIS

METALLOPROTEİNAZ-3 DOKU İNHİBİTÖRÜNÜN (TIMP3) İFADE DÜZEYLERİ İLE ATEROSKLEROZUN ŞİDDETİ ARASINDAKİ İLİŞKİ

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Cite this article as: Celebi G, Guclu Geyik F, Yilmazbayhan D, Ozsoy D, Yildiz CE, Yildiz M, et al. The relationship between the expression levels of tissue inhibitor of metalloproteinases-3 (TIMP3) and severity of atherosclerosis. J Ist Faculty Med 2021;84(4):472-81. doi: 10.26650/IUITFD.2021.896230

ABSTRACT

Objective: Tissue Inhibitor of Metalloproteinase-3 human (TIMP3) is one of tissue inhibitors of metalloproteinases (TIMPs), which binds to the components of the extracellular matrix, and has crucial roles in atherosclerogenesis and adipose tissue differentiation. In this study, it was aimed to determine the effects of *TIMP3* gene expression levels on severity of atherosclerosis in different tissues.

Material and Methods: The first group of the study (evaluated for coronary artery disease) were cases classified as high and low plaque scores according to the degree and location of atherosclerotic lesions. In the second group (post-mortem cases) were male cadavers who died due to coronary heart disease (CHD, n=26) and non-cardiac trauma (T-nonP, n=4). The *TIMP3* expression levels were examined in leukocyte and peri-coronary epicardial adipose tissues (EAT) samples (n=69 and n=34, respectively) of the first group and EAT and coronary artery samples (n=12 and n=30, respectively) of the second group using quantitative RT-PCR. In addition, the protein expressions of TIMP3 were analysed on artery sections by immunofluorestaining.

Results: In the post-mortem study group, the *TIMP3* expression levels were found to increase in no plaque segments of arteries of cases with CHD (CHD-nonP) compared to advanced atherosclerotic arteries (CHD-P) and normal arteries of T-nonP cases

ÖZET

Amaç: Hücre dışı matriksin bileşenlerine bağlanan TIMP3, metaloproteinazların doku inhibitörlerinden (TIMP'ler) biridir ve ateroskleroz gelişimi ile yağ dokusu farklılaşmasında rol oynamaktadır. Bu çalışmada, farklı dokulardaki *TIMP3* gen ifade düzeylerinin ateroskleroz şiddeti üzerine olan etkisinin belirlenmesi amaçlandı.

Gereç ve Yöntemler: Çalışmanın ilk grubu (koroner arter hastalığı için değerlendirilen), aterosklerotik lezyonların derecesine ve yerine göre yüksek ve düşük plak skoru olarak sınıflandırılan vakalardı. İkinci grup (post-mortem vakalar), koroner kalp hastalığı (KKH, n=26) ve kardiyak olmayan travma (T-nonP, n=4) nedeniyle ölen erkek kadavralardı. Birinci grubun lökosit ve peri-koroner epikardiyal yağ doku (EYD) örnekleri (sırasıyla, n=69 ve n=34) ile ikinci grubun EYD ve koroner arter örneklerinde (sırasıyla, n=12 ve n=30) *TIPM3* ifade düzeyleri kantitatif RT-PCR ile incelendi. Ek olarak, TIMP3 protein lokalizasyonları, immunfluoresans tekniği ile belirlendi.

Bulgular: Post-mortem çalışma grubunda, *TIMP3* ifade düzeylerinin KKH'lı vakaların plaksız arter segmentlerinde (CHD-nonP), ileri aterosklerotik arterlere (CHD-P) ve T-nonP vakaların normal arterlere kıyasla arttığı bulundu (sırasıyla, p=0,01 ve p=0,05). Lökosit ve EYD'lerdeki *TIMP3* gen ifadeleri, çalışma grupları arasın-

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Submitted/Başvuru: 13.03.2021 • **Revision Requested/Revizyon Talebi:** 12.04.2021 •
Last Revision Received/Son Revizyon: 24.04.2021 • **Accepted/Kabul:** 03.05.2021 • **Published Online/Online Yayın:** 13.09.2021

(p=0.01 and p=0.05, respectively). The *TIMP3* expressions in EATs and leukocytes were not statistically significant between the study groups. In addition, TIMP3 protein was detected in normal arteries, peri-coronary EATs and mostly in macrophages-rich areas in advanced atherosclerotic arteries.

Conclusion: Tissue Inhibitor of Metalloproteinase-3 human (TIMP3) expression levels increase in normal coronary arterial segments of cases with CHD, which depict a protective role of TIMP3 in development of atherosclerosis.

Keywords: Atherosclerosis, TIMP3, gene expression level, coronary artery, adipocyte, leukocyte

da istatistiksel olarak anlamlı fark göstermedi. Ek olarak, TIMP3 proteini normal arter, peri-koroner EYD ve ileri aterosklerotik arterde makrofajların yoğun olduğu alanlarda tespit edildi.

Sonuç: KKH'lı vakaların normal koroner arteriyel segmentlerinde *TIMP3* ifadesindeki artışın ateroskleroz gelişimine karşı koruyucu bir etkisi olabileceğini göstermektedir.

Anahtar Kelimeler: Ateroskleroz, TIMP3, gen ifade seviyesi, koroner arter, adiposit, lökosit

INTRODUCTION

Atherosclerosis, which is the main causal mechanism underlying coronary heart diseases (CHD), is one of the leading causes of morbidity and mortality in the world. Atherosclerosis is a progressive arterial lesion that develops primarily from a series of reactions induced by endothelial injury. In this inflammatory-fibroproliferative response, the extracellular matrix (ECM) plays an important role in the intercellular network among smooth muscle cells, macrophages, T lymphocytes and endothelial cells (1-3). Tissue inhibitors of metalloproteinase (TIMPs) are responsible for inhibiting matrix metalloproteinases (MMPs), which are the main regulators of ECM (4). The interaction between MMPs and TIMPs regulates balance between ECM and its environment (5). It has been demonstrated in previous studies that this delicate balance is disturbed in cancer, myocardial infarction and inflammatory diseases, including atherosclerosis (6-8). To balance MMP levels in damaged tissues, exogenous therapeutic application of TIMP3 has been found to be beneficial in experimental studies (8). Secreted TIMP3 is the only ECM in the TIMP family in insoluble form and has been associated with decreased inflammation and the atherosclerotic plaque stability (9, 10).

Moreover, *TIMP3* expression remains low during the adipocyte differentiation; however, it increases when cell differentiation is disrupted (11). Adipose tissue has a crucial regulatory role in the development of atherosclerosis and diabetes due to inflammation (12-15). However, the role of TIMP3 in atherosclerosis, which has a modulatory role in adipose tissue formation around the atherosclerotic coronary artery, is unknown.

The influence of *TIMP3* expression, which has therapeutic significance in disease modulation, on different types of tissues and cells and advanced atherosclerosis has thus far not been addressed in the same study design. In this study, it was aimed to determine the effect of the *TIMP3* expression profiles in different cells and tissues on the atherosclerosis process. For this purpose, *TIMP3* expression levels in peripheral blood leukocytes and peri-coronary epicardial adipose tissue (EAT) were investigated in low and high score groups formed by Gensini and Syntax scoring according to the complexity and severity of coronary artery disease in the living cases of the study. On the other hand, we attempted to verify the pre-existing *TIMP3* relationships and determine the association between EAT and coronary arteries with advanced atherosclerotic plaques in post-mortem cases with CHD.

MATERIALS AND METHODS

All procedures performed in the living study groups involving human participants were in accordance with the ethics committee of the Faculty of Medicine Clinical Research Ethics Committee, Istanbul University (Date: 27.10.2011, No: 1822 and Date: 17.01.2014, No: 160) and followed the Declaration of Helsinki. The experimental protocol of post-mortem study groups was evaluated and approved by the Scientific Research Commission of Council of Forensic Medicine (B.03.1.ATK.0.01.00.08/863, 28.12.2010).

Samples of living cases

The first study group, named as living cases (total n=69) consist of participants who were evaluated for coronary artery disease (CAD) by performing invasive coronary angiography due to stable angina pectoris, ischemia, acute coronary syndrome and pre-surgical assessment. All cases in the first study group were divided into two groups according to complexity and severity of their coronary artery disease as low and high plaque score groups. Gensini and Syntax scores were calculated based on the degree and location of atherosclerotic lesions in coronary angiographic evaluation (16). Gensini and Syntax were considered to have a cut-off value of eight for both scores. The group with high plaque score (n=48) consisted of cases with CAD with at least one stenosis of least 50% in any coronary vessel. Twenty-five of 48 patients with CAD in this group were operated on for heart valve repair or replacement (n=5) and coronary artery by-pass surgery (n=20). Nine of the 21 cases in the low plaque score group without CAD (0% or <20% stenosis) were operated on for heart valve repair or replacement. Peripheral blood samples were collected for leukocyte separation from all cases. The peri-coronary epicardial adipose tis-

sues (EAT) from the proximal tract of the right coronary artery were obtained from 34 of 69 cases within the first 20 minutes during heart valve surgery (n=14) and by-pass surgery (n=20). The study design and the number of samples studied in subgroups is given in Figure 1.

Peripheral blood and tissue samples of cases were provided from the Istanbul University-Cerrahpasa, Department of Cardiovascular Surgery and Department of Cardiology. Written informed consent was obtained from every participant before blood and/or tissue samples were taken in surgical operation and coronary angiography.

Cases with atherosclerosis combined with various systemic autoimmune or chronic inflammatory diseases including rheumatoid arthritis, systemic lupus erythematosus, and antiphospholipid syndrome were excluded from the study.

Samples of post-mortem cases

The coronary artery samples and peri-coronary epicardial adipose tissues (EAT) were obtained from male autopsy cases (total n=30) within 24 hours post-mortem. Since female autopsy cases were extremely rare, they were not included in the study, and also cases with appropriate sampling time were not included in the study. All post-mortem samples were provided from the Republic of Turkey, Ministry of Justice Council of Forensic Medicine. The ethical approval was obtained for the post-mortem tissue collection from Scientific Research Commission of Council of Forensic Medicine (Project number and date: B.03.1.ATK.0.01.00.08/863, 28.12.2010).

Post-mortem cases were evaluated according to the autopsy report including the pathological, biochemical and toxicological analysis. The cases were classified according to causes of death as coronary heart disease (CHD, n=26) and non-cardiac trauma (T, n=4). The non-atherosclerotic coronary arteries (T-nonP) group included traumatic cases without coronary heart disease, whose coronary arteries were evaluated as normal in autopsy including histopathologic evaluations. The exclusion criteria for this study were: moderate to advanced putrefaction, drug poisoning or toxicity, and cases of homicide or suspected homicide. The characteristics of the study groups are shown in Table 1. The coronary artery samples were dissected according to their macroscopic features (plaque size, occlusion, presence of calcification for atheroma, and also anatomic location, artery diameter, artery wall thickness and normal appearance, etc.) and grouped as arteries with advanced atherosclerotic plaque (CHD-P) (n=26) and no plaque segments (CHD-nonP) (n=25) obtained from CHD cases and also non-atherosclerotic coronary arteries (T-nonP) (n=4). The peri-coronary EAT and arterial samples of the young T-nonP group who were reported to have no atherosclerosis in the autopsy report were used as controls for gene expression and histopathological examinations.

The post-mortem peri-coronary EAT samples were obtained from the surrounding tissue of the coronary artery of traumatic cases (n=2 from T-nonP group), the surrounding tissue of the coronary artery with plaque (n=10 from CHD-P group) and without plaque (n=10 from CHD-nonP) of CHD cases. Since tissue could not be obtained from some cases and there was insufficient tissue for

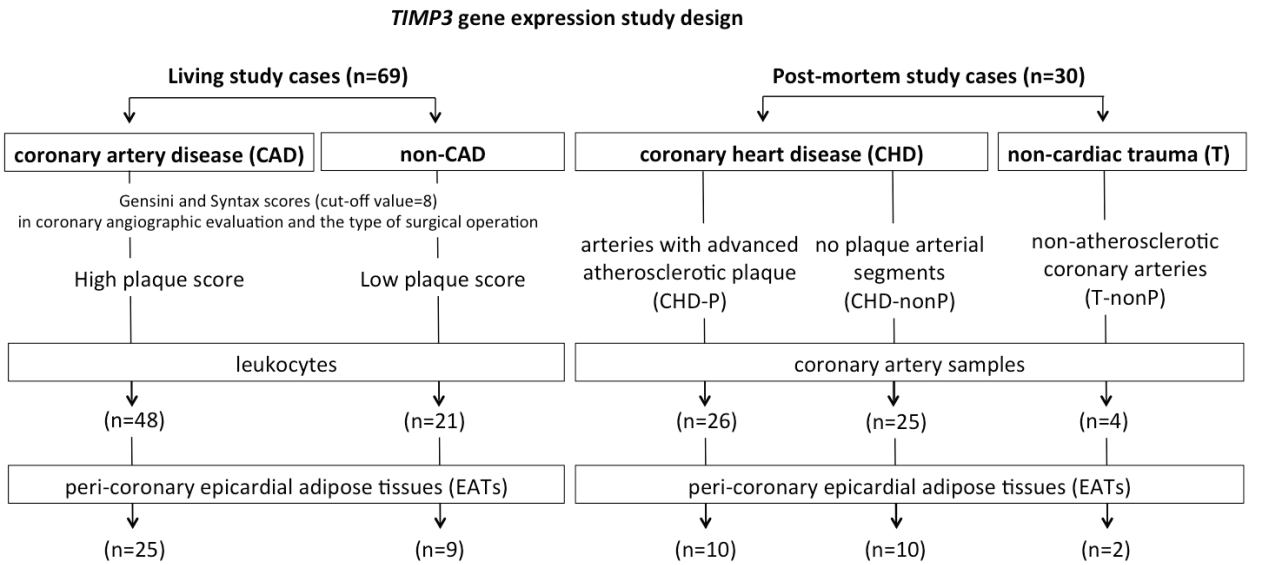

Figure 1: Flowchart of the *TIMP3* gene expression study design. This figure illustrates the study design of living study cases and post-mortem study cases and their subgroups. The numbers of peripheral blood leukocytes, peri-coronary epicardial adipose tissues (EATs) and coronary artery tissues from a total of 99 cases are shown.

RNA isolation in some cases, EAT sample numbers of the study groups were unequal.

Histopathology and immunofluorescence techniques

Small pieces of (approximately 3-4 mm) the non-atherosclerotic segments of coronary arteries (n=2 from T-nonP group and n=2 from CHD-nonP group) and the coronary arteries with advanced plaque including peri-coronary epicardial adipose tissues (n=4 from CHD-P group) of the post-mortem cases were separated to compare protein localizations between the groups for use in the immunofluorescence technique. These tissues were fixed in 5% paraformaldehyde, mounted within OCT Medium (Sakura/Tissue-Tek Company, Torrance), and then were dissected into 10 µm thick cross-sectional segments using a cryostat in -40°C.

In Hematoxylin and Eosin (HE) staining, at least three tissue sections of all fixed tissue samples were confirmed to have advanced atherosclerotic lesions and normal artery morphology with histopathologic evaluation (17).

The other tissue sections on poly-L-lysine coated glass slides were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 15 min at room temperature for immunofluorescence analysis of TIMP3 and tissue specific proteins. After rinsing twice with ice cold PBS, tissues on slides were subjected to heat-induced antigen retrieval by incubation in citrate buffer (10 mM citric acid in 1xPBS, pH 6.0). Next, immunofluorescence protocol was followed for immunostaining. The tissue sections were incubated first with primer antibody (diluted with antibody diluent solution, abcam, ab64211) overnight at 4°C, and then with specific secondary fluorescence antibody in the dark for one hour at room temperature. The sections were washed in PBS (3×2 minutes) and mounted Fluoroshield Mounting Medium with DAPI (4,6-diamino-2-phenyl indole) to identify the nuclei. Monoclonal mouse anti-CD68 antibody [KP1] (1/200 dilution rate, ab955), polyclonal rabbit anti-alpha smooth muscle actin (1/100 dilution rate, ab5694), polyclonal rabbit anti-human TIMP3 antibody (1/200 dilution rate, ab39184), goat anti-rabbit IgG H&L (Alexafluor 488 Green, 1/500 dilution rate, ab181448), goat anti-mouse IgG H&L (FITC, 1/1000 dilution rate, ab6785) and Fluoroshield Mounting Medium with DAPI (ab104139) was obtained from Abcam. Images were acquired using Confocal Microscope (Leica TCS-SPE).

Total RNA isolation

The coronary arteries and perivascular adipose tissues were carefully removed by dissection, immediately frozen in liquid nitrogen and stored at -80°C until further use. Total RNAs were extracted using the Trizol reagent (TRIZOL Reagent, Invitrogen, USA). Trizol reagent was added 1 mL (for each 100 mg) to the tissues and homogenized with tissue homogenizer.

The peripheral blood samples were collected into 10 ml Vacuette K_2EDTA tubes (BD, Franklin Lakes, NJ). Gey's Solution (155 mM NH_4Cl, 10mM $KHCO_3$ in DEPC treated distilled water) was added to a 2:1 ratio on whole blood. Then, samples were incubated at +4°C for 20 minutes. White blood cells (leukocytes) were isolated via centrifugation for 10 min at 1500 rpm at 10°C. The cell pellet was washed a second time in Gey's solution as equal volume of the blood. Next, 1 ml of 1x Phosphate Buffered Saline (PBS) was added to the pellet and centrifuged for 10 minutes at room temperature at 1500 rpm. The leukocytes pellet was homogenized with 1 ml Trizol Reagent.

Total RNA isolation from tissue and leukocyte homogenates was performed according to the Trizol RNA isolation protocol. The concentration and quantity of total RNA samples were measured at 260 nm and 280 nm (A260/280) using a Nanodrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Expression analysis with Quantitative Real Time PCR

The expression levels of *TIMP3* were determined in the leukocytes and peri-coronary EAT obtained from the living cases and in the coronary artery and peri-coronary EAT samples obtained from the post-mortem cases using quantitative Real Time-PCR (qRT-PCR). qRT-PCR were performed with the PCR Master primer-probe mixes of each gene transcripts with the Probe Master Mix in the LC480 instrument for the *TIMP3* and as a control for the *ACTB* (actin, beta, NM_001101.2) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, NM_002046.3) as endogenous controls. cDNA was first synthesized from total RNA samples, and then qRT-PCR was performed, and the relative expression levels were calculated as $2^{-\Delta\Delta Ct}$ method. The difference in the threshold cycle between target (*TIMP3*) and reference genes (the mean of *B-actin* and *GADPH*) as ΔCt was calculated for all samples studied in duplicate. The sample with the lowest Ct mean of endogenous genes selected from the T-nonP group and low score group was used as the calibrator (reference sample) for post-mortem groups and living study groups, respectively. The relative expression of the *TIMP3* in all samples was compared to the calibrator and the results were expressed as relative quantification (RQ) values. Real Time ready Catalog Assay for *TIMP3* (Assay IDs: 101221), *ACTB* (Assay IDs: 143636), *GAPDH* (Assay IDs: 141139) and Light Cycler 480 Probes Master Kit was purchased from Roche Life Science for quantitative RT-PCR.

Statistical analysis

Comparison of expression levels of *TIMP3* was conducted by using RQ values, and results were expressed as mean and standard deviation (S.D.). Normality of distributions of the *TIMP3* expression levels and other continuous variables were assessed using the Shapiro-Wilk test. If vari-

ables were not normally distributed, Mann Whitney U-test was used for comparison between two groups, and Kruskal-Wallis test was used for multiple comparisons among three groups. Student t-test and ANOVA test was used to compare for the means of clinical characteristics that were normally distributed. The Chi Square Test or Fisher's Exact Test was used for categorical variables. Spearman's test was used for correlation analysis of Syntax and Gensini scores that were not normally distributed. All statistical analyses were performed using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). Values of $p<0.05$ were considered statistically significant. There was at least 80% ($\alpha=0.05$) statistical power and an effect size of 0.5 and 0.8 in global effects when total sample size was at least 27 and 12, respectively. Power and sample size calculations were performed using the G*Power statistics software (18).

Table 1: The characteristics of living cases and post-mortem cases

	Living cases		
Characteristics	**Low plaque score (n=21)**	**High plaque score (n=48)**	**p-values**
Gender, % (men)	50%	64%	0.244
Age (means, years)	57.93±10.21	57.7±10.91	0.900
Gensini score	3.42±3.69	54.58±32.24	0.0001
Syntax score	2.33±2.95	19.90±9.12	0.0001
BMI (kg/m^2)	27.53±4.49	27.16±3.79	0.611
Diastolic blood pressure (mmHg)	75.54±9.43	75.83±9.46	0.870
Systolic blood pressure (mmHg)	124.08±16.34	123.13±15.21	0.756
Triglycerides (mg/dl)	163.59±94.22	170.73±95.10	0.645
Total cholesterol (mg/dl)	186.5±43.5	189.23±46.92	0.724
HDL cholesterol (mg/dl)	41.45±12.42	39.20±11.72	0.249
LDL cholesterol (mg/dl)	122.81±36.32	125.94±43.41	0.649
CRP (mg/dl)	7.84±11.38	12.02±20.33	0.214
Glucose (mg/dl)	107.55±29.37	122.96±43.75	0.008
Myocardial infarction, yes (%)	-	58.3%	0.0001
Hypertension, yes (%)	57.1%	47.9%	0.481
Diabetes mellitus, yes (%)	33.3%	31.2%	0.864
Type of surgery operation; Heart valve surgery, yes (%)	42.9%	10.4%	0.0001
Coronary by-pass surgery, yes (%)	-	41.7%	

Post-mortem cases

Characteristics	**CHD group (n=26)**		**T-nonP group with normal arteries (n=4)**	**p-values***	**p-values****
	with plaque segments (CHD-P) (n=26)	**arterial segments without plaque (CHD-nonP) (n=25)[♯]**			
Age (mean, years)	51.3±12.1	51.3±12.1	29.3±16.3	0.003	0.003
BMI (kg/m^2)	27.2±4.4	28.1±3.8	23.3±4.6	0.111	0.03
Heart weight (gr)	458.6±119.4	479.7±135.4	366.0±54.0	0.142	0.113
Positive family history, yes	53.8%	48.0%	0%	0.044	0.07
Plaque in aorta, yes	76.0%	70.8%	0%	0.003	0.007
Plaque in LCA, yes	88.5%	88.0%	0%	0.0001	0.0001
Plaque in RCA, yes	69.2%	60.0%	0%	0.009	0.026

[♯], A sufficient amount of arterial segment without a plaque could not be obtained in one CHD case. *CHD-P group vs. T-nonP group, **CHD-nonP group vs. T-nonP group; P; advanced atherosclerotic plaque, CHD; coronary heart disease

RESULTS

The basic clinical characteristics of living cases (n=69) and post-mortem cases (n=30) were shown in Table 1. There was a strong positive correlation between Gensini and Syntax scores (r=0.92; p<0.0001) of the living cases. The age, body mass index (BMI), blood pressures, and serum lipids except gensini/syntax scores and glucose levels were not statistically different between the low and high plaque score groups. In the post-mortem study groups, the traumatic cases in the T-nonP group (n=4) were younger than the coronary heart disease case groups (n=26) (p=0.003), and the heart and arteries were normal in the autopsy reports. This group (T-nonP) was used for calculation of RQ values in post-mortem samples.

TIMP3 expression levels in leukocytes and peri-coronary EAT of living cases

The leukocytes and peri-coronary EATs of living cases grouped as high and low plaque scores according to surgical operation, and invasive coronary angiography were investigated for the expression levels of *TIMP3*. The high score group consisted of 48 patients with high Gensini and Syntax scores (cut-off value ≥8 for both) who had coronary artery disease. The low plaque score group consisted of 21 cases that underwent heart valve surgery and with low Gensini and Syntax scores (cut-off value <8 for both). The peri-coronary epicardial adipose tissues (EAT) samples were obtained from 34 of the 69 cases, of which nine cases were in the low score group and 25 cases in the high score group.

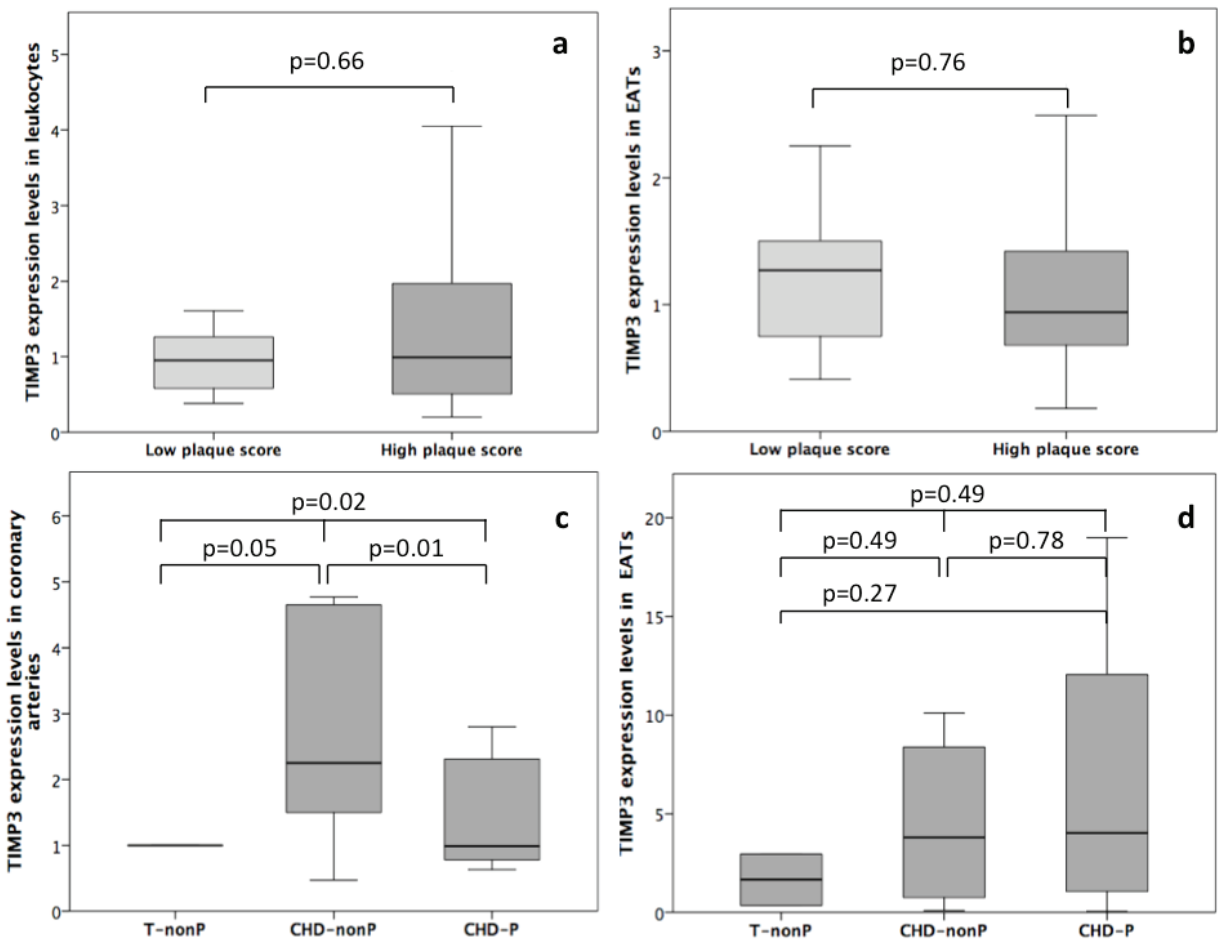

Figure 2: The comparison of *TIMP3* expression levels in study groups. *TIMP3* expression levels in leukocytes (a) and peri-coronary Epicardial Adipose Tissues (EAT) (b) according to plaque scores were shown. In post-mortem, *TIMP3* expression levels in coronary arteries (c) and peri-coronary EAT (d) were compared in tissues with advanced plaque (CHD-P) and without plaque (CHD-nonP) of cases with coronary heart disease (CHD) and non-cardiac trauma (T-nonP). Kruskal-Wallis test was used to make a comparison among three groups. Mann Whitney-U test was used to compare two groups. Mean±standard deviation of the expression levels was shown as the box-plots.

The expression levels of *TIMP3* were analysed in both leukocytes and peri-coronary EAT according to plaque scores. *TIMP3* expression levels of circulating leukocytes were not found statistically different in the high plaque score group (1.47±1.33, n=48) compared to the low plaque score group (1.08±0.70, n=21) (p=0.66) (Figure 2a). Moreover, *TIMP3* expression levels in peri-coronary EAT samples did not show any difference (p=0.76) between two groups. The expression levels of the low and high plaque score groups were 1.18±0.59 (n=9) and 1.29±0.99 (n=25), respectively (Figure 2b).

TIMP3 mRNA expression levels in coronary arteries and peri-coronary EAT of the post-mortem cases

The coronary artery and peri-coronary EAT samples of post-mortem cases were investigated for *TIMP3* mRNA expression levels. The expression levels of *TIMP3* in post-mortem coronary arteries showed statistically significant differences between three groups (2.99±2.62, n=25 for CHD-nonP group, 1.46±1.14, n=26 for CHD-P group and 1.13±0.32, n=4 for T-nonP, p=0.02) (Figure 2c). *TIMP3* expression was significantly higher in coronary artery segments without atherosclerotic plaques (CHD-nonP group) as compared to advanced plaques (CHD-P group) (p=0.01) and also non-atherosclerotic coronary arteries (T-nonP) (p=0.05) (Figure 2c). The expression levels of *TIMP3* in post-mortem EAT samples were shown in Figure 2d. The expression levels of *TIMP3* in peri-coronary EAT samples of CHD-P group (6.21±6.46, n=10) were found 1.4 fold higher compared to EAT samples of CHD-nonP group (4.53±3.85, n=10) and 3.8 fold higher compared to EAT samples of T-nonP group (1.65±1.86, n=2); however, both were statistically non-significant (p=0.27 and p=0.78, respectively). *TIMP3* expression levels in EAT samples were not comparable among three groups (p=0.49) (Figure 2d).

TIMP3 protein detection in coronary arteries of the post-mortem cases

Hematoxylin-Eosin (HE) staining was used in serial sections to determine the histological classification of the coronary arteries with and without plaque. Two randomly selected coronary arteries obtained from the non-cardiac traumatic cases (T-nonP group) and the cases with coronary heart disease (CHD-nonP group) were determined to have histologically normal morphology. Obvious differences between the arterial walls were shown as normal histological morphology (Figure 3a) and advanced lesion atherosclerotic morphology (Figure 3d). TIMP3 protein localization (Figure 3b) had a distribution that was similar to smooth muscle specific α-actin (Figure 3c) in medial and intimal smooth muscle cells of the non-atherosclerotic segments of coronary arteries. Although the TIMP3 protein was not stained as high as the α-actin signal, it was detected in all atherosclerotic plaque and normal arterial sections in areas of smooth muscle cells. On the other hand,

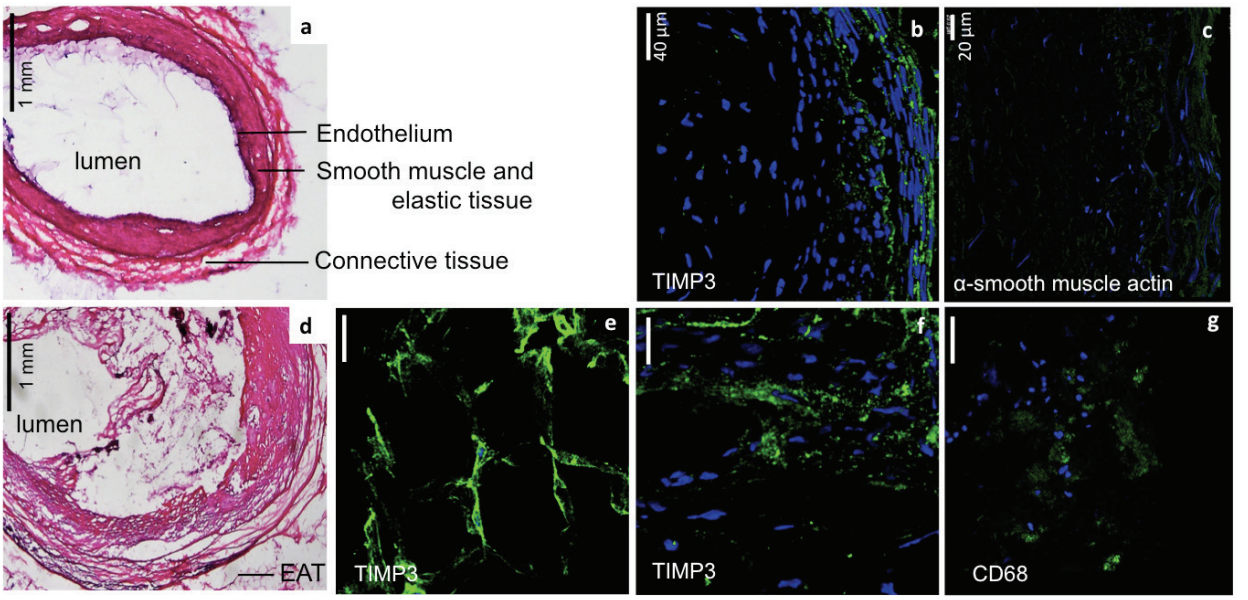

Figure 3: Immunostaining images of TIMP3 in EAT, normal and atherosclerotic artery of the post-mortem samples. Hematoxylin-Eosin (H-E) stained sections of normal artery (T-nonP group) (a) and artery with advanced atherosclerotic plaque (CHD-P) (d) were shown; pink shows the cytoplasmic area and blue represents the nuclei of cells in the artery sections. Post-mortem tissue samples were analyzed to determine specific proteins by staining Alexa Fluor 488 (green) and FITC (green) and also nuclei by staining DAPI (blue). TIMP3 was determined in normal arteries (b), in adipocytes of EAT (e) and in CHD-P (f). Smooth muscle specific α-actin was shown in normal arteryies (c). Macrophage specific CD68 was determined in CHD-P arteries (g)

in all sections obtained from CHD-P cases, the TIMP3 protein signals (Figure 3e and 3f, respectively) were observed at similar intensity than CD68 signal (Figure 3g) around the peri-coronary adipocytes and in macrophages-rich regions within fibrous plaques of advanced atherosclerotic arteries. Immunostaining results for localizations and distributions of all proteins were evaluated comparatively using serial sections obtained from the same tissue of each case.

DISCUSSION

In this study, evidence of reduced *TIMP3* expression in advanced atherosclerotic plaques when compared to histologically normal arterial segments obtained from post-mortem cases with coronary heart disease was demonstrated. In addition, TIMP3 protein was determined in macrophage-rich regions within fibrous plaques of atherosclerotic arteries. These results may indicate that higher macrophage density in advanced atheroma is associated with decreased *TIMP3* gene expression. Aso, although TIMP3 protein was shown around peri-coronary adipocytes for the first time in this study, it was determined that *TIMP3* expression levels in peri-coronary EAT samples and leukocytes had no effect on the severity of atherosclerosis.

Monocytes and macrophages have the crucial immunological roles in atherosclerotic plaque development (19). The circulating monocytes differentiate to macrophages, which migrate to the site of inflammation and disrupt extracellular matrix (ECM) barriers (20). In atheroma plaques, differentiated macrophages are most abundant cells that are formed by migration of circulating monocytes. Systemic insulin resistance and subclinical atherosclerosis was shown to be in association with decreased *TIMP3* expression in circulating monocytes (21). In this study, it was found that *TIMP3* expression levels did not show a statistically significant difference in circulating leukocytes between the plaque score groups. Since monocytes were not separated from peripheral blood leukocytes in this study, a similar association between expression levels of TIMP3 and severity of atherosclerosis could not be determined.

In studies on human advanced plaques, abundant macrophages were observed at the necrotic core and rupture-prone shoulders of the plaques (9). Decreased *TIMP3* expression was characterized by the proliferation of foam cell macrophages (FCMs), which are responsible for atherosclerotic plaque development (9, 10, 21, 22). Decreased *TIMP3* expression mainly was found in intimal macrophages that was previously reported to have higher MMP activities (9). In addition, decreased TIMP3 activity has been found to be associated with increased MMP9 level in the atheroma that is known to be a potential effect for plaque instability (23). In this study, as in previous results, *TIMP3* gene expression levels were decreased in atherosclerotic plaques and also TIMP3 protein was localized in macrophage-rich regions of atheroma. Elevated *TIMP3* expression level in rupture-prone sites of the atherosclerotic plaque has been claimed to play a protective role against plaque rupture (7, 24). In a previous study, TIMP3 protein levels in advanced human atheroma obtained from carotid endarterectomy have been shown to decrease compared to fibrous plaques with a small or absent lipid core (24). In this study, TIMP3 protein localization was confirmed by immunostaining in regions rich in SMC and macrophages. However, protein levels could not be compared between groups. Also, down regulated expression of *TIMP3* has been found in arterial tissues enriched with monocyte/macrophages of patients with metabolic and inflammatory diseases like Type 2 Diabetes Mellitus (10, 21). Other studies also support decreased *TIMP3* expression levels in macrophages differentiated to FCMs (9, 10, 22). Similar to these studies, in the post-mortem samples of this study, two fold decreased *TIMP3* expression was found in advanced atheroma plaque (CHD-P) compared to coronary artery segments without plaque (CHD-nonP). On the other hand, the cases in the T-nonP group were younger and had lower BMI than the cases with CHD. The reason for the lower *TIMP3* expression levels in the normal arteries of this young subject group (mean age 29.3 years) compared to the arteries of both CHD groups might be due to the early onset of the atherosclerotic process or to lifestyle and other unknown metabolic conditions.

It has been shown that EAT causes progression of atherosclerosis and finally cardiac complications (13, 14). EAT thickness and volumes are closely associated with coronary artery disease, metabolic syndrome and insulin resistance (14, 15, 22, 25-27). In addition, in post-mortem and clinical studies, increase in the size of adipose tissue around the atherosclerotic coronary artery was demonstrated (28-30). In the present study, the peri-coronary EAT volume in patients with CHD was higher than the non-atherosclerotic cases. In particular, adipose tissue has a crucial regulatory role for the development of atherosclerosis and provides micro-environmental homeostasis in vasculature (12). It has been reported that *TIMP3* expression decreases during adipocyte differentiation, but increases when cell differentiation is disrupted (11). In a study, it has been shown that overexpression of *TIMP3* in macrophages within white adipose tissue of transgenic mice protects from metabolic inflammation and is related to metabolic disorders such as diabetes and non-alcoholic steatohepatitis (31). However, the contribution of *TIMP3* expression level in peri-coronary EATs to the development of atherosclerosis is unknown. For the first time in this study, the relationship between *TIMP3* expression levels and atherosclerosis was investigated in peri-coronary EATs, but no statistically significant association in both post-mortem and living cases was demonstrated. In addition, TIMP3 protein using immunofluores-

cence technique in peri-coronary adipocytes surrounding advanced atherosclerotic arteries was demonstrated for the first time in this study. As a result, differences in TIMP3 expression profiles in adipocytes within peri-coronary EAT might not significantly contribute to the development of atherosclerosis.

Limitations

The coronary artery plaque samples from living cases using endarterectomy have not been collected for comparison with the post-mortem artery samples. *TIMP3* expression differences could not be investigated as monocytes were not isolated from circulating blood samples of living cases. In this study, data on medication use of the cases were not available. Other limitations of this study include heterogeneity of atherosclerotic plaque characteristics, difficulty of obtaining standard post-mortem tissues, and unknown lifestyles of post-mortem cases. Limitations of using post-mortem samples depend on many conditions such as storage time, temperature, time until it is frozen, pH, thawing, and pain level of death etc. Moreover, lifestyle of the cases also has effects on results such as physical exercise, legal/illegal substance use, and their diet (32). In this study, after the autopsy, a detailed death report was examined, extent of eligibility criteria was assessed, and standard time was applied for tissue supply. The number of young post-mortem non-cardiac trauma cases investigated is a major limitation of this study, and the *TIMP3* expression result of this group could not be discussed since there is no comparison publication on *TIMP3* expression levels in arterial tissues of different age groups. And also, interactions of TIMP3 with other MMPs could not be investigated in this study.

CONCLUSION

TIMP3 expression is significantly decreased in advanced atherosclerotic plaques. Increased *TIMP3* expression levels in normal coronary arterial segments of cases with CHD indicate that TIMP3 plays a protective role in development of atherosclerosis at the molecular level in these arterial areas. Although the association of *TIMP3* expression levels with the severity of atherosclerosis in circulating leukocytes and peri-coronary epicardial adipose tissue could not be demonstrated in this study, in the future, determination of differences in *TIMP3* expression and other interacting extracellular matrix proteins in circulating monocytes and other vascular cells involved in atheroma plaque development will help to understand atherosclerotic pathogenicity. Finally, in this study, the *TIMP3* expression level, which has therapeutic significance, on different types of tissues and cells was investigated with the same workflow, and these results were presented. The balance between MMPs and their inhibitors might be possible with exogenous administration of TIMP3 to ensure plaque stability in advanced atheroma plaque arteries in the future.

Ethics Committee Approval: TThis study was approved by the-Clinical Research Ethical Committee of the Istanbul University, Istanbul Faculty of Medicine (Date: 27.10.2011, No: 1822 and Date: 17.01.2014, No: 160) and Scientific Research Commission of Council of Forensic Medicine (Project number and date: B.03.1.ATK.0.01.00.08/863, 28.12.2010).

Informed Consent: Written consent was obtained from the participants.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- E.K.B., C.E.Y., M.Y.; Data Acquisition- F.G.G., D.Y., D.Ö., C.E.Y., M.Y., D.Ö., M.C., E.K.B.; Data Analysis/Interpretation- G.Ç., E.K.B., M.C., C.E.Y., M.Y.; Drafting Manuscript- G.Ç., E.K.B.; Critical Revision of Manuscript- E.K.B., M.C., C.E.Y., M.Y.; Final Approval and Accountability- G.Ç., F.G.G., D.Y., D.Ö., C.E.Y., M.Y., D.Ö., M.C., E.K.B.

Conflict of Interest: Authors declared no conflict of interest.

Financial Disclosure: The present work was supported by The Research Fund of Istanbul University (Project no: TSA-2016-21496 ve 28473).

Acknowlegment: We are grateful to Dr. Sema Bilgic for her contribution in the application of cryosection method.

Etik Komite Onayı: Bu calışma icin etik komite onayı İstanbul Universitesi, İstanbul Tıp Fakultesi Klinik Araştırmalar Etik Kurulu (Tarih: 27.10.2011, No: 1822 and Tarih: 17.01.2014, No: 160) ve Adli Tıp Kurumu Bilimsel Araştırma Komisyonu'ndan (No: B.03.1.ATK.0.01.00.08/863, 28.12.2010) alınmıştır.

Bilgilendirilmiş Onam: Katılımcılardan bilgilendirilmiş onam alınmıştır.

Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Çalışma Konsepti/Tasarım- E.K.B., C.E.Y., M.Y.; Veri Toplama- F.G.G., D.Y., D.Ö., C.E.Y., M.Y., D.Ö., M.C., E.K.B.; Veri Analizi/Yorumlama- G.Ç., E.K.B., M.C., C.E.Y., M.Y.; Yazı Taslağı- G.Ç., E.K.B.; İçeriğin Eleştirel İncelemesi- E.K.B., M.C., C.E.Y., M.Y.; Son Onay ve Sorumluluk- G.G., F.G.G., D.Y., D.Ö., C.E.Y., M.Y., D.Ö., M.C., E.K.B.

Çıkar Çatışması: Yazarlar çıkar çatışması beyan etmemişlerdir.

Finansal Destek: Bu çalışma İstanbul Üniversitesi Bilimsel Araştırma Projeleri Koordinasyon Birimi tarafından ile desteklenmiştir (Proje no: TSA-2016-21496 ve 28473).

Teşekkür: Donmuş doku kesit alınmasında teknik desteklerinden ötürü Dr. Sema Bilgiç'e teşekkür ederiz.

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