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Serum Endothelin-1, MMP-9, and Myeloperoxidase and Coronary Artery Morphology as Detected by Multi-Slice CT Angiography in Intermediate and High Risk Asymptomatic Subjects

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Abstract: *Objectives*: To study possible correlation between Endothelin-1, Matrix-metallo-proteinase- 9, and Myeloperoxidase serum activity and coronary CT angiography findings of atherosclerotic coronary artery disease in intermediate - high risk asymptomatic subjects.

Methods: 65 consecutive asymptomatic subjects, 53 males and 12 females, mean age 58.8 +/-10.9 s.d., referred by their primary practitioner for the evaluation of coronary artery atherosclerotic disease by Cardiac CT exam were selected for the study. One serum sample was drawn from each participant and examined for the levels of Endothelin -1, MMP-9, MPO serum activity. Obstructive coronary artery disease was defined as at least 50 percent stenosis of one or more coronary segments. Plaques were identified as calcified or soft according to the CT Hounsfield attenuation number.

Results: The Endothelin activity level was significantly elevated in subjects with significant obstructive coronary artery disease (p=0.022). Furthermore, high Endothelin activity level was found to correlate with calcified plaque burden (p=0.006), and to be an independent determinant of lesion severity (R^2 = 0.124**,** R^2 change = 0.006, p= 0.008**)**

Conclusions: Endothelin-1 may be a marker of significant obstructive coronary disease and calcified plaque burden in asymptomatic subjects. The significance of MMP-9 and MPO as markers of atherosclerosis in such subjects has not been definitely clarified.

Keywords: Endothelin 1, metalloproteinase 9, myeloperoxidase, coronary atherosclerosis.

INTRODUCTION

Coronary artery disease (CAD) is one of the leading causes of death worldwide and it is expected that the rate of CAD will accelerate in the next decade due to overall aging of population and increases in the prevalence of cardiovascular risk factors (type 2 diabetes, obesity, metabolic syndrome) in younger generations [1].

Early detection of CAD is of paramount importance for initiating aggressive control of risk factors (smoking cessation, lipid lowering therapy, weight reduction therapy, aggressive therapy for hypertension) and establishing of pharmacologic therapy in order to reduce occurrence of life threatening acute cardiovascular events (anti platelet agents, statins).

The accepted modes for diagnosis of stable coronary artery disease include exercise electrocardiography, stress nuclear myocardial perfusion imaging and dobutamine stress echocardiography. The main disadvantage of these tests is related to the relatively low positive and negative predictive values, so in many cases there is a need for direct assessment of coronary artery anatomy. The gold standard for such an assessment is coronary angiography; however the risk benefit ratio of coronary angiography might be unacceptable for many intermediate or high risk asymptomatic individuals in whom there is a need for some additional diagnostic assessment of coronary artery status. In recent years a new hope has risen from development of "virtual" coronary angiography by multidetector computed tomography (MDCT). Several studies have shown that MDCT angiography of coronary arteries has a highly acceptable sensitivity and specificity as compared with conventional angiography [2, 3]. However, the major withdrawals of this technique are the high X –ray exposure of the patient and high economic cost, so nowadays this technique is not applicable as a screening test for coronary atherosclerosis.

To sum up, there is an obvious need for an additional diagnostic method that would be safe, inexpensive and sensitive enough to become a reliable screening test for the presence of coronary atherosclerosis in broad range of patients.

In recent years, there is ongoing research by different groups of investigators trying to delineate the role of different biologically active substances in the pathogenesis of progression of atherosclerotic coronary artery disease.

Endogenous matrix metalloproteinases (MMPs) are important mediators of extracellular matrix remodelling, which is a crucial process in the pathophysiology of atherosclerotic plaque development and progression from stable to unstable

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plaque [4] There is evidence that circulating levels of MMP-9 are higher in patients with stable coronary artery disease compared to healthy controls [5].

Moreover, it has been shown by several groups of investigators that there is positive correlation between levels of MMP-9 and morphology of atherosclerotic plaques with patients with vulnerable or ruptured plaques as defined by coronary angiography and intravascular ultrasound, having significantly higher circulating levels of MMP-9 than patients with stable plaques [6,7].

Myeloperoxidase (MPO) is a peroxidase enzyme most abundantly present in neutrophil granulocytes where it is stored in azurophilic granules .

It has been shown that Apo A-1, the main apolipoprotein of HDL, is subjected to nitration by myeloperoxidase (MPO) and this oxidative modification renders HDL proatherogenic. There is evidence that plasma levels of MPO is higher in patients with chronic coronary artery disease and there is strong correlation between the levels of MPO and the burden of coronary atherosclerosis as assessed by Gensini scores which are determined by angiography and coronary calcium scores which are determined by CT [8].

Moreover it was shown that higher levels of MPO are correlated with progression from stable coronary artery disease to acute coronary syndromes [9].

Lastly, it was demonstrated that MPO may be an important prognostic factor in patients presenting with chest pain, with higher levels indicating higher risk of myocardial infarction, the need for revascularization and death [10].

Endothelins are a family of peptides, which comprises endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3). ET-1 is a peptide secreted mostly by vascular endothelial cells, it is the predominant isoform expressed in vasculature and the most potent vasoconstrictor currently known. ET-1 also has inotropic, chemotactic and mitogenic properties. In addition, it influences salt and water homeostasis through its effects on the renin-angiotensin-aldosterone system (RAAS), vasopressin and atrial natriuretic peptide and stimulates the sympathetic nervous system. The overall action of endothelin is to increase blood pressure and vascular tone.

Several studies demonstrated that circulating levels of ET-1 are higher in patients with cardiovascular risk factors (diabetes mellitus, hypertension, obesity) compared to healthy controls [11, 12].

One small clinical study determined that ET-1 levels are increased in patients with coronary artery disease compared with controls and are correlated with the clinical severity of the disease with patients suffering from acute myocardial infarction having the highest level and patients suffering from stable angina pectoris having the lowest [13].

Moreover, it was demonstrated that high level of ET- 1 is an independent predictor of 30 day major clinical adverse outcomes (including severe congestive heart failure and mortality) in patients undergoing primary coronary percutaneous intervention (PCI) [14].

In this study, we have sought to find out whether there is any correlation between levels of ET-1, MPO, and MMP-9 in patients with cardiovascular risk factors or patients with known stable coronary artery disease referred for MDCT of coronary arteries and morphologic findings as shown by the virtual coronary angiography, in order to determine if any of the biomarkers might be a suitable marker for the existence of coronary artery disease and for the identification of high risk patients with vulnerable plaques.

SUBJECTS AND METHODS

Subjects

Participants were recruited from The Diagnostic Radiology Service of Bnai Zion Medical Centre. A total of 65 consecutive, asymptomatic subjects who were referred for performance of coronary MDCT by their primary practitioners were included. All subjects were considered to be intermediate to high risk, or high risk candidates for clinically overt CAD due to their coronary risk factors or evidence of previous clinical manifestation of CAD. One sample of peripheral venous blood and clinical information was obtained for all participants at the time of recruitment. Informed consent was obtained from each study participants and the study was approved by the institutional ethical committee.

Study participants were between 38 and 85 years of age with a mean of 58.8 ± 10.9. Eighty two percent (53 out of 65) of the patients were males, 18 percent (12 out of 65) Only 9 subjects had an evidence of a previous clinical manifestation of CAD (2 patients underwent CABG and 7 underwent PCI in the past), 46% (n=30) of the patients had hypertension, 51% (n=33) had hyperlipidemia, 18% (n=12) had diabetes mellitus type 2, 20% (n=13) were obese, and 15% (n=10) of them were current smokers (Table **1**).

Table1. Characteristics of the Study Cohort

No. of Patients	**65**
Age (mean ± SD)	58.8 ± 10.9
Male (%)	82
Patients with known CAD (%)	14
Hypertension, (%)	47
Hyperlipidemia, (%)	50
Smoking, (%)	15
DM, (%)	18
Obesity, (%)	20
% of coronary segments with ≥ 50% lesions	8.6
% of coronary segments with ≤ 50% lesions	91.4
Mean Calcium score	308.9
Patients with ≥ 1 soft plaque (%)	55
Patients with ≥ 1 calcified plaque (%)	83

MDCT Scan Protocol

Subjects received a β-blocker two hours prior to the examination (atenolol 50 to 100 mg orally, based on body mass) if resting heart rate exceeded 70 beats per minutes (bpm). All subjects were in sinus rhythm. The heart rate of all subjects ranged between 50 and 70 bpm with or without

premedication. Coronary computed tomography angiography (CCTA) was performed using 64- row MDCT scanner (VCT, General Electric Medical systems, Milwaukee, WI) and was begun at the level of the aortic root above the coronary ostia and included the entire hear. Each patient first underwent a native scan without contrast media to determine the total calcium burden of the coronary tree. Calcified lesions were identified on the basis of a threshold of 130 H for all applied scoring methods. Coronary stents were identified and excluded during the scoring process. Scores for a normalized Agatston score and calcium mass were obtained [15]. Scanning parameters applied for contrast media enhanced examinations: detector collimation, 64 x 0.625mm; kVp, 120; mAs, 500; pitch range 0.2-0.29; gantry rotation time 0.35; slice thickness, 0.6 mm. ECG modulation was used in all CCTA examinations (ECG pulsing). A bolus of 70-90 ml Iomeron 400 (iomeprol) [(400mgI/ml, Bracco, Milan, Italy)] was intravenously injected (4 ml/s) followed by a bolus of 40 ml of saline. Scan delay was determined according to the Smart Prep program (automatic bolus test; the region of interest was placed on the ascending aorta). The subjects were instructed to maintain an inspiratory breath hold during which the CT data and ECG trace were acquired.

Image Reconstruction

Image reconstruction was done using the retrospective electrocardiographic gating method. Datasets were acquired at phases 40%, 70%, and 80% of the R-R cycle. Other window positions within the cardiac cycle were reconstructed when unsatisfactory results were achieved. The image data sets were processed on a separate workstation (ADW 4.6, General Electric Medical systems, Milwaukee, WI) and analyzed using curved multi-planar reconstruction (MPR) in various planes, thin-slab maximum intensity projection (MIP) and 3D-volume rendered (VR) reconstructions, in addition to the axial source images. Coronary arteries were consensually reviewed by two experienced radiologists and a cardiologist.

Biomarkers' Level Testing

Circulating levels of ET-1 and MMP-9 were determined by ELISA method utilizing R&D Systems Kit (R&D Systems Inc. Minneapolis, MN, USA). The levels of MMP-9 were measured in ng/ml, and the levels of ET-1 were measured in pg/ml. Extracted EDTA plasma was used for measurement. The minimum detectable dose of ET-1 in this test is typically less than 1.0 pg/Ml. The assay recognizes both synthetic and natural human ET-1, and limited levels of cross reactivity (<1%) was found with the Big Endothelin. MMP-9 was determined using heparinized platelet free plasma following appropriate dilution. The minimum detectable dose of MMP-9 is less than 0.156 ng/mL. The assay recognizes recombinant and natural human MMP-9 without significant cross-reactivity or interference.

Circulating levels of MPO were determined by ELISA method utilizing the Immunodiagnostic Ag Kit. The levels of MPO were measured in ng/ml.

Morphologic Characteristics of Atherosclerotic Plaques

We defined obstructive coronary disease as at least 50 percent stenosis of one or more coronary segments. Plaques associated with CT Hounsfield attenuation number of 130 or more were identified as calcified plaques. On the other hand, plaques associated with CT Hounsfield attenuation number of 121 or less were identified as soft plaques. Characteristics of coronary artery disease morphology of our cohort as defined by MDCT are shown in Table **1**.

Statistical Analysis

Statistical analysis was performed using the SPPS (version 10.1 SPPS, Inc., Chicago, Illinois) statistical package.

Several variables were not normally distributed, therefore the Mann Whitney non – parametric test was used for comparisons between patients group stratified by presence or absence of cardiovascular risk factors, presence or absence of obstructive coronary artery disease, presence or absence of soft plaques.

Spearman's correlation was used to define correlation between number of calcified, soft, and combined calcified and soft plaques, and the levels of biomarkers.

The role of ET-1, MMP-9, and MPO as possible independent determinants of lesion severity was assessed using multiple regression analysis.

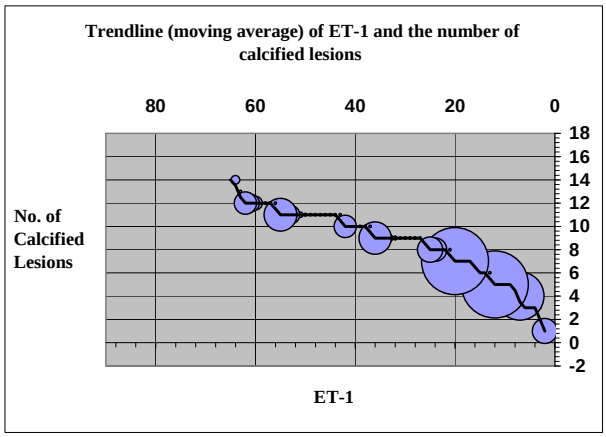

Fig. (1). ET-1 levels and plaque burden (No. of calcified lesions).

RESULTS

A total of 666 diseased segments were found among the 65 subjects studied. Fifty seven (8.6%) segments were significant, ≥50% lesions and 609 (91.4%) segments were mild, ≤50% coronary lesions (Table **1**). Seventy six lesions were described as soft plaques, 11 as calcified only, and 251 as combined lesions, calcified and soft. Most (92.4%) of the minor lesions (irregularities or <25% stenosis) were not characterized as calcified or soft plaques. The mean calcium score of the study group was 308.9+/-106 s.d.

Mean serum level of MMP-9 was 398.9 ng/ml+/-129/9s.d., mean ET-1 was 8.37p/ml +/-22.3, and MPO level was 325.5 ng/ml +/- 257.5 s.d.

Correlation between morphologic characteristics of the atherosclerotic plaque, cardiovascular risk factors, sex and serum levels of endothelin-1, myeloperoxidase and MMP-9 (Tables **2**,**3**,**4**).

Analysis of the levels of three markers stratified for the presence or absence of soft plaques, obstructive coronary

Table 2. Serum Levels of Endothelin 1 Stratified by the Presence or Absence of Obstructive Coronary Artery Disease, Soft Plaques, Hypertension, Hyperlipidemia, Diabetes, Smoking and Male Sex

Plaque morphology/ Cardiovascular risk factors	Absent No. ET-1 (mean rank, pg/ml)	Present No. ET-1 (mean rank, pg/ml)	p
Obstructive coronary disease	52 29.62	13 46.54	0.002
Soft plaques	29 32.50	36 33.40	0.840
Male sex	12 35.25	53 32.49	0.631
Smoking	55 33.23	10 31.75	0.811
Hyperlipidemia	30 32.10	35 33.77	0.708
Hypertension	33 34.12	32 31.84	0.609
Diabetes Mellitus	53 31.78	12 38.38	0.251

Table 3. Serum Levels of MMP-9 Stratified by the Presence or Absence of obstructive Coronary Artery Disease, Soft Plaques, Hypertension, Hyperlipidemia, Diabetes, Smoking and Male Sex

Plaque morphology/ Cardiovascular risk factors	Absent No. MMP-9 (mean rank, ng/ml)	Present No. MMP-9 (mean rank, ng/ml)	p
Obstructive coronary disease	52 31.77	13 37.92	0.294
Soft plaques	29 32.88	36 33.10	0.963
Male sex	12 28.29	53 34.07	0.339
Smoking	55 31.05	10 43.75	0.051
Hyperlipidemia	30 32.97	35 33.03	0.990
Hypertension	33 35.30	32 30.63	0.319
Diabetes Mellitus	53 31.20	12 40.96	0.106

Table 4. Serum Levels of MPO Stratified by Presence or Absence of Obstructive Coronary Artery Disease, Soft Plaques, Hypertension, Hyperlipidemia, Diabetes, Smoking and Male Sex

Plaque morphology/ Cardiovascular risk factors	Absent No. & MPO (mean rank, ng/ml)	Present No. & MPO (mean rank, ng/ml)	p
Obstructive coronary disease	52 33.41	13 31.35	0.724
Soft plaques	29 32.28	36 33.58	0.782
Male sex	12 24.54	53 34.92	0.086
Smoking	55 31.27	10 42.50	0.084
Hyperlipidemia	30 30.63	35 35.03	0.350
Hypertension	33 32.06	32 33.97	0.684
Diabetes Mellitus	53 32.94	12 33.25	0.960

disease (defined as stenosis of more than 50% in at least one segment as determined by CT Angiography), age, male sex, hypertension, hyperlipidemia, diabetes and smoking revealed that there is a statistically significant correlation between the presence of obstructive coronary artery disease and serum levels of ET-1 (Table **2**).

There was a trend for positive correlation between smoking and serum levels of MMP-9, which didn't reach a statistical significance (p=0.051, Table **3**).

Lastly, there was no correlation between any clinical or demographic parameter of our patients and the serum levels of MPO (Table **4**).

By using Spearman's rank correlation, we found no correlation between age and serum levels of any marker ($p = 0.152$ for ET-1, $p=0.195$ for MPO, and $p=0.371$ for MMP-9).

Correlation between calcified plaques burden and levels of biomarkers:

By using Spearman's rank correlation, we found positive correlation between calcified plaques burden (which was defined as a sum of calcified plaques for each patient) and levels of ET-1 ($R=340$, $p=0.006$).

When a parametric test was done for the calcium score, using the Pearson's correlation, then a strong correlation was found between ET-1 and calcium score, $r = 0.87$, $p < 0.01$.

The role of ET-1, MMP-9, and MPO as possible independent determinants of lesion severity using multiple regression analysis:

Regression analysis model of factors affecting calcified plaque burden and soft plaques presence revealed that only ET-1 serum level and older age (>60 years) can be considered independent determinants of calcified plaque burden, $R^2 = 0.124$**,** and 0.131, R^2 change = 0.006, and 0.007, $p= 0.008$, and 0.005, B (SEM) = -0.011, and -0.142, Beta = -0.080, and -0.111, $p= 0.007$ and $p<0.001$ respectively.

DISCUSSION

In our cohort of asymptomatic subjects at high risk for angiographic evidence of obstructive coronary artery disease, a statistically significant correlation between serum levels of ET-1 and calcified plaque burden and calcium score as defined by MDCT was found. Among the three biomarkers tested, only ET-1 serum level (along with older age, >60 years) can be considered an independent determinant the of calcified plaque burden.

It has found that this association is independent of any of the other risk factors which were assessed.

Our findings are in concordance with findings of several previous studies.

Unlike ET-1, we were unable to determine any correlation between circulating levels of MMP- 9 and existence of obstructive coronary artery disease, burden of calcified plaques or existence of vulnerable soft plaques which is in contrast to results of previous two studies that showed positive correlation between existence of vulnerable plaques as defined by coronary angiography and IVUS (intravascular ultrasound) and circulating levels of MMP-9 (6.7). One possible explanation for this discrepancy may be the fact that definition of soft plaques by MDCT coronary angiography is not sensitive and accurate enough in comparison with coronary angiography and IVUS.

Lastly, we didn't find any correlation between MPO levels and coronary atherosclerosis, which, again, is in contrast to findings of one previous study that demonstrated a positive correlation between levels of MPO and burden of coronary atherosclerosis. One possible reason for this discrepancy may be the fact that we had a very low number of patients with significant coronary artery disease in our study cohort.

Coronary artery calcium score especially when combined with the Framingham score is accepted for risk prediction in intermediate and high risk asymptomatic individuals [16,18]. In a study comparing coronary calcium score to carotid intima-media thickness in asymptomatic patients aged 45-84 years, it has been found that after adjustment for traditional risk factors, the hazard ratio of cardiovascular disease was increased by 2.1-fold for each 1-standard deviation increment of log-transformed CAC score [17].

In view of study findings, the strong correlation between serum endothelin-1 level the calcified plaque burden, and calcium score, and endothelin-1 being an independent determinant of lesion severity, we suggest possible use of ET-1 as a possible risk assessment tool along with the traditional risk factors in asymptomatic subjects.

In summary, we found positive correlation between circulating levels of endothelin-1 and coronary atherosclerosis. There is a need for further large scale studies aiming to define feasibility and value of measuring of ET-1 as a screening method for the presence of significant angiographic coronary artery disease and for risk prediction in asymptomatic subjects.

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