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Original Article

Increased Circulatory Levels of Ischemia Modified Albumin, Protein Carbonyl, Malondialdehyde and Total Antioxidant Capacity as Prognostic Biomarkers for Non-ST-segment Elevation Myocardial Infarction: A ROC Curve Analysis

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Abstract
Objectives: Oxidative stress by cardiac ischemia causes protein modifications in serum albumin to create Ischemia Modified Albumin (IMA) and other circulatory proteins to create protein carbonyl (PC), whose increase from the baseline could be used as a diagnostic tool for cardiac ischemia disease. We aimed to evaluate the differentiation of IMA, PC, malondialdehyde (MDA) and total antioxidant capacity (TAC) between patients with early stage non-ST-segment elevation myocardial infarction (NSTEMI) and healthy control subjects and test their sole and integrative recognition efficiency as rapid predictive biomarkers for NSTEMI.
Materials and Methods: We selected 52 patients of both sexes with the diagnosis of NSTEMI within 6 hours. Fifty-two healthy individuals without significant differences in sex and age having normal cardiac troponin I (cTnI) levels were enrolled as control group. Serum samples were collected and IMA, PC, MDA, and TAC levels were quantified and then, their findings were compared with serum cTnI levels as the "gold standard".
Results: Linear regression, correlation, and receiver operating characteristic (ROC) curve analyses showed that both circulatory levels of PC and IMA were statistically elevated in NSTEMI patients compared to control group, and both had statistically high sensitivity and specificity for rapid prediction of NSTEMI. Combinatorial determination of both biomarkers increased the test specificity and negative predictive value (NPV).
Conclusions: Both PC and IMA contents could be used as early biomarkers for diagnosis of NSTEMI and integrative determination of both biomarkers could be used in emergency departments for the fast diagnosis of NSTEMI.
Keywords: Ischemia modified albumin, Protein carbonyl, Biomarker, Non-ST-segment Elevation myocardial infarction.

Introduction

As the leading cause of global death, cardiovascular diseases (CVDs) caused 30% of global death in 2008 (1). Depending on the mechanism of action and location of confliction, CVDs have various types, the most serious of which with the highest morbidity and mortality is coronary artery disease (CAD) that is also mentioned as coronary heart disease (CHD) and ischemic heart disease (IHD) (2). It is responsible for about 12.2% of worldwide deaths in developed and semi-developed countries (3).

CADs are divided into myocardial infarction (MI or more commonly known as heart attack) and angina, however, MI has a higher incidence. Main risk factors for coronary artery diseases are hypercholesterolemia, hypertension, inadequate physical activity, smoking,

obesity, and some disorders like diabetes, among others (4,5). Along with clinical assessments such as a history of chest pain and interpretation of electrocardiogram with/without coronary angiogram, blood tests such as cardiac troponins T and I (cTnT and cTnI) without/with creatine kinase MB (CK-MB) are the main clinical tests that are currently used to diagnose CADs (6). Elevation in electrocardiogram led to the classification of myocardial infarctions to ST-segment Elevation Myocardial Infarction (STEMI) and non-alteration in electrocardiogram as non-ST-segment elevation myocardial infarction (NSTEMI) (7). It has been suggested that NSTEMI had a higher prevalence than STEMI with very similar 6-month mortality (8-11). Although the infarct-related artery is almost totally occluded by clots in STEMI, its occlusion in

NSTEMI is partial (12).

Although cardiac troponins have high sensitivities and specificities to diagnose CADs, their detectable increase in serum delays about 6 hours after the attack and this could retard early lifesaving diagnosis of heart diseases (13,14). Therefore, introducing biomarkers that reflect early-phase injury of the cardiac cell could support more quick diagnosis and initiation of medical interventions and treatments.

Cardiac ischemia causes oxidative stress and reactive oxygen species (ROS) are one of the main cell-damaging products of oxidative stress that could cause subsequent modifications in nucleic acids, lipids and proteins (15). Changes in malondialdehyde (MDA) (16), total antioxidant capacity (TAC) (17), protein carbonyl (PC) as well as ischemia-modified albumin (IMA) (18-20) are among the modifications that are induced by ischemia, and due to their early onsets in ischemic heart diseases, they could be used as early biomarkers of ischemia and CADs (21-23).

The objective of this study was to evaluate PC and IMA as biomarkers for medical diagnosis of NSTEMI and test capability of increasing diagnosis efficiency by combining test results of these two biomarkers.

Materials and Methods
Study Population
Fifty-two patients (26 males and 26 females) who attended the emergency room within 6 hours after the diagnosis of NSTEMI were chosen and a quick blood sampling was performed. To minimize interfering factors, we chose 52 healthy individuals (26 males and 26 females) with normal cTnI levels as the control group. The inclusion criteria included a complaint of chest pain and diagnosis of NSTEMI by cardiologists of the hospital within 6 hours. Touchstones for exclusion from this study included the presence of other diseases like stroke, malignancy, muscle injury, trauma, cirrhosis, renal diseases, infectious diseases, serum albumin levels below 2 g/dL, patients with complaints lasting more than 6 hours, hypertension, diabetes mellitus, and patients who were unable to relate the time of beginning or ending their symptoms. Patients complaining of chest pain and dyspnea were referred to serum cTnI measurement. Serum cTnI levels ≤0.05 ng/L were diagnosed as unstable angina (UA) and did not enter this study. Then electrocardiography was carried out on the patients who had cTnI levels above 0.05 ng/L (MI patients), and the individuals who did not have elevated ST segment were enrolled in the current study as NSTEMI patients.

Sample Collection and Storage
Mediplus tubes (Sunphoria Co. Ltd., Taiwan) containing a clot activator were used to collect blood samples. After 10 minutes of incubation at room temperature for coagulation, serum was separated by centrifugation for 7 minutes at 10 000 rpm and kept frozen in −80°C.

Measurement of Ischemia Modified Albumin
We used a spectrophotometric method based on the changes in cobalt binding properties of serum albumin after ischemia-induced conversion to IMA, introduced by Bar-Or et al (20). This albumin cobalt binding (ACB) test is rapid and uses just 200 μL of human serum to measure IMA content. First of all, we prepared a stock solution of 0.1% cobalt (II) chloride 6-hydrate (CoCl2) (product no. 7791-13-1, Alfa Aesar, Karlsruhe, Germany). Frozen serum samples were thawed and vortexed gently, and 200 μL of it was instilled with 50 μL of stock solution of 0.1% CoCl2. Then, the mixture was gently mixed and incubated for 10 minutes at room temperature. During this process, the Co (II) ions bind to the N-terminus of albumin but not altered N-terminus of ischemia modified albumin (IMA). Then, 50 μL of Dithiothreitol (DTT) (product no. D9779, Sigma–Aldrich Chemie, Steinheim, Germany) was added as colorizing reagent that bound to unbound Co(II) ions and was incubated again at room temperature for 120 seconds. Then, 1000 μL of 0.9% NaCl was added to stop the reaction. For each sample (test and control), we used a mixture of serum plus CoCl2 as a serum blank. The absorbance of the mixture (that is the absorbance of unbound cobalt after binding to free albumin) was then measured against serum blank at 470 nm by spectrophotometry (Spekol 2000, Analytik Jena, Germany). Each serum sample was measured three times to get a mean and within-run coefficient of variation (CV) for each sample. Following out the procedure introduced by Bar-Or et al in 2000 (20), the values above 0.400 absorbance units (ABSU) were considered as positive and values under or equal to that were considered as negative results for the ischemia.

Measurement of Protein Carbonyl
The old colorimetric method for PC analysis based on the conversion of 2,4-dinitrophenylhydrazine (DNP) to the blue colored complex of dinitrophenylhydrazone by the effect PC groups introduced separately in 1994 by Levine et al (24) and Reznick et al (25) has low reproducibility and sensitivity and was then replaced by high sensitive enzyme-linked immunosorbent assay (ELISA) method presented by Buss et al in 1997 (26, 27) that was later commercialized by Zenith Technology Corporation and then Northwest Life Science Specialties.

PC content of the samples in this study was measured using a high sensitive (intra-assay and inter-assay variation variations are both as low as 5% and sensitivity of the test was 0.1 nmol/mg) ELISA method (Northwest Life Science Specialties, Vancouver, WA, USA; Product ID: NWK-PCK01). Briefly, EIA Buffer (reagent C), Blocking reagent (reagent D), Dinitrophenylhydrazine (DNP) (reagent E), Anti-DNP-biotin-antibody (reagent G), Streptavidin-horseradish peroxidase (reagent H), chromatin reagent

(reagent I), stopping reagent (reagent J), oxidized protein standards (reagent K1-6), and carbonyl control sample (reagent L) were prepared following the instructions of the kit. 200 μL of diluted DNP solution was added to pre-labeled 1.5 mL reaction tubes of standards, carbonyl controls and samples. Then 5 μL of each standard, control or sample was poured and left for 45 minutes at room temperature. 200 μLl of each standard, control or sample was added to the assigned ELISA wells, sealed and left for 120 minutes at 37°C. The plate was then washed five times with approximately 300 μl per well of EIA Buffer. Then 250 μL of diluted blocking solution per well was added and left for 30 minutes at room temperature. The washing process was repeated 5 times with EIA buffer. 200 μl of diluted anti-DNP-biotin-antibody was added to each well and waited for 1 hour at 37°C, ensuing by washing process as above. 200 μl of diluted streptavidin-horseradish-peroxidase was poured, incubated for 1 hour at room temperature, and washed as above. For color Development, 200 μl of chromatin reagent was added per well and incubated for 5-10 minutes at room temperature. By using 100 μl of stopping reagent, the reaction was terminated and read at 450 nm. The standard regression curve was obtained by plotting the PC concentrations of K1-K6 against their absorbances.

Measurement of cTnI

Chemiluminescent analyzer (Architect i1000 SR, Abbott, USA) that uses commercial kits (Pars Azmoon, Tehran, Iran) was applied to measure cTnI levels.

Measurement of MDA, TAC and Other Circulating Substances

Serum MDA levels of the patient and control groups were measured by the methods of Placer et al (28) and Buege and Aust (29), which are based on the reaction of MDA with thiobarbituric acid (TBA) in acidic environment and measurement of the resulted pink product by photometry at 535 nm, which was then divided by 1.56×10^5 M^{-1} cm^{-1} as extinction coefficient and reported as μmole/liters. TAC levels were estimated by Benzie's "The Ferric Reducing Ability of Plasma (FRAP)" method (30), which is described as the reduction of colorless ferric tripyridyltriazine (Fe^{III}-TPTZ) complex to the intense blue colored complex of ferrous tripyridyltriazine (Fe^{II}-TPTZ) in hydrochloric acid solution, measurement of optical densities (ODs) at 593 nm, and calculation of TAC concentrations of unknown samples against an standard curve drawn by OD measurements of serial dilutions (0.25, 0.5, 1 and 2 mM) of ferrous sulfate ($FeSO_4.7H_2O$). To determine soluble analytes we used an auto analyzer system (Autolab, BT 3500, Autoanalyzer Medical System, Rome, Italy) available in the clinical laboratory of the hospital using commercial kits (Pars Azmoon, Tehran, Iran).

Statistical Analysis

SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) as well as Microsoft Excel version 2013 (Microsoft Corp., Redmond, WA, USA) and XLSTAT Version 2016.02.28451 (Addinsoft, Paris, France) was used for statistical analysis and plotting graphs. Although the sample size was big enough to admit normal distribution of the patient and normal control groups, a confirmatory Kolmogorov–Smirnov test (K-S test) was also done. Two-tailed P values below 0.005 were considered statistically significant. Correlation between biomarkers was estimated using Pearson correlation coefficient. Receiver operating characteristic (ROC) curve and area under the curve (AUC) values were used to determine the sensitivity and specificity (with 95% CI) of the changes in AMI and PC as a discriminative predictor of NSTEMI.

Results

Subject Characteristics

Clinical data for the patient and healthy control groups are summarized in Table 1. No significant differences were seen in age, sex, BMI, and serum analytes like sodium, potassium, creatinine, glucose, cholesterol, TG, and ALP, HDL, AST, LDL, TSH, and FT4 levels. There were higher serum concentrations of CKMB and Urea in the NSTEMI group, compared to the healthy control group.

Serum Level of Soluble IMA, PC, MDA, and TAC in NSTEMI Patient and Normal Healthy Groups

Figure 1 shows the comparisons of the results of IMA, PC, cTnI, CKMB, MDA, and TAC between NSTEMI patient and healthy control groups (mean ± SD). The patients attended the emergency room within 6 hours (3.52 ± 1.86) after the diagnosis of NSTEMI. To determine the intra-assay coefficient of variation (CV) for IMA test, we performed all analyses (totally 104 tests, 52 patients and 52 controls) in triplicate. Similarly, to find out intra-assay CV, we accomplished 10 runs of triplicate determination of IMA for one of the samples. The intra-assay CV was 6.545% and the inter-assay CV was 7.38%, which means acceptability of the test.

Table 2 summarizes circulatory concentrations of IMA and PC in patient and healthy individuals.

Circulatory IMA values were significantly elevated in NSTEMI (0.83 ± 0.33 ABSU) compared to normal individuals (0.39 ± 0.23 ABSU) ($P < 0.001$). Serum PC concentrations were also statistically higher in NSTEMI patients (6.77 ± 2.37 nmol/mg) compared to normal healthy people (3.40 ± 2.01 nmol/mg) ($P < 0.001$).

cTnI (ng/L) levels that were used as gold standard were 26.43 ± 59.17 and 0.136 ± 0.141 ng/L, respectively ($P = 0.002$). CKMB (IU/L) levels were also elevated in the serum of the patients with NSTEMI (33.94 ± 24.78 IU/L) compared to normal healthy controls (25.00 ± 15.07 IU/L) ($P = 0.002$).

Serum concentrations of MDA (μM) were statistically

Table 1. Clinical Characteristics of NSTEMI Patient and Control Groups

Characteristics	NSTEMI Patients (n=52)	Healthy Controls (n=52)	P Value[a]
Age (y)	62.58 ± 9.25	60.42 ± 10.29	0.264
Male/Female	26/26	26/26	1
Body mass index (kg/m²)	25.32 ± 6.72	25.21 ± 4.28	0.473
Urea (mg/dL)	38.71 ± 16.61	33.90 ± 8.94	0.069
Creatinine (mg/dL)	1.18 ± 0.31	1.09 ± 0.222	0.113
Glucose (mg/dL)	143.38 ± 68.45	121.21 ± 31.87	0.037
Na⁺ (mEq/L)	141.21 ± 1.74	140.79 ± 1.38	0.172
K⁺ (mEq/L)	4.15 ± 0.36	4.22 ± 0.33	0.360
Cholesterol (mg/dL)	179.88 ± 37.80	174.98 ± 29.93	0.465
TG (mg/dL)	185.53 ± 59.96	170.35 ± 29.79	0.105
HDL (mg/dL)	39.77 ± 8.48	39.52 ± 8.21	0.879
AST (U/L)	27.98 ± 33.29	20.69 ± 7.55	0.127
ALT (U/L)	21.73 ± 13.48	25.83 ± 12.93	0.117
ALP (U/L)	112.88 ± 53.33	102.85 ± 51.15	0.330
LDL (mg/dL)	90.27 ± 28.36	95.86 ± 32.27	0.350
TSH (mg/dL)	3.43 ± 11.21	3.13 ± 9.28	0.884
FT4 (ng/dL)	2.06 ± 2.04	1.88 ± 1.73	0.641

Na⁺=sodium, K⁺=potassium, TG=Triglyceride, HDL=high density lipoprotein, AST=aspartate transaminase, ALT=alanine transaminase, ALP=alkaline phosphatase, LDL=low-density lipoprotein, TSH= Thyroid-stimulating hormone, FT4=free thyroxine.
[a]Two-tailed (*t* test).

elevated and TAC (mM) was diminished in NSTEMI patients compared with normal individuals (2.35 ± 0.76 μM vs. 1.92 ± 0.83 μM (P = 0.007) and 1.82 ± 0.41 mM vs. 2.05 ± 0.38 mM (P = 0.004)), respectively.

Significant positive correlation was observed between serum IMA (ABSU) and PC (nmol/mg) concentrations, which is described by the following equation: Mean IMA (ABSU) = 0.433 + 0.035 × PC (nmol/mg) (R^2 = 0.075; P = 0.005).

There was also a meaningful positive correlation between serum IMA (ABSU) and CKMB (IU/L) (R^2 = 0.118; $P \leq$ 0.001) and between PC (nmol/mg) and cTnI (ng/L) (R^2 = 0.051; P = 0.021). The equations are as follows: Mean IMA (ABSU) = 0.433 + 0.0063 × CKMB (IU/L), and PC (nmol/mg) = 4.89 + 0.014 × cTnI (ng/L). Nevertheless, the correlation between serum IMA (ABSU) and cTnI (ng/L) (R^2 = 0.013; P = 0.246) was not statistically significant.

Positive correlation was also observed between oxidative-stress marker, MDA (μM) with serum IMA (ABSU) (R^2 = 0.039; P = 0.043) and with serum PC (nmol/
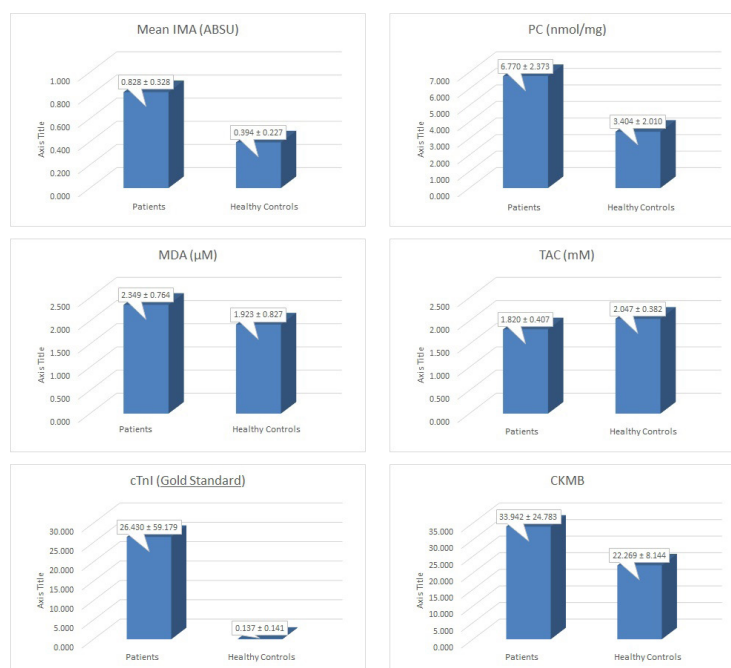

Figure 1. Comparison of Mean ± SD of IMA, PC, MDA, TAC, cTnI (Gold Standard), and CKMB, Between NSTEMI Patient and Healthy Control Groups.

Table 2. Serum Levels of Ischemia Modified Albumin, Protein Carbonyl, cTnI (Gold Standard), and CKMB in NSTEMI Patient and Control Groups

Characteristics	NSTEMI Patients (n=52)	Healthy Controls (n=52)	P Value[a]
Mean absorbance of IMA (ABSU)	0.83 ± 0.33	0.39 ± 0.23	<0.001
PC (nmol/mg)	6.77 ± 2.37	3.40 ± 2.01	<0.001
MDA (μM)	2.35 ± 0.76	1.92 ± 0.83	0.007
TAC (mM)	1.82 ± 0.41	2.05 ± 0.38	0.004
cTnI (ng/L) (gold standard)	26.43 ± 59.17	0.136 ± 0.141	0.002
CKMB (IU/L)	33.94 ± 24.78	22.27 ± 8.14	0.002

IMA = Ischemia Modified Albumin, PC = Protein Carbonyl, MDA = Malondialdehyde, TAC = Total Antioxidant Capacity, cTnI = cardiac troponin I, CKMB = Creatine Kinase-MB isozyme.
[a]Two-tailed (*t* test).

mg) ($R^2 = 0.061$; $P = 0.011$). The equations are as follows: Mean IMA (ABSU) = 0.428 + 0.086 × MDA (μM) and PC (nmol/mg) = 3.303 + 0.835 × MDA (μM), respectively.

The other oxidative-stress marker, TAC (mM) did not display any meaningful correlation with mean IMA (ABSU) ($R^2 = 0.018$; $P = 0.178$) and with PC (nmol/mg) ($R^2 = 0.016$; $P = 0.201$).

Figure 2 shows the regression plots of these correlations.

The Predictive Value of IMA and PC for NSTEMI

To measure specificity and sensitivity of the quantification of serum IMA and PC as tests to predict early stage NSTEMI, receiver operating characteristic curves (ROC curves) were plotted and the area under the ROC curves (AUCs) were measured. Results of the ROC curve analysis for IMA showed a sensitivity of 88.46%, specificity of 76.9%, and AUC of 0.880 (95% CI is from 0.315 to 0.445; two-tailed $P < 0.0001$). Similarly the results of the sensitivity, specificity, and AUC for PC to predict NSTEMI were 80.8%, 88.5%, and 0.827, respectively (95% CI is from 0.241 to 0.412; two-tailed $P < 0.0001$). Figure 3 shows these statistical correlations.

The results of sensitivity, specificity, along with negative predictive value (NPV), positive predictive value (PPV), and accuracy (diagnostic efficiency) of single IMA and PC tests as well as combination of these 2 tests for the diagnosis of NSTEMI were displayed in Table 3. As shown in Table 3, integrative quantification of circulatory IMA and PC tests increased specificity and NPV for the diagnosis of NSTEMI.

Discussion

One of the main complications of the reduction in blood flow due to atherosclerotic plaque in coronary vessels is inadequate oxygen transport to the heart tissue and subsequent diminish in pH of the tissue. This phenomenon causes triggering a chain reaction that begins with the liberation of copper ions (Cu^{++}) from impotent binding sites on plasma proteins, conversion of it to Cu^+ with the aid of reductants like ascorbic acid, reaction of Cu^+ with O_2 to form superoxide radicals (O^-_2), and dismutation of superoxide radicals to hydrogen peroxide (H_2O_2) in the presence of superoxide dismutase (SOD). H_2O_2 could follow through the Fenton chain reaction to form
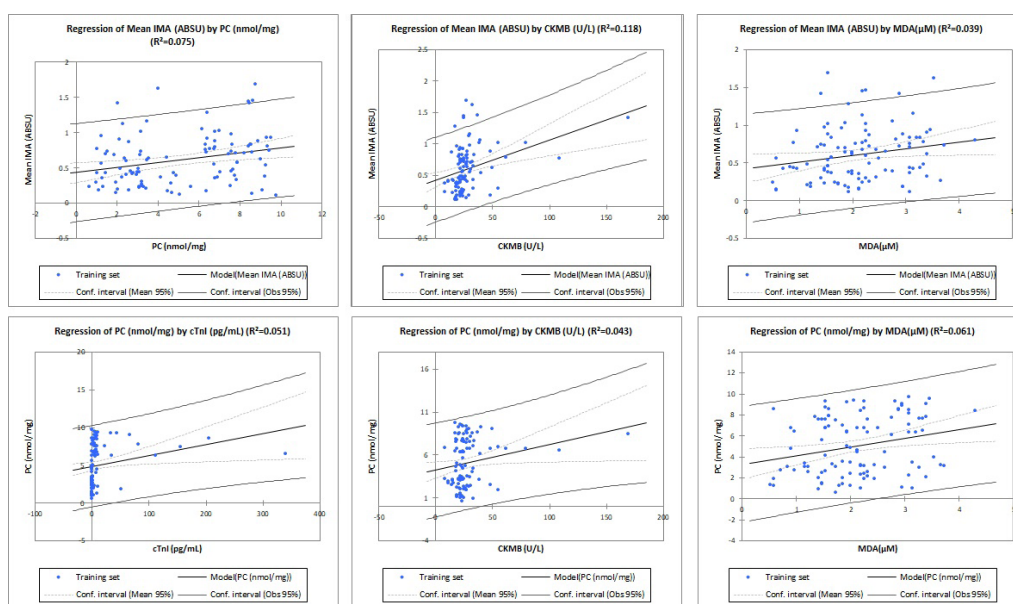

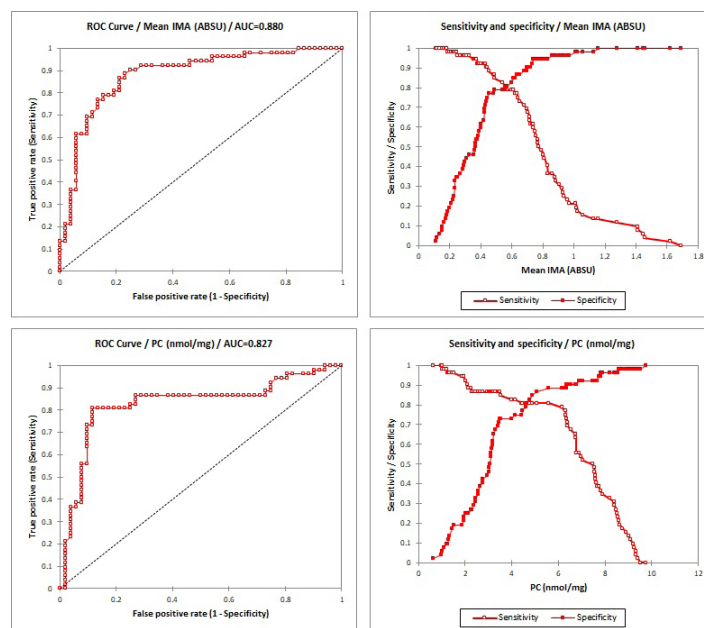**Figure 2.** Regression Analyses Between IMA, PC, cTnI, CKMB, and MDA.

Figure 3. ROC Curve Analyses of IMA and PC for the Diagnosis of NSTEMI.

hydroxyl free radicals ($^{\cdot}$OH). $^{\cdot}$OH is highly reactive and attacks serum albumin and alters its natural N-terminal binding site to metal ions like cobalt and copper, and other proteins of the serum and the biofluids to convert some of the amino acid side chains to carbonyl groups (21). Relative quantification of altered albumin (named IMA) is possible by measuring the relative diminish in the binding of cobalt by the albumin cobalt binding (ACB) test. The carbonyl groups of soluble proteins could be quantified directly by especially designed highly sensitive ELISA kits. The results of this study supported very early increase in IMA and PC concentrations, which is in accordance with the results of Maneewong et al (31), it is also in statistically meaningful conformity with MDA and TAC as the other oxidative stress biomarkers that we determined in the current study. Both IMA and PC are further downstream products of ischemia induced by oxidative stress in addition to other markers like nitric oxide, free fatty acids, myeloperoxidase, lactate, glutathione, and TBARS, and so may offer more specificity for cardiac tissue than them. Not a direct comparison study was done between IMA and PC on one hand and other oxidative markers on the other hand in heart patients, which could be an open field for further studies. The results of this study showed a meaningful increase in IMA and PC levels in NSTEMI

patients as direct products of ischemia in heart tissue. As the rise in IMA and PC concentrations happens very earlier than the rise of cardiac troponins, CKMB, and other cardiac biomarkers like natriuretic peptides, and so they serve as potential biomarkers for ischemic cardiac failures including NSTEMI. They not only rise sooner than routine cardiac disease biomarkers but also have much more specificity than other ischemia markers. These characteristics make them good biomarkers for ischemic cardiopathies, especially when used together since concurrent measurement of them increases their specificity and efficiency to diagnose NSTEMI. We insistently advise using IMA and PC, singly or together, as routine laboratory examinations along with electrocardiography and echocardiography in emergency departments of heart hospitals for quick and reliable diagnosis of heart diseases.

Conflict of Interests
None.

Ethical Issues
Each individual participant in the study signed a written consent. The information gained was kept confidential. This study was carried out according to the principles of WMA Declaration of Helsinki and guidelines of the Ethics

Table 3. Sensitivity, PPV, NPV and Accuracy of IMA and PC Tests for the Diagnosis of NSTEMI

	Sensitivity (%)	PPV (%)	NPV (%)	Accuracy (%)
IMA test	88.46	79.3	87	82.7
PC test	80.8	87.5	82.1	84.6
IMA + PC tests (in Parallel)	97.78	75.35	96.84	82.9

Committee of Tabriz University of Medical Sciences ((Ethics Code: IR.TBZMED.REC.1395.43; April 24, 2016) as well as the principles of Seyedoshohada Hospital of Urmia. Blood sampling complied completely with relevant rules of the hospital.

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