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Trace element, immune and opioid biomarkers of unstable angina, increased atherogenicity and insulin resistance: results of machine learning

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Abstract

Background. Aberrations in endothelial cells, immune and oxidative pathways are associated with atherosclerosis (ATS) and unstable angina (UA). The role of trace elements, minerals, and the endogenous opioid system (EOS) in UA are less well established.

Methods. We measured lipid, insulin resistance (IR), and immune, trace element (copper and zinc), mineral (magnesium, calcium), EOS (β-endorphin and mu-opioid receptor (MOR)) and antioxidant (vitamin D3) biomarkers in patients with ATS (n=60) and UA (n=60) and healthy controls (n=58).

Results. ATS patients showed increased atherogenic and IR indices, IL-6, IL-10, β-endorphin, copper and magnesium, and lower zinc than healthy controls. Logistic regression showed that UA was significantly discriminated from ATS without UA with an accuracy of 85.5% using calcium, IL-10, β-endorphin, MOR, triglycerides, IR (all positively), and copper and vitamin D3 (inversely). Neural networks showed that UA was discriminated from ATS without UA with an area under the ROC curve of 0.942 using MOR, β-endorphin, calcium, insulin resistance, vitamin D3 and copper as input variables. We found that 50.0% of the variance in IR was explained by the regression on copper, IL-10, IL-6 (all positively), and zinc (inversely), while 32.9% of the variance in the atherogenic index of plasma was explained by copper, IL-10 (both positively), and magnesium (inversely).

Conclusion. UA is not only mediated by insulin resistance, atherogenicity, and immune disorders, but also by aberrations in the endogenous opioid system and trace elements as well as lowered antioxidant levels. Copper appears to play a key role in IR and atherogenicity.

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Introduction

Cardiovascular diseases (CVD) are a prominent cause of mortality worldwide [1] with atherosclerosis (ATS) being the most important risk factor [2]. In a population with low to intermediate risk of CVD, the prevalence of ATS was 5.3% while about half of the participants showed at least one vessel with stenosis [3]. Moreover, 38% of women and 75% of men start to develop ATS at the age of 30–35 years [4]. ATS may remain asymptomatic as long as the arteries are functioning well and until atherosclerotic plaques obstruct more than 40% of the lumen of blood vessels [5]. Unstable angina (UA), one of the syndromes caused by ATS, is an acute coronary syndrome that is defined as myocardial ischemia without significant myocardial necrosis [6]. Most UA patients will develop myocardial infarction (MI) [7]. Currently, UA is a clinical diagnosis as there are no biomarkers to externally validate the clinical diagnosis [8]. The incidence and prevalence of UA are not easy to determine because UA is an exclusion diagnosis [9]. Nevertheless, in the Middle East, UA represents about half of patients with acute coronary syndrome [10]. Therefore, the early diagnosis of ATS and UA are essential to avoid the development of UA and myocardial infarction, respectively. Nevertheless, there are no data whether machine learning models combining different biomarkers may be used to externally validate the clinical diagnosis of UA.

One of the earliest events in ATS is vascular endothelial dysfunction [11] whereby a damaged endothelium may become more permeable and facilitates the entrance of lipids into the arterial wall thereby initiating an immune- inflammatory response with the involvement of peripheral blood immunocytes, which stick to the endothelium lesions [12]. Proinflammatory cytokines, including interleukin-6 (IL-6), stimulate the development and progression of ATS, whereas anti-inflammatory cytokines, including IL-10, may have anti-atherogenic activities [13].

 doi:10.20944/preprints202007.0260.v1

Moreover, immune activation may impact the ATS plaques, thereby causing a rupture and accompanying clinical symptoms, including myocardial infarction [14, 15]. The pathogenesis of ATS not only encompasses hyperlipidemia coupled with aberrations in immune cells and inflammatory responses [16, 17], but also increased oxidative stress toxicity. The latter is associated with damage to lipids, proteins and DNA, and formation of oxidized lipids including low-density lipoprotein (LDL) cholesterol and, consequently, IgG-mediated autoimmune responses to oxidized LDL and other oxidative specific epitopes [18, 19]. Increased oxidative toxicity and lowered antioxidant defenses not only participate in the pathogenesis of ATS, but also in UA [20] while gradual increases in oxidative damage to lipids and proteins and gradually decreasing antioxidant defenses are associated with the development of ST-segment myocardial infarction from UA [21].

Low vitamin D status is accompanied by an increased incidence of slow coronary flow rate while treatment with vitamin D may reduce angina episodes [22]. Zinc, another antioxidant with anti-inflammatory properties, has some anti-atherogenic effects [23]. Zinc deficiency increases the oxidative and inflammatory potential and may cause endothelial cell dysfunctions while zinc supplementation may improve inflammation, LDL oxidation, and vascular endothelial cell functions [23]. Insulin resistance (IR) is another independent risk factor of CVD in nondiabetic subjects [24, 25]. IR may directly contribute to the development of ATS by stimulating the mitogen-activated protein kinase (MAPK) pathway and inhibiting nitric oxide production (endothelial dysfunction) [26].

Minerals may also contribute to the mechanism leading to ATS or UA, including calcium, magnesium, and copper. Calcium mineralization of atherosclerotic artery lumen may promote plaque formation and calcification of plaques, thereby narrowing blood vessels [27]. Moreover,

 doi:10.20944/preprints202007.0260.v1

increased calcification may cause increased blood vessel rigidity and, consequently, elevated arterial vascular resistance and left ventricular hypertrophy [28]. Lowered serum magnesium levels and nutritional magnesium intake are associated with ATS, arrhythmias, and heart failure [29, 30]. Magnesium deficiency is accompanied by elevated vascular inflammatory responsivity [31] and lowered expression or activity of antioxidant enzymes including glutathione peroxidase, superoxide dismutase, catalase, and lowered levels of vitamin C, vitamin E, and selenium [32-34]. Moreover, magnesium deficiency is associated with the development of insulin resistance, hyperglycemia, and changes in lipid metabolism, which enhance atherosclerotic changes and arterial stiffness [35-37]. The results on copper in relation to ATS did not show consistent results [38, 39]. For example, some results show that serum copper is increased in atherosclerotic patients and is correlated with the severity of illness [40]. On the other hand, there is evidence that copper deficiency may impact almost all risk factor of ischemic heart disease including cholesterol levels, glucose tolerance, and inflammatory and oxidative stress processes [41].

Finally, the role of the endogenous opioid system (EOS) in ATS and UA is mostly unknown. Recently, a report showed that β-endorphins and agonists at the mu-opioid receptor (MOR) may mediate the development of ATS and additionally play a role in the instability of the plaques [42]. The same authors showed that β-endorphin is accompanied by IR and endothelial dysfunctions. Nevertheless, there are no data on whether UA is associated with alterations in the above-mentioned trace elements and in the EOS system.

Hence, the present study was performed to examine whether a) minerals / trace elements and β-endorphins and MOR are associated with UA and with ATS independently from the classical biomarkers (IR and atherogenicity), and b) whether combinations of the various biomarkers may be used to externally validate the clinical diagnosis of ATS and UA.

 doi:10.20944/preprints202007.0260.v1

Participants and Methods

Participants

The current study recruited 120 patients with ATS divided into two groups, namely those with UA (UA group) and without UA (ATS group). The participants were recruited at the Sadr-Teaching Hospital, Najaf city, Iraq from November 2019 to the mid-January 2020. The diagnosis of ATS was made according to ICD-10 criteria of CM-170 by using a complete medical history and physical exam coupled with Doppler sonography, electrocardiogram (ECG), echocardiography, and blood pressure measurements. The UA patients were diagnosed according to the established guidelines [43] and further classified by cardiologists as having a severe form of the disease, namely class III (n=34) or IV (n=26) according to the Canadian Cardiovascular Society (CCS) grading of angina pectoris [44]. Diabetes type 2 (T2DM) was diagnosed according to the World Health Organization criteria [45, 46] when plasma glucose ≥ 7.0 mM, and glycated haemoglobin (HbA1c) > 6.5%. Hypertension was diagnosed according to the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH) guidelines [47]. All hypertension patients had blood pressure measurements of > 140/95 mmHg using a conventional sphygmomanometer in a seated posture and with the arm in the horizontal position after fifteen minutes of quiet sitting.

We also recruited 58 healthy controls, age and sex-matched to the patient groups. All controls were free from any systemic disease. Exclusion criteria for patients and controls were major medical illness including liver disease, renal diseases, stroke, myocardial infarction, and cancer. We also excluded patients with an albumin/creatinine ratio above 30 mg/g [48] and with serum CRP concentrations > 6 mg/dL thereby excluding subjects with overt inflammation. All patients showed serum FBG <25 mM and fasting insulin <400 pM to comply with the requirements

 doi:10.20944/preprints202007.0260.v1

of the Homeostatic Model Assessment (HOMA) calculator software and TG <4.5 mM to comply with the Friedewald's equation requirements. Written informed consent was obtained from all subjects before participating in the study. Approval for the study was obtained from the IRB of the University of Kufa (487/2019), which complies with the International Guidelines for Human Research protection as required by the Declaration of Helsinki.

Methods

Ten milliliters of venous blood were drawn between 8-10 a.m. from patients and controls after an overnight fast. The blood was separated, after complete clotting by centrifugation at 3000 rpm for 10 min, and then stored at -80 ^{o}C until assay. Total calcium, total cholesterol (TC), triglycerides (TG), glucose, albumin, urea, creatinine, and magnesium were measured spectrophotometrically by kits supplied by Biolabo® (Maizy, France). Serum high-density lipoprotein cholesterol (HDLc) was measured after precipitation of all other lipoproteins by a reagent consisting of sodium phosphotungstate and $MgCl_2$; the cholesterol level in the supernatant was measured using the automated analyzer. Low-density lipoprotein cholesterol (LDLc) was computed from Friedewald's formula: LDLc= TC − HDLc – TG/2.19. Serum CRP was measured using a kit based on the principle of latex agglutination supplied by Spinreact® (Barcelona, Spain). Copper and zinc in serum were measured spectrophotometrically by kits supplied by Giesse Diagnostics (Rome, Italy).

Commercial ELISA sandwich kits were used to measure serum IL-6 and β-endorphin (Melsin Medical Co, Jilin, China), IL-10 (Elabscience®, Hubei, China), insulin (DRG® International Inc., NJ, USA), and MOR (Mybiosource® Inc. San Diego, California, USA). The sensitivities of the kits were for IL-6: 0.1 pg/mL, β-endorphin: 0.1pg/mL, insulin: 12.22 pM, IL-

 doi:10.20944/preprints202007.0260.v1

10 4.69 pg/mL, and MOR: 7.18 pg/mL. The intra-assay CVs (precision within-assay) of all assays were less than 10%. The procedures were followed exactly without modifications according to the manufacturer's instructions.

Three atherogenic indices were calculated by using z unit-weighted composite scores, namely zTC – z HDLc (zTC-zHDLc, reflecting the Castelli risk index 1), z LDLc – zHDLc (zLDLc-zHDLc, reflecting Castelli risk index 2), and z triglycerides – z HDLc (zTG–zHDLc reflecting the atherogenic index of plasma (AIP)) [49, 50]. The zTC-zHDLc score was significantly correlated with Castelli risk index 1 ($r=0.988$, $p<0.001$, $n=161$), zLDLc-zHDLc, with Castelli risk index 2 ($r=0.945$, $p<0.001$, $n=161$) and zTG-zHDLc with the atherogenic index of plasma ($r=0.939$, $p<0.001$, $n=161$). Likewise, we also computed indices reflecting IR as z glucose + z insulin (zIR), β cell function as z insulin–z glucose (zβCell), and glucose toxicity as z Glucose –z Insulin (zGLUTOX). There were significant associations between zIR and HOMA2IR as defined with HOMA2 Calculator© (Diabetes Trials Unit, University of Oxford) ($r=0.623$, $p<0.001$, $n=161$) and between zβCell and HOMA%B ($r=0.991$, $p<0.001$, $n=161$). Body mass index (BMI) was calculated from the standard formula by weight in kilograms divided by square of height in meters.

Statistical analysis

Categorical data are shown as frequencies, and continuous variables are displayed as mean ± standard deviation (or standard error). The normality of continuous data was tested using the Kolmogorov-Smirnov test. Differences in the continuous variables between study groups were assessed by analysis of variance (ANOVA) and associations between nominal variables using analysis of contingency tables (using χ^2 test or ψ coefficient). We employed univariate generalized

 doi:10.20944/preprints202007.0260.v1

linear model (GLM) analysis to check the relationship among the biomarkers and the diagnosis (controls versus ATS with and without UA) while controlling for background variables including age, BMI, nicotine dependence, and sex. We also computed effect sizes using partial η^2 values, and GLM-generated estimated marginal mean (SE) values and conducted protected pairwise comparisons among treatment means. We used p-correction for false discovery rate (FDR) to adjust for multiple comparisons [51]. Binary logistic regression analysis was used to delineate the essential explanatory variables that predict UA versus no UA (as the reference group). We used multiple regression analysis to predict atherogenicity and IR indices (output variables) using immune, trace element and opioid biomarkers as dependent variables. Tests were 2-tailed, and a p-value of 0.05 was used for statistical significance.

Multilayer perceptron (MLP) Neural Network (NN) models were used to assess the complex associations between the diagnoses of UA and ATS and between ATS and healthy controls as output variables and the measured biomarkers as input variables. We used automated feedforward architecture models to train the neural networks and employed two hidden layers with up to 10 nodes and up to 250 epochs and mini-batch training with gradient descent. The stopping criterion was one consecutive step with no decrease in the error term. We used three samples, namely a training sample (46.7%) to estimate the network parameters, a testing sample (20%) to prevent overtraining, and a holdout set (33.3%) to compute the predictive value of the neural network. Finally, we computed error, relative error, the importance of the explanatory variables (in an importance chart) and the area under the ROC curve with diagnostic performance. All statistical analyses were performed using IBM SPSS windows version 25, 2017.

Results.

 doi:10.20944/preprints202007.0260.v1

Socio-demographic data

Table 1 shows the socio-demographic data of the three different study groups. There were no significant differences in age, sex ratio, body weight, BMI, and TUD between the three study groups. Systolic and diastolic blood pressure were significantly higher in patients than in controls as were the frequencies of T2DM and hypertension.

Intergroup differences in biomarkers

Table 2 shows the measurements of the different biomarkers in the three study groups. Univariate GLM showed that total cholesterol, zTC-zHDLc, FPG, insulin, zIR, and IL-6 were significantly higher in patients than in controls. HDLc was significantly lower in both patient groups than in controls. The levels of TG, LDLc, zTG-zHDLc, zLDLc-zHDLc, zinc, copper, IL-10, and β-endorphin were significantly different among the three study groups whereby TG, zTG-zHDLc, and IL-10 increased from controls → ATS → UA, and zinc decreased from controls → TS → UA. LDLc, zLDLc-zHDLc, and copper were higher in patients than controls, but lower in UA than in ATS. Serum vitamin D3 was significantly lower in UA than in controls while MOR was significantly higher in UA than in controls and ATS. Total calcium was significantly higher in UA than in controls and ATS, while total Mg was higher in ATS than in controls and UA. The differences in those biomarkers as shown in Table 1 remained significant after p-correction for FDR.

We have also examined the effects of the drug state of the patients on the biomarkers. After FDR p-correction there were significant effects of Glibenclamide on TG (p=0.019; partial eta squared=0.062) and MOR (p=0.040, partial eta squared=0.051), Bisoprolol on Castelli risk index 1 (p=0.045, partial eta squared=0.044), IR (p=0.03, partial eta-squared=0.055), MOR (p=0.045,

 doi:10.20944/preprints202007.0260.v1

partial eta-squared=0.042) and urea (p=0.019, partial eta squared=0.0094), and Atenolol on magnesium (p=0.019, partial eta squared=0.066). The levels of all those biomarkers were increased in subjects who took the drugs, except IR and MOR which were decreased. The other drugs, namely Aspirin, Clopidogrel, Atorvastatin, Isosorbide Dinitrate, and Trimetazidine, did not have any significant effect. By inference, the drug state of the patients has only minimal effects on a few biomarkers (partial eta squared < 0.1).

Best predictions of UA versus ATS and ATS versus controls

Table 3 shows the outcome of binary logistic regression analysis with UA as dependent variable (and ATS without UA as the reference group). We found that 8 input variables significantly discriminated UA from ATS (χ^2=93.43, df=8, p<0.001), namely total calcium, IL-10, β-endorphin, MOR, TG, zIR (all positively associated) and copper and vitamin D3 (both inversely associated). The Nagelkerke effect size was 0.721, and the accuracy of the classification was 85.8% with a sensitivity of 88.3% and a specificity of 83.3%. Table 3 shows also that interleukin-6 was the single best predictor of CCS class IV (versus CCS class III) with a pseudo-R^2 Nagelkerke value of 0.612 and an overall accuracy of 86.7% with a sensitivity=88.5% and specificity=85.3%.

Table 4 shows the results of the most accurate neural network (NN#1) discriminating UA patients from ATS without UA using the biomarkers as input variables (the zTG-zHDLc index was included as lipid variable rather than the separate lipids). We trained the feedforward network with 2 hidden layers, with 4 units in layer 1 and 3 units in layer 2. Hyperbolic tangent was the activation function in the hidden layers and identity in the output layer. The sum of squares error term was much lower in the testing than in the training sample, while the percentage of incorrect classifications was reasonably constant in the three samples, indicating that the model learned to

 doi:10.20944/preprints202007.0260.v1

generalize from the trend and is not over fitted. The AUC ROC was 0.942 with a sensitivity of 71.4% and specificity of 93.3%. **Figure 1** displays the importance chart and shows that MOR has the highest predictive power of the model, followed by β-endorphins, total calcium, zIR, and vitamin D3, again followed at a distance by copper. IL-10, zTG-zHDLc and zGLUTOX were less significant.

The prediction of ATS (with and without UA) versus no ATS (controls) yielded a perfect (100%) prediction using logistic regression analysis as well as neural networks. However, since logistic regression analysis shows a 100% accuracy, the regression parameters cannot be estimated. **Table 4** shows the characteristics of NN#2 discriminating ATS from controls. This model was trained using 2 hidden layers, with 4 units in layer 1 and 3 units in layer 2, again with hyperbolic tangent and identity as activation functions in the hidden layers and output layer, respectively. The percentage of incorrect classifications was 0% in the three sets while the AUC ROC curve was 1.00. **Figure 2** displays the importance and shows that zTG-zHDLc was by far the most important predictor of the power of the model, followed by urea and IL-10, again at a distance by zIR, and again at a distance by MOR, vitamin D3 and IL-6.

Prediction of atherogenicity and IR by biomarkers

Table 5 shows the results of different multiple regression analyses with lipid and glucose-associated biomarkers as dependent variables and the biomarkers as explanatory variables. Regression #1 shows that 35.8% of the variance in zTG-zHDLc could be explained by copper, IL-10 and MOR (all positively associated) and Mg (inversely). **Figure 3** shows the partial regression of zTG-zHDLc on copper after adjusting for the variables listed in Table 5, regression #1. We found that 32.9% of the variance in zTC-zHDLc (Regression #2) and 24.3% of that in zLDLc-

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zHDLc (regression #3) was explained by copper and IL-10 (positively) and Mg (inversely). Regression #4 shows 50.0% of the variance in zIR was explained by copper, IL-10, IL-6 (positively) and zinc (inversely). **Figure 4** shows the partial regression of zIR on copper after adjusting for the variables listed in Table 5, regression #5.

Discussion

The first major finding of the current study is that the combination of increased atherogenicity, IR, urea, IL-10, IL-6, MOR, and lowered vitamin D3 in a machine learning model may be used to externally validate the clinical diagnosis of ATS. Moreover, simple ANOVAs showed that also increased copper, β-endorphin, magnesium, and creatinine and lowered zinc are other features of ATS. These findings extend the results described in the introduction indicating that increased atherogenicity, IR, immune-inflammatory pathways (increased IL-6 and IL-10, and lowered zinc), oxidative-antioxidant misbalances (lowered vitamin D3 and zinc) are involved in ATS.

Hyperlipidemia as characterized by elevated total cholesterol, triglycerides or LDL-cholesterol coupled with lowered HDL-cholesterol significantly contributes to the development of ATS [52-55]. IR in peripheral tissues including in adipocytes may induce a proatherogenic phenotype through different mechanism including suppression of lipolysis, defective storage of lipids in adipose cells, increased lipid levels, limited degradation of apoB, and lowered lipoprotein lipase activity [56]. Hyperinsulinemia may accelerate atherosclerotic processes through stimulation of lipogenesis leading to increased LDL synthesis, enhanced LDL-cholesterol transport into smooth muscle cells of arteries, activation of inflammatory genes, stimulation of growth and proliferation of vascular smooth muscle cells, and increased collagen synthesis [57-61]. As described in the introduction, activated immune pathways, including increased levels of

 doi:10.20944/preprints202007.0260.v1

IL-6, take part in the onset of ATS [62-67]. Activated macrophages secrete proinflammatory cytokines and chemokines that recruit other immune cells into the atherosclerotic lesions thereby intensifying the inflammatory environment [68, 69]. Also, during an inflammatory response, macrophages secrete various enzymes that damage the extracellular matrix of the atherosclerotic plaque contributing to the pathogenesis of ATS [70]. One study reported a robust inverse correlation between ATS severity and the frequency of IL-10^+ B cells [71]. IL-10 can inhibit macrophage foam cell formation [72] by enhancing the uptake of modified LDL and cholesterol efflux [73]. IL-10 inhibits macrophage activation as well as matrix metalloproteinase, proinflammatory cytokines, and cyclooxygenase-2 expression in lipid-loaded and activated macrophage foam cells [74]. Therefore, increased IL-10 in ATS is probably a compensatory mechanism to attenuate the inflammatory response.

In the present study we found that lowered levels of the key antioxidants zinc and vitamin D3 were inversely associated with ATS. As discussed in the introduction vitamin D deficiency may increase risk to ATS and cardio-vascular disease [22, 75-78]. Vitamin D may protect against ATS trough antioxidant, anti-inflammatory and anti-atherogenic effects as well as modulation of endothelial functions, IR, and vascular cell differentiation and growth [76, 77]. Our findings on zinc extend those of previous papers reporting lowered serum zinc in ATS [79]. Zinc deficiency is associated with an increased vulnerability to formation of reactive oxygen species and consequent oxidative stress toxicity, endothelial cell apoptosis, and atherogenesis [80], while lipid and protein peroxidation frequently occur in ATS and play a role in mechanical and bioenergetic incompatibilities [81, 82]. In addition, zinc protects against LDL oxidation and atherogenesis [83, 84] and loss of metallothionein in zinc as a consequence of ROS [85]. Moreover, zinc antagonizes copper driven pro-atherogenic effects via balancing the bioavailability and metabolism of copper

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[39, 86]. This is important as we found that ATS is associated with increased copper levels. In obese patients, however, copper levels were inversely associated with intima-media thickness and predicted early ATS [87]. Copper ions may participate in LDL oxidation and are a constituent of antioxidant enzymes including superoxide dismutase and caeruloplasmin [88]. In our study, ATS was associated with increased magnesium levels while previous clinical studies, which examined the effects of magnesium supplementation, yielded inconsistent results but raised potential adverse effects of magnesium overload [89].

The positive association between β-endorphin and ATS found in the current study may be explained by the known effects of β-endorphins on the development of atherosclerotic plaques [42]. For example, chronic administration of β-endorphin to Apoe −/− mice increases vascular inflammation and has an atherogenic effect thereby participating in the development of ATS lesions [42]. Moreover, β-endorphins contribute to a disbalance between lowered nitric oxide release and increased endothelin (ET)-1, which appears to be mediated by MOR [90]. In humans, opioid receptors are present in the heart [91] and cardiac MORs are substantially up-regulated during heart failure [92]. Finally, the association between increased urea and creatinine and ATS extend previous reports that increased serum creatinine is associated with ischemic heart disease [93] and that proteinuria is associated with intima-media thickness [94].

The second major finding of this study is that a neural network algorithm allowed to externally validate the clinical diagnosis of UA CCS class III + IV *vs.* ATS without UA with an AUC ROC curve of 0.942 and with MOR, β-endorphin, calcium, IR, vitamin D3 and copper as most important discriminatory variables, while logistic regression showed that IL-10 and triglyceride levels had additional effects. Moreover, simple ANOVA showed that also the AIP was significantly and

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positively associated with UA, whereas zinc and magnesium were lowered in UA as compared with ATS without UA.

Our results indicate that the development of UA CCS class III + IV from ATS is associated with a further increase in atherogenicity which is determined by increased triglyceride levels. It is interesting that, in our study, lowered LDL-cholesterol increased risk to UA while total cholesterol was not associated with UA *vs.* ATS without UA. These findings do not support the theory that the "LDL-cholesterol paradox" [95] or the "cholesterol paradox" [96] may be involved in UA. Hyperglycemia, hyperinsulinemia and IR can all cause disorders in lipid metabolism, oxidative stress toxicity and vascular endothelial damage, ultimately leading to the aggravation of coronary ATS [97]. As such, hyperlipidemia may contribute to the development of UA [98] while interactions between immune-inflammatory and atherogenic pathways may further contribute to UA [99]. However, in our study, IL-6 was not significantly increased in UA *vs.* ATS without UA, although our results agree with previous data, which reported that IL-6 (and TNF-α and CRP) levels are higher in UA patients than in healthy controls [100, 101]. Moreover, increased IL-6 is an independent predictor of myocardial infarction or cardiac death in UA [102]. This is further substantiated by our findings that IL-6 is the single best predictor of CCS UA class IV *vs.* class III. We found that IL-10 is not only associated with ATS (*vs.* controls) but also with UA *vs.* ATS without UA. Previous papers reported a positive association between IL-10 and proinflammatory cytokines (TNF-α) in patients with UA [103]. As such, increased IL-10 in UA may be a compensatory mechanism which offers protection against the immune-inflammatory response [104], which is one of the critical factors in the progression of atherosclerotic plaques, plaque rupture, and atherothrombosis [105, 106].

 doi:10.20944/preprints202007.0260.v1

The most important biomarkers of UA CSS classes III and IV were increased levels of β-endorphin and MOR. Previous data showed that β-endorphin levels may be raised in a meaningful part of patients with UA and in most patients with myocardial infarction [107] [108]. Patients undergoing pacing-induced angina pectoris show a local myocardial release of β-endorphin (and leu-enkephalin, met-enkephalin, and dynorphin) in the human heart [109] while patients with acute myocardial infarction show increased plasma β-endorphin concentrations [110]. On the other hand, other authors reported lower or no changes in β-endorphin levels in patients with myocardial infarction [108]. In humans, cardiac opioid receptors play an important role in myocardial ischemia [91], whereby MOR may have cardioprotective properties as detected in an in vitro ischemia-reperfusion model [92]. In this respect, it is thought that activation of peripheral opioid receptors may protect cardiac tissues by suppressing cardiac nociceptive signaling [111].

In our study, lowered levels of vitamin D3 and zinc were other hallmarks of UA indicating that the latter condition is accompanied by an increased vulnerability to immune-inflammatory and oxidative toxicity. Vitamin D deficiency has been reported in patients with chronic stable angina [112] and acute coronary syndrome [113]. Nevertheless, in patients with acute coronary syndrome no significant association could be detected between 25-OH-Vitamin D3 and proinflammatory cytokines including IL-6 and TNF-α [114]. Lowered levels of zinc were previously reported in UA [115] while zinc replenishing has protective effects in coronary artery disease [116].

Our results that lowered magnesium is associated with UA extend those of a previous report that lower serum magnesium levels predict major adverse cardiac events [37, 117]. Our data that calcium is increased in UA do not agree with a previous report that men with UA show significant decreases in plasma Ca [115]. It is interesting to note that, in our study, copper was

 doi:10.20944/preprints202007.0260.v1

significantly increased in ATS versus controls, but was lower in UA than in ATS without UA. These results in UA may be explained by the knowledge that lowered copper may impact many risk factors of ischemic heart disease including glucose tolerance, cholesterol levels, and immune and oxidative mechanisms [41].

The third major finding of this study is that IR and atherogenicity indices were strongly associated with different biomarkers, namely atherogenicity with increased copper, IL-10, and MOR, and lowered magnesium, and IR with increased copper, IL-6, and IL10, and lowered magnesium. These associations with the above-mentioned immune biomarkers and MOR and magnesium may be explained by the different properties of those molecules as discussed above and indicate that their effects on ATS and UA are in part mediated by increased atherogenicity and/or IR. Nevertheless, the strong associations between copper and both IR and atherogenicity need further discussion.

There are some papers that lowered copper or marginal copper deficiency is associated with an increased risk of atherosclerosis and ischemic heart disease [41, 118], although other data showed that high copper levels may contribute to atherosclerosis [119]. In physiological conditions, copper levels are tightly regulated while copper deficits and excess are both deleterious to the body, a phenomenon described as the “Blue Janus” effect [88, 120]. Thus, in inflammatory conditions, increased copper is associated with increased ROS and oxidative stress toxicity while copper may induce inflammatory responses thereby playing a role in atherogenesis [88, 120]. Moreover, increased copper levels are reported in diabetes [121]. Copper overload-induced oxidative stress is associated with T2DM thereby causing diabetes complications [122]. Nevertheless, as the latter authors describe, currently it is unknown whether changes in copper homeostasis are the consequence of diabetes or whether copper overload contributes to diabetes.

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In any case, selective copper chelation may improve the function of diabetic hearts by removal of copper, reducing ROS and attenuating IR and glucose intolerance [122].

Conclusion. Figure 5 reviews the findings of the present study. The development of ATS is characterized by increased atherogenicity (higher triglycerides and LDL-cholesterol but lowered HDL cholesterol), IR (higher insulin and glucose), immune activation (increased IL-6 and IL-10), lowered antioxidants (zinc and vitamin D3), EOS dysfunctions (increased β-endorphin and MOR), disorders in trace elements/minerals (increased copper and magnesium), and increased urea and creatinine. The development of UA CCS Classes III and IV is characterized by increased atherogenicity (increased triglyceride levels), IR, immune activation (elevated IL-10), lowered vitamin D3 and zinc, aberrations in the EOS (increased MOR and β-endorphins), increased calcium but lowered zinc, copper and magnesium, and increased urea levels. Increased IL-6 is, additionally, associated with the transition of CCS class III into class IV UA. Combinations of these biomarkers in machine learning models allow to differentiate ATS from normal controls, UA from ATS without UA, and UA CCS class IV from class III. Treatments of ATS and UA should use a combinatorial approach targeting not only atherogenicity and IR, but also immune activation, especially IL-6, redox imbalances including lower vitamin D3, the EOS, and calcium and copper metabolism.

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Declaration of interest

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Table 1. Demographic and clinical data in healthy controls (HC), and patients with atherosclerosis (ATS) and ATS with unstable angina (UA).

Variables		**HC [A] N=58**	**ATS [B] N=60**	**UA [C] N=60**	**F/Ψ/χ^2**	**df**	**p**
Age	years	55.9±9.6	55.6±10.0	56.6±8.1	0.182	2/175	0.834
Sex	Female/Male	18/40	29/31	22/38	3.886	2	0.143
Weight	kg	72.7±9.4	74.6±11.1	73.5±11.6	0.463	2/175	0.630
BMI	kg/m^2	26.2±3.1	26.8±4.6	27.2±4.6	0.757	2/175	0.471
TUD	Yes/No	15/43	20/40	21/39	1.289	2	0.525
Systolic BP	mmHg	120.0±1.5 [B,C]	132.3±14.3 [A]	135.3±18.9 [A]	20.388	2/175	<0.001
Diastolic BP	mmHg	79.8±1.4 [B,C]	85.2±6.7 [A]	83.7±8.0 [A]	11.801	2/175	<0.001
T2DM	Yes/No	0/58	22/38	25/35	31.253	2	<0.001
Hypertension	Yes/No	0/58	29/31	30/30	42.693	2	<0.001

[A, B, C]: Pairwise comparison, BMI: Body mass index, TUD: Tobacco use disorder, ATS: atherosclerosis patients, and UA: unstable angina, BP: blood pressure, T2DM: type 2 diabetes mellitus.

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Table 2. Results of univariate GLM analyses examining the associations between diagnosis and biomarkers after adjusting for age, sex, and body mass index.

Variables	HC [A] N=58	ATS [B] N=60	UA [C] N=60	F	df	p	Partial η^2
TC mM	4.46(0.14) [B,C]	5.87(0.13) [A]	5.80(0.13) [A]	35.455	2/172	<0.001	0.292
TG mM	1.87(0.10) [B,C]	2.85(0.10) [A,C]	3.46(0.10) [A,B]	66.281	2/172	<0.001	0.435
HDLc mM	1.18(0.03) [B,C]	0.94(0.03) [A]	0.97(0.03) [A]	26.004	2/172	<0.001	0.232
LDLc mM	2.43(0.12) [B,C]	3.63(0.11) [A,C]	3.25(0.12) [A,B]	28.154	2/172	<0.001	0.247
zTG – z HDLc (AIP)	-1.561(0.156) [B,C]	0.525(0.150) [A,C]	0.969(0.152) [A,B]	78.370	2/172	<0.001	0.477
zTC – z HDLc (Castelli 1)	-1.471(0.151) [B,C]	0.814(0.144) [A]	0.593(0.147) [A]	72.884	2/172	<0.001	0.459
zLDLc – z HDLc (Castelli 2)	-1.374(0.170) [B,C]	0.932(0.163) [A,C]	0.386(0.166) [A,B]	52.303	2/172	<0.001	0.378
Zinc mg/l	0.77(0.02) [B,C]	0.62(0.02) [A,C]	0.53(0.02) [A,B]	29.661	2/172	<0.001	0.256
Copper mg/l	0.74(0.03) [B,C]	1.11(0.03) [A,C]	1.02(0.03) [A,B]	51.979	2/172	<0.001	0.377
FPG mM	5.39(0.22) [B,C]	7.01(0. 21) [A]	7.26(0.21) [A]	22.871	2/172	<0.001	0.210
Insulin pM	75.19(7.56) [B,C]	132.13(7.24) [A]	142.84(7.35) [A]	24.298	2/172	<0.001	0.220
zFPG+z insulin (Insulin resistance)	-1.360(0.127) [B,C]	0.441(0.122) [A]	0.747(0.124) [A]	84.026	2/172	<0.001	0.494
z Insulin – z FPG (β cell function)	-0.028(0.204)	-0.041(0.195)	-0.011(0.198)	0.006	2/172	0.994	<0.001
z FPG – z Insulin (GLUTOX)	0.028(0.204)	0.041(0.195)	0.011(0.198)	0.006	2/172	0.994	<0.001
Albumin g/l	46.28(0.58)	45.76(0.56)	46.14(0.57)	0.224	2/172	0.799	0.003
T.Ca mM	2.43(0.03) [C]	2.38(0.03) [C]	2.51(0.03) [A]	7.042	2/172	0.001	0.076
T.Mg mM	0.727(0.032) [B]	0.834(0.031) [A,C]	0.692(0.031) [B]	5.656	2/172	0.004	0.062
Vitamin D3 nM	64.22(3.31) [C]	56.22(3.17)	51.55(3.22) [A]	3.955	2/172	0.021	0.044
IL-6 pg/ml	8.76(1.95) [B,C]	17.07(1.87) [A]	15.38(1.90) [A]	8.724	2/172	<0.001	0.092
IL-10 pg/ml	7.40(1.20) [B,C]	17.73(1.15) [A,C]	22.68(1.17) [A,B]	80.809	2/172	<0.001	0.484
βEP pg/ml	17.51(1.70) [B,C]	23.09(1.63) [A,C]	31.58(1.66) [A,B]	15.065	2/172	<0.001	0.149
MOR pg/ml	4.31(0.55) [C]	4.03(0.53) [C]	9.42(0.54) [A,B]	34.086	2/172	<0.001	0.284
Urea mg/dl	32.03(1.264) [B,C]	38.05(1.21) [A]	36.14(1.23) [A,B]	6.198	2/172	0.003	0.067
Creatinine mg/dl	0.764(0.028) [B,C]	0.931(0.027) [A]	0.921(0.027) [A]	11.753	2/172	<0.001	0.120

[A, B, C]: Pairwise comparisons among treatment means, BMI: Body mass index, TUD: Tobacco use disorder, ATS: atherosclerosis patients, UA: unstable angina, Z: Results in z-score (standardized results), HDLc: high-density lipoprotein cholesterol, LDLc: low-density lipoprotein cholesterol, TG: triglycerides, TC: total cholesterol, HbA1c: glycated haemoglobin, FPG: fasting blood glucose, IL-6: interleukine-6, IL-10: interleukine-10, βEP: beta-endorphin, MOR: Mu-opioid receptor, T.Ca: total calcium, T.Mg: total magnesium,

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zFPG+zINS: Insulin resistance composite, zINS–zFPG: beta-cell function composite, zFPG–zINS (GLUTOX): glucose toxicity composite.

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Table 3. Results of automatic binary logistic regression with unstable angina (UA) or the Canadian Cardiovascular Society (CCS) grading class IV as the dependent variables.

Dichotomies	Explanatory variables	B	SE	Wald	df	p	OR	95% CI
UA *vs.* ATS*	Copper	-1.419	0.501	8.01	1	0.005	0.242	0.091-0.646
	Total calcium	1.635	0.471	12.07	1	0.001	5.130	2.039-12.904
	Vitamin D3	-0.944	0.386	5.98	1	0.014	0.389	0.182-0.829
	Interleukin-10	0.982	0.452	4.72	1	0.030	2.671	1.101-6.478
	β-endorphin	0.920	0.411	5.01	1	0.025	2.510	1.121-5.616
	Mu-opioid receptors	1.597	0.409	15.23	1	<0.001	4.938	2.214-11.014
	Triglycerides	1.832	0.580	9.98	1	0.002	6.247	2.004-19.471
	Z FPG + z insulin (IR index)	1.330	0.515	6.67	1	0.010	3.779	1.377-10.370
CCS Class IV *vs.* III*	Interleukin-6	2.643	0.621	18.10	1	<0.001	14.050	4.158-47.472

* Reference groups are ATS: atherosclerosis without UA, and CCS grading class III.
z FGG + z insulin: z unit weighted composite score using z-scores of FPG (fasting blood glucose) and insulin. This score is an index of insulin resistance (IR).
Class III or IV: based on the Canadian Cardiovascular Society (CCS) grading of angina pectoris.

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Table 4. Results of neural network models examining the differentiation of atherosclerosis with unstable angina (ATS+UA) *versus* atherosclerosis without UA (ATS) *versus* healthy controls (HC)

	Models	**NN#1 ATS+UA *vs.* ATS**	**NN#2 ATS *vs.* HC**
Input Layer	Number of units	9 (Biomarkers)	12 (Biomarkers)
	Rescaling method	Normalized	Normalized
Hidden layers	Number of hidden layers	2	2
	Number of units in hidden layer 1	4	4
	Number of units in hidden layer 2	3	3
	Activation Function	Hyperbolic tangent	Hyperbolic tangent
Output layer	Dependent variables	ATS+UA *vs.* ASC	ASC *vs.* HC
	Number of units	2	2
	Activation function	Identity	Identity
	Error function	Sum of squares	Sum of squares
Training	Sum of squares error term	6.777	0.859
	% incorrect or relative error	13.8%	0.0%
	Prediction (spec, sens)	82.8%, 89.7%	100%, 100%
Testing	Sum of Squares error	3.939	0.301
	% incorrect or relative error	15.2%	0.0%
	Prediction (specificity-sensitivity)	75.0%-94.1%	100%, 100%
	AUC ROC	0.942	1.00
Holdout	% incorrect or relative error	17.2%	0.0%
	Prediction (spec-sens)	93.3%, 71.4%	100%, 100%

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Table 5. Results of multiple regression analysis with lipid and glucose-associated biomarkers as dependent variables.

Dependent variables	Explanatory variables	β	t	p	F_{model}	df	p	R^2
#1. z triglycerides – z HDLc	**Model**				24.103	4/173	<0.001	0.358
	Copper	0.459	6.433	<0.001				
	Interleukin-10	0.224	3.322	0.001				
	Magnesium	-0.205	-3.036	0.003				
	Mu-opioid receptors	0.142	2.250	0.026				
#2. z Total cholesterol – z HDLc	**Model**				28.475	3/174	<0.001	0.329
	Copper	0.413	5.682	<0.001				
	Interleukin-10	0.299	4.419	<0.001				
	Magnesium	-0.179	-2.633	.009				
#3. z LDLc – z HDLc	**Model**				18.571	3/174	<0.001	0.243
	Interleukin-10	0.264	3.667	<0.001				
	Copper	0.348	4.507	<0.001				
	Magnesium	-0.143	-1.983	0.049				
#4. HDLc	**Model**				9.205	4/173	<0.001	0.175
	Copper	-0.284	-3.693	<0.001				
	β-endorphin	-0.192	-2.697	0.008				
	Interleukin-6	-0.155	-2.198	0.029				
	Magnesium	0.157	2.106	0.037				
#5. z FPG + z insulin	**Model**				43.314	4/173	<0.001	0.500
	Copper	0.451	7.661	<0.001				
	Interleukin-10	0.276	4.431	<0.001				
	Interleukin-6	0.119	2.075	0.039				
	Zinc	-0.119	-2.007	0.046				
#6. FPG	**Model**				16.114	3/174	<0.001	0.217
	Copper	0.243	3.415	0.001				
	Zinc	-0.223	-3.130	0.002				
	Interleukin-6	0.202	2.933	0.004				
#7. HOMA2%S	**Model**				11.788	2/175	<0.001	0.119
	Copper	-0.344	-4.512	<0.001				
	Magnesium	0.253	3.316	0.001				

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HDLc: high-density lipoprotein cholesterol, LDL: low-density lipoprotein cholesterol, FPG: fasting plasma glucose, HOMA2%S: homeostasis model assessment of insulin sensitivity percentage.

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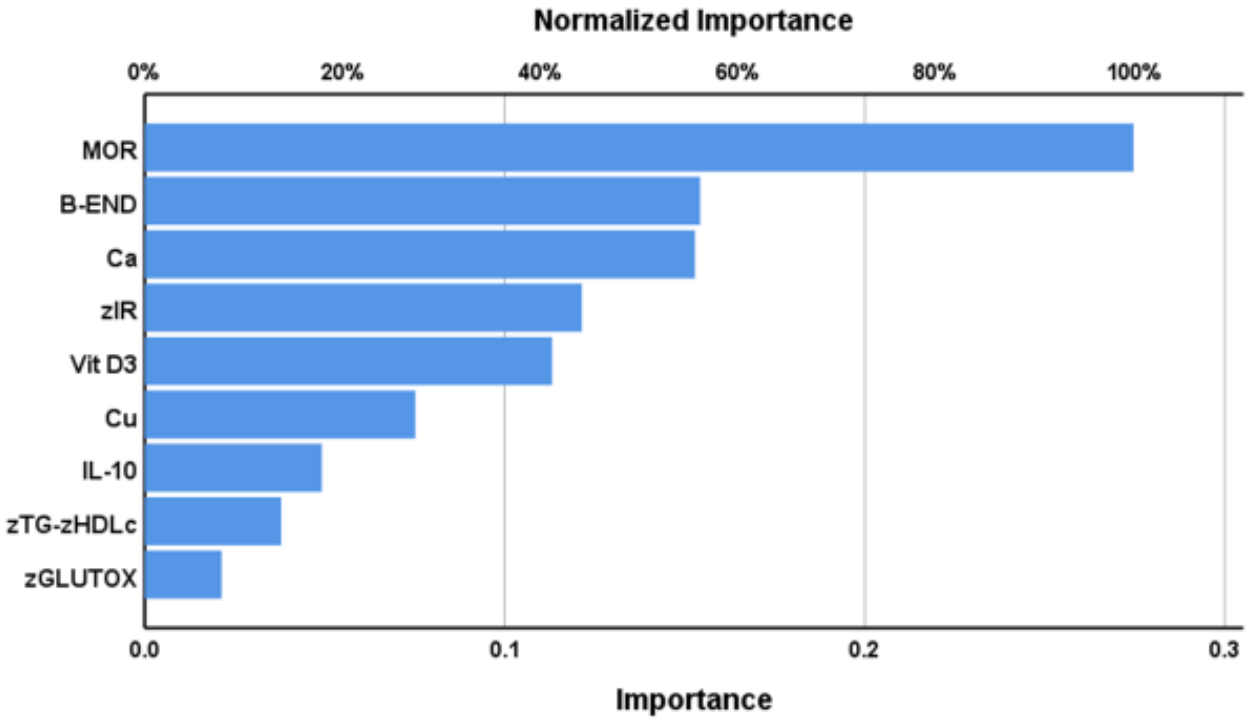

Figure 1. Results of a neural network (importance chart) with unstable angina (UA) and atherosclerosis without UA as output variables and biomarkers as input variables. MOR: mu-opioid receptors, B-END: β-endorphin, Ca: calcium, zIR: index of insulin resistance, vit D3: vitamin D3, Cu: copper, IL: interleukin, zTG-zHDLc: reflects the atherogenic index of plasma; zGLUTOX: reflects glucose toxicity.

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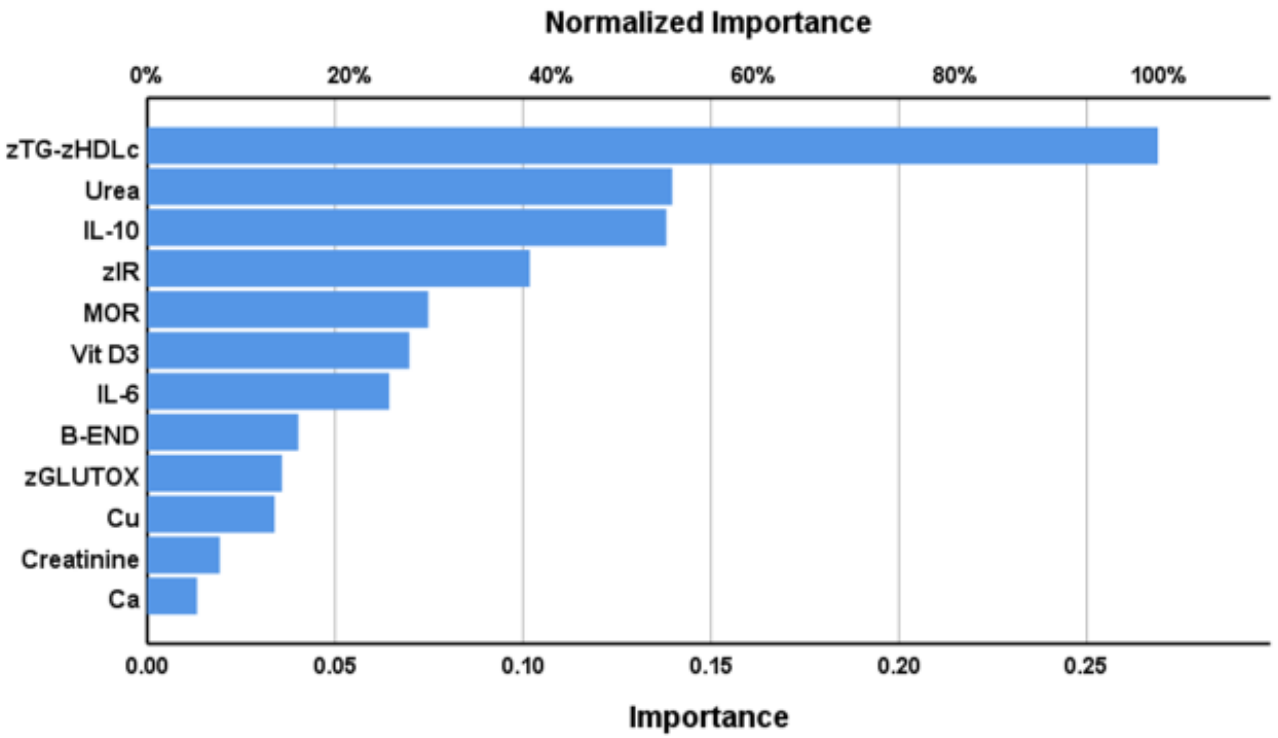

Figure 2. Results of a neural network (importance chart) with atherosclerosis and healthy controls as output variables and biomarkers as input variables. zTG-zHDLc: reflects the atherogenic index of plasma, IL: interleukin, zIR: index of insulin resistance, MOR: mu-opioid receptor, vit D3: vitamin D3, B-END: β-endorphin, zGLUTOX: index of glucose toxicity, Cu: copper, Ca: calcium

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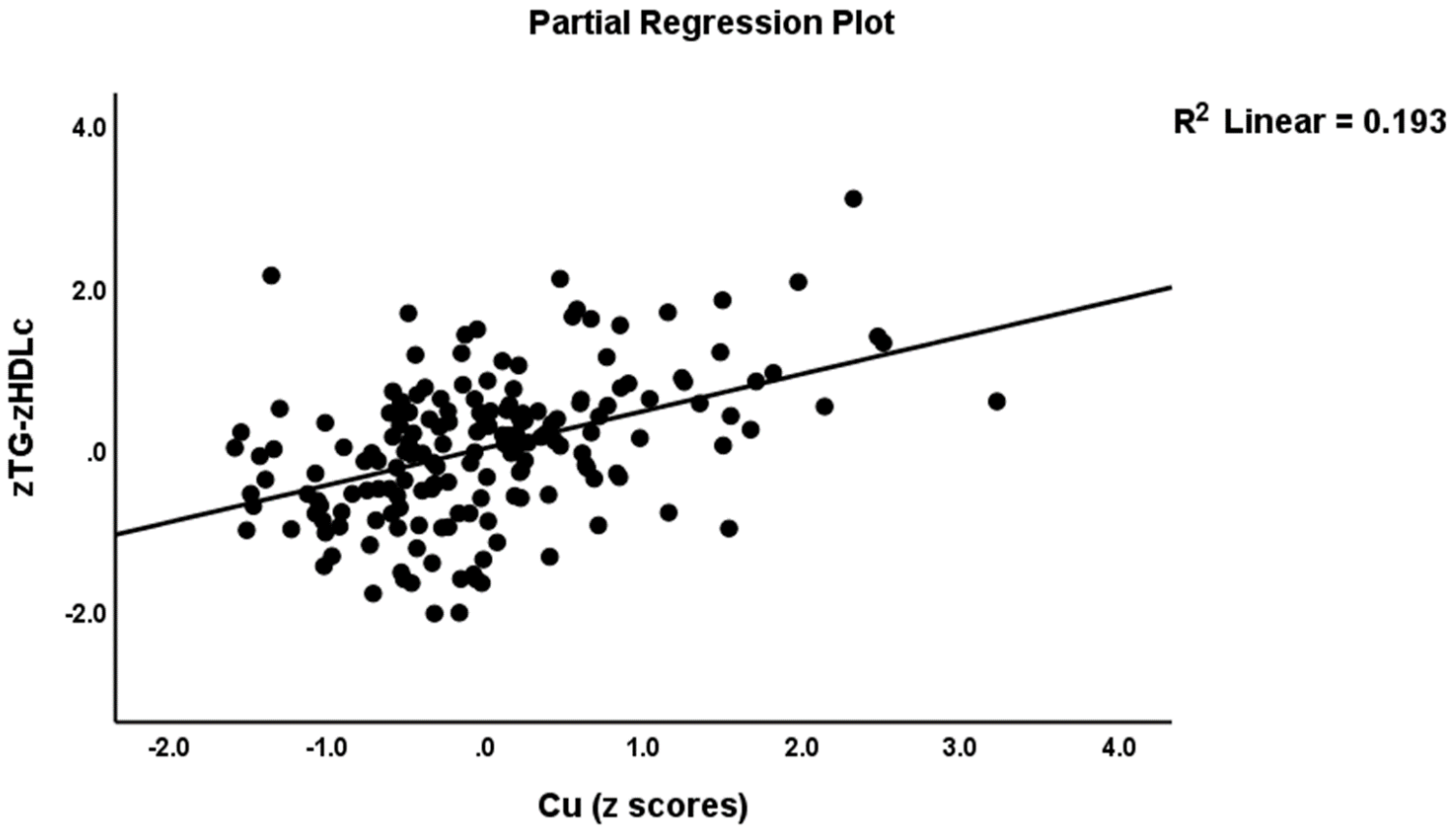

Figure 3. Partial regression plot of a z unit-weighted composite score reflecting the atherogenic index of plasma (computed as z triglycerides – z high density lipoprotein cholesterol) on copper.

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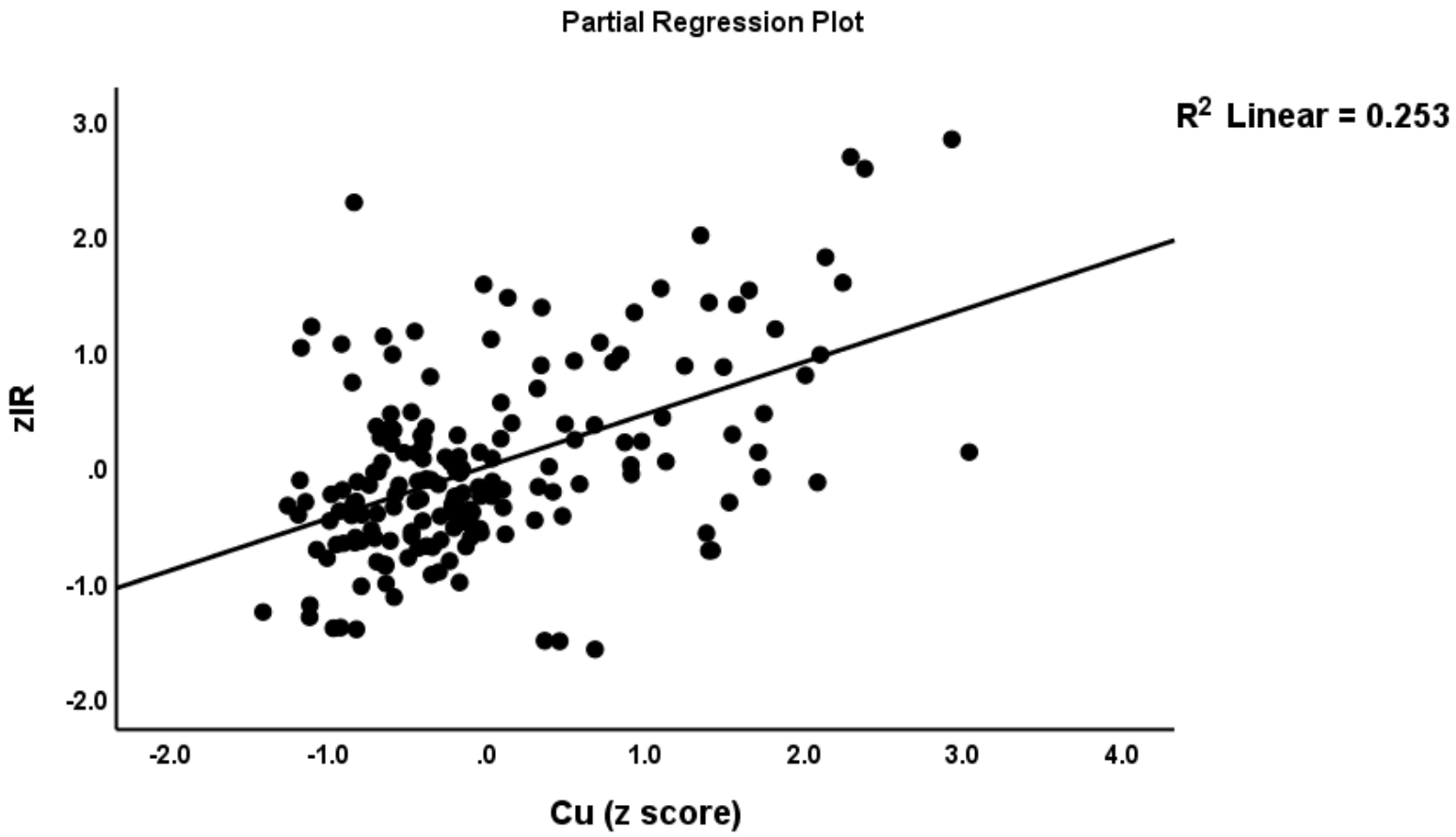

Figure 4. Partial regression plot of a z unit-weighted composite score reflecting insulin resistance (zIR, computed as z insulin + z glucose) on copper.

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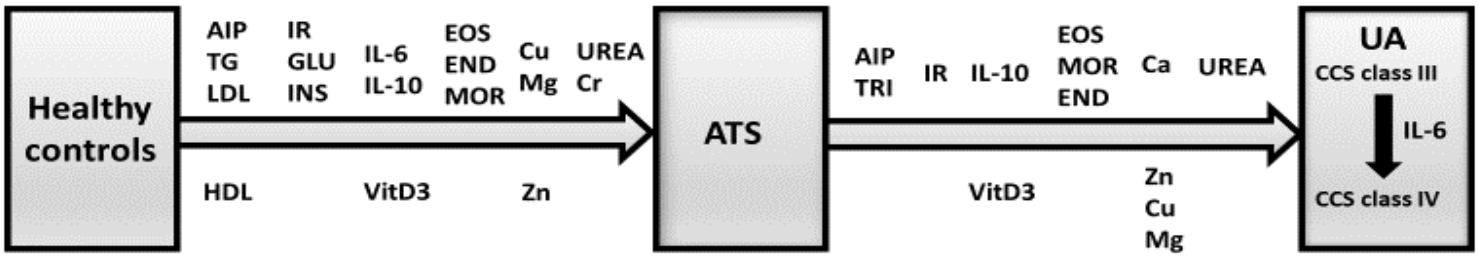

Figure 5. Summary of the present study. The development of atherosclerosis (ATS) is characterized by increased atherogenicity (higher triglycerides (TG) and LDL-cholesterol but lower HDL-cholesterol), insulin resistance (IR, higher insulin (INS) and glucose (GLU), increased interleukin (IL)-6 and IL-10, lower zinc (Zn) and vitamin D3 (vitD3), increased β-endorphin (B-END) and mu-opioid receptors (MOR), increased copper (Cu) and magnesium (Mg), and increased urea and creatinine (Cr).

The development of unstable angina (UA) is characterized by increased atherogenicity (increased TG levels), IR, and elevated IL-10, MOR, β-endorphins, calcium, and urea, and lower zinc, copper and magnesium.

The transition of CCS class III into class IV is associated with increased IL-6.