Predictive value of ceruloplasmin for metabolic syndrome in adolescents

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Abstract

The metabolic syndrome (MetS) is precisely defined and the cardiovascular risk associated with the clustering of its components has been demonstrated in adults. However, data on children and adolescents are still scarce, in part, due to difficulties in transposing the definition from adults. The identification of risk factors for the development of MetS at an early age is essential for prevention purposes with low-grade inflammation acting as a determinant for the association among the MetS components. The aim of this study was to investigate the associations of the MetS with systemic markers of inflammation and ceruloplasmin in a population of adolescents. The present is a cross-sectional study whose sample population consisted of 976 adolescents, 13.2 ± 1.2 years of age. Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were determined by ELISA. High-sensitivity C-reactive protein (hs-CRP) was determined by a solid-phase chemiluminiscent immunometric assay. Ceruloplasmin was measured by immunoturbidimetry. MetS adolescents exhibited higher levels of TNF-α, IL-6, CRP and ceruloplasmin compared to non-MetS individuals. TNF-α, IL-6 and CRP showed strong correlations with the MetS components and insulin resistance but not relevant predictive values according to ROC curves (AUC values 0.544- 0.555). In contrast, ceruloplasmin only showed significant correlations in non-Mets individuals, but exhibited a very high predictive value (AUC=0.941, P < 0.001). The determination of serum ceruloplasmin in adolescents might be a useful tool to identify patients with the highest risk of future cardiovascular disease.

Keywords: inflammation; metabolic syndrome; adolescents; obesity; ceruloplasmin
1. Introduction

The metabolic syndrome (MetS) is defined as a set of risk factors of metabolic precursors of atherosclerotic cardiovascular disease and type 2 diabetes mellitus (DM2) (1-3). Until recently, the cluster of metabolic risk factors that are considered part of the MetS had only been reported in adults, but increasingly numerous reports have shown the presence of MetS during adolescence, probably driven by the high prevalence of obesity from early age (4). According to the International Diabetes Federation (IDF), MetS can be diagnosed in adolescent subjects when the following criteria are fulfilled: a waist circumference ≥ 94 cm in boys and ≥80 cm in girls, together with two more risk factors being these ones: fasting blood glucose levels 100-125 mg/dl, serum triglyceride levels ≥150 mg/dl, HDL-cholesterol levels < 40 mg/dl in boys and < than 50 mg/dl in girls and blood pressure ≥ 130/85 mmHg (5).

The identification of risk factors for the development of MetS at an early age is essential for prevention and early detection of the syndrome (6). In this regard, recent studies raised the importance of the existence of a chronic state of low-grade inflammation in these subjects, which appears to act as a determinant for the association among the components of the MetS (7). This low-grade inflammation seems to be expressed by alterations in serum levels of certain inflammation markers, such as C-reactive protein (CRP), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α). Elevated levels of CRP are associated with increased expression and release of IL-6 by adipose tissue (8) and can be considered independent predictors of future cardiovascular events (9). In the case of TNF-α, despite being a classical marker in adults, for the moment the evidence is not solid enough to support an association between serum levels and nutritional status of in adolescents (10). More recently, Aguilar et al. (11) and...
Giordano et al. (12) found a correlation between increased serum levels of ceruloplasmin, the main copper-carrying protein and one of the acute-phase reactants in serum (13), and early development of cardiovascular disorders in obese adolescents. There is evidence indicating that ceruloplasmin is associated with various components of the MetS, like fasting glucose, triglycerides and HDL-cholesterol (14).

Furthermore, the evidence suggests that insulin resistance (IR) is another key factor in the development of MetS, as it is believed that a number of MetS components, such as high blood pressure, dyslipidemia and glucose intolerance are caused by the IR (15,16). However, studies on the relationship between IR and inflammatory markers are limited to date, especially in adolescents. In this context, the objective of this study was to investigate the associations of the MetS with the main markers of inflammation, especially ceruloplasmin, and IR in adolescents.
2. Methods

2.1 Data Source, Study Design and Recruitment

The present is a cross-sectional study whose sample population consisted of 976 adolescents, 13.2 ± 1.2 years of age. The subjects attended 18 schools in the provinces of Granada and Almeria (south-east of Spain). The study was previously approved by the Board of Education of the Andalusian Regional Government (Granada and Almeria Delegations), and also authorized by the school principals. The study, including the model of informed consent used, was approved by the Ethics Committee of the University of Granada. At the beginning of the study, an informed consent was obtained from the subjects' parents or guardians. This research was performed in strict compliance with the international code of medical ethics established by the World Medical Association and the Declaration of Helsinki. An important condition for inclusion in the study was that subjects had to be healthy and not have any type of endocrine dysfunction or physical disorder. All students who did not meet these criteria were not candidates to participate in the study.

2.2 Serum Biochemical Examination

At 8:00 A.M. after a 12-hour overnight fast, 10 ml of blood was extracted by venipuncture in the antecubital fossa of the right arm with a disposable vacuum blood collection tube. In the four hours after the extraction, all samples were centrifuged at 1300g for 15 minutes (Z400 K, Hermle, Wehingen, Germany). The red blood cells were thus separated and the serum was finally frozen at -80°C for its subsequent analysis. Only glucose was measured immediately after collection. IL-6 and TNF-α were
determined by ELISA (Terumo Corp, Newark, USA; R&D Systems, Minneapolis, USA). hs-CRP was determined by solid-phase chemiluminiscient immunometric assay. Ceruloplasmin was measured by immunoturbidimetry (AU2700 biochemistry analyzer; Olympus). Glucose was measured using a colorimetric enzymatic method (GOD-PAP Method, Human Diagnostics, Germany). HbA1c (glycosylated hemoglobin) was measured with high-performance liquid chromatography using a National Glycohemoglobin Standardization Program certified automated analyzer (model HLC-723 G7; Tosoh, Tokyo, Japan, intra-assay coefficient of variation <0.8 %, inter-assay coefficient of variation <0.5 %), and was standardized according to the Diabetes Control and Complications Trial. Serum insulin was determined by radioimmunoanalysis (Insulin Kit, DPC, Los Angeles, EEUU). Insulin resistance was quantified with HOMA (Homeostasis Model Assessment) (17) by applying the following formula: fasting glucose (mmol/L) x fasting insulin (mU/L)/ 22.5.

2.3 Statistical Analyses

Continuous variables are expressed as mean and SD. CRP, TNF-α and IL-6 values were log transformed to correct for non-normality. Normality was then assessed using the Kolmogorov-Smirnov's test. Statistical differences among Non-MetS and MetS boys and girls were assessed using ANOVA with post-hoc Tukey's test. Multivariate linear regression analysis of HOMA-IR, the number of MetS components and inflammatory markers were performed adjusting by age, sex, BMI, weight at birth, smoking mother, maternal educational level and maternal obesity. Receiver-operator curves (ROC) were created to determine the predictive value for MetS of inflammation markers and the
MetS components. K-mean cluster analyses were performed to group cases according to BMI and ceruloplasmin levels. Differences were considered significant at $P < 0.05$.

Data were analyzed using SPSS software for Linux version 21.0 (IBM Corp, Amonk, USA).
3. Results

Differences in markers of insulin resistance between boys and girls with or without a diagnosis of MetS are depicted in Table 1. As expected, MetS subjects exhibited higher glucose, insulin, HBA1c and HOMA-IR values. Most of these parameters, except for glucose, also differed according to sex, but only in the non-MetS group. Insulin, HBA1c, and HOMA-IR values were higher in boys compared to girls. Likewise, MetS boys and girls exhibited higher levels of all markers of inflammation studied (TNF-α, IL-6, CRP and ceruloplasmin) compared to non-MetS individuals (Table 2). Within the MetS group, boys presented higher plasma concentrations of TNF-α, IL-6, CRP but not ceruloplasmin.

Correlations among markers of inflammation and HOMA-IR are shown in Table 3. HOMA-IR values correlated with TNF-α, IL-6, CRP and ceruloplasmin in both MetS and non-MetS subjects with a high level of significance and independently of BMI. However, the level of significance was lower in boys than in girls suffering from MetS, which might be related to the lower number of subjects recruited in this group (n=16). We also correlated the levels of inflammatory markers with the number of MetS components (serum glucose, triglyceride and HDL-cholesterol concentrations, blood pressure and waist circumference) (Table 4). TNF-α, IL-6 and CRP correlated positively with the number MetS components in both MetS and non-MetS subjects. However, ceruloplasmin levels only showed significant correlations in non-MetS. No differences were observed regarding to sex.
In order to identify which of the MetS components had a higher predictive value for the syndrome, we performed a receiver operating characteristic (ROC) analysis (Figure 1A). As expected, waist circumference showed the highest area under the curve (AUC) value (0.970, $P < 0.001$), since it is a prerequisite for the diagnosis. The predictive value of blood pressure was also high (AUC=0.784, $P < 0.001$), but glucose, triglycerides and HDL-cholesterol exhibited a lower contribution to the prediction of MetS. The same analysis was performed for the markers of inflammation (Figure 1B). TNF-α, IL-6 and CRP showed very little predictive value for the MetS with very similar AUC values, ranging from 0.544 to 0.555. Remarkably, serum ceruloplasmin concentrations showed a high predictive value (AUC=0.941, $P < 0.001$), which was almost as high as that of waist circumference. The cut-off point for ceruloplasmin was established in 22.5 mg/dL, with a sensitivity of 90.9% and a specificity of 83.0%. The contingency analysis showed an asymptotic significance of $p<0.001$ for the Chi-square test and the linearity of the association.

Taking into account these results, and in order to visualize possible outliers that would explain the sensitivity and specificity values, individual ceruloplasmin levels were plotted as a dot graph (Figure 2A). The graph clearly shows that dots are grouped into three categories of ceruloplasmin levels. The k-means cluster analysis confirmed the presence of three distinct groups that were associated with BMI (Figure 2B). In the first group (n=872) we found subjects with low BMI and ceruloplasmin levels (center located at 22 mg/dl for ceruloplasmin and 20.3 kg/m$^2$ for BMI). The second group, consisted of subjects (n=28) with intermediate BMI and ceruloplasmin values (center located at 63 mg/dl for ceruloplasmin and 26.5 kg/m$^2$ for BMI). And the third group
(n=76) showed high BMI and ceruloplasmin values (center located at 81 mg/dl for ceruloplasmin and 29.8 kg/m\(^2\) for BMI).

4. Discussion

A chronic state of low-grade inflammation appears to be determinant for the association among the MetS components. A number of inflammation markers have been associated with the presence of MetS (18) or with the different components that are used in the diagnosis of the MetS, i.e. triglycerides, HDL-c, glucose, blood pressure and waist circumference (7). The results of the present study support these observations in adolescents, since classical markers of inflammation (TNF-\(\alpha\), IL-6 and CRP) correlated with the number of MetS components and with the HOMA index of insulin resistance. However, the most outstanding result was that ceruloplasmin showed the highest predictive value among markers of inflammation, being almost as good as that of the waist circumference, which is considered a prerequisite for the diagnosis of MetS. This might be of great importance for obese adolescents as ceruloplasmin has been suggested as a potent marker of inflammation and insulin resistance in this population (15,16).

Adolescents with MetS showed higher levels of TNF-\(\alpha\), IL-6 and CRP compared to non-MetS individuals, which is in agreement with previous observations. Cytokines have been strongly associated with the MetS in the whole population (19) but also in the childhood. CRP has been related with MetS and its components in adolescents (20,21), being considered as a good marker of cardiovascular risk in this population (22). Makni et al. (23) compared a group of obese adolescents with and without MetS with a groups of controls, finding significant differences among the three
groups for TNF–α, IL-6 and CRP. Unfortunately, these authors did not include sex as a discriminatory variable. In the present study, sex appeared to be determinant for the presence of insulin resistance and inflammation, both in MetS and non-MetS adolescents. Boys showed higher HOMA-IR values in the group of non-MetS individuals and higher TNF–α, IL-6 and CRP values in the group of MetS. Serum ceruloplasmin concentrations resulted to be higher also in boys, but only in the non-MetS group. Although there was a lack of significance in the MetS group, which might be related to the low number of subjects, sex might be an important characteristic when determining the presence of MetS. In adults, women with MetS exhibit higher levels of CRP (24,25) but the reasons for the influence of sex remain unclear. It has been proposed that they might be associated with differences in body fat distribution (26) or hormone levels (27), but other authors found no differences in CRP levels between women with or without hormone replacement (24,25). In adolescents with MetS, González et al. (28) also observed higher levels of inflammatory markers in boys compared to girls, although differences were only significant for PAI-1.

All markers of inflammation correlated strongly with the HOMA-IR and with the numbers of MetS components. Makni et al. (23) also reported significant correlations between TNF–α, IL-6 and CRP and the number of MetS components in obese adolescents with or without MetS, although they included up to three components in the model, the minimum necessary for the classification of MetS, whereas we included the five components. In obese and normal-weight adolescents, Moon et al. (29) found correlations between TNF–α and some, but not all, MetS components. In
particular, systolic blood pressure and fasting plasma concentrations did not correlate with TNF–α.

Despite the lack of correlation between ceruloplasmin and the MetS, this marker of inflammation showed the strongest predictive value according to ROC analyses, being similar to that of waist circumference and better than other MetS components. In fact, glucose, triglycerides and HDL-c showed a low predictive value in adolescents when considered individually. In the present study we employed the IDF criteria for the diagnosis of MetS in adolescents (5). However, although the MetS in adults is precisely defined and the cardiovascular risk associated with the clustering of its components has been demonstrated (30), data on children and adolescents are still scarce. In part, this is due to difficulties encountered in transposing the definition from adults and, thus, the cluster of components (5). In this regard, ROC curves can be employed to discriminate between individuals with and without a certain disease, as they describe the performance of a model across the range of classification thresholds. The use of the AUC facilitates the task, as it aggregates performance across the entire range, giving higher the AUC values for variables with better predictive capacity. Interestingly, AUC values of ceruloplasmin (0.941) were similar to those of waist circumference (0.970) and considerably higher that other MetS components and markers of inflammation. Indeed, TNF–α, IL-6 and CRP, despite showing strong correlations with the components of MetS and insulin resistance, did not show relevant prediction values according to ROC curves.

Ceruloplasmin, the main copper-carrying protein, is one of the acute-phase reactants and antioxidant proteins in serum, and has been associated with inflammatory
events occurring in atherosclerosis and diabetes mellitus (31). It is known that serum ceruloplasmin levels are elevated in subjects with MetS, as well as in subjects with Type 2 diabetes mellitus, and there is some evidence indicating associations with various components of the MetS, like fasting glucose, triglycerides and HDL-cholesterol (13). However, ceruloplasmin has not been independently associated with the other MetS components, that is, blood pressure (13) and central obesity (14), although patients with central obesity have characteristically higher ceruloplasmin serum levels, (14) due to overexpression of the protein (32). Therefore, the lack of association with some MetS components in previous studies might explain the lack of correlation of ceruloplasmin with the number of MetS components in the present one.

As stated above, ceruloplasmin has been reported to strongly correlate with cardiovascular risk factors of lipid nature, particularly triglycerides (13, 33), which might be related to increased oxidative stress in the cell. Long-chain acyl-CoA derived from triglycerides can inhibit mitochondrial adenine nucleotide translocator, the rate-limiting enzyme for exchange between mitochondrial adenosine triphosphate (ATP) and cytosolic adenine diphosphate (ADP). When the mitochondrial ADP concentration is reduced, the proton gradient becomes higher, increasing the likelihood of accidental transfer of a single electron to oxygen, thereby increasing ROS generation in the cells. In this regard, it has been suggested that increased ceruloplasmin levels in MetS subjects might be a compensatory mechanism for increased oxidative stress associated with high serum triglycerides (13).

Nevertheless, and despite it has been proposed that serum ceruloplasmin level could be an independent risk factor for cardiovascular disease (34), the mechanism by which this protein is linked to cardiovascular disease is not established. Ceruloplasmin
might be involved in the inflammatory pathway linked to oxidative stress, in conditions promoting the copper release of the molecule, allowing the reaction of free copper with pro-oxidants factors (35). In contrast, others have proposed an antioxidant effect, as ceruloplasmin can reduce oxidative stress through its ferroxidase activity and inhibition of Fenton reaction, which uses the ferrous iron to generate reactive oxygen species (31). Ceruloplasmin has an unspecific oxidase activity, participating in oxidation reactions with multiple organic and inorganic substrate, including Fe2+ ion, benzidine, p-phenylenediamine or N-dimethylphenylenediamine. Currently, there is controversy on the association of iron concentrations in the body and its metabolism with the development of the MetS in both genders, both in adults and adolescents. Iron is usually considered as an independent factor for the development of MetS and, thus, not in all cases associated with ceruloplasmin levels (36-41).

Another important finding of the present study was that ceruloplasmin levels were dependent on BMI. We found that ceruloplasmin levels were distributed into three groups according to ceruloplasmin levels and BMI, which suggests that BMI is the leading factor by which the MetS and ceruloplasmin are associated. In addition, they show that normal-weight subjects and obese subject had very different ceruloplasmin values, with overweight subjects falling within the three groups. In Spanish adolescents, Wärnberg et al. (42) found significant correlations between BMI and WC with ceruloplasmin and, more recently, we reported associations between ceruloplasmin and BMI z-score, in non-obese and obese subjects (43).

The present study has some strengths and limitations. Among the strengths, the number of participants in this study is high. In addition, all participants belonged to the same geographical region, with similar culture, life-style and dietary habits, making the
sample more homogeneous. The recruitment was designed to select a representative sample of the entire population for the age groups studied in the region, for which we consider that the study is of great importance in epidemiological terms. Potential confounders such as age, sex, BMI, weight at birth, smoking mother, maternal educational level and maternal obesity were included in the regression model. However, other potential confounders like the socioeconomic status or physical activity could not be included. Nevertheless, all participants were of a similar socioeconomic status and attended middle-class high-schools.

In conclusion, adolescents diagnosed with the MetS showed a higher level of low-grade inflammation compared with non-MetS. Classical markers of inflammation (TNF-α, IL-6 and CRP) exhibited strong correlations with insulin resistance and the number of MetS components but a low predictive value for MetS. In contrast, serum ceruloplasmin concentration revealed as a potent predictor of MetS, although did not correlate with the number of MetS components in MetS subjects. In adolescents, we and others have reported that ceruloplasmin correlates with indexes of obesity, such as BMI and the waist-to-hip ratio (11,42) and with markers of insulin resistance (43). In the present study we give evidence for the first time that ceruloplasmin can provide an elevated predicted value for MetS in a large group of adolescents. Therefore, the determination of serum ceruloplasmin in adolescents with central obesity might be a useful tool to identify patients with the highest risk of future cardiovascular disease.
Acknowledgements

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References


**Figure 1.** Receiver operating characteristic (ROC) curves of the components of the metabolic syndrome (A) and inflammation markers (B) for prediction of MetS. AUC values: IL-6: 0.555, TNF-α: 0.544, CRP: 0.548, ceruloplasmin: 0.941, glucose: 0.548, triglycerides: 0.538, HDL-cholesterol: 0.446, waist circumference: 0.970, systolic blood pressure (SBP): 0.784 and diastolic blood pressure (DBP): 0.740.

**Figure 2.** Plasma ceruloplasmin concentrations in all participants (A) and according to their BMI (B).
Table 1. Markers of insulin resistance in adolescents classified according to the absence (non MetS) or presence (MetS) of the metabolic syndrome.

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<th>MetS</th>
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<tr>
<td></td>
<td>Girls (n=489)</td>
<td>Boys (n=441)</td>
<td>Girls (n=30)</td>
<td>Boys (n=16)</td>
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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>84.4</td>
<td>27.4</td>
<td>84.6</td>
<td>29.1</td>
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<tr>
<td>Insulin (mU/ml)</td>
<td>18.5</td>
<td>5.9</td>
<td>20.2</td>
<td>9.1**</td>
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<tr>
<td>HBA1c (%)</td>
<td>4.4</td>
<td>0.7</td>
<td>4.7</td>
<td>2.2**</td>
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<tr>
<td>HOMA-IR</td>
<td>3.9</td>
<td>1.9</td>
<td>4.2</td>
<td>2.0*</td>
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</table>

HBA1c, glycosylated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance.

*, P < 0.05 vs. girls; **, P < 0.01 vs. Girls; †, P < 0.05 vs. non MetS; †††, P < 0.001 vs. non MetS.
Table 2. Serum concentrations of inflammatory markers in adolescents classified according to the absence (non MetS) or presence (MetS) of the metabolic syndrome.

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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>11.7</td>
<td>2.2</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>12.7</td>
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</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>1.10</td>
<td>1.05</td>
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<tr>
<td>Ceruloplasmin (mg/dl)</td>
<td>23.9</td>
<td>10.9</td>
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**, P < 0.01 vs. girls; ***, P < 0.001 vs. girls; †, P < 0.05 vs. non MetS; †††, P < 0.001 vs. non MetS.
Table 3. Correlation parameters of the serum concentration of inflammatory markers and HOMA-IR index in adolescents without (non MetS) or with (MetS) the metabolic syndrome.

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<td>Boys (n=16)</td>
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<tr>
<td></td>
<td>β</td>
<td>P</td>
<td>β</td>
<td>P</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>0.596</td>
<td>&lt;0.001</td>
<td>0.487</td>
<td>&lt;0.001</td>
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<tr>
<td>IL-6 (pg/ml)</td>
<td>0.628</td>
<td>&lt;0.001</td>
<td>0.436</td>
<td>&lt;0.001</td>
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<tr>
<td>CRP (mg/dl)</td>
<td>0.646</td>
<td>&lt;0.001</td>
<td>0.490</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ceruloplasmin (mg/dl)</td>
<td>0.643</td>
<td>&lt;0.001</td>
<td>0.763</td>
<td>&lt;0.001</td>
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Regression co-efficient (β, (CI-95%)) is the effect size per increase in serum concentration of inflammatory markers and the HOMA-IR index.
Table 4. Correlation parameters of the serum concentration of inflammatory markers and the number of metabolic syndrome components in adolescents without (non MetS) or with (MetS) the metabolic syndrome.

<table>
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<tr>
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<td>Boys (n=16)</td>
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<tr>
<td><strong>β</strong></td>
<td><strong>P</strong></td>
<td><strong>β</strong></td>
<td><strong>P</strong></td>
<td><strong>β</strong></td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>0.612</td>
<td>&lt; 0.001</td>
<td>0.679</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>0.653</td>
<td>&lt; 0.001</td>
<td>0.580</td>
<td>&lt; 0.001</td>
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<tr>
<td>CRP (mg/dl)</td>
<td>0.695</td>
<td>&lt; 0.001</td>
<td>0.689</td>
<td>&lt; 0.001</td>
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<tr>
<td>Ceruloplasmin (mg/dl)</td>
<td>0.276</td>
<td>&lt; 0.001</td>
<td>0.372</td>
<td>&lt; 0.001</td>
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Regression co-efficient (β, (CI-95%)) is the effect size per increase in serum concentration of inflammatory markers and the number of components of the metabolic syndrome. Components of the metabolic syndrome: serum glucose, triglyceride and HDL-cholesterol concentrations, blood pressure and waist circumference.