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Increased nitro-oxidative toxicity in association with metabolic syndrome, atherogenicity and insulin resistance in patients with affective disorders.

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Abstract

Background: There is a strong comorbidity between mood disorders and metabolic syndrome (MetS). Increased levels of reactive oxygen and nitrogen species (RONS) and nitro-oxidative stress toxicity (NOSTOX) partially underpin this comorbidity.

Aims: To examine the associations of RONS/NOSTOX biomarkers with MetS after adjusting for the significant effects of mood disorders (major depression, and bipolar type 1 and 2), generalized anxiety disorder (GAD), tobacco use disorder (TUD), and male sex.

Methods: The study included subjects with (n=65) and without (n=107) MetS and measured levels of superoxide dismutase 1 (SOD1), lipid hydroperoxides (LOOH), nitric oxide metabolites (NOx), malondialdehyde (MDA), and advanced oxidation protein products (AOPP) and computed z unit-weighted composite scores which reflect RONS/NOSTOX. The study included 105 patients with mood disorders, 46 with GAD, and 95 with TUD.

Results: MetS was associated with increased levels of MDA and AOPP, independently from mood disorders, TUD, sex and GAD. Atherogenicity and insulin resistance (IR) were significantly associated with a NOSTOX composite score. Mood disorders, TUD, GAD, male sex and MetS independently contribute to increased RONS/NOSTOX. The RONS/NOSTOX profile of MetS was different from that of GAD, which showed increased SOD1 and NOx levels. TUD was accompanied by increased SOD1, LOOH and MDA, and male sex by increased LOOH and AOPP.

Conclusions: MetS is characterized by increased lipid peroxidation with aldehyde formation and chlorinative stress, and atherogenicity and IR are strongly mediated by RONS/NOSTOX. Partially shared RONS/NOSTOX pathways underpin the comorbidity of MetS with mood disorders, GAD, and TUD.

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Introduction

Evidence shows that there is a strong comorbidity between mood disorders, such as Major Depressive Disorder (MDD) and Bipolar Disorder (BD), and Metabolic Syndrome (MetS) (de Melo et al., 2017). Not only mood disorders, but also anxiety disorders, especially Generalized Anxiety Disorder (GAD), co-occur with MetS (Moreira et al., 2019). MetS increases the risk to cardiovascular disease through a number of risk factors including abdominal obesity, blood pressure, lowered levels of high-density lipoprotein (HDL) cholesterol, and increased levels of triglycerides and glucose (Grundy et al., 2005). These components reflect the two important characteristics of MetS, namely increased atherogenicity and insulin resistance (IR) (de Melo et al., 2017).

Recent studies and reviews found that mood disorders are accompanied by increased atherogenicity. An early study found a decreased ratio of HDL cholesterol/total cholesterol in patients with a major depressive episode, indicating increased atherogenicity (Maes et al., 1994). A study conducted by Nunes et al. (2015) showed that the atherogenic index of plasma (AIP) was significantly increased in both MDD and BD. Vargas et al. (2014) reported significantly increased Castelli 1 and 2 indices in MDD, but not in BD. Furthermore, some but not all studies showed that mood disorders may be accompanied by increased IR. For example, Bortolasci et al. (2015) were unable to find a significant association between IR and mood disorders (MDD or BD), whereas Bonifacio et al. (2017) reported that number of depressive and manic episodes were associated with increased IR.

There is now evidence that MDD and BD are accompanied by activation of the immune-inflammatory response system (IRS) as indicated by increased levels of pro-inflammatory cytokines and acute phase proteins, including tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β) and interleukin 6 (IL-6) and high sensitivity C-reactive protein (hsCRP) (Maes and Carvalho, 2018). Nevertheless, the findings on hsCRP are strongly affected

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by an increased elevated body mass index (BMI) and early life trauma (ELT) explaining the increased hsCRP levels in BD (Moraes et al., 2017). In addition, the IRS response is accompanied by increased nitro-oxidative stress toxicity (NOSTOX), due to high levels of reactive oxygen and nitrogen species (RONS) that have damaged lipids and proteins, and lowered antioxidant defenses, establishing an imbalance between pro- and anti-oxidants (Maes et al., 2011; Moylan et al., 2014). In MDD, activated RONS/NOSTOX pathways lead to lipid peroxidation with the generation of aldehydes including malondialdehyde (MDA) and protein damage with the formation of advanced oxidation protein products (AOPP) (Maes et al., 2019a). Moreover, increased AOPP formation is associated with bipolar type 1 (BP1), whereas in bipolar type 2 (BP2) the RONS/NOSTOX pathways seem to be attenuated (Maes et al., 2019a).

There is a strong comorbidity between MDD/BD and GAD. Anxiety disorders, including GAD, represent 59.2% and 74.9% of lifetime comorbidity in MDD and BD, respectively (Kessler et al., 2003; Merikangas et al., 2007). GAD is also accompanied by RONS/NOSTOX with increased lipid hydroperoxides (LOOH) production and lowered antioxidant activity (Maes et al., 2018). The co-occurrence between MDD and GAD is characterized by a highly increased activation of RONS/NOSTOX pathways as indicated by increased MDA and AOPP formation (Maes et al., 2018).

MetS is associated with activation of immune cells and mild chronic inflammation and increased NOSTOX (Zafar et al., 2018; Vona et al., 2019). Lowered levels of HDL cholesterol, which have antioxidant activity, may not only contribute to atherogenicity, but also be responsible for the activation of RONS pathways in MetS (de Melo et al., 2017). IR is associated with increased levels of uric acid which can trigger inflammatory and RONS pathways (de Melo et al., 2017). In addition, a recent study found that, in mood disorders, NOSTOX is an important predictor of increased blood pressure (Bonifacio et al., 2021).

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Nevertheless, there are no data whether the comorbidity between MetS and mood disorders may share activated RONS/NOSTOX pathways.

Hence, the aim of the present study was to examine the associations of RONS/NOSTOX biomarkers, including superoxide dismutase 1 (SOD1), LOOH, nitric oxide metabolites (NOx), MDA and AOPP, as well as its composite scores, with MetS in patients with mood disorders, after considering the effects of affective disorders, GAD, smoking and sex. The a priori hypothesis was that the comorbidity of MetS, atherogenicity and IR are accompanied by increased RONS and NOSTOX.

Subjects and Methods

Participants

The patients were recruited at the Psychiatry outpatient clinic, University Hospital of the State University of Londrina (UEL), Londrina, Brazil. Healthy controls (HC) were recruited by word of mouth from the same catchment area. We included both men and women, aged 20-65 years old. We excluded patients and controls with neurodegenerative and neuroinflammatory disorders (Alzheimer's disease, Parkinson's disease, multiple sclerosis); immune-inflammatory disorders, including psoriasis, rheumatoid arthritis, chronic obstructive pulmonary disease, cancer, chronic kidney disease, systemic lupus erythematosus, type 1 diabetes, hepatitis B and C virus, HIV infection; other axis I disorders according to Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (DSM-IV-TR) (autism, schizophrenia, schizo-affective disorder, psycho-organic syndromes); and pregnant or lactating women. Treatments with non-steroidal anti-inflammatory drugs, interferon, glucocorticoids, herbal supplements, antioxidants and omega-3 for the past 4 weeks before the study were other exclusion criteria. Written informed consent was obtained from all the participants. The study

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was approved by the Ethics Committee on Research from UEL, Londrina, Brazil (CAAE 34935814.2.0000.5231).

Methods

The diagnosis of BP1, BP2 and MDD were made by psychiatrists using a validated Brazilian Portuguese version of the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) (Del-Ben et al., 2001) and DSM-IV-TR diagnostic criteria (APA, 2000). The participants completed a structured interview comprising socio-demographic and clinical data. GAD and Tobacco Use Disorder (TUD) were also assessed using the Brazilian Portuguese version of SCID-I (Del-Ben et al., 2001) and DSM-IV-TR diagnostic criteria (APA, 2000). The translated and adapted version for Brazilian subjects of the 17-item Hamilton Depression Rating Scale (HAM-D) (Moreno and Moreno, 1998) and the Hamilton Anxiety Rating Scale (HAM-A) (Hamilton, 1959) were used to measure the severity of depression and anxiety, respectively. Based on the number of manic and depression episodes, number of suicidal attempts and suicidal ideation, patients were classified in three different staging phases, as explained previously (Maes et al., 2019b).

The diagnosis of MetS was made using the International Diabetes Federation (IDF) criteria (Alberti et al., 2006), namely presence of three out of the following criteria: (a) abdominal obesity (waist circumference ≥ 90 cm for men and ≥ 80 cm for women in South Asian and South Americans and ≥ 94 cm for men and ≥ 80 cm for women in Caucasians); (b) low HDL cholesterol (< 40 mg/dL in men and < 50 mg/dL in women) or use of hypolipidemic drugs; (c) hypertriglyceridemia (triglycerides ≥ 150 mg/dL) or use of hypolipidemic agent; (d) increased fasting glucose (≥ 100 mg/dL) or use of oral antidiabetic medications; (e) increased average blood pressure (≥ 130/85 mmHg) or currently taking antihypertensive medication. The BMI was calculated following the formula: weight (in kg) divided by square of height (in m^2).

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The waist circumference (WCF) was measured at the midway between the margin of the lower rib and the iliac crest and the blood pressure was assessed using a mercury sphygmomanometer.

Assays

After 12h of overnight fasting, blood samples from the participants were collected. The levels of glucose, triglycerides, total cholesterol, HDL cholesterol, and uric acid were assayed using an automated clinical chemistry system (Dimension® RxL, Siemens Healthcare Diagnostics Inc, USA). The results were expressed in mmol/L for glucose and total cholesterol and mg/dL for triglycerides, HDL cholesterol, and uric acid. The levels of insulin were determined by microparticle enzyme immunoassay (MEIA) (AXSYM, Abbott Laboratory, Germany). The levels of hsCRP were determined using a turbidimetric assay (ARCHITECT c8000, Architect, Abbott Laboratory, Abbott Park, IL, USA). The results for insulin and hsCRP were expressed in µU/mL and mg/L, respectively. The inter-assay coefficients of variability for all analytes were less than 10%.

IR and insulin secretion (IS) were computed using the HOMA calculator©, Version 2.2.3 (Diabetes Trials Unit of University of Oxford) (Bonifacio et al., 2017). Nevertheless, in the current study we show the results using z unit weighted composite scores, namely z insulin + z glucose (reflecting IR) and z insulin – z glucose (reflecting IS). For the assessment of atherogenicity, the AIP {log (triglycerides/HDL cholesterol)} (Nunes et al., 2015) and the Castelli 1 index (total cholesterol/HDL cholesterol) (Vargas et al., 2014) were calculated. Nevertheless, in the current study we show the results using z unit weighted composite scores, namely z triglycerides - z HDL cholesterol (reflecting AIP) and z total cholesterol – z HDL cholesterol (reflecting Castelli 1 index).

The activity of the enzyme SOD1 and the concentrations of LOOH, NOx, MDA and AOPP were measured. SOD1 activity in erythrocytes was determined using the pyrogallol

method described by Marklund and Marklund (1974). This technique is based on the inhibition of pyrogallol selfoxidation by SOD1 in aqueous solution. The assay was conducted in a spectrophotometer Helios α, Thermo Spectronic (Waltham, MA, USA) at 420 nm and 37 °C. During 5 min, variation in optical density (OD) was recorded every minute. The level of SOD1 that inhibited 50% of the pyrogallol oxidation was defined as one unit of enzymatic activity. The results were expressed in U/mg of hemoglobin (Hb). LOOH were assayed by chemiluminescence (CL-LOOH) (Gonzalez-Flecha et al., 1991; Panis et al., 2012). This method uses the compound tert-butyl hydroperoxide to start a lipid chain reaction that can be detected by photon emission during the formation of lipid hydroperoxides. Readings were performed in a Glomax luminometer (TD 20/20 Turner Designers, USA) over 1 h at one reading per second. Results were expressed as relative light units (RLU). NOx levels were assessed indirectly by determining the plasma nitrite concentration using an adaptation of the technique described by Navarro-Gonzálvez et al. (1998). This method is based on the reduction of the nitrate present in the sample to nitrite by oxidation-reduction reactions mediated by the system cadmium-copper reagent. Thereafter, Griess reagent was added to induce diazotization, forming a colored complex and subsequent detection at 540 nm. The quantification of NOx was made in a microplate reader Asys Expert Plus, Biochrom (Holliston, MA, USA). NOx concentrations were expressed in µM. MDA levels were measured through complexation with two molecules of thiobarbituric acid (TBA) using MDA estimation through high performance liquid chromatography (HPLC Alliance e2695, Waters', Barueri, SP, Brasil) (Bastos et al., 2012). Experimental conditions included the use of a column Eclipse XDB-C18 (Agilent, USA), mobile phase consisting of 65% phosphate buffer (50 nM pH 7.0) and 35% HPLC grade methanol, flow rate of 1.0 mL/min, temperature of 30°C, and wavelength of 532 nm. MDA concentrations in the samples was quantified based on a calibration curve and is expressed in mmol of MDA/mg of proteins. AOPP were quantified using the method described by Hanasand

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et al. (2012) in a microplate reader, PerkinElmer, model EnSpire (Waltham, MA, USA) at a wavelength of 340 nm. AOPP concentrations were expressed in µM of equivalent chloramine T.

In order to examine the activated RONS/NOSTOX pathways, as explained previously (Maes et al., 2018, 2019a), the raw data were transformed in z scores and used to compute five z unit-weighted composite scores, namely SOD+LOOH (computed as z score SOD (zSOD) + zLOOH), SOD+LOOH+NOx (computed as zSOD+zLOOH+zNOx), SOD+LOOH+NOx+MDA (computed as zSOD+zLOOH+zNOx+zMDA), SOD+LOOH+NOx+AOPP (computed as zSOD+zLOOH+zNOx+zAOPP) and SOD+LOOH+NOx+MDA+AOPP (computed as zSOD+zLOOH+zNOx+zMDA+zAOPP).

Statistical analysis

The Kolmogorov-Smirnov test was used to analyze the normal distribution of the variables. Pearson chi-squared test (analyses of contingency tables) was used to examine the associations between nominal variables and the one-way analysis of variance (ANOVA) to examine differences in scale variables among groups. Multivariate GLM analysis was employed to examine the associations between the RONS/NOSTOX biomarkers and MetS while adjusting for effects of mood disorders, GAD, TUD, age and sex (mood diagnosis, GAD, sex and TUD were entered as factors and age as covariate). Partial eta squared values were used as estimates of effect size. Consequently, we computed model-generated estimated marginal means (SE) values, thus, the values after adjustment for mood diagnosis, GAD, sex, TUD and age. A second multivariate GLM analysis examined the effects of RONS/NOSTOX biomarkers on IR, IS and atherogenicity indices while adjusting for TUD, sex, age and WCF. Binary logistic regression analysis was performed to delineate the best predictors of MetS as dependent variable and no MetS as reference group. Odds ratios with 95% confidence interval

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were computed and Nagelkerke pseudo-R^2 was used as estimate of the effect size. IBM SPSS 25 for windows was used to performed all analysis. Statistical significance was set at $p < 0.05$, two-tailed.

Results

Socio-demographic and clinical data

Table 1 shows the socio-demographic data of the subjects in this study divided into those with and without MetS. There were no significant differences in sex ratio and education between both groups, although individuals with MetS were somewhat older than those without MetS. All affective disorder features were not significantly different between both groups, including number of mood disorder episodes, HAM-D scores, frequencies of mood disorder subtypes and GAD, and staging. Nevertheless, the HAM-A score was significantly higher in subjects with MetS compared to those without MetS. All MetS features were significantly different between both groups with significantly higher BMI, WCF, insulin, glucose, IR, triglycerides, total cholesterol, the AIP and Castelli 1 index, hsCRP, and uric acid in those with MetS. HDL cholesterol was significantly lower in MetS subjects, and there were no differences in IS between both groups.

RONS/NOSTOX biomarkers in MetS

Table 2 shows the results of multivariate GLM analysis, which examines the association between the 5 RONS/NOSTOX biomarkers and MetS while adjusting for the effects of mood disorders, GAD, TUD, and sex. Age was not significant in this regression and thus deleted from the analysis. There was a significant association between the biomarkers and MetS with an effect size of 0.177. Test for between-subjects effects showed significant associations between MetS and three composites, namely SOD+LOOH+NOx+MDA,

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SOD+LOOH+NOx+AOPP, and SOD+LOOH+NOx+MDA+AOPP. The second multivariate analysis displayed in Table 2 shows that there were associations between MetS and the separate biomarkers MDA and AOPP, but not SOD1, LOOH, or NOx. Table 3 shows the model-generated estimated marginal means (SE) indicating that three composites, MDA, and AOPP were significantly higher in subjects with MetS than in those without. **Figure 1** shows a clustered bar graph of the residualised RONS/NOSTOX values (all in z scores) in MetS and no-MetS, after partialling out the effects of mood disorders, TUD, GAD, and sex. **Table 4** shows the results of two logistic regression analysis with MetS as dependent variable. The first one entered all composites (and age, BMI, WCF, sex, TUD, mood disorders, GAD, hsCRP, and uric acid) and showed that MetS was best predicted by SOD+LOOH+NOx+MDA+AOPP and WCF combined ($\chi 2 = 67.83$, d f= 2, $p < 0.001$. Nagelkerke = 0.491). The second regression entered all 5 separate biomarkers and detected that 4 variables best predicted MetS, namely MDA, AOPP, hsCRP, and uric acid (all positively associated; $\chi 2 = 51.84$, df = 4, $p < 0.001$. Nagelkerke = 0.354).

Prediction of IR, IS and atherogenicity by RONS/NOSTOX biomarkers

Table 5 shows the results of a multivariate GLM analyses which examined the effects of the RONS/NOSTOX composite scores on the IR, IS, and atherogenicity indices. We found that the SOD+LOOH+NOx+MDA+AOPP composite score had a significant effect on IR (effect size of 0.159), AIP (effect size 0.138), and Castelli 1 index (effect size 0.076) after adjusting for the significant effects of TUD, sex, age and WCF. **Figure 2** shows the partial regression of the AIP on the SOD+LOOH+NOx+MDA+AOPP composite score. There was no significant association between the RONS/NOSTOX composite score and IS.

Effects of background variables

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The multivariate GLM analysis in Table 2 showed that there were significant effects of mood disorders, GAD, TUD and sex on the biomarkers. The associations with mood disorders and GAD have been described previously (Maes et al., 2018, 2019a). There were significant associations between sex and all composite scores (all at $p < 0.033$), except SOD+LOOH+NOx+MDA with significantly higher values in men than women. Tests for between subject effects showed significant effects of sex on LOOH ($t = +2.58$, $p < 0.001$) and AOPP ($t = +4.96$, $p < 0.001$). **Figure 3** shows a clustered bar graph of the residualised RONS/NOSTOX data (all in z scores) in males and females, after partialling out the effects of MetS, mood disorders, TUD, and GAD. There were significant associations between TUD and all composite scores (all at $p < 0.001$), except SOD+LOOH+NOx+AOPP with significantly higher values in smokers than non-smokers. Tests for between subject effects showed significant effects of TUD on SOD1 ($t = +2.95$, $p = 0.004$), LOOH ($t = +3.84$, $p < 0.001$) and MDA ($t = +2.65$, $p < 0.001$). **Figure 4** shows a clustered bar graph of the residualised RONS/NOSTOX data (all in z scores) in TUD and no-TUD, after partialling out the effects of MetS, mood disorders, GAD, and sex.

The multivariate GLM analysis in Table 5 showed that there were significant effects of TUD, sex, age, and WCF on the biomarkers. TUD was significantly and positively associated with IR only ($F = 7.45$, 1/166, $p = 0.007$, partial eta squared = 0.043). There was a significant positive effect of female sex on IS ($F = 10.39$, df = 1/166, $p = 0.002$, partial eta squared = 0.059). Age was significantly and inversely associated with IS ($F = 7.05$, df = 1/166, $p = 0.009$, partial eta squared = 0.041).

Discussion

Increased RONS/NOSTOX in MetS

The first major finding of this study is that RONS/NOSTOX indices were significantly increased in subjects with MetS as compared with those without MetS. The concept that RONS/NOTOX are associated with MetS and its outcomes, such as coronary artery disease and type 2 diabetes, was previously described (Roberts and Sindhu, 2009). Recently, lower levels of antioxidant defenses and increased levels of MDA and protein carbonyl, another biomarker for protein damage, were found in subjects with MetS in comparison to subjects without MetS (Awadallah et al., 2019). We found that MetS was associated with increased levels of MDA and AOPP, but not with changes in SOD1, LOOH and NOx.

The hydroxyl radical ($OH^{\bullet}$) and hydroperoxyl radical ($HO_2^{\bullet}$) are the main ROS responsible for triggering the lipid peroxidation chain reaction in polyunsaturated fatty acids (PUFAs), leading to the production of LOOH as initial products, which can continue reacting until the generation of end products including MDA (Ayala et al., 2014). The AOPP formation is related mainly with the production of hypochlorous acid (HOCl), a potent oxidant generated through the reaction between hydrogen peroxide (H_2O_2) and ions chloride, catalyzed by myeloperoxidases (MPO) (Witko-Sarsat et al., 1996; Ndrepepa, 2019).

Our findings on increased AOPP, but not LOOH, levels in MetS extend the findings of Venturini et al. (2015) who found that AOPP levels were more strongly associated with MetS than LOOH levels. Although no difference in the levels of LOOH was observed between the groups with and without MetS, we found increased levels of MDA in the group with MetS. Moreto et al. (2014) found that hyperglycemia, IR, and hypertriglyceridemia are determinants for increased levels of MDA in the MetS.

We also observed no difference in the levels of NOx between individuals with and without MetS. Peroxynitrite ($ONOO^-$), a RNS produced by the reaction between nitric oxide ($NO^{\bullet}$) and superoxide anion ($O_2^{\bullet-}$), is also involved with the lipid peroxidation process (Szabó et al., 2007; Morita et al., 2016). The fact that LOOH is a substrate for MDA generation during

lipid peroxidation (Ayala et al., 2014) may explain why our results showed increased levels of MDA but no changes in LOOH in MetS.

The enzyme SOD performs an important role on the pathways leading to lipid peroxidation and AOPP formation (Maes et al., 2018). SOD dismutates $O_2^{\bullet-}$ into H_2O_2, which can be converted into water and oxygen by the enzyme catalase or continue reacting, for example, with transition metals to produce $OH^{\bullet}$ (Fukai and Ushio-Fukai, 2011). Increased levels of SOD activity coupled with insufficient catalase activity leads to increased ROS production (Maes et al., 2018) and the pathways to lipid peroxidation and protein damage might be activated. Our study did not show any changes in the SOD1 activity in MetS. The enzyme SOD has three isoforms found in different sites (Fukai and Ushio-Fukai, 2011). We measured only the Cu/ZnSOD isoform (SOD1) (Marklund and Marklund, 1974) and, perhaps, the other isoforms could provide more information on SOD activity in MetS.

RONS/NOSTOX and atherogenicity

The second major finding of this study is that atherogenicity was significantly predicted by an index of RONS/NOSTOX, namely SOD+LOOH+NOx+MDA+AOPP, reflecting the activated pathway from ROS/RNS production to MDA and AOPP formation. Atherogenesis is a complex process which results from several mechanisms involving RONS pathways, activation of immune cells, and inflammation. The oxidation of low-density lipoprotein (LDL) cholesterol and its uptake by macrophages becoming foam cells are a hallmark in the atherogenic process (Marchio et al., 2019).

Our findings are in line with the study conducted by Bortolasci et al. (2015), showing that increased AIP was predicted by MDA levels. LDL oxidation can lead to MDA production which modifies LDL particles by reacting with lysine residues from apolipoprotein B (ApoB) to generate MDA-modified LDL (MDA-LDL) (Orekhov et al., 2014). Tanaga et al. (2002)

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showed higher levels of MDA-LDL in subjects with coronary artery disease. MDA-LDL levels significantly predict the presence of vulnerable plaques in subjects not receiving lipid-lowering therapy (Ito et al., 2018).

Our result shows a relationship between AOPP formation and atherogenicity. Marsche et al. (2009) showed the ability of AOPP to impair HDL metabolism by blocking the receptor scavenger receptor class B type I (SR-BI). SR-BI is expressed in different cell types, including macrophages into the atheroma plaque, which is responsible for the cholesterol efflux from the macrophages to HDL, an important antiatherogenic mechanism (Ma et al., 2020) that can be compromised by AOPP. A previous study conducted by Mo et al. (2014) found that AOPP exacerbated atherosclerotic lesions, increased macrophages accumulation in these lesions and interfered in the reverse cholesterol transport in mice.

RONS/NOSTOX and IR and IS

The third major finding is that IR, but not IS, was significantly predicted by the SOD+LOOH+NOx+MDA+AOPP composite score. Fazakerley et al. (2018) found that the induction of mitochondrial $O_2^{\bullet-}$ and H_2O_2 production with no changes in the mitochondrial respiratory chain was sufficient to cause IR in adipocytes and muscle cells. IR can be induced by RONS/NOSTOX affecting the insulin signal transduction and glucose transporter type 4 (GLUT4) expression in the peripheral tissues (Yaribeygi et al., 2020). RONS/NOSTOX can also affect pancreatic β-cells function leading to IS impairment (Yaribeygi et al., 2020), although, in our study, IS was not significantly lowered in MetS suggesting no β-cell dysfunction. AOPPs are able to induce IR in adipocytes by decreasing Akt phosphorylation, a pivotal process in the insulin signal transduction (Zhou et al., 2010). MDA is a reactive aldehyde as well as the 4-hydroxynonenal (4-HNE), another end product generated through lipid peroxidation (Ayala et al., 2014). 4-HNE may impair insulin signaling and glucose uptake

(Pillon et al., 2012) and, perhaps, MDA could be associated with IR through a similar mechanism.

Other RONS/NOTOX modifying variables

The fourth major finding is that other intervening variables, including mood disorders, GAD, TUD, and sex significantly affected RONS/NOSTOX. Our study showed significant associations between mood disorders and GAD with RONS/NOSTOX biomarkers. The strong increase in RONS/NOSTOX due to the co-occurrence of MDD and GAD has been described in previous studies (Maes et al., 2018). However, the pathways leading to MetS, on the one hand, and mood disorders/GAD, on the other did not completely overlap. Thus, this study could not find increased SOD1 levels in MetS, whereas SOD1 activity was highly significantly increased in MDD and GAD (Maes et al., 2018, 2019a) were not found in the MetS.

All composite scores, except SOD+LOOH+NOx+MDA (thus aldehyde generation), were significantly higher in men than women and there were significant effects of male sex on LOOH and AOPP. In an animal model, AOPP levels were significantly higher in males than females, but no difference was found in LOOH levels (Kayali et al., 2007).

We found that all composite scores, except SOD+LOOH+NOx+AOPP, were higher in smokers than in non-smokers and that TUD had a strong effect of SOD1, LOOH, and MDA. These findings suggest that TUD is specially associated with lipid peroxidation, but not AOPP formation. In fact, increased lipid peroxidation, measured by LOOH and thiobarbituric acid reactive substances (TBARS), was found in the plasma and tissues of animals treated with nicotine (Muthukumaran et al., 2008). Both TUD and depression share oxidative stress pathways and lowered levels of antioxidants and these pathways may explain increased risk to develop depression in TUD (Nunes et al., 2013).

Limitations

The findings of this study should be interpreted with regard to its limitations. This is a cross-sectional study and therefore no firm causal inferences can be established. It would have been more interesting if we had measured other biomarkers which are disordered in mood disorders and contribute to atherogenicity and thus MeS, including the reverse cholesterol transport and LCAT activity (Maes et al., 2014) and oxidized LDL, IgG responses to oxidized LDL and IgM responses to oxidative modified neoepitopes (Maes et al., 2011).

Conclusions

This study shows that MDA and AOPP are hallmarks for MetS and its tow characteristics, atherogenicity and IR. Although MetS shows a strong comorbidity with mood disorders, these conditions share different activated RONS/NOSTOX pathways, as for example increased SOD1 activity in GAD and major depression, but not in MetS.

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Authorships

All authors contributed to the writing up of the paper. The work was designed by SOVN, MM, DSB, EGM and HOV. Data were collected by SOVN and HOV. Laboratory analyses were conducted by KLB, NRM, EGM and DSB. Statistics were performed by MM and NRM. All authors revised and approved the final draft.

Compliance with ethical standards

Disclosure of potential conflicts of interests

The authors report no conflict of interest with any commercial or other association in connection with the submitted article.

Research involving human participants

Approval for the study was obtained from the Institutional Review Board of the State University of Londrina, Londrina, Brasil.

Informed consent.

All controls and patients gave written informed consent before participation in our study.

Data Access Statement.

The dataset generated during and/or analyzed during the current study will be available from MM upon reasonable request and once the dataset has been fully exploited by the authors.

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Table 1: Socio-demographic, clinical and biomarker features of subjects with and without Metabolic Syndrome (MetS).

Variables	**No MetS (n = 107)**	**MetS (n = 65)**	**F/X^2**	**df**	**p**
Age (years)	41.5 (11.2)	45.0 (10.5)	4.20	1/170	0.042
Sex (female/male)	85/22	44/21	2.98	1	0.085
BMI (kg/m^2)	24.5 (3.8)	29.5 (4.7)	53.57	1/162	< 0.001
WCF (cm)	86.0 (11.2)	100.8 (10.2)	66.66	1/143	< 0.001
Education (years)	11.7 (5.4)	10.3 (4.7)	3.02	1/169	0.084
Number of episodes	5.4 (8.0)	7.1 (9.2)	1.51	1/151	0.221
HAM-D	6.6 (6.5)	8.1 (6.7)	2.23	1/170	0.137
HAM-A	10.5 (9.7)	14.1 (10.3)	5.05	1/159	0.026
HC/BP2/BP1/MDD	41/27/12/27	26/18/11/10	2.93	3	0.402
GAD (no/yes)	79/28	47/18	0.05	1	0.827
Staging (0/1/2/3)	67/15/17/6	34/8/12/9	0.413	3	0.247
TUD (no/yes)	51/56	26/39	0.960	1	0.327
Glucose (mmol/L)	4.91 (0.53)	5.85 (1.46)	40.08	1/153	< 0.001
Insulin (µU/mL)	7.91 (5.00)	11.68 (7.88)	23.09	1/154	< 0.001
IR (z scores)	-0.611 (1.293)	1.006 (1.442)	57.87	1/170	< 0.001
IS (z scores)	-0.070 (0.959)	0.115 (1.626)	0.89	1/170	0.348

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Triglycerides (mg/dL)	93.5 (51.2)	173.9 (91.2)	69.15	1/157	< 0.001
Total cholesterol (mmol/L)	5.20 (0.18)	5.29 (0.19)	8.97	1/157	0.003
HDL cholesterol (mg/dL)	54.51 (14.89)	39.25 (11.34)	63.13	1/157	< 0.001
AIP (z scores)	-0.823 (1.223)	1.354 (1.447)	111.27	1/170	< 0.001
Castelli 1 index (z scores)	-0.558 (1.046)	0.918 (1.201)	71.86	1/170	< 0.001
hsCRP (mg/L)	3.20 (4.30)	5.17 (6.50)	10.95	1/158	< 0.001
Uric acid (mg/dL)	4.23 (1.33)	5.26 (1.33)	21.73	1/151	< 0.001

BMI: Body mass index; WCF: Waist circumference; HAM-D: 17-item Hamilton Depression Rating Scale; HAM-A: Hamilton Anxiety Rating Scale; HC: Healthy controls; BP: Bipolar type 1 and type 2; MDD: Major depressive disorder; GAD: Generalized anxiety disorder; TUD: Tobacco use disorder; IR: Insulin resistance; IS: Insulin secretion; HDL: High-density lipoprotein; AIP: Atherogenic index of plasma; hsCRP: High sensitivity C-reactive protein.

Table 2: Results of multivariate general linear model (GLM) analysis, which examines the associations between nitro-oxidative stress toxicity (NOSTOX) biomarkers and Metabolic Syndrome (MetS), mood disorders (MOOD), generalized anxiety disorder (GAD), tobacco use disorder (TUD) and sex.

Type	Dependent variables	Explanatory variables	F	df	p	R^2
Multivariate	All 5 NOSTOX biomarkers composite scores	MetS	6.90	5/160	< 0.001	0.177
		MOOD	2.05	15/486	0.011	0.060
		GAD	3.30	5/160	0.007	0.094
		TUD	5.46	5/160	< 0.001	0.146
		Sex	5.92	5/160	< 0.001	0.156
Between-subject effects	SOD+LOOH	MetS	0.03	1/164	0.870	< 0.001
	SOD+LOOH+NOx	MetS	1.89	1/164	0.172	0.011
	SOD+LOOH+NOx+MDA	MetS	7.68	1/164	0.006	0.045
	SOD+LOOH+NOx+AOPP	MetS	13.91	1/164	< 0.001	0.078
	SOD+LOOH+NOx+MDA+AOPP	MetS	21.64	1/164	< 0.001	0.117
Multivariate	All 5 separate NOSTOX biomarkers	MetS	7.47	5/160	< 0.001	0.189
		MOOD	2.40	15/486	0.002	0.069
		GAD	3.46	5/160	0.005	0.098
		TUD	5.49	5/160	< 0.001	0.147

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		Sex	5.57	5/160	< 0.001	0.148
Between-subject effects	SOD1	MetS	0.32	1/164	0.575	0.002
	LOOH	MetS	0.13	1/164	0.715	0.001
	NOx	MetS	2.14	1/164	0.145	0.013
	MDA	MetS	9.09	1/164	0.003	0.053
	AOPP	MetS	17.99	1/164	< 0.001	0.099

MOOD: Mood disorders: 4 diagnostic categories, namely normal control, bipolar type 1 and type 2, and major depressive disorder; SOD1: Superoxide dismutase 1; LOOH: Lipid hydroperoxides; NOx: Nitric oxide metabolites; MDA: Malondialdehyde; AOPP: Advanced oxidation protein products.

Table 3: GLM-generated estimated marginal mean values of the nitro-oxidative stress toxicity biomarkers in subjects with Metabolic Syndrome (MetS) versus subjects without MetS after adjusting for effects of mood disorders, generalized anxiety disorder, tobacco use disorder, and sex.

Variables	No MetS	MetS
SOD+LOOH (z scores)	0.160 (0.147)	0.191 (0.166)
SOD+LOOH+NOx (z scores)	0.083 (0.093)	0.252 (0.105)
SOD+LOOH+NOx+MDA (z scores)	-0.003 (0.091)	0.328 (0.103)
SOD+LOOH+NOx+AOPP (z scores)	0.056 (0.104)	0.506 (0.104)
SOD+LOOH+NOx+MDA+AOPP (z scores)	-0.005 (0.088)	0.533 (0.099)
SOD1 (U/mg Hb)	99.8 (5.1)	102.1 (5.3)
LOOH (RLU)	1810453 (140717)	1656563 (149175)
NOx (µM)	6.46 (0.46)	7.51 (0.49)
MDA (mmol/mg protein)	61.3 (2.8)	71.7 (2.9)
AOPP (µM)	81.0 (5.5)	108.5 (5.8)

SOD1: Superoxide dismutase 1; LOOH: Lipid hydroperoxides; NOx: Nitric oxide metabolites; MDA: Malondialdehyde; AOPP: Advanced oxidation protein products.

Table 4: Results of linear logistic regression analysis with Metabolic Syndrome (MetS) as dependent variables and no MetS as reference group.

Explanatory variables	β	SE	Wald (df = 1)	p	OR	95% CI
SOD+LOOH+NOx+MDA+AOPP	0.911	0.274	11.07	0.001	2.49	1.45 – 4.25
WCF	1.684	0.321	27.44	< 0.001	2.87	2.87 – 10.12
hsCRP	0.503	0.200	6.31	0.012	1.65	1.12 – 2.45
MDA	0.650	0.204	10.13	0.001	1.92	1.28 – 2.86
AOPP	0.853	0.327	6.80	0.009	2.35	1.24 – 4.46
Uric acid	0.790	0.252	9.81	0.002	2.20	1.34 – 3.61

OR: Odd's ratio, with 95% confidence intervals (CI)

WCF: Waist circumference; hsCRP: High sensitivity C-reactive protein; SOD1: Superoxide dismutase 1; LOOH: Lipid hydroperoxides; NOx: Nitric oxide metabolites; MDA: Malondialdehyde; AOPP: Advanced oxidation protein products.

Table 5: Results of multivariate general linear model (GLM) analysis with insulin resistance (IR), insulin sensitivity (IS), and atherogenicity indices as dependent variables.

Type	Dependent variables	Explanatory variables	F	df	p	R^2
Multivariate	IR, IS and	SOD+LOOH+NOx+MDA+AOPP	7.70	4/163	< 0.001	0.159
	atherogenicity data	TUD	2.90	4/163	0.024	0.067
		Sex	2.78	4/163	0.028	0.064
		Age	5.31	4/163	< 0.001	0.115
		WCF	21.58	4/163	< 0.001	0.346
Between-subject effects	IR	SOD+LOOH+NOx+MDA+AOPP	8.83	1/163	0.003	0.051
	IS	SOD+LOOH+NOx+MDA+AOPP	1.79	1/163	0.183	0.011
	AIP	SOD+LOOH+NOx+MDA+AOPP	26.51	1/163	< 0.001	0.138
	Castelli 1 index	SOD+LOOH+NOx+MDA+AOPP	13.56	1/163	<0.001	0.076

WCF: Waist circumference; TUD: Tobacco use disorder; AIP: Atherogenic Index of Plasma; SOD1: Superoxide dismutase 1; LOOH: Lipid hydroperoxides; NOx: Nitric oxide metabolites; MDA: Malondialdehyde; AOPP: Advanced oxidation protein products.

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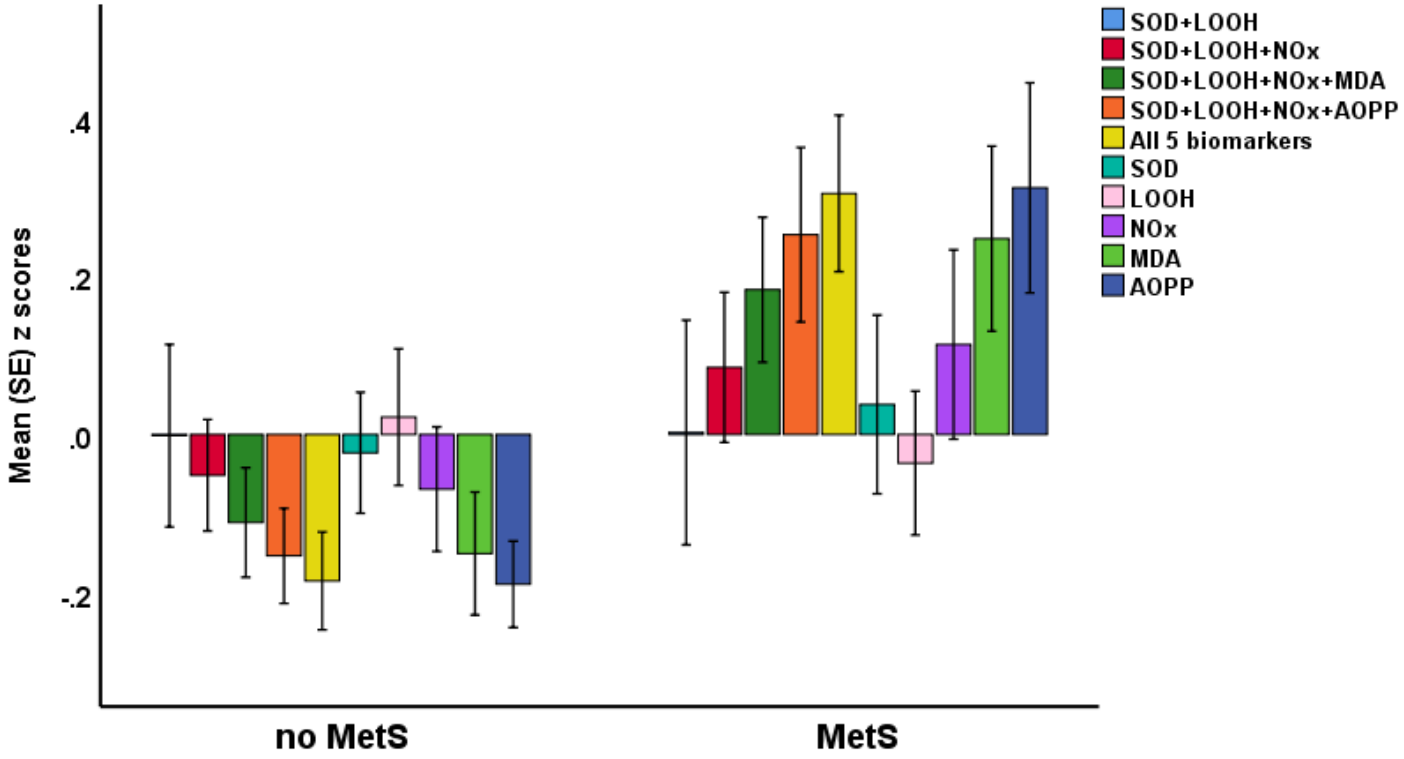

Figure 1 Clustered bar graph of the residualised biomarker levels (all in z scores) in metabolic syndrome (MetS) after partialling out the effects of mood disorders, tobacco use disorder, generalized anxiety disorder, and sex. Shown are the levels of superoxide dismutase 1 (SOD1), lipid

hydroperoxides (LOOH), nitric oxide metabolites (NOx), malondialdehyde (MDA), and advanced oxidation protein products (AOPP) as well as z unit weighted composite scores based on their sums.

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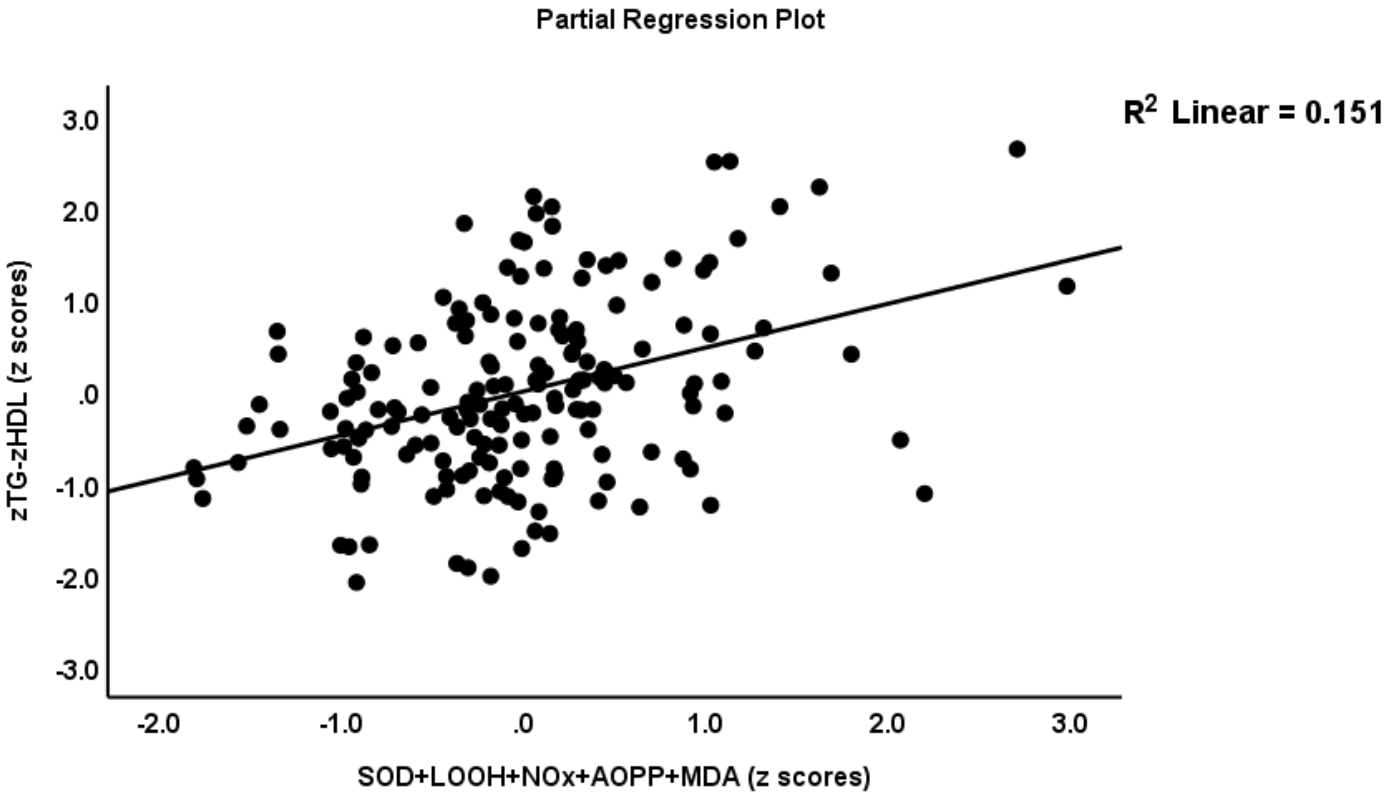

Figure 2 Partial regression plot of z triglycerides (TG) – z high density lipoprotein cholesterol (HDL) on an index of nitro-oxidative stress toxicity computed as a z unit weighted composite scores summing up the z scores of superoxide dismutase 1 (SOD1), lipid hydroperoxides (LOOH), nitric oxide metabolites (NOx), malondialdehyde (MDA), and advanced oxidation protein products (AOPP).

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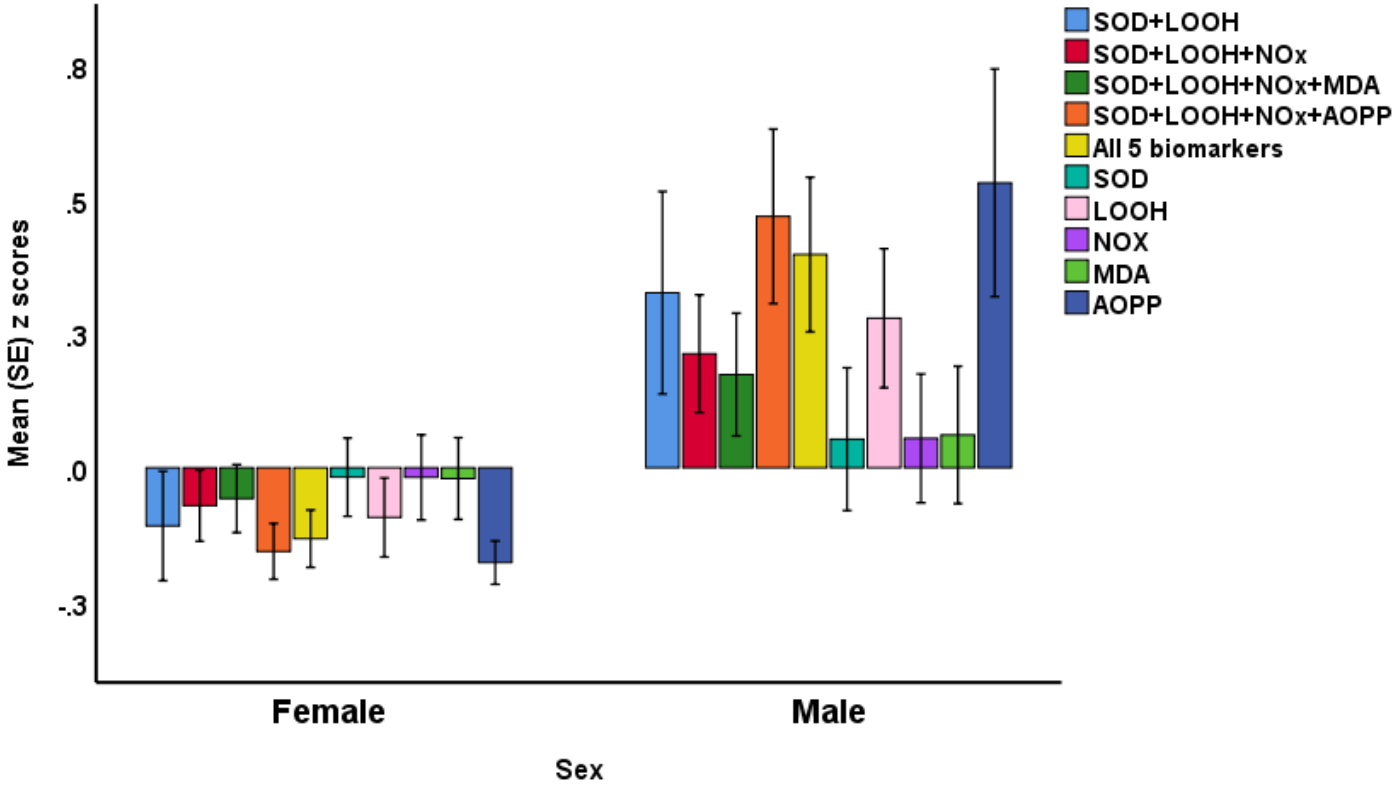

Figure 3 Clustered bar graph of the residualised biomarker data (all in z scores) in males and females, after partialling out the effects of metabolic syndrome, mood disorders, generalized anxiety disorder, and TUD. Shown are the levels of superoxide dismutase 1 (SOD1), lipid hydroperoxides (LOOH), nitric oxide metabolites (NOx), malondialdehyde (MDA), and advanced oxidation protein products (AOPP) as well as z unit weighted composite scores based on their sums.

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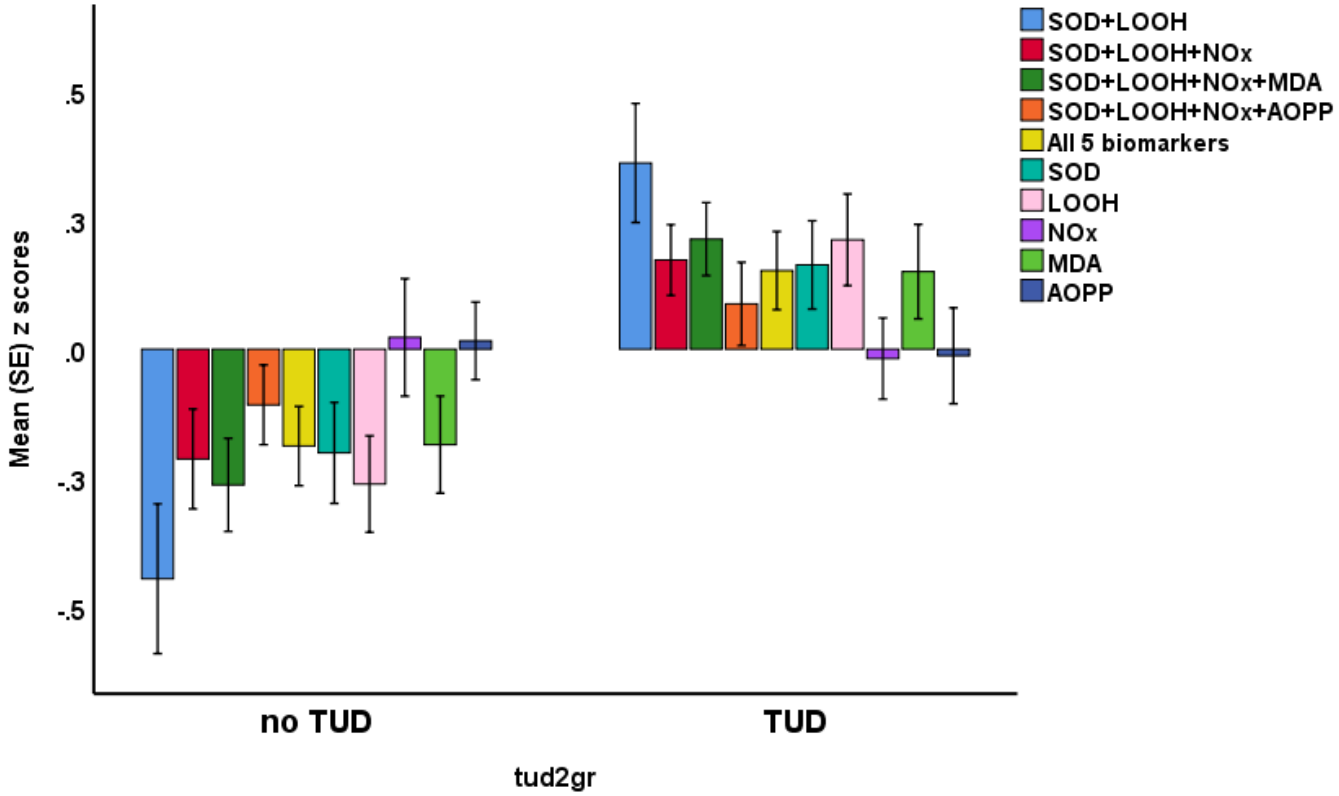

Figure 4 Clustered bar graph of the residualised biomarker data (all in z scores) in tobacco use disorder (TUD) and no-TUD, after partialling out the effects of metabolic syndrome, mood disorders, generalized anxiety disorder, and sex. Shown are the levels of superoxide dismutase 1 (SOD1),

lipid hydroperoxides (LOOH), nitric oxide metabolites (NOx), malondialdehyde (MDA), and advanced oxidation protein products (AOPP) as well as z unit weighted composite scores based on their sums.