Lowered zinc and copper levels in drug-naïve patients with major depression: effects of antidepressants, ketoprofen and immune activation

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Abstract

There is now evidence that major depression is accompanied by lowered serum zinc, an immune-inflammatory biomarker. However, the effect of anti-inflammatory drugs as adjuvant to antidepressants on serum zinc and copper in relation to pro- and anti-inflammatory cytokines are not studied. The aim of the present work is to examine the effects of treatment with sertraline with and without ketoprofen on serum levels of zinc and copper in association with immune-inflammatory biomarkers in drug-naïve major depressed patients. We measured serum zinc and copper, interleukin (IL)-1β, IL-4, IL-6, IL-18, interferon (IFN)-γ, and transforming growth factor (TGF)-β1 in 40 controls and 133 depressed patients. The clinical efficacy of the treatment was measured using the Beck Depression Inventory-II (BDI-II) at baseline and 8 weeks later. In drug-naïve major depressed patients we found significantly reduced baseline levels of serum zinc and copper in association with upregulation of all cytokines, indicating activation of the immune-inflammatory responses system (IRS) as well as the compensatory immune regulatory system (CIRS). Treatment with sertraline significantly increased zinc and decreased copper levels, while ketoprofen did not have a significant add-on effect on zinc but attenuated the suppressant effects of sertraline on copper levels. During treatment, there was a significant inverse association between serum zinc and activation of the IRS/CIRS. The improvement in the BDI-II during treatment was significantly associated with increments in serum zinc coupled with attenuation of the IRS/CIRS. In conclusion, lower serum zinc is a hallmark of depression, while increments in serum zinc and attenuation of the immune-inflammatory response during treatment appear to play a role in the

clinical efficacy of sertraline. Intertwined changes in zinc levels and the immune response play a

role in the pathophysiology of major depression and participate in the mechanisms underpinning

the clinical efficacy of antidepressants.

Introduction

Major depression (MDD) represents a significant public health concern that in 2013-2016 affected 8.1% of adults in the USA (Brody et al., 2018) and MDD is expected to become the leading cause of disease burden by 2030 (Zunszain et al., 2010). Dysregulations in immune responses are a hallmark of MDD with changes in both cell-mediated and humoral immunity (Maes 1995; Schiepers et al., 2005; Al-Hakeim 2008; Goyal et al., 2017). Various immune markers were reported to be present in the sera of patients with MDD (Haapakoski et al., 2015; Cassano et al., 2017; Al-Hakeim et al., 2018), including higher serum levels of positive acute phase reactants, (e.g. haptoglobin), and lowered levels of negative acute phase reactants (e.g. albumin) (Maes, 1993; Liu et al., 2012). Activation of immune-inflammatory pathways may contribute to the development of MDD in at least a subcategory of patients (Maes 2008; Miller & Raison, 2016). A recent review shows that MDD is accompanied by activation of M1 macrophages (with increased production of interleukin (IL)-1β and IL-6), T helper (Th)1 cells (with increased production of interferon (IFN)-γ, Th2 activation (with increased production of IL-4), and a T regulatory (Treg) response (with increased production of IL-10 and transforming growth factor (TGF)-β1) (Maes and Carvalho, 2018). IL-4, IL-10 and TGF-β1 have immune-regulatory effects attenuating activation of the pro-inflammatory Th1 and M1 macrophagic phenotypes, indicating that MDD is accompanied by activation of the immune-inflammatory response system (IRS) and increased immune-regulatory or anti-inflammatory effects that serve an adaptive purpose down-regulating the primary IRS, called the "compensatory immune regulatory system" (CIRS) (Maes and Carvalho, 2018). Accordingly,

anti-inflammatory drugs were trialed as adjuvants to antidepressant drugs to augment their efficiency in the treatment of MDD (Köhler et al., 2016; Al-Hakeim et al., 2018a). However, the effects of the combination of antidepressant and anti-inflammatory drugs on trace elements in relation to immune-inflammatory markers have not been studied well.

Zinc (Zn) and copper (Cu) are essential trace elements which play an important role in numerous physiological processes necessary for life and normal development (Mehri & Marjan 2013). Moreover, Zn is involved in a variety of biochemical processes that modulate the function of the central nervous and immune systems (Gower-Winter & Levenson 2012; Maares & Haase 2016). Zn is tightly regulated in the brain, with higher levels in the amygdala, hippocampus and cortex and is predominantly located within glutamatergic neurons (Prakash et al., 2015). This explains that aberrations in Zn and Cu metabolism participate in the pathophysiology of psychiatric disorders (Maes et al., 1999a; Siwek et al., 2005; Młyniec et al., 2017) and that Zn insufficiency can present as altered behaviors and cognition (Petrilli et al., 2017; Szewczyk 2013) as well as major depression, treatment resistant depression and prenatal and postpartum depression (Maes et al., 1994; 1997; Wójcik et al., 2006; Roomruangwong et al., 2017). Zn has antidepressant activity in animal models and in clinical studies (Nowak et al., 2003, Sowa-Kućma et al 2008; Szewczyk et al., 2008; Szewczyk 2013; Szewczyk et al., 2017) due to its effect on the N-methyl-D-aspartate /glutamate pathway (Poleszak et al., 2008; Doboszewska et al., 2015). Moreover, Zn monotherapy is effective in reducing depressive symptoms in humans (Solati et al., 2015). Lowered levels of

serum Zn are at least in part mediated by immune-inflammatory processes including lowered albumin levels and increased IL-6 levels (Maes et al., 1997; 1999a).

Cu is necessary for the proper development and functioning of the central nervous system whereby low Cu levels may result in incomplete development, while excess concentration may be injurious (Sharma et al., 2014). The brain is the one of the most Cu-rich tissues (next to the heart and liver) (Desai & Kaler 2008; Scheiber et al., 2014). Dopamine to norepinephrine conversion is Cu dependent as Cu ions interact with dopamine β-hydroxylase (Crayton et al., 2007).

The aim of the present study is to examine Zn and Cu in major depression and the effects of sertraline with and without ketoprofen (an anti-inflammatory drug) on Zn and Cu levels in association with immune-inflammatory biomarkers. The specific hypotheses are that depression is accompanied by lowered serum Zn levels and that antidepressants increase serum Zn while suppressing the primary immune-inflammatory response.

Materials and Methods

Participants

The present study recruited one hundred and thirty-three MDD patients and forty healthy controls. Patients were recruited at the Psychiatry Unit, Al-Hakeem General Hospital in Najaf Governorate-Iraq and a private psychiatric clinic during the period from November 2016 till August 2017. MDD patients were diagnosed by psychiatrists using a semi-structured psychiatric interview schedule for the diagnosis of MDD based on the ICD-10 (10th revision of the International Statistical Classification of Diseases and Related Health Problems). The healthy controls were

recruited from the same catchment area. These subjects were free from psychiatric (axis-1) and somatic diseases. All subjects were evaluated through a complete medical history to exclude any systemic diseases that may affect the biomarkers, including diabetes mellitus, liver and kidney diseases, (auto)immune disorders and neurological disorders, including multiple sclerosis, stroke and Parkinson's disorder. Furthermore, in all participants, C-reactive protein (CRP) was < 6 mg/L excluding subjects with overt inflammation.

The present study is part of a prospective, 2-months, randomized double-blind study of parallel groups of patients with MDD with or without ketoprofen administration in addition to sertraline. In this prospective part of the study, we included 41 of the 133 MDD patients, namely those with biomarker measurements both before and after treatment. All biomarker assays and BDI-II scores were completed in these 41 subjects. Among the follow up group, fourteen patients received sertraline, orally 50 mg once daily, and twenty seven patients were additionally treated with the anti-inflammatory drug ketoprofen, 100 mg orally once daily, as adjuvant to sertraline. Patients were instructed to take one capsule daily after breakfast. The coloured empty capsules (Caps & Chemicals, India) were filled either with sertraline (Actavis, Italy) alone, for the placebo group or filled with sertraline + ketoprofen (Menarini Int., Italy) for drug adjuvant group. The laboratory analysts were blinded to the treatment modalities. The protocol was approved by the IRB of the University of Kufa (#221, June 2016). The patients or their close first-degree relatives provided informed consent in accordance with the procedures outlined by the current IRB. The study is recorded in the NIH US Library of Medicine, ClinicalTrials.gov Identifier: NCT03514810.

Measurements

The total score on the Beck-Depression Inventory (BDI-II) (Beck et al., 1996) was used to rate severity of illness. The BDI-II score was obtained in all MDD patients at baseline and was also rated two months after starting treatment in the 41 patients who were included in the biomarker treatment study. Five milliliters of venous blood samples was drawn by utilizing disposable needles and plastic syringes. The samples were transferred into clean plain tubes. Haemolyzed samples were discarded. The blood was left at room temperature for 10 minutes for clotting, centrifuged 3000 rpm for 5 minutes, and then serum was separated and transported into three new Eppindrof tubes until assay. Serum IL-4, IFNγ, and TGF-β1 were measured by using ELISA kits supplied by MyBioSource® Inc., USA, while IL-1β, IL-6, and IL-18 ELISA kits were supplied by BioAssay Systems®, USA. All kits were based on a sandwich technique and showed an inter-assay CV less than 12%. Briefly, serum containing analyte was added to the wells to bind with the monoclonal antibody of the analyte pre-coated on the microwells. After incubation, a biotin-conjugated antibodies were added to bind the human analyte. After washing, streptavidin-HRP was added to bind to the biotin-conjugated antibodies and after incubation, the substrate solution (TMB) was added followed by color development proportionally to the amount of human analyte. The reaction was terminated by addition of acidic stop solution (0.1N HCl) and absorbance was measured at 450 nm and transformed into concentration by using logarithmic standard curves.

Serum Cu and Zn were measured by flame atomic absorption spectrophotometry AA990 (PG Instruments Ltd.). Samples were diluted 1:10 with 6% n-butanol as diluent before measurement.

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This method achieved 30% increase in sensitivity compared to use of water only due to decrease viscosity and difference in droplet formation (Meret & Henkin 1971).

Statistical Analysis

Analysis of contingency tables (χ^2 test) was used to assess associations between nominal variables and analysis of variance (ANOVAs) to assess differences in scale variables among diagnostic groups. Associations between scale variables were computed using Pearson's product moment correlation coefficients. We used multivariate general linear model (GLM) analysis to investigate the effects of MDD (versus controls) on the biomarkers (dependent variables). The primary outcome analysis is a generalized estimating equation (GEE) analysis (repeated measurements) used to assess the effects of time on the biomarkers (treatment with sertraline with or without ketoprofen from baseline to endpoint), while the interaction time X treatment was introduced in the GEE analysis to examine differences between the effects of sertraline + ketoprofen versus sertraline + placebo. The demographic and clinical baseline (pre-treatment) data were assessed for balance between both treatment groups employing analysis of contingency tables and ANOVAs. Power analysis to examine the add-on effects of ketoprofen on BDI-II scores shows that the required sample size was around 142 patients using a 2-tailed test at $\alpha=0.05$ and assuming an effect size of 0.13 with power of 0.80. However, only 41 major depressed patients were included in this prospective part of this study because the grant support was limited in time, indicating that this part of the study was underpowered. Tests were 2-tailed and a p-value of 0.05 was used for statistical significance. All statistical analyses were performed using IBM SPSS windows version

25. Statistical analyses were conducted in accordance with the International Conference on Harmonisation E9 statistical principles (November 2005).

We computed four z unit weighted composite scores based on the cytokine levels (Maes and Carvalho, 2018). A first one reflected immune activation (M1+Th1+Th2+Treg) and was computed as zIL-1 + zIL-6 + zIL-18 + zIFN-γ + zIL-4 + zTGF-β1; b) a composite score reflecting pro-inflammatory phenotypes (M1+Th1) computed as zIL-1 + zIL-6 + zIL-18 + zIFN-γ; c) a composite score reflecting more regulatory phenotypes (Th2+Treg), computed as zIL-4 + zTGF-β1; and d) pro-inflammatory / immune regulatory ratio (M1+Th1/Th2+Treg), computed as z(zIL-1 + zIL-6 +zIL-18 +zIFN-γ) + / z(zIL4 + zTGF-β1). The Cu / Zn ratio was computed as zCu - zZn.

Results

Socio-demographic data

Table 1 displays the demographic, clinical and biomarker data of the patients and controls recruited in this study. This table shows that there were no significant differences in age, BMI, sex, and employment status between the study groups. There were significantly more smoking and single participants in the major depression group as compared with healthy controls. Serum levels of Cu and Zn were significantly lower in major depressed subjects than in controls, while there were no significant differences in the Cu / Zn ratio. Serum levels of IL-1β, IL-6, IL-18, IFN-γ, IL-4 and TGF-β1 were significantly greater in major depression than in controls.

There were no significant correlations between Cu and any of the cytokine levels. Serum Zn was significantly and inversely associated with IL-1β (r=-0.154, p=0.044, n=172), IL-18 (r=-0.154,

p=0.042, n=173), IFN-γ (r=-0.215, p=0.004, n=173) and TGF-β1 (r=-0.177, p=0.020, n=0.173), but not IL-6 (r=-0.029, p=0.708, n=173). There were no significant correlations between age or BMI and levels of Cu, Zn, the Cu / Zn ratio and any of the cytokine levels. In patients there were no significant associations between the baseline BDI-II scores and Cu, Zn, the Cu/Zn ratio and any of the cytokine levels.

Differences in the biomarkers between major depressed patients at baseline and controls

Table 2 shows the results of a first multivariate GLM analysis with Cu, Zn and the Cu / Zn ratio as dependent variables and diagnosis (MDD versus controls) as explanatory variable, while adjusting for sex, smoking, age and BMI. There was a highly significant association between the dependent variables (biomarkers at baseline) and diagnosis, while also age (but not sex, BMI and smoking) had a significant effect. Tests for between-subjects effects showed that Cu and Zn, but not the Cu / Zn ratio, were significantly associated with diagnosis. Tests for between-subject effects showed that there was a significant positive correlation between age and Cu (F=11.44, df=1/166, p=0.001) and the Cu / Zn ratio (F=7.83, df=1/166, p=0.006), but not Zn (F=0.11, df=1/166, p=0.739). Moreover, adding other extraneous variables in this GLM analysis showed that there were no significant effects of employment status (F=0.35, df=2/165, p=0.703) and marital status (F=0.15, df=2/165, p=0.862). **Table 3** shows the model-generated estimated marginal mean values (all in z transformations) of Cu, Zn and the Cu / Zn ratio obtained by this multivariate GLM analysis. Cu and Zn were significantly lower in MDD than in controls and the differences in the mean Cu and Zn values between both groups were 0.584 and 0.667 standard deviations, respectively.

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The second multivariate in Table 2 examined the effects of diagnosis (and age, sex, smoking and BMI) on four z composite scores reflecting 4 different cytokine ratios. There was a highly significant effect of diagnosis on the cytokine scores with an effect size of 0.527. Tests for between-subject effects showed a highly significant effect of diagnosis on M1+Th1+Th2+Treg, M1+Th1, Th2+Treg and M1+Th1/Th2+Treg, with a very strong impact on the first two z composite scores (effect sizes 0.513 and 0.524, respectively). There were no significant effects of the extraneous variables (age, sex, BMI and smoking) on these composite scores. Table 3 shows that all 4 composite scores were significantly higher in MDD than in controls.

Effects of treatments on the BDI-II score and biomarkers.

Table 4 shows the baseline characteristics of patients treated with sertraline + placebo versus sertraline + ketoprofen. There were no significant differences in age, BMI, sex, marital and employment status, smoking behavior, BDI-II, Cu, Zn, the Cu / Zn ratio and any of the cytokine scores between both treatment groups.

Table 5 shows the results of GEE analyses with effects of time (treatment) and the time X treatment interaction on BDI-II, Cu, Zn, Cu / Zn ratio and the 4 cytokine composite scores (the results were adjusted for sex, age, BMI, smoking, marital and employment status). This table also shows the pre- and post-treatment mean (SE) values. We found that the BDI-II score was significantly lower after treatment and that there was a significant time X treatment interaction showing that sertraline + ketoprofen (pre-treatment and post-treatment means (SE): 49.3 ±1.9 and

13.5 ±1.3, respectively) had a significantly greater effects on the BDI-II score than sertraline + placebo (50.9 ±2.1 and 20.4 ±1.8, respectively). There was a suppressant effect of time (treatment) on Cu and a significant time X treatment interaction with a significantly greater effect of sertraline + placebo (pre-treatment versus post-treatment means (SE): 0.837 ±0.035 mg/l and 0.616 ±0.027 mg/l, respectively) than sertraline + ketoprofen (0.756 ±0.039 mg/l and 0.738 ±0.034 mg/l, respectively). Serum Zn was significantly increased after treatment while there was no significant time X treatment interaction. This explains that there was also a suppressant effect of treatment on the Cu / Zn ratio as well as a significant interaction time X treatment with a similar pattern as that described for Cu. GEE analyses showed that the cytokine composite scores (except M1+Th1 / Th2+Treg) were significantly suppressed after treatment. No significant interaction patterns could be established.

Using GEE analyses we investigated the associations between changes in the BDI-II and biomarker levels (**Table** 6). GEE analysis #1 and #2 show that there were no significant associations between the BDI-II scores and either Cu or Zn. GEE analyses #3 and #4 shows that there was a significant association between the BDI-II score and the M1+Th1+Th2+Treg composite score (positively), but not with the M1+Th1 / Th2+Treg ratio. GEE analysis #5 shows that after considering the effects of the M1+Th1+Th2+Treg composite score, also Zn was significantly and inversely associated with the BDI-II score. GEE analyses #6 and #7 show that Zn levels were significantly associated with the M1+Th1+Th2+Treg composite score (inversely), while there was no significant effect of IL-6.

Discussion

Baseline levels of zinc and copper in depression

The first major finding of this study is that MDD is accompanied by **s**ignificantly lower serum levels of Cu and Zn as compared with controls, while there were no significant differences in the Cu / Zn ratio. These findings are in agreement with previous reports which consistently reported hypozincaemia in MDD patients (Maes 1994; 1997; Maes et al., 1999a; Siwek et al. 2013; Alghadir et al., 2016; Styczeń et al., 2017). Maes et al. (1994) reported that low serum Zn was significantly and negatively correlated with the severity of depressive symptoms. Relative zinc deficiency may contribute to the core symptomatology of depression because Zn deficiency may impair normal brain functions (Sandstead 2012) and cause neuroprogression comprising aberrations in neuroplasticity, immune functions, monoamine metabolism, stress response dysregulation, oxidative and nitrosative stress, neurotrophic deficits and transcriptional/epigenetic regulation of neural networks (Maes et al., 2009; Leonard and Maes, 2012; Siwek et al., 2013; Swardfager et al., 2013).

Previous studies also reported decreased Cu levels in depression (Siwek et al., 2005). Styczeń et al. (2016) reported a 11% reduction in the mean serum Cu levels in depressed patients, which was not significantly different from controls, whilst no significant differences in Cu were found between acute depression and the remission stage and controls. Other papers reported a significant increase in serum Cu as compared with controls (Russo 2011; Islam et al., 2018). Nevertheless, studies that did not control for overt inflammation (e.g. using increased CRP levels as an exclusion criterion) are more difficult to interpret as overt inflammation is accompanied by increased levels of ceruloplasmin, an acute phase protein that transports Cu, and consequently, increments in Cu levels.

Our findings that serum levels of IL-1β, IL-6, IL-18, IFN-γ, IL-4 and TGF-β1 are significantly increased in MDD patients as compared with healthy controls are in accordance with many other papers reporting an immune-inflammatory respose in depression (Su et al., 2009; Liu et al., 2012; Maes et al., 2012a; Al-Hakeim, et al., 2018; Köhler et al., 2018; Maes and Carvalho, 2018; Sowa-Kućma et al., 2018; Zou et al., 2018). Moreover, our results indicate that depression is accompanied by an upregulation of the IRS as well as the CIRS (Maes and Carvalho, 2018), however with a predominant IRS (IL-1β, IL-6, IL-18, IFN-γ) and a relatively lower CIRS (IL-4, TGF-β1) activation. Phrased differently, there is a net pro-inflammatory M1 and Th1 response in MDD. It is thought that increased levels of pro-inflammatory cytokines may participate in the pathophysiology of MDD (Maes, 1995; Maes and Carvalho, 2018). For example, elevated IL-1β, IL-6, TNF-α and IL-18 are more likely to be risk factors for depression rather than a consequence (Huang et al., 2019; Raison et al., 2006; Fan et al., 2017; Köhler et al., 2017). Nevertheless, IRS (M1 and Th1) as well as CIRS (Th2 cytokines, including IL-4, IL-13, CCL-11) may have detrimental effects leading to neuroprogression thereby contributing to MDD (Maes and Carvalho, 2018).

Effects of treatments on serum Zn and immune biomarkers

The second major finding of this study is that treatment with sertraline significantly increased serum zinc concentrations and decreased copper levels thereby significantly decreasing the Cu / Zn ratio. The post-treatment levels of zinc were even higher than the concentrations observed in controls. In a previous study, Ciubotariu and Nechifor (2007) found a decrease in plasma zinc concentrations in MDD which was significantly increased following sertraline or

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amitriptyline treatment. Repeated administration of imipramine or citalopram induces a 20% increase in Zn concentrations in the hippocampus and other regions of the brain (Nowak et al., 1999).

In our study, the increase in serum Zn during treatment was accompanied by a suppression of the immune response, including IRS and CIRS. In fact, antidepressant treatment significantly reduced immune activation, including M1 plus Th1 and Th2 plus Treg, but not the pro-inflammatory / immune regulatory ratio. Antidepressants have known immune regulatory effects by reducing the ex vivo production of M1 and Th1 cytokines and increasing the production of IL-10 (Xia et al., 1996; Maes et al., 1999b), whilst also modulating the in vivo immune status of depressed patients (Goyal et al., 2017; Köhler et al., 2018).

The significant inverse association between serum Zn and the immune activation index during treatment and between baseline Zn and selected cytokines is in accordance with the knowledge that there are reciprocal relationships between both zinc metabolism and immune activation. Previous research showed significant inverse associations between Zn and immune biomarkers in depression (Maes et al., 1997; 1999a) and between Zn and IL-1β in rheumatoid arthritis (Zoli et al., 1998). Firstly, lowered Zn levels in depression may be caused by the immune-inflammatory response, especially by increased IL-6 production and by lowered levels of albumin, a Zn-binding protein (Maes et al., 1997; 1999). Because of the involvement of Zn in about 400 enzymes, and over 2000 zinc-containing proteins (Andreini et al., 2006), lower levels of Zn may be due to an accumulation of Zn-containing proteins in the liver and in the inflamed areas.

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Nevertheless, also loss of appetite and weight loss may affect Zn storage systems in the human body (Baltaci & Mogulkoc 2008). Secondly, Zn is an anti-inflammatory and anti-oxidant compound and therefore Zn deficiency may be associated with immune disturbances including increased production of pro-inflammatory cytokines (Wessels et al., 2013; Maywald and Rink, 2015) and reduced immune responses with increased susceptibility to infections (Maes et al., 1997; Malavolta et al 2015; Maywald et al., 2015; 2017). Zn plays a vital role in immune system homeostasis affecting both innate and adaptive immunity (Maywald et al., 2017; Gao et al., 2018), and modulates T cell activation and the number and function of immune cells, including T and B cells, macrophages, dendritic cells, mast cells, and neutrophils (Prasad 1998; Daaboul et al., 2012; Maywald et al., 2017; Gao et al., 2018). This may explain that low Zn levels increase T-cell autoreactivity and alloreactivity, whereas high zinc concentrations suppress T-cells (Rink L, Gabriel 2001). Furthermore, maintaining normal Zn levels seem to be essential to avoid negative concentration-dependent effects of Zn on T-cell activation (Daaboul et al., 2012) and enhance the symptoms of MDD (Nowak et al., 2003).

The third major finding of this study revolves around the clinical efficacy of treatment with sertraline (with or without ketoprofen) which is associated with changes in biomarkers. Firstly, treatment with sertraline plus ketoprofen had a significantly greater effect on the BDI-II score than sertraline alone, indicating that ketoprofen enhances the antidepressant efficacy of sertraline. Secondly, the treatment-induced changes in Zn levels (increased) and the immune activation index (attenuated) were significantly associated with clinical improvement as measured with the BDI-II.

These findings suggest that increments in Zn and suppression of the immune rresponse may mediate at least in part the clinical efficacy of antidepressants. The mechanism underpinning the putative antidepressant activity of zinc may be related with its neuroprotective, immune-regulatory and anti-oxidant properties (Szewczyk et al. 2011; Leonard and Maes, 2012; Siwek et al. 2013; Swardfager et al., 2013; Tyszka-Czochara et al. 2014; Nowak 2015; Doboszewska et al., 2016; Maurya et al. 2016). Another possibility is that Zn modulates serotonergic receptors in both preclinical and clinical studies (Szewczyk et al., 2009).

Finally, the Cu / Zn ratio provides additional information on the health status of patients (Malavolta et al., 2015) and, therefore, our findings on this ratio merit further discussion. In baseline conditions, we could not find differences in the ratio between patients and controls (because both Zn and Cu were decreased in MDD), but treatment with antidepressants lowered the Cu / Zn ratio. There is some consensus that an imbalance between both trace elements with increased Cu but low Zn levels may contribute to the pathophysiology of medical disorders including depression, schizophrenia, autism, fatigue, childhood hyperactivity and premenstrual syndrome (Osredkar and Sustar, 2011). The increase in the Cu / Zn ratio usually reflects detrimental effects because low zinc indicates immune disturbances (Maywald et al., 2017), while increased copper usually indicates hypercerruloplasminaemia as a consequence of an inflammatory response (Reunanen et al., 1996; Böckerman et al., 2016). In this regard, we found that ketoprofen did not have a significant add-on effect on Zn levels but attenuated the suppressant effects of sertraline on Cu levels. As such, ketoprofen may counteract the effects of sertraline by increasing the Cu / Zn ratio. This could be

another side effect of COX-2 inhibitors that should be added to the long list of detrimental side effects that may aggravate the pathophysiology of major depression, including increased Th1 responses and lipid peroxidation, induction of neuroinflammation, reduction of key antioxidant levels and damage to mitochondria (Maes et al., 2012b).

Conclusions

The present study further confirms that the immune-inflammatory system is disturbed in MDD patients as evidenced by lowered levels of Zn and a robust intercorrelated IRS (M1, Th1) and CIRS (Th2 and Treg) response. Treatment with sertraline with or without ketoprofen increases serum Zn levels, lowers Cu levels and attenuates the immune response. During treatment, there is a significant inverse correlation between lower Zn and immune activation, which may be explained by reciprocal relationships between both systems. After considering the effects of immune activation it appeared that both increased levels of Zn and attenuation of the immune response were associated with the clinical efficacy of antidepressants, suggesting that both increasing Zn and suppressing the baseline immune-response may yield antidepressant activities.

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Conflict of interest

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

Author's contributions

All the contributing authors have participated in preparation of the manuscript.

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Table 1: Socio-demographic, clinical and pre-treatment biomarker data in healthy controls (HC) and major depressed (MDD) patients.

Variables		Control n=40	MDD n=133	$F/\chi^2/\psi$	df	P
Age	(Years)	40.3 (7.9)	39.4 (10.7)	0.25	1/171	0.621
BMI	Kg/m²	27.7 (4.2)	26.1 (5.2)	3.3	1/171	0.071
Sex	(F/M)	18/22	59/74	0	1	0.943
Smokers	Y/N	40/0	78/55	ψ=0.374		<0.001
Employment	Y/N	18/22	76/57	1.83	1	0.180
Married / Single		22/18	48/85	4.57	1	0.033
Cu	mg/l	0.840 (0.182)	0.742 (0.147)	12.06	1/171	0.001
Zn	mg/l	0.598 (0.146)	0.519 (0.103)	14.68	1/171	<0.001
Cu / Zn ratio		1.465 (0.402)	1.492 (0.441)	0.12	1/171	0.734
IL-1*	pg/ml	362.3 (76.1)	589 (235.6)	40.91	1/170	<0.001
IL-6 *	pg/ml	12.6 (6.4)	25.8 (12.4)	49.92	1/171	<0.001
IL-18 *	pg/ml	16.4 (7.8)	35.8 (16.5)	80.22	1/171	<0.001
IFN-γ*	pg/ml	32.3 (9.3)	46.7 (18.0)	25.13	1/171	<0.001
IL-4*	pg/ml	198.3 (51.5)	397.7 (185.8)	53.51	1/171	<0.001
TGF-β1*	pg/ml	2102.4 (589.9)	2883.3 (1933.1)	12.03	1/171	0.001

Results are shown as mean (SD).

BMI: body mass index.

Cu: copper, Zn:zinc.

IL: interleukin, IFN-γ: interferon-gamma, TGF-β1: Transforming growth factor-beta 1.

*Processed in Ln transformation.

Table 2: Results of multivariate GLM analysis with the biomarkers as dependent variables and diagnosis as explanatory variable while adjusting for extraneous variables.

Tests	Dependent Variables	Explanatory Variables	F	df	P	Partial η^2
#1. Multivariate	Cu, Zn, zCu-zZn	Diagnosis	9.23	2/166	<0.001	0.100
		Sex	0.61	2/166	0.542	0.007
		Smoking	0.13	2/166	0.875	0.002
		Age	6.47	2/166	0.002	0.072
		BMI	0.30	2/166	0.739	0.004
Between-subjects effects	Cu	Diagnosis	11.91	1/170	0.001	0.065
	Zn	Diagnosis	14.67	1/170	<0.001	0.079
	zCu-zZn	Diagnosis	0.13	1/170	0.715	0.001
#2. Multivariate	M1+Th1+Th2+Treg, M1+Th1, Th2+Treg, M1+Th1/Th2+Treg	Diagnosis	91.96	2/165	<0.001	0.527
		Sex	0.64	2/165	0.58	0.008
		Smoking	0.21	2/165	0.815	0.002
		Age	1.14	2/165	0.322	0.014
		BMI	1.84	2/165	0.162	0.022
Between-subject effects	M1+Th1+Th2+Treg	Diagnosis	179.32	1/170	<0.001	0.513
	M1+Th1	Diagnosis	186.98	1/170	<0.001	0.524
	Th2+Treg	Diagnosis	42.37	1/170	<0.001	0.200
	M1+Th1/Th2+Treg	Diagnosis	12.68	1/170	<0.001	0.069

BMI: body mass index.

Cu: copper, Zn: zinc.

M1: Macrophage M1, Th1: T-helper 1, Th2: T-helper 2, and Treg: T-regulatory phenotypes.

M1+Th1+Th2+Treg: index of immune activation.

M1+Th1: index of pro-inflammatory M1 and Th1 phenotypes.

Th2+Treg: index of immune-regulatory phenotypes.

M1+Th1/Th2+Treg: index of pro-inflammatory / immune regulatory ratio.

zCu - zZn: reflects the Cu/Zn ratio, computed as z score Cu (zCu) - zZn.

Table 3: Model-generated estimated marginal means values (obtained by the GLM analyses shown in Table 2) in controls and major depressed (MDD) patients.

Variables	Controls	MDD
Cu (z score)	0.449 (0.148)	-0.135 (0.081)
Zn (z score)	0.513 (0.153)	-0.154 (0.084)
zCu-zZn (z score)	-0.05 (0.155)	0.015 (0.085)
M1+Th1+Th2+Treg (z score)	-1.319 (0.112)	0.387 (0.061)
M1+Th1 (z score)	-1.333 (0.111)	0.391 (0.060)
Th2+Treg (z score)	-0.811 (0.143)	0.249 (0.078)
M1+Th1/Th2+Treg (z score)	-0.485 (0.155)	0.142 (0.084)

All data are shown as z scores **(SE).**

Cu**:** copper, Zn**:** Zinc

zCu - zZn: reflects the Cu/Zn ratio, computed as z score Cu (zCu) - zZn.

M1**:** Macrophage M1, Th1**:** T-helper 1, Th2**:** T-helper 2, and Treg**:** T-regulatory phenotypes**.**

M1+Th1+Th2+Treg: index of immune activation.

M1+Th1: index of pro-inflammatory M1 and Th1 phenotypes.

Th2+Treg: index of immune-regulatory phenotypes.

M1+Th1/Th2+Treg: index of pro-inflammatory / immune regulatory ratio.

Table 4: Sociodemographic, clinical, and baseline biomarker data in depressed patients allocated to the sertraline+placebo or sertraline+ketoprofen study groups.

Variables	Sertraline+Placebo n=14	Sertraline+Ketoprofen n=27	F/χ^2	df	P
BDI_II	50.6 (8.3)	49.4 (10.4)	0.12	1/39	0.726
Age (years)	39.9(12.0)	39.7 (10.2)	0.01	1/39	0.950
BMI Kg/m2	25.7 (5.5)	26.9 (4.4)	0.48	1/39	0.491
Sex (F/M)	5/9	10/17	0.01	1	0.934
Smokers Y/N	12/2	23/4	ψ=0.01	-	0.964
Employment Y/N	9/5	20/7	0.43	1	0.514
Married/Single	6/8	16/11	1.00	0	0.318
Cu mg/l	0.826 (0.148)	0.742 (0.211)	1.80	1/39	0.187
Zinc mg/l	0.485 (0.172)	0.505 (0.118)	0.20	1/39	0.661
zCu – zZn (z score)	0.331 (0.676)	-0.171 (1.105)	2.40	1/39	0.129
M1+Th1+Th2+Treg (z score)	-0.044 (1.065)	0.023 (0.985)	0.04	1/39	0.843
M1+Th1 (z score)	-0.065 (0.985)	0.034 (1.024)	0.09	1/39	0.768
Th2+Treg (z score)	0.001 (1.269)	-0.000 (0.856)	0.00	1/39	0.996
M1+Th1/Th2+Treg (z score)	-0.053 (1.219)	0.028 (0.890)	0.06	1/39	0.808

Results are shown as mean (SD).

BDI-II: Beck depression inventory scale II.

BMI: body mass index.

Cu: copper, Zn: zinc.

zCu - zZn: reflects the Cu/Zn ratio, computed as z score Cu (zCu) - zZn.

M1: Macrophage 1, Th1:T-helper cell 1, Th2:T-helper cell 2, and Treg: T-regulatory phenotypes.

M1+Th1+Th2+Treg: index of immune activation.

M1+Th1: index of pro-inflammatory M1 and Th1 phenotypes.

Th2+Treg: index of immune-regulatory phenotypes.

M1+Th1/Th2+Treg: index of pro-inflammatory / immune regulatory ratio.

Table 5: Results of generalized estimating equation (GEE) analyses, repeated measurements, examining the effects of treatments on the Beck Depression rating scale II (BDI-II) score and biomarkers.

	Time				Time X treatment		
	t_0	$t_{end-point}$	W	p	W	df	p
BDI-II	50.0 (1.4)	6.9 (1.1)	296.50	<0.001	5.72	1	0.017
Cu mg/l	0.796 (0.269)	0.677 (0.022)	12.04	0.001	8.61	1	0.003
Zinc mg/l	0.492 (0.023)	0.713 (0.027)	36.55	<0.001	0.84	1	0.359
zCu – zZn (z score)	0.718 (0.114)	-0.631 (0.124)	66.49	<0.001	10.38	1	0.001
M1+Th1+Th2+Treg (z score)	0.711 (0.141)	-0.547 (0.080)	63.91	<0.001	2.54	1	0.111
M1+Th1 (z score)	0.573 (0.159)	-0.377 (0.102)	30.92	<0.001	2.15	1	0.143
Th2+Treg (z score)	0.700 (0.158)	-0.623 (0.083)	47.03	<0.001	0.92	1	0.338
M1+Th1/Th2+Treg (z score)	-0.135 (0.207)	0.260 (0.132)	2.59	0.108	0.08	1	0.779

Result are shown as mean (SE)

T_0: measurement at baseline; t endpoint: after treatment with sertraline with or without ketoprofen

Time: effect of time (or treatment) on the dependent variables; Time X treatment: differences in the effects between sertraline + placebo versus sertraline + ketoprofen

Cu: copper, Zn: zinc

zCu - zZn: reflects the Cu/Zn ratio.

M1**:** Macrophage M1, Th1**:** T-helper 1, Th2**:** T-helper 2 and Treg**:** T-regulatory phenotypes**.**

M1+Th1+Th2+Treg: index of immune activation.

M1+Th1: index of pro-inflammatory M1 and Th1 phenotypes.

Th2+Treg: index of immune-regulatory phenotypes.

M1+Th1/Th2+Treg: index of pro-inflammatory / immune regulatory ratio.

Table 6: Associations between the Beck Depression Inventory rating scale II (BDI-II) score and biomarkers. Results of generalized estimating equation (GEE) analyses, repeated measurements.

# GEE	Dependent variable	Explanatory variables	B	SE	W	df	p
#1	BDI-II	Cu	0.106	0.097	1.19	1	0.275
#2	BDI-II	Zn	-0.112	0.095	1.39	1	0.239
#3	BDI-II	M1+Th1+Th2+Treg	0.492	0.085	33.76	1	<0.001
#4	BDI-II	M1+Th1/Th2+Treg	0.015	0.084	0.00	1	0.954
#5	BDI-II	M1+Th1+Th2+Treg	0.507	0.087	34.20	1	<0.001
		Zn	-0.247	0.094	6.88	1	0.009
#6	Zn	M1+Th1+Th2+Treg	-0.306	0.105	8.55	1	0.003
#7	Zn	IL-6	0.020	0.112	0.03	1	0.856

Cu: copper, Zn: zinc.

M1: Macrophage M1, Th1: T-helper 1, Th2: T-helper 2 and Treg: T-regulatory phenotypes.

M1+Th1+Th2+Treg: index of immune activation.

IL: interleukin.