Total and ionized calcium and magnesium are significantly lowered in drug-naïve depressed patients: effects of antidepressants and associations with immune activation.

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Abstract

Major depressive disorder (MDD) is associated with changes in the levels of the cations calcium (Ca) and magnesium (Mg) as well as circulating pro- and anti-inflammatory cytokines. The immune-inflammatory nature of MDD has encouraged researchers to use anti-inflammatory drugs as an adjuvant treatment for MDD. However, the effect of this treatment on cation levels has not been studied.

The present study examined a) differences in both cations between drug-naïve MDD patients and controls, and b) the effects of a combination of sertraline and ketoprofen, an anti-inflammatory drug, on Ca and Mg (both total and ionized). In the same patients we also examined the associations between both cations and IL-1β, IL-4, IL-6, IL-18, IFN-γ, TGF-β1, zinc and indoleamine 2,3-dioxygenase (IDO). Clinical improvement was estimated using the Beck Depression Inventory-II (BDI-II) at baseline and after follow up for two months.

Serum Ca and Mg (total and ionized) were significantly lower in MDD patients as compared with controls, while treatment significantly increased calcium but decreased magnesium levels. There were significant and inverse correlations between the BDI-II scores from baseline to endpoint and Ca (both total and ionized), but not Mg, levels. The effects of calcium on the BDI-II score remained significant after considering the effects of zinc, IDO and an immune activation z unit weighted composite score based on the sum of all cytokines. There was a significant and inverse association between this immune activation index and calcium levels from baseline to endpoint.

In conclusion, reduced levels of both cations play a role in the pathophysiology of major depression. Increased calcium levels are coupled to the clinical efficacy of antidepressants and attenuation of immune activation. The suppressant effect of antidepressants on Mg levels may be

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a side effect of those drugs. New antidepressant treatments should be developed that increase the levels both Ca and Mg.

Keywords: Depression, neuro-immune, cytokines, inflammation, indoleamine 2,3-dioxygenase.

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Introduction

Major depressive disorder (MDD) is the most common mental disorder with a prevalence varying between 0.4 and 15.7% across countries (Rai et al., 2013). The etiology and pathophysiology of MDD is associated with many different factors including psychological stress (Dold et al., 2019), immune activation (Maes & Carvalho 2018), changes in endogenous opioids (Al-Fadhel et al., 2019), genetic factors (Czarny et al., 2015), oxidative and nitrosative stress (Maes et al., 2010) and changes in minerals, elements and anti-oxidants (Maes et al., 1999). Among the elements examined in MDD, calcium (Ca) and magnesium (Mg) have been examined showing inconsistent results (Joffe et al., 1996; Jamilian et al., 2013; Styczeń et al., 2015; Deb et al., 2016; Islam et al., 2018).

Several studies have shown that lower Mg levels can cause depression, anxiety, and behavioral and personality changes (Wacker & Parisi 1968). Mg is an essential mineral that plays a key role in many bodily functions and regulates over 300 biochemical reactions, while 3571 human proteins potentially bind to Mg (Piovesan et al., 2012). Mg deficiency increases risk to depression and is often accompanied by a variety of depressive symptoms (Serefko et al. 2013; Boyle et al., 2017). An association between a reduced content of Mg in the brain and depression was reported by Sowa-Kucma et al., (2013). Indeed, Mg deficiency affects brain chemistry, membrane fluidity and inflammation (De Baaij et al., 2015; Phelan et al., 2018) all of which are associated with depression. Furthermore, Mg is also involved in the glutamatergic system, regulating learning, memory, neuroplasticity and perhaps antidepressant activity (Marsden 2011). Mg supplements may be used as an adjuvant in the treatment of MDD (Schwalfenberg & Genuis 2017).

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Ca exists in blood in three forms: bound to proteins mainly albumin, complexed with organic and inorganic anions, or in ionized form, which represents about half of the total calcium (Baird 2011). The biologically active ionized form has the highest physiological relevance for disturbances in Ca homeostasis as seen in many disorders (Sava et al., 2005; Al-Hakeim & Alhillawi 2018a). There is much less direct evidence for the role of calcium related processes in depression, although indirect evidence shows an association between depression and either lowered vitamin D (Milaneschi et al., 2014) or hypoparathyroidism (Rosa et al., 2014), two conditions that are both associated with altered Ca homeostasis. Ca plays an important role in the activation of various neurophysiological pathways explaining that it is negatively associated with neuropsychological performance in MDD (Grützner et al., 2018). Mandel (1960) noticed that abnormal levels of Ca, both high and low, can lead to neuropsychiatric disorders including psychosis and depression. Sharma et al. (2017) reported a positive relationship between serum Ca and neuropsychological functions and daily-life activities in depression.

There is now evidence that proinflammatory and anti-inflammatory cytokines are elevated in sera of patients with MDD (Schiepers et al., 2005; Al-Hakeim et al., 2015; 2018c; Maes & Carvalho 2018). These results indicate that MDD is not only accompanied by increased production of pro-inflammatory mediators, but also by mediators that have immune regulatory or anti-inflammatory effects and that serve an adaptive purpose down-regulating the primary immune-inflammatory response. This new concept was called the “compensatory immune regulatory system” (CIRS) (Maes and Carvalho, 2018). However, the associations between changed cation levels and activation of IRS/CIRS pathways has not been studied yet.

The role of immune-inflammatory pathways in MDD has encouraged clinicians and researchers to use anti-inflammatory drugs as an adjuvant to antidepressants for the treatment of

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MDD (Kohler et al., 2016; Al-Hakeim et al., 2018b). A meta-analysis testing NSAIDs and cytokine inhibitors as antidepressants showed that anti-inflammatory treatments reduced depressive symptoms compared with placebo (Köhler et al., 2014; 2018). Nevertheless, there are no data whether treatments with sertraline with or without ketoprofen significantly affect the levels of Ca and Mg in association with clinical recovery. Ketoprofen is a non-specific COX inhibitor that has anti-inflammatory properties by inhibiting COX-1 and 2 thereby decreasing the production of proinflammatory prostaglandin precursors (Cryer B, Feldman 1998*)*. Previously, we have shown that the clinical improvement in depression scores during treatment with sertraline (with or without ketoprofen) was significantly associated with increments in zinc levels and reductions in indoleamine 2,3-dioxygenase (IDO) and immune activation (Al-Hakeim et al 2018d; Twayej et al., 2019). However, no research examined whether changes in Ca and Mg levels during antidepressant treatment are associated with clinical improvement beyond and above the effects of zinc, immune induces and IDO.

Hence, the present study was carried out to examine a) differences in both cations (total and ionized forms) between drug-naïve MDD patients and controls; b) the effects of a combination of sertraline and ketoprofen on Ca and Mg (both total and ionized) levels and c) whether changes in Ca and Mg during this treatment are associated with clinical improvement beyond and above the effects of zinc, immune activation (based on assays of IL-1β, IL-4, IL-6, IL-18, IFN-γ and TGF-β1) and IDO.

Subjects and Methods

Participants

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The present study recruited one hundred and forty MDD patients in addition to 40 healthy controls. The samples were collected at the Psychiatry Unit, Al-Hakeem General Hospital in Najaf Governorate-Iraq and from a private psychiatric clinic during the period from November 2016 till August 2017. The MDD patients were diagnosed by psychiatrists according to a semi-structured psychiatric interview schedule for the diagnosis of MDD based on the ICD-10 (10^{th} revision of the International Statistical Classification of Diseases and Related Health Problems). Controls 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, C-reactive protein (CRP) was not less than 6 mg/L which excludes overt inflammation.

The present study consists of two parts: a) a case control study including 140 MDD patients and 40 controls; and b) a prospective study that examines 44 of the 140 depressed patients in a prospective study during 2 months with blood samplings both before (baseline) and during treatment with sertraline with and without ketoprofen. The latter was part of a randomized trial recorded in the NIH US Library of Medicine, ClinicalTrials.gov Identifier: NCT03514810. All biomarker assays and BDI-II scores were completed in these 44 subjects. Among the follow up group, sixteen patients received sertraline (orally 50 mg once daily) and twenty eight patients were treated with ketoprofen (100 mg orally once daily) as adjuvant to sertraline. Patients took one capsule daily after breakfast. The colored empty capsules (Caps & Chemicals, India) were filled either with sertraline (Actavis, Italy) or sertraline + ketoprofen (Menarini Int., Italy). The laboratory analysts were blinded to the treatment modalities. The protocol was approved by the

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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.

Measurements

The total score on the Beck-Depression Inventory (BDI-II) (Hautzinger et al., 2006) 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 44 patients who were included in the biomarker treatment study. The blood samples were aspirated without tourniquet and left at room temperature for 10 minutes for clotting, centrifuged 3000 rpm for 5 minutes, and then sera were separated and transported into three new Eppendorf tubes until assay. Serum Calcium was measured using a ready for use kit supplied by Biolabo® Co, France. Ca with Arsenazo III [1,8-Dihydroxy-3,6-disulpho-2,7-naphthalene-bis (azo)-dibenzenearsonic acid], at slightly acidic pH (6.8), yields a blue colored complex, whose intensity is proportional to the calcium concentration. Serum albumin was measured in order to correct or adjust the Ca and Mg levels for changes in albumin. Serum albumin was assayed by bromocresol green at 630 nm. Corrected calcium was calculated from the following formula: Corrected Ca (mg/dl) = T.Ca+ 0.8[4-Albumin] (Ohbal et al., 2014). Ionized calcium was calculated from the following formula: $I.Ca^{2+} = 0.813 \times T.Ca^{0.5} - 0.006 \times Albumin^{0.75} + 0.079$ (Mateu-de Antonio 2016), which give the best approximate result. Serum Mg was estimated using the calmagite method supplied by Biolabo® Co, France. Mg forms a purple colored complex when treated with calmagite in alkaline solution measured at 520 nm. In presence of EGTA, the reaction is specific and the intensity of the purple color is proportional to the Mg concentration. Serum ionized Mg levels

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were calculated according to the following formula: I.Mg (in mM) = (0.66 × (T.Mg in mM)) + 0.039 (Koch et al., 2002).

Serum indoleamine-2,3-Dioxygenase activity (IDO), IL-4, INFγ, 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 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. This method achieved 30% increase in sensitivity compared to use of deionized water only due to decrease viscosity and difference in droplet formation (Meret & Henkin 1971).

Statistical Analysis

Analysis of variance (ANOVA) was employed to assess differences in scale variables between diagnostic groups and analysis of contingency tables (χ^2 test) to check associations between categorical variables. We used Pearson's product moment correlation coefficients to examine associations between scale variables. Multivariate general linear model (GLM) analysis

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was used to assess the effects of diagnosis (independent variable) on biomarkers (dependent variables), while adjusting for extraneous variables (age, sex, BMI). Repeated measurement data were analyzed using generalized estimating equation (GEE) tests examining the effects of treatment on Ca and Mg levels with effects of time (pre- versus post-treatment), treatment (sertraline versus sertraline + ketoprofen) and time X treatment. Tests were two-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). As explained previously, we used z unit weighted composite scores based on cytokine levels (Maes and Carvalho, 2018) to obtain an index of general immune activation. This index was computed as z value of IL-1 (zIL-1) + zIL-6 + zIL-18 + zIFN-γ + zIL-4 + zTGF-β1 (M1+Th1+Th2+Treg denoting that this index reflect the combined activity of macrophagic M1, Thelper-1, Thelper-2 and Tregulatory functions. We also computed zIL-1 + zIL-6 + zIL-18 + zIFN-γ, which reflects activation of M1 and Th1 phenotypes (M1+Th1). Another index used here is the zIL-4 + zTGF-β1 composite score reflecting regulatory mechanisms (Th2 + Treg). We also computed the ratio of z(zIL-1 + zIL-6 +zIL-18 +zIFN-γ) - z(zIL4 + zTGF-β1), which reflects the pro-inflammatory / immune regulatory ratio.

Results

Socio-demographic data

Table 1 shows the socio-demographic and clinical data as well as Ca and Mg measurements in MDD patients and controls. There were no significant differences in age, BMI, sex ratio, and employment status between the both categories. There were significantly more

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smokers and single subjects in the MDD group as compared with controls. Serum T.Mg and I.Mg were significantly lower in MDD than in controls. T.Ca, corrected Ca and I.Ca were all significantly lower in MDD than in controls. There were no significant differences in the T.Mg / T.Ca ratio between the two study groups, while the I.Mg / I.Ca ratio was significantly lower in MDD patients than in controls. There were no significant differences in serum albumin between both study groups.

There was no significant correlation between T.Mg and T.Ca ($r=0.105$, $p=0.159$, $n=180$) while there was a weak significant inverse correlation between I.Mg and I.Ca ($r=-0.223$, $p=0.003$, $n=180$). There were no significant correlations between the BDI-II score and any of the Mg and Ca data. There was a significant positive correlation between albumin and T.Mg ($r=0.504$, $p<0.001$, $n=180$) and a weak but significant inverse correlation between albumin and T.Ca ($r=-0.157$, $p=0.035$, $n=180$). There were significant associations between albumin and I.Ca ($r=-0.707$, $p<0.001$, $n=180$) and corrected Ca ($r=-0.640$, $p<0.001$, $n=180$). Thus, the "corrected" values are strongly and inversely associated with albumin values, indicating that the "correction" equations used are not very adequate. Therefore, we will present the ionized and corrected Ca data in baseline conditions and compute the residualized Mg and Ca values obtained by regression of the cations on albumin. Also, because I.Mg is statistically redundant we will show the I.Mg values only in baseline conditions, but will compute the residualized Mg values after regression on albumin.

Differences in the Ca and Mg between MDD and controls

Table 2 displays the results of a multivariate GLM analysis with T.Mg, T.Ca, corrected Ca, I.Ca, and the T.Ca / T.Mg and I.Ca / I.Mg ratios as dependent variables, while diagnosis was

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the primary explanatory variable. The results were controlled for possible effects of sex, age and BMI. There was a highly significant effect of diagnosis with an effect size of 0.233 and also a moderate effect of age with an effect size of 0.106. Sex and BMI had no significant effects on the biomarkers. Tests for between-subjects effects showed that diagnosis was significantly associated with T.Mg, T.Ca, corrected Ca, I.Ca and I.Ca / I.Ca ratio. Tests for between-subject effects showed that there was a very weak albeit significant negative association between age and I.Ca / I.Mg ratio (F=4.50, df=1/175, p=0.035). Smoking (F=2.00, df=7/168, p=0.057) did not have a significant effect on these 6 biomarkers, while also tests for between-subjects effects showed that there were no significant effects of smoking on any of the 6 biomarkers (even without p-correction). **Table 3** shows the model-generated estimated marginal mean values of the 6 biomarkers. Here we present the z values of the biomarker values, which were additionally adjusted for age, sex and BMI. We found that T.Mg, T.Ca, I.Ca and corrected Ca and the I.Ca / I.Mg ratio were significantly lower in MDD than in controls and that the distance between both groups for T.Ca was 0.886 standard deviations.

Table 2 shows also the results of univariate GLM analyses with the other immune biomarkers as dependent variable and diagnosis as primary explanatory variable while adjusting for possible effects of age, sex and BMI. Table 3 shows the model-generated estimated marginal means in z scores. The univariate analyses showed significantly higher composite scores of immune activation, M1+Th1, Th2+Treg, M1+Th1/Th2+Treg ratio and IDO in MDD versus controls, while zinc was significantly lower in MDD than controls. There was a particularly strong impact of diagnosis on the M1+Th1+Th2+Treg and M1+Th1 indices with effect sizes of 0.511 and 0.524, respectively. There were no significant differences in albumin between both groups.

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Table 2 shows also the T.Ca and T.Mg values corrected for serum albumin levels, thus reflecting the unbound (or ionized) forms of Ca and Mg. Toward this end we have performed a multivariate GLM analysis with T.Ca and T.Mg as dependent variables and diagnosis and albumin as primary explanatory variables, while adjusting for age, sex and BMI. This GLM analysis showed that both T.Ca and T.Mg were significantly predicted by diagnosis and albumin. Table 3 shows the residualized T.Ca and T.Mg values (reflecting the unbound levels independent of albumin). Both the residualized T.Ca and T.Mg values were significantly lower in MDD than in controls and the difference between MDD and controls was 0.901 and 0.702 standard deviations, respectively.

Effects of treatments on the Ca and Mg biomarkers

Table 4 shows the baseline characteristics of the 44 patients who were treated with sertraline alone or sertraline + ketoprofen. We found no significant differences in BDI-II, age, BMI, sex ratio, and smoking behavior between both treatment groups. There were no significant differences in T.Mg and the residualized Mg (adjusted for albumin) and T.Ca and residualized Ca (adjusted for albumin) values between both study groups. There were no differences in albumin levels between both study groups.

Table 5 displays the results of GEE analyses, repeated measures, with effects of time (pre- versus post-treatment), treatment (sertraline *versus* sertraline + ketoprofen) and the time X treatment interaction on BDI-II and the biomarkers, while adjusting for sex, age, smoking and BMI. We published previously (Al-Hakeim et al., 2018b) that sertraline + ketoprofen (pre-treatment and post-treatment means (SE): 48.82 ±1.98 and 13.43±1.26, respectively) reduced the BDI-II more than sertraline + placebo (49.94 ±2.00 and 19.69 ±1.70, respectively). There was a

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weak albeit significant effect of time (treatment) on T.Mg and the residualized Mg values (both lowered) and a significant positive effect of time on T.Ca and the residualized Ca values, while no significant time X treatment interactions could be established for both Mg and Ca values.

Using GEE analysis, repeated measurements, we have also examined the associations between BDI-II values from pre- to post-treatment condition (as dependent variables) and the Ca and Mg biomarkers (explanatory variables). **Table 6**, analysis #1 shows that there was no significant association between BDI-II values and T.Mg values (as well as residualized Mg, not shown). There was a significant inverse association between T.Ca (GEE #2), but not residualized Ca (GEE #3), and the BDI-II score from baseline to post-treatment. We have also examined whether the pre- and post-treatment Ca values contribute to the prediction of the BDI-score and, therefore, entered the residualized Ca values together with M1+Th1+Th2+Treg index, IDO and zinc as explanatory variables. GEE analysis #4 shows that these 4 different biomarkers had a significant effect on the BDI-II score. Finally, GEE analysis #5 shows that the residualized Ca data are significantly and inversely associated with the M1+Th1+Th2+Treg index.

Discussion

Differences in Mg and Ca between MDD patients and controls

The first major finding of this study is that MDD patients show significantly lower levels of serum total and unbound Mg than controls. These results are in accordance with previous studies reporting that serum concentrations of Mg were substantially reduced in depressed patients (Zieba et al., 2000; Cheungpasitporn et al., 2015) and that the incidence of hypomagnesemia is elevated in depressed patients as compared with controls (Levine et al., 1999). Chronic stress, alcohol abuse and diets rich in carbohydrates and fats may cause prolonged Mg deficiency

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leading to development of depressive symptoms (Tarleton et al., 2017), suggesting that reduced Mg levels participate in the pathophysiology of MDD (Rajizadeh et al., 2016; Islam et al., 2018). Possible causes of the lowered Mg levels in MDD are decreased appetite and Mg-impoverished diets (Jacka et al., 2009), whereas Mg rich diets may reduce depressive symptoms (Derom et al., 2013). One pathway explaining the effects of Mg is that this cation regulates the N-methyl-D-aspartate (NMDA) channels (Jung et al., 2010).

Serum total and unbound Ca were significantly lower in MDD than in controls. These findings extent those of previous authors (Bowden et al., 1988; Islam et al., 2018). Jung et al. (2010) and Paul (2001) proposed that lowered Ca levels in the peripheral blood and abnormal neuronal calcium homeostasis may cause depressive symptoms. Extracellular and intracellular Ca levels play a role in mood and cognition through effects on neuronal signaling pathways modulating neuroplasticity and cognitive functions including learning (Hurst, 2010; Grützner et al., 2018; Toescu and Verkhratsky, 2007). Upon activation, NMDA receptors allow the entry of Ca ions into the neuron activating downstream signaling pathways whereby Ca enhances synaptic transmission (Lisman et al., 2002) and induces long-term depression via calcineurin (Mulkey et al., 1994). NMDA dysfunctions have been implicated in depression (Iadarola et al., 2015). Nevertheless, some authors found that the incidence of hypercalcemia was elevated in depressed patients as compared with controls (Levine et al., 1999), while other studies found that a higher serum Ca/Mg ratio may be associated with risk to develop depression (Jung et al., 2010). Mg is known to play a role in calcium balance as well as vitamin D metabolism (Deng et al., 2013) and dysregulation of the balance between these cations has been implicated in the onset of depression (Dittman et al., 2000; Anglin et al., 2013).

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In our study we could not find a significant associations between baseline Mg and Ca levels and severity of illness. Previously, it was shown that severity of depression was significantly associated with serum levels of Mg (Styczeń et al., 2015; Rajizadeh et al., 2016), while another study could not detect significant correlations between severity of illness and serum Mg in MDD (Ram et al., 2016). Previous data show that serum total Ca was significantly and positively correlated with neuropsychological composite scores, including information processing speed, executive functions and global assessment of functioning in MDD patients (Sharma et al., 2017). However, the study of Ram et al. (2016) found no correlations between severity of depressive symptoms and serum Ca levels (Ram et al., 2016).

The significant positive correlation between albumin and total Mg detected in our study may be explained by the knowledge that a large part of Mg (around 25%) is bound to albumin (Kroll and Elin, 1985). Surprisingly, we found highly significant associations between albumin and ionized Ca and the corrected Ca index indicating that those “corrected” values (Ohbal et al., 2014; Mateu-de Antonio 2016) cannot be applied to our population. Therefore, we have adjusted our total Ca and Mg data for albumin using regression analysis and used the residualized values as indices of unbound Ca and Mg, respectively. As such, we found that the unbound (residualized) Ca and Mg values were significantly lowered in MDD patients than in controls, indicating that total as well as unbound cation levels are decreased.

In our study, there were no significant correlations between serum total Mg and Ca levels. Previously, a strong correlation between ionized Ca and Mg has been observed (Ryden et al., 1976) reflecting a close physiologic relationship between these divalent cations in serum (Altman 1977).

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Effects of treatments on the Ca and Mg biomarkers

The second major finding of this study is that antidepressants (with or without ketoprofen) significantly increased total Ca and that the changes over time in Ca were significantly and inversely associated with clinical efficacy of the antidepressants. Moreover, the effects of increased unbound Ca levels on severity of illness remained significant after considering the effects of immune activation and IDO (both positively associated with the BDI score) and zinc (inversely associated with the BDI score). Elevated production of IFN-γ and M1 macrophagic cytokines may lead to activation of peripheral and brain levels of IDO (Maes et al., 1994; 2011b), while lowered levels of zinc show intertwined association with immune activation (Twayej et al., 2019). Importantly, in the present study we found that unbound Ca was significantly and inversely associated with the immune activation index. Previously it was shown that the metabolism and processing of cytokines is calcium dependent and that calcium signaling plays a key role in diverse immune responses (Ainscough et al., 2015; Fracchia et al., 2013). These data suggest that lowered Ca levels may contribute to immune activation in MDD and that intertwined associations between calcium, zinc and immune activation participate in the pathophysiology of MDD.

In the current study, we found that antidepressant treatments had a weak albeit suppressant effect on total and unbound Mg values. Previously, contradictory findings were reported. Camerdese et al. (2012) reported that Mg levels correlated with the patient's responses to antidepressant treatment, whilst Young et al., (1996) found no significant differences in Mg levels or Ca/Mg ratio in drug-free MDD patients compared with control group independently of responsiveness to the antidepressant treatment. In the remission phase, Mg levels of patients were not significantly different from the concentrations of the healthy controls

(Styczeń et al., 2015), suggesting that lowered Mg levels during the acute phase may be state markers of depression. Nevertheless, the suppressant effect of antidepressants on Mg levels may be another side effect of these type of drugs. Although there are well-known effects of Mg on the development of systemic immune responses (Moslehi et al., 2012), the current study could not find a significant association between Mg levels and the immune activation index. Nevertheless, extracellular Mg concentrations may not reflect their intracellular levels and therefore the current assays of Mg status may not be satisfactory to reflect intracellular Mg (Serefko et al., 2016).

Conclusions

Baseline serum total and ionized Mg and Ca were significantly lower in MDD than in controls, but were not associated with severity of illness. Treatment with antidepressants (with or without ketoprofen) significantly reduced serum Mg and increased Ca levels. The increments in the latter were significantly associated with the clinical efficacy of antidepressants. The results sugegst that lowered Mg and Ca levels may contribute to the pathophysiology of depression and that lowered Ca may contribute to immune activation and that intertwined associations between calcium, zinc and immune activation participate in the pathophysiology of MDD and the clinical response to antidepressants.

Conflict of interest

The authors declare that there is no conflict of interest.

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Conflict of interest

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Author's contributions

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

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Table 1: Socio-demographic, clinical and biomarker data in healthy controls (HC) and major depressed (MDD) patients both before (baseline) and after (post-) treatment.

Variables	HC n=40	MDD n=140	F/X/ψ	df	p
Age (years)	40.3(7.9)	39.0(10.6)	0.53	1/178	0.469
BMI (kg/m^2)	27.72(4.16)	27.55(16.81)	0	1/178	0.949
Sex (F/M)	18/22	62/78	0	1	0.936
Smoker (Y/N)	40/0	82/58	24.45	1	<0.001
Employment (Y/N)	22/18	59/81	2.08	1	0.149
Married / Single	18/22	89/51	4.45	1	0.035
T.Mg (mM)	0.805(0.147)	0.714(0.104)	19.51	1/178	<0.001
I.Mg (mM)	0.571(0.097)	0.511(0.688)	19.51	1/178	<0.001
T.Ca (mM)	2.400(0.107)	2.249(0.171)	28.04	1/178	<0.001
Corrected Ca (mM)	2.394(0.138)	2.255(0.222)	13.98	1/178	<0.001
I.Ca (mM)	1.240(0.040)	1.204(0.067)	10.76	1/178	0.001
T.Mg / T.Ca	0.336(0.060)	0.320(0.053)	2.77	1/178	0.098
I.Mg / I.Ca	0.461(0.082)	0.427(0.069)	6.98	1/178	0.009
Albumin (g/l)	40.31(3.41)	39.69(5.72)	0.42	1/178	0.520

All data are shown as mean (SD)

T. Mg: Total magnesium; T. Ca: Total calcium; I. Mg: Ionized magnesium; I. Ca: Ionized calcium

Corrected Ca: Corrected calcium

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Table 2: Results of multivariate GLM analysis with the biomarkers as dependent variables and diagnosis as explanatory variable while adjusting for extraneous variables including sex, age and body mass index(BMI)

Tests	Dependent variables	Independent variables	F	Df	P	Partial η^2
Multivariate	T.Mg, T.Ca, Corrected Ca, I.Ca, T.Ca / T.Mg, I.Ca / I.Mg	Diagnosis	8.59	2/166	<0.001	0.233
		Sex	0.89	2/166	0.503	0.030
		Age	3.35	2/166	0.004	0.106
		BMI	0.54	2/166	0.775	0.019
Between-subject effects	T.Mg	Diagnosis	18.75	1/175	<0.001	0.097
	T.Ca	Diagnosis	27.61	1/175	<0.001	0.136
	Corrected Ca	Diagnosis	14.67	1/175	<0.001	0.077
	I.Ca	Diagnosis	11.54	1/175	0.001	0.062
	T.Ca / T.Mg	Diagnosis	2.59	1/175	0.11	0.015
	I.Ca / I.Mg	Diagnosis	6.54	1/175	0.011	0.036
Univariate	M1+Th1+Th2+Treg	Diagnosis*	122.14	1/174	<0.001	0.511
	M1+Th1	Diagnosis*	191.19	1/174	<0.001	0.524
	Th2+Treg	Diagnosis	45.77	1/175	<0.001	0.207
	M1+Th1/Th2+Treg	Diagnosis	13.34	1/174	<0.001	0.071
	IDO	Diagnosis	11.7	1/175	0.001	0.059
	Zinc	Diagnosis	14.51	1/168	<0.001	0.080
	Albumin	Diagnosis	0.23	1/175	0.63	0.001
Multivariate	Ca, Mg	Diagnosis*	23.41	2/173	<0.001	0.213
		Albumin*	36.45	2/173	<0.001	0.296
Between-subject effects	Mg	Diagnosis*	22.45	1/174	<0.001	0.114
		Albumin*	61.91	1/174	<0.001	0.262
	Ca	Diagnosis*	29.44	1/174	<0.001	0.145
		Albumin*	6.49	1/174	0.012	0.036

(*): All analysis are adjusted for age, BMI, and sex.

T. Mg: Total magnesium; T. Ca: Total calcium; I. Mg: Ionized magnesium; I. Ca: Ionized calcium; Corrected Ca: Corrected calcium

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

Variables	Controls	MDD
T. Mg	0.577(0.152)	-0.167(0.149)
T. Ca	0.694(0.149)	-0.192(0.08)
Corrected Ca	0.521(0.153)	-0.142(0.082)
I. Ca	0.466(0.154)	-0.125(0.082)
T. Ca / T. Mg	0.220(0.159)	-0.069(0.085)
I. Ca / I. Mg	0.349(0.156)	-0.104(0.084)
M1+Th1+Th2+Treg	-1.345(0.114)	0.386(0.062)
M1+Th1	-1.357(0.113)	0.389(0.061)
Th2+Treg	-0.831(0.146)	0.250(0.079)
M1+Th1/Th2+Treg	-0.492(0.158)	0.140(0.086)
IDO	-0.455(0.152)	0.119(0.082)
Zinc	0.538(0.156)	-0.147(0.085)
Albumin	0.063(0.155)	-0.022(0.083)
Residualized Mg *	0.546(0.131)	-0.156(0.07)
Residualized Ca *	0.705(0.147)	-0.196(0.079)

All data are shows as z scores (SE)

* These are residualized values obtained after the regression on albumin levels.

T. Mg: Total magnesium; T. Ca: Total calcium; I. Mg: Ionized magnesium; I. Ca: Ionized calcium;

Corrected Ca: Corrected calcium

IDO: indeoleamine-2,3-dioxygenase activity

M1+Th1+Th2+Treg: zIL-10 + zIFNγ+zIL-4+zTGF-β1 computed as z Ln IL-10 z Ln IFN-γ + z Ln IL-4 + z Ln TGF-β1.

M1+Th1: zIL-10 + zIFNγ computed as z Ln IL-4 + z Ln IFNγ. .

Th2+Treg: zIL-4+zTGF-β1 computed as z Ln IL-4 + z Ln TGF-β1.

M1+Th1/Th2+Treg: zIL-10 + zIFNγ-zIL-4+zTGF-β1 computed as z Ln IL-10 z Ln IFN-γ – z(z Ln IL-4 + z Ln TGF-β1).

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

Variable	Sertraline alone	Sertraline + Ketoprofen	F/χ^2	df	p
BDI-II	49.9(8.3)	48.8(10.7)	0.13	1/42	0.720
Age (years)	38.5(11.8)	39.4(10.1)	0.07	1/42	0.801
BMI (kg/m^2)	25.61(5.37)	26.89(4.35)	0.75	1/42	0.392
Sex (F/M)	6/10	10/18	0.01	1	0.906
Smoker (Y/N)	10/21	6/7	0.76	1	0.382
T. Mg (mM)	0.743(0.077)	0.748(0.078)	0.04	1/42	0.840
T. Ca (mM)	2.161 (0.159)	2.222 (0.153)	1.55	1/42	0.219
T. Ca / T. Mg	2.936 (0.0351)	3.001 (0.424)	0.52	1/42	0.475
EMM Mg (mM)	0.739 (0.020)	0.750 (0.015)	0.17	1/41	0.682
EMM Ca (mM)	2.177 (0.039)	2.213 (0.029)	0.55	1/41	0.462
Albumin (g/l)	44.21(3.72)	42.21(3.36)	3.32	1/42	0.075

All data are shown as means (SD), except the EMM data

T. Mg: Total magnesium; T. Ca: Total Calcium; T. Mg: Ionized magnesium

* EMM (SE): estimated marginal means after adjusting for albumin

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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.

Variable	Time					Time x treatment		
	t_0	t end-point	W	df	p	W	df	p
BDI-II	49.38 (1.41)	16.56 (1.06)	356.89	1	<0.001	9.71	2	0.002
T. Mg (mM)	0.753 (0.012)	0.687 (0.029)	3.98	1	0.046	0.64	2	0.727
T. Ca (mM)	2.176 (0.024)	2.328 (0.035)	14.19	1	<0.001	5.05	2	0.080
residualized Mg	0.344 (0.087)	-0.170 (0.211)	4.33	1	0.038	0.61	2	0.736
residualized Ca	-0.354 (0.115)	0.272 (0.181)	9.54	1	0.002	3.18	2	0.204

Data are shown as mean (SE)

T. Mg: Total magnesium; T. Ca: Total Calcium

Residualized: residual valued obtained by regression on albumin levels

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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 variables	Explanatory variables	B	SE	W	df	P
#1	BDI-II	T.Mg	0.044	0.111	0.16	1	0.690
#2	BDI-II	T.Ca	-0.162	0.063	6.67	1	0.010
#3	BDI-II	Residualized Ca	-0.105	0.064	2.66	1	0.103
#4	BDI-II	Residualized Ca	-0.147	0.065	5.09	1	0.024
		M1+Th1+Th2+Treg	0.329	0.053	38.28	1	<0.001
		Zinc	-0.177	0.043	16.77	1	<0.001
		IDO	0.257	0.046	31.93	1	<0.001
#5	M1+Th1+Th2+Treg	Residualized Ca	-0.207	0.087	5.62	1	0.018

BDI-II: Beck Depression Inventory score

T.Mg: total magnesium; T.Ca: total calcium; Residualized Ca: the residualized Calcium values obtained after regression on albumin

M1+Th1+Th2+Treg: zIL-10 + zIFNγ+zIL-4+zTGF-β1 computed as z Ln IL-10 z Ln IFN-γ + z Ln IL-4 + z Ln TGF-β1.