Upregulation of the nitrosylome in bipolar disorder type 1 (BP1), but not BP2, and major depression: increased IgM antibodies to nitrosylated conjugates are associated with indicants of leaky gut.

Running Title: nitrosylation in bipolar disorder

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Abstract

Objective: Major depression (MDD) and a lifetime history of MDD are characterized by increased nitrosylation, while bipolar disorder type 1 (BP1), but not BP2, is accompanied by highly increased levels of oxidative stress and nitric oxide (NO) production. Nevertheless, it is unknown whether nitrosylation is involved in BP and whether there are differences in nitrosylation between BP1 and BP2.

Methods: Serum IgM antibodies directed against nitroso (NO)-adducts were examined in MDD, BP1, BP2 and healthy controls, namely IgM responses to NO-cysteine, NO-tryptophan (NOW), NO-arginine and NO-albumin (SBA) in association with IgA/IgM responses to Gram-negative bacteria, IgG responses to oxidized low-density lipoprotein (oxLDL) and serum peroxides.

Results: Serum IgM levels against NO adducts were significantly higher in BP1 and MDD as compared with healthy controls, whereas BP2 patients occupied an intermediate position. IgM responses to NO-albumin were significantly higher in BP1 and MDD than in BP2 patients. There were highly significant associations between the IgM responses to NO-adducts and IgG responses to oxLDL and IgA/IgM responses to Gram-negative bacteria.

Conclusions: BP1 and MDD are characterized by an upregulation of the nitrosylome (the proteome of nitrosylated proteins), and increased IgM responses to nitrosylated conjugates. Increased nitrosylation may be driven by increased bacterial translocation and is associated with lipid peroxidation processes. Innate like (B1 and marginal zone) B cells and increased nitrosylation may play a key

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role in the major affective disorders through activation of immune-inflammatory and oxidative pathways, cardiovascular comorbidity and impairments in antioxidant defenses, neuro-glial interactions, synaptic plasticity, neuroprotection, neurogenesis, etc.

Key words: depression, bipolar disorder, oxidative and nitrosative stress, neuro-immune, inflammation, cytokines

Introduction.

There is now evidence that activated immune-inflammatory and oxidative stress pathways play an important role in the pathophysiology of major depression (MDD) and bipolar (BP) disorder. [1-5] Moreover, recently we reported that O&NS biomarkers may aid in the differentiation of MDD, and BP disorder type 1 (BPI) and type 2 (BP2) with increasing nitro-oxidative stress, aldehyde production and protein oxidation from BP2 → BP2 → MDD in (partially) remitted patients.[6] Thus, MDD patients showed higher superoxide dismutase (SOD1) activity, NO metabolite (NOx) production, lipid peroxides and malondialdehyde (MDA) levels than patients with BP1 and BP2 and controls, while protein oxidation (measured with advanced oxidation protein products, AOPP) was higher in BP1 patients than in BP2 patients and healthy controls.[6] There are not many studies that directly compare oxidative biomarkers among patients with BP1 and BP2 in an acute depressive state. For example, Sowa-Kucma et al.[7] reported that there are no significant differences in aldehyde formation (TBARS levels) between subjects with MDD and BP disorder (BP1 and BP2) in an acute phase of illness.

It is understood that MDD and BP disorder are progressive disorders whereby increasing recurrent depressive/manic episodes and suicidal behaviors are associated with disabilities, lowered quality of life, neurocognitive decline and increased suicidal ideation and suicide attempts.[8] Furthermore, a new staging model of MDD and BP disorder indicates that this staging dimension (recurrent episodes and recurrent suicidal behaviors, increasing disabilities and lowered quality of life) is strongly associated with deficits in

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paraoxonase 1 (PON1), a strong antioxidant, and indicants of increased oxidative stress including aldehyde formation and protein oxidation.[8]

Activated immune-inflammatory and oxidative pathways are usually accompanied by increased nitrosylation of proteins.[9-11] Nitrosylation involves the covalent addition of a nitroso (NO) group to cysteine thiolate anions (S-nitrosylation) or NH_2 groups (N-nitrosylation), reactions that are reversible and mediated by increased levels of mainly N_2O_3.[9,10] Nitrosylation plays a key role in cellular adaptation to nitro-oxidative stress and regulates the biological activity of many proteins in a similar manner to palmitoylation and phosphorylation thereby protecting against irreversible cysteine oxidation with permanent changes in its secondary and tertiary structure.[9,10] Increased reactive oxygen (e.g. superoxide) and nitrogen (e.g. NO) species (ROS and RNS, respectively) may induce this type of protective nitrosylation. Nevertheless, increases in ROS/RNS and oxidative damage leads to breakdown of processes that counterbalance protein nitrosylation (including denitrosylation and transnitrosylation) causing irreversible oxidative damage to organosulfur oxoacids (RSOH, RSO2H and RSO3H).[9,10] As such, hypernitrosylation may be accompanied by nitrosative stress (cellular injury due to nitrosylation) with consequent inactivation of SIRT-1, which compromises cortisol responses to stress, neuronal apoptosis, formation of damage-associated molecular patterns, autoimmunity, activated immune-inflammatory pathways, glutamate excitotoxicity, synaptic plasticity and impaired neuro-glial interactions and neurogenesis, and loss of antioxidant defenses and neuroprotection, which are all hallmarks of MDD and BP disorder.[3-5,9,10,12]

Recently, we have provided evidence in different study samples that MDD is accompanied by increased nitrosylation as measured with IgM responses to conjugated nitroso-adducts including NO-albumin (serum bovine albumin), NO-tryptophan (NOW), NO-cysteine, NO-creatinine, NO-aspartate and NO-phenylalanine.[13-16] These results indicate that post-translational nitrosative modifications (NO-adducts), which have triggered an IgM-mediated autoimmune response to these adducts, occur in patients with MDD. Moreover, in women with prenatal depression, a lifetime history of MDD was significantly associated with increased IgM responses directed against NO-cysteine.[17] As such, increased nitrosylation may underpin and orchestrate many neuro-immune aberrations observed in MDD as reviewed above. Moreover, there are also data that indicants of increased bacterial translocation in MDD (as assessed by IgA/IgM responses to sonicated Gram-negative bacteria) are significantly associated with elevated IgM responses to NO-adducts (including NOW), suggesting that bacterial translocation may drive Nitrosylation.[18] Also, in pregnant women at the end of term significant associations were detected between indices of bacterial translocation and IgM to NO-cysteine.[17] Nevertheless, there are no studies that have directly compared alterations in nitrosylation among patients with MDD, BP1 and BP2 or that have examined associations between indices of bacterial translocation and nitrosylation in subjects with BP disorder.

Hence, we have examined IgM responses to NO-cysteine, NO-arginine, NO-albumin and NOW in subjects with MDD, BP1 and BP2 versus normal controls and examined the associations between these IgM responses to NO-adducts and indices of bacterial translocation (as assessed with IgA/IgM responses to 6 sonicated Gram-negative gut commensal bacteria) and oxidative stress (as assessed with peroxide levels and IgG responses to oxidized low-density lipoprotein (oxLDL)). Plasma peroxide is one of the ROS

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that can be measured in peripheral blood, while IgG autoantibodies to oxLDL not only reflect lipid peroxidation processes but also autoimmune responses directed against neoepitopes.[19]

Subjects and Methods

Subjects

One-hundred and eighteen individuals participated in the current study, namely 22 healthy controls, 25 patients with BP2 and 27 with BP1 and 44 MDD patients. Healthy controls were recruited by word of mouth and consisted of personnel of the clinic or affiliated laboratories and /or their friends or family members. The BP disorder and MDD patients were all outpatients admitted to the Clinics of the first author, Belgium. We recruited male and female Caucasian individuals with Flemish nationality between 18 and 71 years of age. They were recruited from the same catchment area and showed a similar socio-economical level namely higher middle class and this in the Benelux where social class differences are minimal. The diagnoses of BP1, BP2 and MDD were made employing a semistructured interview according to DSM-IV-TR criteria.[20] (APA, 2002). In addition, we measured the Hamilton Depression Rating Scale (HAM-D) to assess severity of depression.[21] In the current study, we included patients in an acute depressive phase of illness with a HAM-D score > 15 and excluded those with chronic depression.

Exclusion criteria for normal controls were any axis-1 psychiatric disorder, current or lifetime, while patients were excluded for axis-1 disorders except BD and MDD but including schizophrenia, substance use disorders, post-traumatic stress disorder, and

obsessive compulsive disorder. Exclusion criteria for controls and patients were: a) neuroinflammatory disorders including Alzheimer's disorder, Parkinson's disorder, stroke and multiple sclerosis; b) medical and (auto)immune disorders, including COPD, inflammatory bowel disease, psoriasis, diabetes type 1 and 2, rheumatoid arthritis, and chronic kidney disease; subjects who showed inflammatory (e.g. flu and bronchitis) or allergic responses 2 months prior to the study; and c) subjects who ever had taken immunomodulatory drugs, including glucocorticoids, or subjects who were treated with antioxidant supplements (including omega-3 poly-unsaturated fatty acids). In the present study we exclude subjects with a body mass index (BMI) > 30. BMI was calculated using the formula: weight (kg) / body height (in meter)^2. The study has been approved by the ethical committee of the Medical University of Plovdiv (2/19.04.2018). All controls and patients gave written informed consent after the study protocol was explained and before starting the study.

Methods.

In all participants we sampled fasting blood at 8.00 a.m. for the assay of IgM antibody responses to NO-adducts, IgA/IgM responses to Gram-negative bacteria, IgG responses directed against oxLDL and peroxides. We described previously the assay to measure IgM to NO-adducts.[22] In brief, NO-arginine, NOW and NO-cysteinyl were synthesized by linking haptens to BSA (Sigma-Aldrich) using glutaraldehyde.[23-25] The synthesis of these conjugates has been described previously.[26] Each hapten conjugate was nitrosylated using sodium nitrite)$NaNO_2$(dissolved in 2 ml of each conjugate, in 0.5 M HCl at 37°C for 2 h, while shaking in the

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dark. Conjugates were then dialyzed at 4°C for 24 h against a Phosphate Buffered Saline)PBS: 10^{-2} M NaH_2PO_4, $12H_2O$; 0.15M NaCl; pH 7.4(solution. The detection of IgM autoantibodies to the conjugates was performed by indirect ELISA tests.[26,27] Briefly, polystyrene 96-well plates)NUNC(were coated with 200 µl solution containing the conjugates or BSA in 0.05 M carbonate buffer at pH 9.6. Well plates were incubated at 4°C for 16 h under agitation. Then, a 200 µl of blocking solution)PBS, 2.5 g/l BSA(was added for 1 h and placed at 37°C. Following three washes with PBS, plates were filled up with 100 µl of sera diluted at 1:1000 in the blocking buffer A)PBS, 0.05% Tween 20, 10% Glycerol, 2.5 g/l BSA, 1 g/l BSA-G(and incubated at 37°C for 2 h. After three washes with PBS-0.05% Tween 20, plates were incubated at 37°C for 1 h with peroxidase-labeled anti-human IgM secondary antibodies diluted respectively at 1: 15,000, in the blocking buffer)PBS, 0.05% Tween 20, 2.5 g/l BSA(. They were then washed three times with PBS-0.05% Tween 20, and incubated with the detection solution for 10 min in the dark. Chromogen detection solution was used for the peroxidase assay at 8% in 0.1 M acetate and 0.01 M phosphate buffer)pH 5.0(containing 0.01% H_2O_2. The reaction was stopped with 25 µl 2-N HCl. S-nitrosothiol bond formation was determined by spectrophotometry. The S-nitrosothiol compounds possess two absorbance maxima, at 336 and 550 nm, respectively: e_{336} nm= 900 $M^{-1}cm^{-1}$ for the conjugates, e_{550}nm=4000 $M^{-1}cm^{-1}$ for BSA. Absorbance was evaluated in order to determine NO concentrations linked to the compounds. All assays were carried out in duplicate. The inter-assay coefficients of variation)CV(were < 10%. In the current study we computed a z unit weighted composite score (zNOadducts) reflecting overall nitrosylation as: z (z IgM NO-Arginine + z IgM Albumin + z NOW + z NO-cysteine).

A description of the measurements of IgA/IgM antibodies directed to Gram-negative bacteria is described somewhere else.[18,28] Antigens derived from six Gram-negative bacteria were assayed after sonication, namely *Hafnia alvei, Klebsiella pneumonia, Morganella morganii, Pseudomonas aeruginosa, Pseudomonas putida* and *Citrobacter koseri.* Polystyrene 96-well plates)NUNC(were coated with 200 µl solution containing bacterial components at 4 µg/ml in 0.05 M carbonate buffer at pH 9.6. Well plates were incubated at 4°C for 16 h under agitation. Then, we added 200 µl blocking solution)PBS, Tween 20 0.05%, 5 g/l BSA(for 1 h and placed at 37°C. Following two washes with PBS, plates were filled up with 100 µl of sera diluted at 1:1000 in the blocking buffer A)PBS, 0.05% Tween 20, 2.5 g/l BSA(and incubated at 37°C for 105 minutes. After three washes with PBS-0.05% Tween 20, plates were incubated at 37°C for 1 h with peroxidase-labeled anti-human IgM or IgA secondary antibodies diluted respectively at 1: 15,000 and 1: 10,000 in the blocking buffer)PBS, 0.05% Tween 20, 2.5 g/l BSA(. Afterwards, plates were washed three times with PBS-0.05%Tween 20, and incubated with the detection solution for 10 min in the dark. Chromogen detection solution)Tetramethylbenzedine(was used for the peroxidase assay at 16.6 ml per liter in 0.11 M sodium acetate trihydrate buffer)pH 5.5(containing 0.01% H_2O_2. The reaction was stopped with 25 µl 2-N HCl. After addition of stop solution)H_2SO_4 or HCl(, the obtained, proportional absorbance in the tested sample)compared to established concentration of respective antibodies(, was measured at 450 nm with one alpha of correction at 660 nm. The inter-assay coefficients of variation)CV(were < 10%. In the current study we computed a z unit weighted composite score (z Gram- bacteria) reflecting overall bacterial load as: z (sum of z values of IgM to the 6 Gram-negative bacteria + sum of z values of IgA to the 6 Gram-negative bacteria).

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The assays of IgG responses to oxidized LDL and peroxides have been described previously.[19] IgG to oxLDL was measured by means of an enzyme immunoassay (EIA; Biomedica Medizinproducte GmbH & Co; A-1210 Wien, Austria; Cat. no: BI-20032; 12 x 8 tests; conventional 96-well ELISA format). The principle of the assay is microtiterplate solid phase which is coated with oxLDL after which diluted samples and calibrators are added to the microtiter plate wells, incubated for 1.5 hours at 37 C, washed, incubated 30 minutes at room temperature with the conjugate i.e. a monoclonal anti-human IgG-HRPO, washed again after incubation and reacted for 15 minutes with TMB substrate. The absorbance measured at 450 nm is proportionally to the amount of oxLDL antibodies in the sample or calibrator. The standard range is 37-1200 mU/ml and the detection limit of this assay is 48 mU/ml. The interassay coefficient of variation is 4.0%. Peroxides were determined using a colorimetrixtric assay Oxystat (Biomedica Medizinproducte GmBH & Co KG, A-1210 Wien) for the quantitative determination of peroxides in EDTA plasma (Cat No BI-5007). The peroxide concentration is determined by reaction of the biological peroxides with the enzyme peroxidase and a subsequent color-reaction using tetra-methyl-benzidine (TMB) as substrate. After addition of a stop solution, the developed colour is measured photometrically at 450 nm. A calibrator is measured in parallel and used to calculate the concentration of circulating biological peroxides in the sample, in a one point calibration protocol. This method determines the total peroxide concentration due to oxidative stress. The detection limit of the assay is 7 µmol/l and the interassay CV is 5.1%.

Statistics

Analysis of variance (ANOVAs) was used to examine differences in scale variables between groups, whereas contingency analysis (Chi-square test) was used to check associations between two categories. Associations between variables were assessed using Pearson's product-moment correlation coefficients and Spearman's rank order correlation analyses. Univariate and multivariate GLM analyses were used to examine the effects of diagnosis (four groups, namely controls, BP1, BP2 and MDD) on the NO values and other biomarkers (entered as dependent variables), while adjusting for extraneous variables (including age and sex). Test for between-subject analyses were used to assess the effects of diagnosis on the separate NO values while adjusting for the same extraneous variables, whilst protected pairwise post-hoc tests were used to assess differences between the 4 study groups. Multinomial logistic regression analysis was used to examine the best predictors of the diagnostic classes; Odds ratios and 95% confidence intervals were computed. Tests were 2-tailed and a p-value of 0.05 was used for statistical significance. All abovementioned statistical analyses were performed using IBM SPSS windows version 25.

Results.

Descriptive statistics

Table 1 presents the socio-demographic data of the participants divided into controls versus mood disorders patients. There were no differences in age, sex ratio and BMI between both diagnostic groups. This table also presents the measurements of peroxide levels, IgG to oxLDL and zGram-bacteria. Univariate GLM analysis with age and sex as covariates showed that peroxide levels were

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significantly higher in patients with mood disorders than in controls with an effects size of 0.078. IgG responses to oxLDL were

significantly higher in patients with mood disorders than in controls with an effect size of 0.126. IgM responses to NO-arginine, NO-albumin and NOW were significantly higher in mood disordered patients than in controls. The z composite scores reflecting total nitrosylation and bacterial translocation were also significantly increased in mood disorder patients as compared with controls. **Figure 1** shows the oxidative and nitrosative profiles in controls, MDD and BP1 and BP2 patients. Shown are the mean z scores (with SE) obtained in the four diagnostic groups. It can be seen that the profiles in MDD and BP1 are quite similar and differ considerably from those in controls and BP2, while the latter show somewhat higher values than controls.

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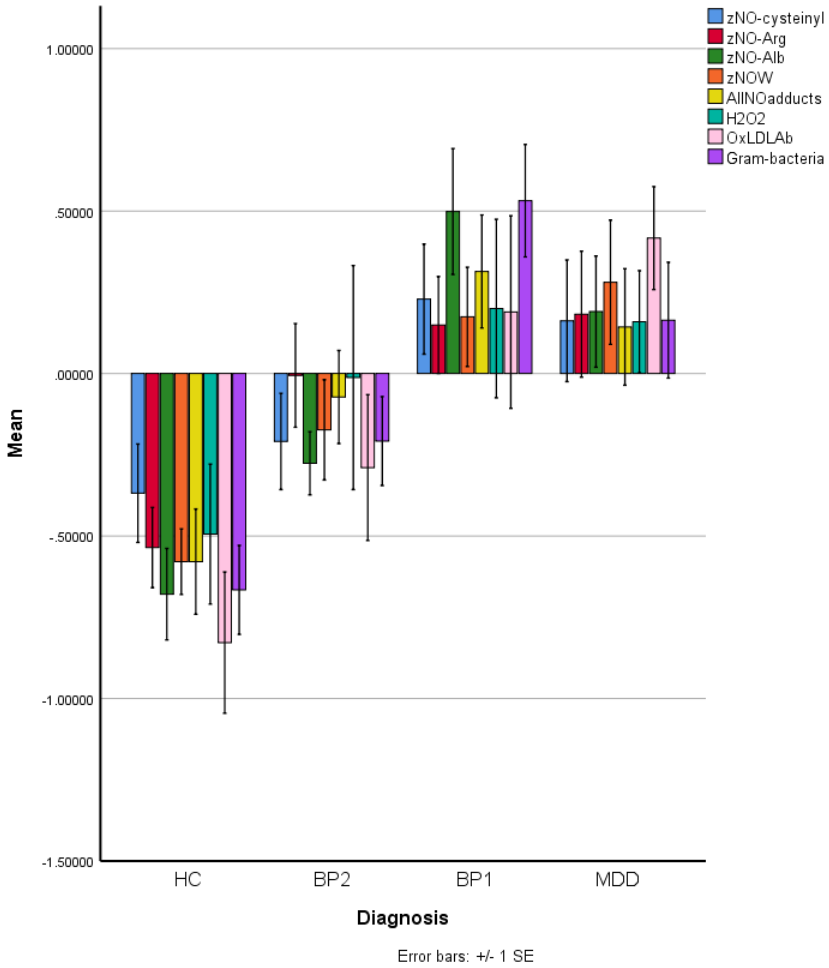

Figure 1. Oxidative and nitrosative profiles in controls, BP2 (bipolar 2), BP1 (bipolar 1) and MDD (major depressed) patients. Shown are the mean z scores (with SE) of IgM responses to NO-adducts in those four diagnostic groups.
zNO-Arg: zNO-arginine; zNO-Alb: zNO-albumin; zNOW: zNO-tryptophan; AllNOadducts: z sum all IgM responses to NO-adducts; H2O2: peroxides; oxLDLAb: IgG to oxidized low densitity lipoprotein.

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Table 1. Socio-demographic, clinical and biomarker data of patients with mood disorders (MOOD) and healthy controls (HC)

Variables	**HC (n=22)**	**MOOD (n=96)**	**F/Ψ/X^2**	**df**	**p**
Age (years)	38.2 (13.3)	42.4 (13.2)	1.80	1/116	0.183
Sex (M/F)	14/8	51/45	0.80	1	0.371
Body Mass Index (kg/m^2)	25.3 (3.8)	25.4 (2.8)	0.00	1/63	0.951
HAM-D	-	22.0 (2.9)	-	-	-
TUD (Y/N)	21/1	76/16	Ψ=0.142.		0.129
Melancholia (N/Y)	-	83/13	-	-	-
Number episodes	-	6.52 (6.05)	-	-	-
Peroxides (µmoL/L)*	239.2 (136.3)	528.5 (502.8)	5.39	1/64	0.023
IgG oxidized LDL (mU/mL)*	148.4 (84.0)	487.2 (404.2)	9.66	1/67	0.003
IgM NO-cysteine (z scores)*	-0.302 (0.871)	0.486 (1.850)	3.78	1/116	0.054
IgM NO-arginine (z scores)*	-0.662 (0.670)	0.321 (1.752)	6.66	1/116	0.011
IgM NO-Albumin (z scores)*	-0.820 (0.944)	0.789 (2.364)	9.77	1/116	0.002
IgM NOW (z scores)*	-0.643 (0.694)	0.399 (1.526)	9.74	1/116	0.002
zNOadducts (z scores)*	-0.579 (0.760)	0.136 (1.004)	9.80	1/116	0.002
IgM/IgA Gram- bacteria (z scores)	-0.666 (0.642)	0.171 (1.024)	13.43	1/116	<0.001

All results are shown as mean (±SD). F: results of analyses of variance; X^2: results of analyses of contingency tables

* These data are processed in Ln transformation

HAM-D: Hamilton Depression rating Scale score

TUD: tobacco use disorder

NOW: NO-tryptophan; zNOadducts: computed as z(sum of all z scores of the 4 IgM NO-adducts)

IgM/IgA Gram- bacteria: computed as z(sum of all z scores of IgM and IgA values to 6 different Gram- bacteria).

There were no significant associations between age, BMI, number of episodes and HAM-D score and the oxidative and nitrosative stress biomarkers. The index zGram-bacteria was significantly correlated with number of episodes (r=0.229, p=0.013,

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p=118) and HAM-D score (r=0.213, p=0.037, n=96) (without p correction). Peroxide levels were significantly associated with IgM to NO-arginine (r=0.242, p=0.050, n=66) and NOW (r=0.306, p=0.011, n=38), while IgG levels to oxLDL were significantly correlated with IgM to NO-arginine (r=0.237, p=0.050, n=69), NO-albumin (r=0.306, p=0.010, n=71) and NOW (r=0.253, p=0.033, n=71). The index zGram-bacteria was significantly associated with IgM to NO-cysteine (r=0.481, p<0.001, n=118), NO-arginine (r=0.486, p<0.001, n=116), NO-albumin (r=0.556, p<0.001, n=118) and NOW (r=0.561, p<0.001, n=118). **Figure 2** shows the association between IgM NO-albumin and IgA/IgM Gram- bacteria. **Figure 3** shows the positive correlation between IgM NO-albumin and IgG oxLDL.

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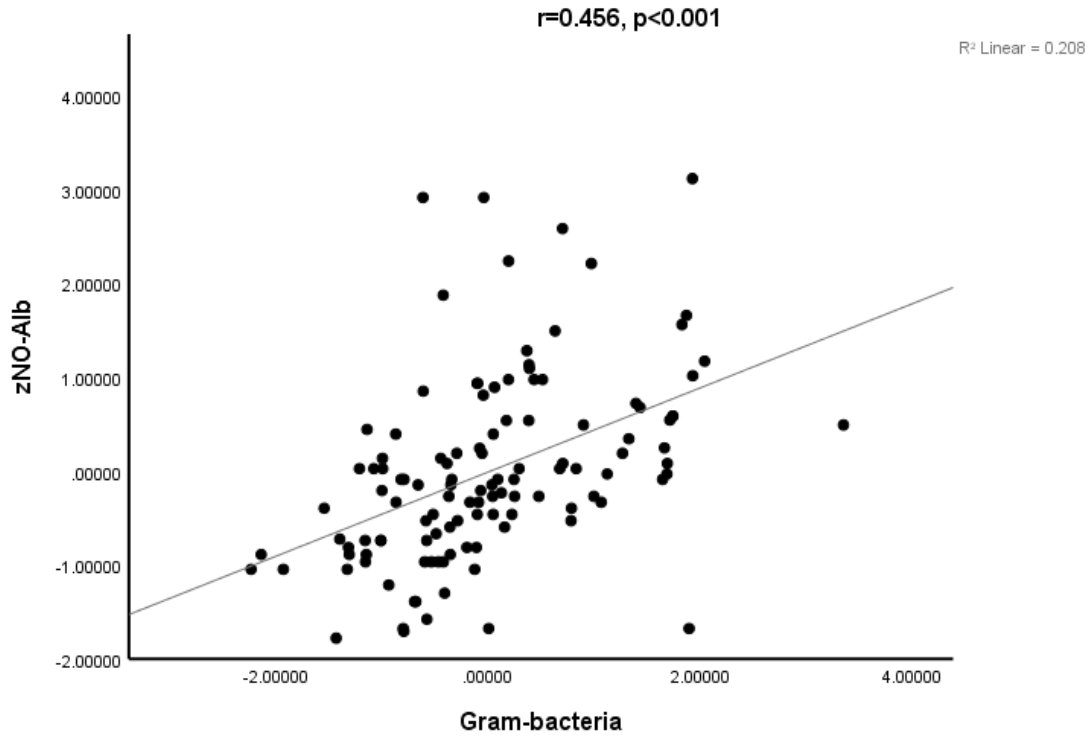

Figure 2. Association between IgM directed to NO-albumin (zNO-Alb) and sum of z values of IgA/IgM directed to Gram- bacteria.

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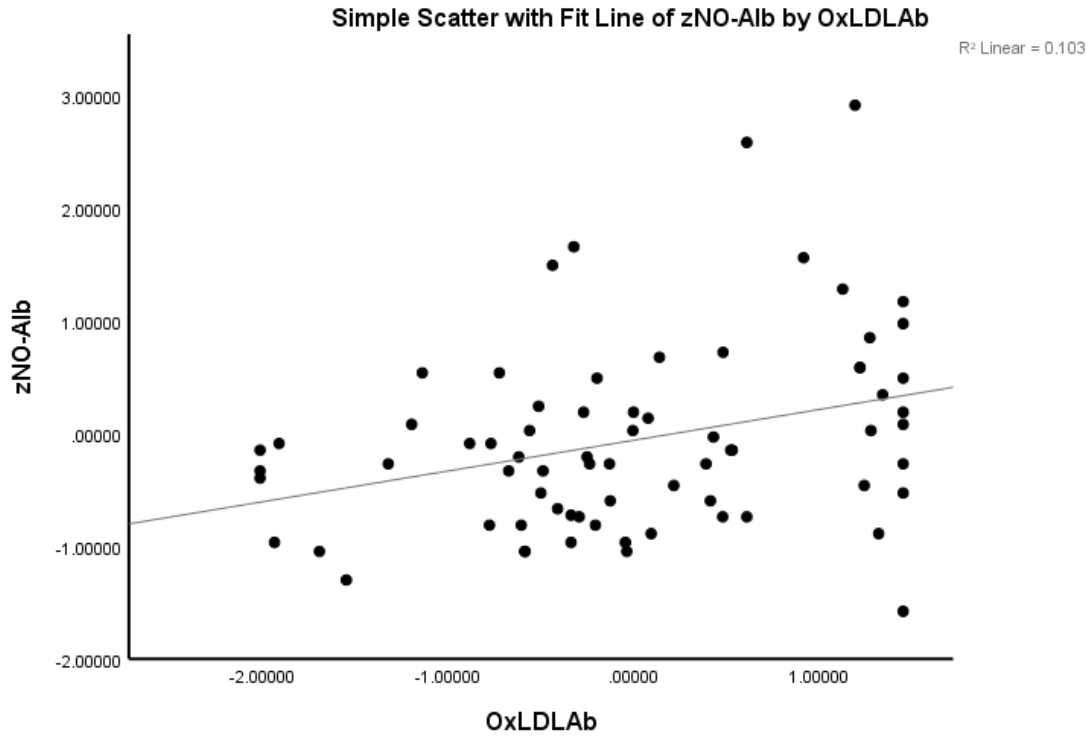

Figure 3. Positive correlation between IgM directed to NO-albumin (zNO-Alb) and IgG to oxidized low density lipoprotein (oxLDLAb)

Table 2 shows the results of a multivariate GLM analysis with the IgM responses to NO-adducts as dependent variables and diagnosis (4 groups, namely HC, BP1, BP2 and MDD) as primary explanatory variable and age and sex as covariates). We found a highly significant effect of diagnosis with an impact size of 0.107, while also sex yielded a significant effect with an effect size of 0.108. Tests for between-subjects effects showed that there was a significant association between diagnosis and IgM NO-arginine,

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NO-albumin, NOW and IgM NOadducts. The highest effect size was observed for NO-albumin, namely 0.153 followed by NOW (0.102). **Table 3** shows the model-generated estimated marginal mean (SE) values (expressed as z values) obtained by this analysis. IgM NO-arginine was significantly higher in BP1 and MDD than in controls. IgM NO-albumin was significantly higher in BP1 patients than in controls and BP2 patients, while IgM NO-albumin was higher in MDD than in controls. IgM NOW and IgM NOadducts were significantly higher in BP1 and MDD than in controls. There were no significant differences between BP2 and controls and between BP1 and MDD for any of the IgM NO-adducts.

Table 3. Model-generated estimated marginal mean (SE) values (expressed in z values) obtained by the general linear model analyses shown in table 2

Variables	**Healthy controls** [A]	**BP2** [B]	**BP1** [C]	**MDD** [D]
IgM NO-cysteine	-0.371 (0.221) [D]	-0.207 (0.212)	0.144 (0.201)	0.219 (0.157) [A]
IgM NO-arginine	-0.560 (0.208) [C,D]	-0.021 (0.200)	0.137 (0.189) [A]	0.162 (0.148) [A]
IgM NO-albumin	-0.631 (0.200) [C,D]	-0.194 (0.192) [C]	0.516 (0.181) [A,B]	0.149 (0.142) [A]
IgM NOW	-0.602 (0.204) [C,D]	-0.142 (0.196)	0.139 (0.185) [A]	0.256 (0.145) [A]
zNOadducts	-0.573 (0.208) [C,D]	-0.058 (0.200)	0.309 (0.189) [A]	0.112 (0.148) [A]
Peroxides	-0.533 (0.240) [C,D]	-0.011 (0.220)	0.200 (0.270) [A]	0.106 (0.164) [A]
IgG oxLDL	-0.900 (0.281) [C,D]	-0.306 (0.223) [D]	0.210 (0.248) [A]	0.458 (0.177) [A,B]
IgA/IgM Gram- bacteria	-0.686 (0.205) [C,D]	-0.221 (0.193) [C]	0.523 (0.182) [A,B]	0.166 (0.145) [A]

[A,B,C,D]: results of pair-wise comparisons between group mean values

NOW: NO-tryptophan; zNO-adducts: z(sum of all z scores of the 4 IgM NO-adducts)

IgM/IgA Gram- bacteria: z(sum of all z scores of IgM and IgA levels directed to 6 different Gram- bacteria).
IgG oxLDL: IgG directed against oxidized LDL

Table 2. Results of (multivariate or univariate) general linear model (GLM) analysis with the IgM directed against nitrosylated adducts and peroxides, IgG to oxidized LDL and an index of bacterial translocation as dependent variables and diagnostic groups, namely controls (HC), bipolar 1 (BPI), bipolar 2 (BPII) and major depression (MDD) as primary explanatory variables, while adjusting for age and sex.

Type Test	**Dependent variables**	**Explanatory variables**	**F**	**df**	**P**	**Partial Eta Squared**
Multivariate ≠1	IgM to NO-cysteine, NO-arginine, NO-albumin, NOW and zNOadducts	HC, BPI, BPII, MDD Age Sex	2.59 0.48 2.57	15/324 5/106 5/106	0.001 0.787 0.031	0.107 0.022 0.108
Between-subject effects	IgM NO-cysteine IgM NO-arginine IgM NO-albumin IgM NOW zNOadducts	HC, BPI, BPII, MDD HC, BPI, BPII, MDD HC, BPI, BPII, MDD HC, BPI, BPII, MDD HC, BPI, BPII, MDD	1.98 2.94 6.63 4.17 3.62	3/110 3/110 3/110 3/110 3/110	0.121 0.036 <0.001 0.008 0.016	0.051 0.074 0.153 0.102 0.090
Between-subject effects	NO-arginine NOW	Sex Sex	3.98 4.42	1/110 1/110	0.048 0.038	0.035 0.039
Univariate #1	Peroxides	HC, BPI, BPII, MDD Age Sex	1.87 0.00 24.24	3/62 1/62 1/62	0.143 0.988 <0.001	0.083 0.000 0.281
Univariate #2	IgG oxLDL	HC, BPI, BPII, MDD Age Sex	6.13 1.32 0.38	3/65 1/65 1/65	0.001 0.254 0.542	0.220 0.020 0.006

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Univariate#3	IgA/IgM Gram-bacteria	HC, BPI, BPII, MDD Age Sex	7.22 0.01 0.78	3/112 1/112 1/112	<0.001 0.945 0.379	0.162 0.000 0.007

NOW: NO-tryptophan; zNOadducts: z(sum of all z scores of the 4 IgM NO-adducts)

IgM/IgA Gram- bacteria: z(sum of all z scores of IgM and IgA values to 6 different Gram- bacteria).

Table 2 shows also the results of univariate GLM analyses with peroxides, IgG oxLDL and IgA/IgM Gram- bacteria as dependent variables and diagnosis as explanatory variable, while adjusting for age and sex. There was no significant effect of diagnosis on peroxide levels, while there was a very strong effect of sex with an effect size of 0.281. There were strong associations between diagnosis and either IgG oxLDL or IgA/IgM Gram- bacteria with effect sizes of 0.220 and 0.162, respectively. Table 3 shows that IgG oxLDL were significantly higher in BP1 than in controls and higher in MDD than in controls and BP2. IgA/IgM Gram-bacteria was higher in MDD than in controls and higher in BP1 than in controls and BP2.

Effects of extraneous variables

We have also examined the effect of extraneous variables. Firstly, as shown in Table 2 there were significant effects of sex on IgM NO-arginine and NOW with higher levels in females than in males. **Figure 4** shows the oxidative and nitrosative profiles in females versus males with significantly higher mean z scores (with SE) of NO-arginine and NOW and peroxides in females than in males. No effects of age on any of the biomarkers could be observed. There were no significant effects of TUD on any of the NO-

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adducts, namely IgM NO-cysteine (F=0.01, df=1/105, p=0.905), NO-arginine (F=3.80, df=1/105, p=0.054), NO-albumin (F=0.02, df=1/105, p=0.877), NOW (F=0.95, df=1/105, p=0.331) and NO-adducts (F=0.79, df=1/105, p=0.376). Multivariate GLM analysis showed that there were no significant effects of use of mood stabilizers (F=0.23, df=5/103, p=0.950; 28 used mood stabilizers while 67 did not take mood stabilizers) and antidepressants (F=1.73, df=5/103, p=0.134; 40 subjects used antidepressants while 55 were free of antidepressants). Multivariate GLM analysis did not show a significant effects of BMI on the 5 IgM NO-adduct values (F=1.93, df=5/54, p=0.104).

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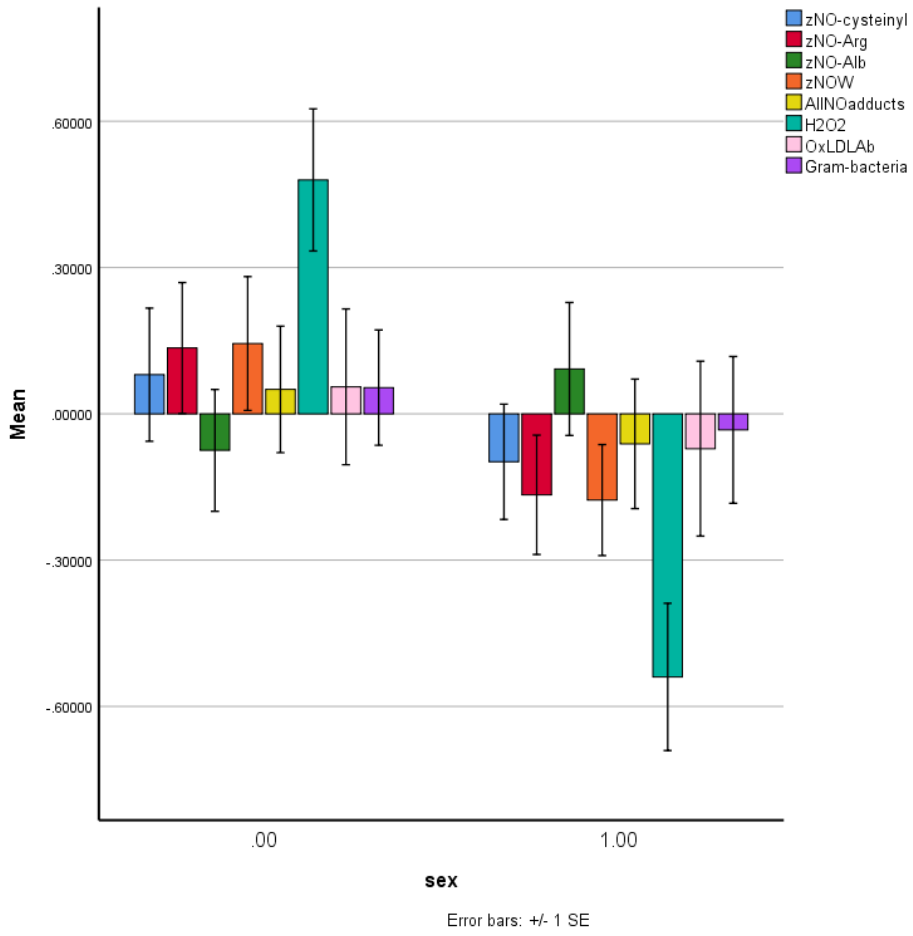

Figure 4. Oxidative and nitrosative profile of females and males
Shown are mean z scores (with SE) in both genders.
zNO-Arg: zNO-arginine; zNO-Alb: zNO-albumin; zNOW: zNO-tryptophan; AllNOadducts: z sum all IgM responses to NO-adducts; H2O2: peroxides; oxLDLAb: IgG to oxidized low densitity lipoprotein.

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Best prediction of the diagnostic classes.

In order to delineate the best predictors of the diagnostic classes we entered the 5 IgM NO-adduct values in a multinomial logistic regression analysis with the 4 diagnostic classes as dependent variables. **Table 4** shows that IgM NO-albumin was the single best predictor of the diagnostic classes with an effect size of 0.216. Increased IgM NO-albumin was significantly associated with BP1 versus controls (Odds ratio=10.02) and MDD versus controls (Odds ratio=8.37). Furthermore, higher IgM NO-albumin was associated with BP1 versus BP2 (Odds ratio=2.67) and MDD versus BP2 (Odds ratio=2.67).

Table 4. Results of multinomial logistic regression analysis with diagnosis as dependent variable and IgM to nitroso-adducts as explanatory variables. Diagnostic groups are: healthy controls (HC) and patients with major depression (MDD) and bipolar type 1 (BP1) and type 2 (BP2).

Dependent Variables	Nagelkerke (model) X^2, df, p (independent)	Explanatory variables	Wald	df	P	OR	95% CI intervals

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BP2/HC	0.216	IgM NO-albumin	2.99	1	0.084	3.13	0.86 – 11.41
BP1/HC	X^2=26.58, df=3,		13.10	1	<0.001	10.02	2.88 – 34.93
MDD/HC	p<0.001		11.54	1	<0.001	8.37	2.46 – 28.53
BP1/BP2			6.80	1	0.009	3.20	1.34 – 7.67
MDD/BP2			5.18	1	0.023	2.67	1.15 – 6.24
MDD/BP1			0.70	1	0.402	0.84	0.55 – 1.27

OR: Odds ratio, 95%CI: 95% confidence intervals with upper and lower limits

Discussion

The first major finding of this study is that IgM responses to NO-adducts were significantly higher in patients with MDD and BP1 than in controls. Previously, we have published that those IgM responses are significantly increased in acute and chronic MDD as compared with controls,[13-16] while the current study is the first to show that IgM antibody levels to NO-adducts are significantly associated with BP1. Furthermore, IgM antibodies directed to albumin showed the best prediction of the diagnostic classes followed by NOW, indicating that the nitrosylated protein (BSA) showed a higher circulating antibody response than the nitrosylated conjugates NO-cysteine-BSA, NO-arginine-BSA and NOW-BSA. This contrasts for example humoral responses in trypanosome-infected mice in which the nitrosylated conjugates illicit a higher humoral IgM response as compared with nitrosylated BSA as

antigen[29] In plasma, nitrosylated albumin is a major reservoir of NO,[30] whereas tryptophan residues in proteins show resistance to nitrosylation with protein-associated mechanisms preventing NOW accumulation.[31] Therefore, it is interesting to note that both IgM antibodies to albumin and NOW are highly significantly associated with MDD and BP1, indicating accumulation of NO, NO-adducts and even NOW in these mood disorders. As described in the Introduction, increased IgM antibodies to nitroso epitopes suggest increased RNS synthesis with subsequent binding of nitroso molecules to proteins thereby generating immunogenic neo-nitrosylated epitopes.[29] Recently, we reported that in pregnant women these kind of IgM responses to nitroso epitopes are strongly associated with serum NOx levels, indicating increased production of NO and derivatives, especially H2NO3.[9,10,17]

In the present study we could not detect any differences in anti-NO epitope antibodies between BP2 patients and controls, whilst IgM antibodies to albumin were significantly higher in BP1 and MDD patients than in BP2 patients. These finding further support our data that in (partially) remitted patients there are highly significant differences in nitro-oxidative stress biomarkers between MDD/BP1 versus BP2 (see Introduction). Thus, both MDD and BP1 are accompanied by increased levels of nitrosative stress (this study) and nitro-oxidative stress[6] as indicated by higher levels of NO metabolites, SOD1 activity, lipid peroxidation, aldehyde formation and protein oxidation. Other studies were unable to find differences in aldehyde formation between BP1 and BP2 patients in an acute depressive state.[7] It is possible that these differences between studies may be explained by nitrosylation being a trait biomarker and aldehyde formation being a state and trait biomarker. Thus, aldehyde formation is significantly associated with severity of illness and staging of illness as indicated by recurrent depressive and manic episodes and suicidal behaviors,[7] while

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increased nitrosylation is not associated with severity of illness and staging (this study) but is associated with a lifetime history of depression.[17]

Nitrosylating agents, administered intravenously, may react with -SH groups on albumin and simple thiols including glutathione thereby forming S-nitrosoglutathione.[32] Moreover, the reaction between the latter and albumin may result in a rapid depletion of –SH groups in albumin. It should be underscored that MDD and BP disorder are characterized by reduced –SH groups and glutathione levels in serum or brain.[33-34] By inference, MDD and BP1, but not BP2, appear to be associated with increased production of NO metabolites which may induce increased nitrosylation, which in turn may damage both the glutathione system and SH-groups thereby attenuating key components of the antioxidant defense. These findings further extent our recent report that the (partially) remitted phase of MDD and BP1, but not BP2, is accompanied by increased signs of nitro-oxidative stress and breakdown of antioxidant defenses.[6]

Moderately increased levels of nitrosylation may be protective by forming immune complexes thereby removing NO excess[29] and preventing irreversible cysteine oxidation, which may cause changes in the secondary and tertiary structure of proteins.[9,10] In contrast, further increases in S-nitrosylation may not only deplete -SH and glutathione antioxidant defences (see above), but also induce pro-cell death effects (in part through caspase activation).[35] Increased activities of inducible NO synthase (iNOS), another characteristic of MDD,[36] coupled with elevated NO metabolites and S-nitrosylation are associated with neuronal loss and microglial activation.[35] In addition, S-nitrosylation suppresses presynaptic metabolism with long-lasting attenuation of synaptic GABA and

glutamate transmission, and loss of neuronal communication, synaptic functions and neuronal projections.[37] Moreover, anti-nitrosylated epitope antibodies may have intrinsic toxic effects especially in conditions characterized by increased permeability of the blood-brain barrier because anti-nitrosylated epitope antibodies could bind to nitrosylated neoepitopes present in the brain.[39] For example, the IgM antibodies directed to NO-cysteine may be involved in the immunopathology of multiple sclerosis by causing demyelination.[39] Nevertheless, in our current study sample there were only marginal differences in IgM directed against NO-cysteine in MDD patients versus controls. These findings are in agreement with our previous papers that IgM NO-cysteine is increased in MDD and in (pregnant) women with a lifetime history of MDD.[22] It should be added that circulating antibodies to nitroso epitopes are significantly increased in a number of other neuro-inflammatory and neurodegenerative disorders including relapsing remitting multiple sclerosis and amyotrophic lateral sclerosis and in immune-inflammatory disorders such as rheumatoid polyarthritis.[39,40] All in all, it is probable that increased nitrosylation may play a key role in the impaired antioxidant defenses, neuro-glial interactions, neuroprotection and neurogenesis as well as activation of immune-inflammatory and oxidative pathways which are frequently observed in both MDD and BP1 disorder.[3-5,9,10]

The second major finding of this study is that increased nitrosylation is significantly associated with increased bacterial translocation (as indicated by IgA/IgM responses to 6 sonicated Gram-negative bacteria) and signs of lipid oxidation, namely increased IgG responses to oxLDL and peroxides. The highly significant association with increased bacterial load in BP1 and MDD (but not BP2) suggests that bacterial translocation (or other pathogens or parasites antigens accompanying Gram-negative bacteria

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antigens through breakdown of the gut paracellular tight and adherens junctions) is causally related to increased nitrosylation. Firstly,

increased LPS and other antigens of Gram- bacteria may activate the Toll-Like Receptor (TLR)2/4 – radical cycle thereby stimulating iNOS and NO production as well as chronic inflammation thereby increasing NO production and nitrosylation.[41] Secondly, LPS stimulation of macrophages increases NO synthesis, which in turn is accompanied by increased nitrosylation of many proteins collectively called the nitrosylome, i.e. the proteome of nitrosylated proteins.[42] Thirdly, infected mice with for example *Trypanosome brucei brucei* show increased macrophage NO production and nitrosylated compounds and circulating IgM antibodies directed to NO modified epitopes.[29]

The significant associations between IgM to NO-adducts and signs of ROS and oxidative damage to lipids indicate that oxidative and nitrosative stress in mood disorders are intimately related phenomena.[3,5] The association with IgG to oxLDL is of particular interest because the latter is directly associated with the development of coronary artery disease (CAD) and arteriosclerosis, which shows a strong comorbidity with mood disorders.[43] It is well known that LDL, oxLDL and IgG (and also IgM) responses to oxLDL play a key role in the development of CAD.[43] This is important as S-nitrosylation mediates most effects of NO on the endothelium including its vascular and cardiac protective activities.[44] Nevertheless, depending on the context of endothelial and cardiac cells (including redox state and duration of nitrosylation), prolonged nitrosylation shows many detrimental effects contributing to degenerative processes,[45] while binding of anti-nitrosylated epitope antibodies to nitrosylated epitopes in the endothelium could interfere with cardiac functions.

Another finding of this study is that women show increased levels of peroxides and IgM to NOW and NO-arginine indicating that ROS/RNS and nitrosative stress may in part contribute to sex differences in depression and BP1 for example to the increased incidence of depression in females.[8] Such effects could be associated with increased early life trauma and other psychosocial triggers that are associated with nitro-oxidative processes and with staging of depression and mood disorders.

In conclusion, BP1 and MDD are accompanied by upregulation of the nitrosylome and increased IgM antibody responses to nitrosylated conjugates and these biomarkers may aid to differentiate MDD/BP1 from BP2. It is probably that such disorders indicate a response of innate like B cells, namely marginal zone B and B1 cells, producing increased natural IgM, directed to self antigens (including oxidative-specific epitopes), which in turn have strong anti-inflammatory and immune-regulatory effects and constitute a first-line defense of innate immunity against pathogens including Gram- negative bacteria.[22,46] Moreover, increased bacterial translocation may drive nitrosylation and thus the production of IgM responses to NO neoepitopes. Induction of the nitrosylome may play a key role in the impairments in neuro-glial interactions, synaptic plasticity, neuroprotection, neurogenesis and antioxidant defenses, the activation of immune-inflammatory and oxidative pathways in mood disorders as well as in comorbid CAD.

Conflict of interest

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

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Author's contributions

All the contributing authors have participated in the manuscript. MM designed the study, recruited patients, completed diagnostic interviews and rating scale measurements and carried out the statistical analyses. J-C Leunis performed the assays of IgM to NO-adducts. All authors contributed to interpretation of the data and writing of the manuscript. All authors approved the final version of the manuscript.

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