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Nitric Oxide Synthase activity in major depressive episodes before and after antidepressant treatment: Results of a large case-control treatment study

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Abstract

Background. Nitric oxide synthase (NOS) activity, an enzyme potentially involved in the major depressive episodes (MDE), could be indirectly measured by the L-Citrulline/L-Arginine ratio (L-Cit/L-Arg). The aim of this study was: (1) to compare the NOS activity of patients with a MDE to that of healthy controls (HC); (2) to assess its change after antidepressant treatment.

Methods. A total of 460 patients with a current MDE in a context of major depressive disorder (MDD) were compared to 895 HC for NOS activity (L-Cit/L-Arg plasma ratio). L-Arg and L-Cit plasma levels were measured using a MS-based liquid chromatography method. Depressed patients were assessed at baseline, and after 3 and 6 months of antidepressant treatment for depression severity and clinical response.

Results. Depressed patients had a lower NOS activity than HC at baseline [0.31 ± 0.09 *v.* 0.38 ± 0.12; 95% confidence interval (CI) −0.084 to −0.062, $p < 0.0001$]. Lower NOS activity at baseline predicted a higher response rate [odds ratio (OR) = 29.20; 95% CI 1.58–536.37; $p = 0.023$]. NOS activity in depressed patients increased significantly up to 0.34 ± 0.08 after antidepressant treatment (Est = 0.0034; 95% CI 0.0002–0.0067; $p = 0.03$).

Conclusions. Depressed patients have a decreased NOS activity that improves after antidepressant treatment and predicts drug response. NOS activity may be a promising biomarker for MDE in a context of MDD.

Introduction

Major depressive disorder (MDD) is the most widespread mental disorder (Wang, Simon, & Kessler, 2003) that needs to be better understood. Recently, the role of the nitric oxide (NO) and the nitric oxide synthase (NOS) enzymes has been highlighted as one of the pathophysiological pathways involved in MDD (Dhir & Kulkarni, 2011; Kudlow, Cha, Carvalho, & McIntyre, 2016). In their review, Wiesinger (2001) described the metabolism of nitric oxide. They reported that L-Arginine (L-Arg) is a 'semi-essential' amino acid and L-Citrulline (L-Cit) as a non-essential amino acid. Both L-Arg and L-Cit belong to the same metabolism cycle, i.e. the urea cycle. Furthermore, NOS enzymes which are dimeric flavoproteins, convert L-Arg into NO and L-Cit. NO is a small and freely diffusible molecule with a 3–6 s half-life (Dhir & Kulkarni, 2011). It was initially identified for its role in the relaxation of blood vessels (Furchgott & Zawadzki J, 1980). The total production of NO depends on the activity of these NOS enzymes (Förstermann et al., 1991; Schmidt & Murad, 1991; Stuehr et al., 1989). There are three isoforms of NOS: neuronal NOS (NOS1 or nNOS), inducible NOS (NOS2 or iNOS), and endothelial NOS (NOS3 or eNOS) (Knowles & Moncada, 1994). Plasma nitric oxide metabolites (NOx) are a valid index of NOS activity (Kleinbongard et al., 2003; Rhodes, Leone, Francis, Struthers, & Moncada, 1995), which could be indirectly represented by the ratio L-Citrulline/L-Arginine (L-Cit/LArg) (Benedetto et al., 2000; Sethuraman, Lee, Chui, & Tachibana, 2006). The plasma concentration of NOx has been used to assess NO production by eNOS (Archer, 1993). However, some authors have suggested that peripheral circulating

NOx levels could also reflect NO production by iNOS and nNOS (Murray, Bullimore, & Long, 2003; Suzuki & Colasanti, 2001). Besides its action on the vascular system, NO is also implicated in some central nervous system (CNS) functions (Yun, Dawson, & Dawson, 1997) including behavior modulation (Calabrese et al., 2007).

Animal studies suggested a role for NO in MDD (Dhir & Kulkarni, 2011). However, clinical studies provided conflicting results about the NOS activity in patients with major depressive episodes (MDE) or depressive symptoms: Suzuki and Colasanti (2001) found increased plasma NOx concentrations in 17 MDE patients treated with antidepressant drugs (imipramine, amitriptyline, or mianserine), compared to healthy controls (HC). In addition, Kim et al. (2006) reported increased plasma NOx levels in 39 depressed patients with suicide attempts. Lu et al. (2014) showed that 27 MDE/MDD patients with melancholic features had an increased plasma NOS activity. Moreover, it should be noted that Mayoral-Mariles et al. (2012) reported opposite results between NOx and NOS activity in 21 elderly women with mild depression. Thus, they showed a significantly lower plasma NOS activity ratio and a higher plasma nitrite concentration. García et al. (2011) found significantly lower plasma nitrite/nitrate concentrations in depressed individuals ($n = 50$) compared with HC ($n = 50$). An additional large study in the general population ($n = 14\,276$) evidenced such an association between NOS activity and depressive symptoms (Cepeda, Stang, & Makadia, 2016). Besides, several studies with post-mortem examinations of patients with MDE showed lower levels of both total amount and density of nNOS immunoreactive neurons in paraventricular and suprachiasmatic nuclei (Bernstein et al., 2002), prefrontal cortex (Xing, Chavko, Zhang, Yang, & Post, 2002) and locus coeruleus (Karolewicz et al., 2004). Their authors suggested a generalized reduction in NO production in the CNS of patients with MDE. Furthermore, previous clinical studies have shown that, along with a decrease of depressive symptoms, the administration of selective serotonin reuptake inhibitors (SSRI) increased or decreased plasma NOx, suggesting a role of NO in the therapeutic effect of these antidepressants (Finkel, Laghrissi-Thode, Pollock, & Rong, 1996; Lara, Archer, Baker, & Le Mell??do, 2003; Ikenouchi-Sugita et al., 2009). Moreover, the non-selective NOS inhibitor, l-N^G-nitroarginine methyl ester (l-NAME), and the selective neuronal NOS inhibitor, 7-nitroindazole, induced dose-dependent antidepressant-like effects in the rodent forced swimming test (Ferreira et al., 2012; Spiacci, Kanamaru, Guimarães, & Oliveira, 2008; Yildiz, Erden, Ulak, Utkan, & Gacar, 2000). In addition, several animal and preclinical studies have shown antidepressant and/or anxiolytic effects after administration of related amino-acids-based NOS inhibitors, L-NAME, L-NG-Nitroarginine (L-NNA), L-NG-Methyl-L-arginine (L-NMMA), and agmatine (Wegener & Volke, 2010).

According to these findings, the role of NOS and its evolution in patients treated for a MDE need to be clarified by a more in-depth study, using metabolomics, a systematic study of endogenous and exogenous small chemical molecules (substrates, metabolites, products of metabolism) in body fluids (blood, urine, etc) that represent the fingerprint of specific metabolic pathways. It could be helpful to identify molecular biomarkers for diagnosing and assessing the therapeutic effects of antidepressant drugs. Indeed, metabolomic research, which is based on gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) coupled with multivariate statistical methods (Li et al., 2014), offers a promising approach to simultaneously monitor the plasma levels of the NO pathway's metabolites and thus the NOS activity.

The main aim of this study was to assess the NOS activity in a large sample of depressed patients with a current MDE in a context of MDD, as compared to HC, in order to identify candidate biomarkers of MDE and MDD. The secondary objective was to provide a follow-up of NOS activity after antidepressant treatment, especially regarding response to treatment.

Materials and methods

Participants

Patients with major depression

In the 6-month prospective, multicentric, real-world treatment observational METADAP cohort study (Corruble et al., 2015) of patients with a current MDE in a context of MDD, we performed a metabolomic analysis among 460 subjects with available data. Data were collected from November 2009 to March 2013 in four university psychiatry departments in France. Experimented psychiatrists made the diagnosis of MDE, based on the MINI section for MDE and MDD.

Consecutive in- or out-patients, aged 18–65 years, with a current MDE in a context of MDD (with a minimum score of 18 at the Hamilton Depression Rating Scale-17, HDRS-17) were assessed for depression severity using the HDRS-17, the Quick-Inventory for Depressive Symptomatology – Clinician (QIDS-C) and the Quick-Inventory for Depressive Symptomatology – Self Report (QIDS-SR) and for biochemical variables, at the introduction of the index antidepressant treatment (M0), and after 3 (M3) and 6 (M6) months of antidepressant treatment. Participants had to be fasting and abstained from strenuous physical activity for 8 h before examination. Fasting plasma levels of triglycerides, HDL cholesterol, and glucose were assessed using routine standardized laboratory methods. The smoking status (smoker or non-smoker) was checked at each assessment time of the study (M0, M3, M6). All patients provided written informed consent. Two subgroups of patients were focused on: (1) 'drug-free' i.e. patients who did not receive any antidepressant treatment since at least 1 year before inclusion; and (2) 'completers', i.e. patients who completed the study until the sixth month of antidepressant treatment. The responder status was defined based on a greater than 50% improvement in the HDRS, QIDS-C, and QIDS-SR scores and the remitter status was defined by a HDRS, QIDS-C, and QIDS-SR total score below 7.

Moreover, patients with, bipolar disorders, psychotic symptoms, psychotic disorders, eating disorders, current substance abuse or dependence, pregnancy, organic brain syndromes, severe unstable medical conditions, and patients receiving antipsychotics or mood stabilizers before inclusion and/or for 4 months or more during the last year could not be included. Benzodiazepines at the minimum effective dose and for the minimum time period and psychotherapies were allowed. The index antidepressant treatment had to be a monotherapy. The drug and its dose were left to the treating psychiatrist, using 'real world' treatment options. This study was registered by the French National Agency for Medicine and Health Products Safety (ANSM) and the Commission Nationale de l'Informatique et des Libertés (CNIL), was approved by the Ethics Committee of Paris-Boulogne, France, and conformed to international ethical standards. The METADAP study was funded by a national grant (PHRC, AOM06022) and sponsored by Assistance Publique-Hôpitaux de Paris (APHP) (ClinicalTrials.gov Identifier: NCT00526383).

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Healthy controls

HC were those of the VARIETE study, a population-based cross-sectional study that was conducted to establish reference values for Insulin-like Growth Factor 1 (IGF-1) in the general population(Chanson et al., 2016). Subjects were recruited in 10 French university hospitals and enrolled between January 2011 and February 2012. To be included in the study, adult subjects (aged between 18 and 89 years) had to be considered healthy based on medical history, had no previous or ongoing mental illness, and clinical examination including the nutritional status evaluation and of gonadal/sexual status, normal routine biology performed after an overnight fast. The study included 895 subjects. All participants gave written informed consent before entering the study. This study was approved by the French National Agency for Medicine and Health Products Safety (ANSM) and the Ethics Committee (Ile de France VII). The VARIETE study was funded by a national grant (PHRC, AOM09122) and sponsored by Assistance Publique-Hôpitaux de Paris (APHP) (NCT01831648).

NOS activity

Metabolomics is an emerging "-omics" approach which is particularly relevant to identify pathophysiologically affected processes and elucidate novel physiological and pathological mechanisms (Jia et al., 2013). In this study, metabolomic analyses were performed to assess the main variables included in the NO pathway with the same procedures in depressed patients and HC.

EDTA blood samples were obtained from each subject in fasting standardized conditions for both depressed patients and HC. Blood samples were obtained between 8:00 and 10:00 a.m. after an overnight fast, before any drug intake as previously detailed (Trabado et al., 2017). For depressed patients, EDTA samples were obtained at each time of the study (M0, M3, M6). A measure of 30 mL of EDTA blood was obtained from each subject and immediately centrifuged (10 min, 3000 RPM at 4 °C) after blood sampling and plasma was aliquoted into separate polypropylen tubes that were immediately stored at −80 °C. And a frozen 1 mL aliquot from every subject was obtained and stored at −80 °C until analysis. The received aliquot was further aliquoted according to the required volume for each analytical method. One aliquot of 10 μL was analyzed with Biocrates AbsoluteIDQ p180 Kit (Biocrates Life Science AG, Austria) that quantified 185 plasma metabolites. Among the metabolites of the NOS pathway, only plasma L-Arg and L-Cit levels were used to quantify NOS activity (L-Arg/L-CIt). The plasma samples were processed according to the manufacturer procedure and analyzed on aAPI 4000 Q-TRAP mass spectrometer (AB Sciex) coupled to an ACQUITY UPLC I Class system (Waters) equipped with an Agilent C18 HPLC column.

Identification and quantification were performed with internal standards and multiple reaction monitoring (MRM) detection. After pre-processing with Multiquant software (AB Sciex) (peak integration and concentration determination from calibration curves), LC data were uploaded into Biocrates Met*IDQ* software (included in the kit). Concentrations of metabolites monitored by FIA were directly calculated in Met*IDQ*.

Statistical analyses

Descriptive statistics were produced based on demographic, treatment-related, biochemical and clinical assessments data and are presented as mean (m), standard deviation (s.d.), median, min, max, and Interquartile-Range (IQR). Since the two cohorts, METADAP (depressed patients) and VARIETE (healthy individuals) were independent, it seemed to us more appropriate and rigorous to show the extensive data, rather than propose an a-posteriori matching, that would have been unperfect regarding the number of relevant factors required in the matching process. Outliers were studied using the Grubbs test and the percentile method.

Bivariate analyses were performed according to the continuous or categorical nature of each variable and according to the normality of their distribution. Thus, we used Chi-square tests to evaluate group differences in categorical variables (Gender, Smoker) and *t* tests (with or without the Welch approximation) to evaluate group differences in continuous variables (Age, BMI, L-Arg, L-Cit, NOS activity).

At baseline, multiple linear regressions were used to assess differences between patients with MDE and HC on NOS activity while controlling for several potential confounders: the models were adjusted on variables significantly different between the two groups in bivariate analyses ($p < 0.05$) or with a known effect on the NO pathway, e.g. glycaemia, total cholesterol dietary, age and smoking (Di Massimo et al., 2006; Feron, Dessy, Moniotte, Desager, & Balligand, 1999; Ishibashi, Yoshida, & Nishio, 1999; Kelm, 1999; Selley, 2004).

Thus, simple and multiple logistic regressions were performed to assess potential associations between the responder status and the NOS activity (using the hundredth of a unit).

Moreover, mixed-effects multivariate models were used in depressed patients to assess the NOS activity change during the study period and if this variation was correlated with the change in clinical symptoms. Mixed-effects multivariate models were used because they are a well-accepted method for analyzing longitudinal clinical data in which missing or mistimed observations are present (Fitzmaurice, Laird, & Shneyer, 2001). The previous analyses were conducted in the whole sample of depressed patients and in both subgroups of 'drug-free' and 'completers' patients.

For the entire statistics, the significant set was fixed such as $p < 0.05$. All statistical analyses were performed using the R Project for Statistical Computing [R version 3.2.3 (2015–12–10)].

Results

Socio-demographic characteristics at baseline

In the 460 patients of the METADAP cohort, the mean age was 46.0 ± 13.0 years; 68.4% were women; 87.5% were inpatients at baseline; 36.9% were smokers. Body mass index (BMI) was 24.13 ± 4.95 kg/m^2; total cholesterol was 5.10 ± 1.10 mmol/L and glycaemia was 4.94 ± 1.12 mmol/L. Mean HDRS-17 score at baseline was 24.7 ± 5.0. The mean number of previous MDE was 2.3 ± 1.0. The average lifetime duration of MDD before inclusion was 11.5 ± 12.2 years. The mean lifetime duration of antidepressant treatment before inclusion was 2.3 ± 4.1 years. Upon inclusion, 22.7% of patients were antidepressant naïve and belonged to the drugfree subgroup. The prescribed antidepressant at baseline was a selective serotonin reuptake inhibitor (SSRI) in 38.9% of patients, a serotonin-norepinephrine reuptake inhibitor (SNRI) in 38.3%, and another one in 22.8% [tricyclic antidepressants, monoamine oxidase inhibitors and alpha-2 antagonists (mianserine and mirtazapine)]. The mean duration of follow-up was 4.9 ±

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Table 1. Demographics, clinical, and biological characteristics

	Healthy controls ($n = 895$)	MDD patients ($n = 460$)	p
Gender (male)	457 (51%)	145 (31.6%)	<0.0001
Age (years)	39.82 ± 18.61	46.0 ± 13.0	<0.0001
Smoker (no)	799 (90.1%)	290 (63.1%)	<0.0001
BMI	23.07 ± 2.43	24.13 ± 4.95	<0.0001
Total cholesterol (mmol/L)	4.95 ± 1.11	5.10 ± 1.10	<0.0001
Glycaemia (mmol/L)	4.68 ± 0.60	4.94 ± 1.12	<0.0001

Table 2. Mean (standard deviation) of NOS activity ratio in healthy controls and MDE patients

	Healthy controls ($n = 895$)	MDE patients ($n = 460$)	p
L-Arginine (μmol/L) m (s.d.)	81.80 ± 19.47	90.91 ± 22.85	<0.0001
L-Citrulline (μmol/L) m (s.d.)	30.11 ± 8.08	27.25 ± 8.02	<0.0001
NOS activity m (s.d.)	0.38 ± 0.12	0.31 ± 0.09	<0.0001

4.6 months. The drop-out rate was 25.9% before M1, 21.8% between M1 and M3, and 14.3% later. The main reasons for drop-outs were antidepressant change (28.4%), prescription of antipsychotics or mood stabilizers (29.4%) and lost to follow-up (20.4%).

In the 895 HC, the mean age was 39.8 ± 18.6 years; 438 HC were women (49%); 9.9% were smokers. Their metabolic variables were BMI (kg/m^2) = 23.07 ± 2.43; total cholesterol = 4.95 ± 1.11 mmol/L and glycaemia = 4.68 ± 0.60 mmol/L. They differed significantly from those of depressed patients in bivariate analyses (Table 1).

Comparison of NOS activity at baseline between depressed patients and HC

NOS activity (L-Cit/L-Arg) had a normal distribution even taking account the outliers.

In bivariate analyses, at baseline, NOS activity (L-Cit/L-Arg) was significantly lower in the whole sample of depressed patients (0.31 ± 0.09) [and in each subgroup: i.e. in the subgroup of completers (0.30 ± 0.08) [95% confidence interval (CI) −0.0940 to −0.0653, $p < 0.0001$] and in the subgroup of drug-free patients (0.31 ± 0.1) (95% CI −0.0877 to −0.0536, $p < 0.0001$)] than in HC (0.38 ± 0.12) (95% CI −0.0845 to −0.0617, $p < 0.0001$) (Table 2; Figure 1).

No significant difference in NOS activity (L-Cit/L-Arg) was found at baseline neither between antidepressant free and non-antidepressant-free patients ($p = 0.83$), nor between patients treated with benzodiazepines (0.3 ± 0.09) or not (0.31 ± 0.10) ($p = 0.41$), nor between completers and non-completers ($p = 0.22$).

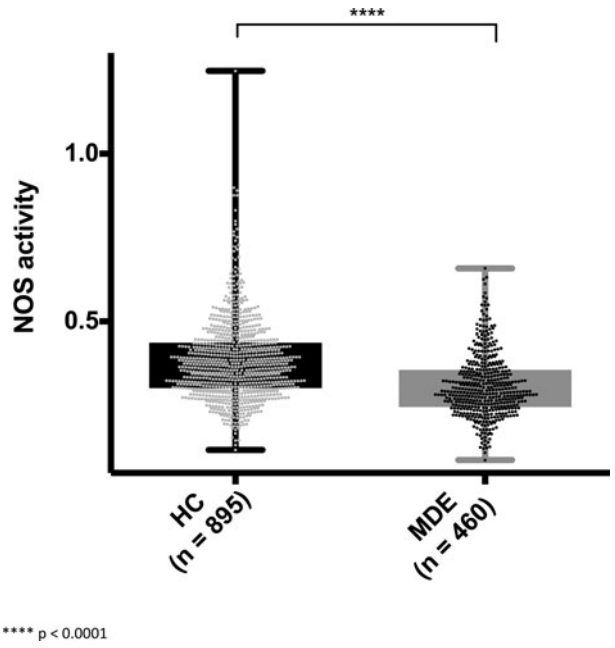

Fig. 1. Box plots (jitter) of NOS activity in patients with MDE and healthy controls (HC) at baseline. **** $p < 0.0001$.

In multivariate analyses adjusted for age, gender, BMI, smoking status, total cholesterol and glycaemia, these results remained significant (Table 3).

No association was found between NOS activity and the HDRS total or item scores at baseline.

NOS activity at baseline as a predictor of response after 3 months of antidepressant treatment

Using logistic regression adjusted for age, gender, BMI, smoking status, total cholesterol and glycaemia, responders at M3 had a lower baseline NOS activity (L-Cit/L-Arg) (0.30 ± 0.08) than non-responders (0.32 ± 0.10) [odds ratio (OR) = 29.20; 95% CI 1.5896–536.3785; $p = 0.023$] (Table 4). This result was confirmed with the QIDS-C scale (QIDS-C) (OR = 0.05; 95% CI −6.1829 to −0.1399; $p = 0.043$), but not with the self-rating assessment (QIDS-SR) (OR = 0.56; 95% CI −3.6371 to 2.4326; $p = 0.707$)(online Supplementary Table S1).

At last, the NOS activity did not predict the remitter status after 3 months of antidepressant treatment (OR = 0.65; 95% CI −3.6982 to 2.7468; $p = 0.794$) (online Supplementary Table S2).

NOS activity in depressed patients after treatment

The demographics, clinical, and biological characteristics over the time in each subgroup (i.e. drugfree, completers, responders, and non-responders) are reported in Tables 5 and 6.

In bivariate analyses, NOS activity (L-Cit/L-Arg) differed significantly between baseline (0.31 ± 0.09) and M3 (0.33 ± 0.09) ($p = 0.016$) and between M0 and M6 (0.34 ± 0.08) ($p < 0.0001$). It remained significantly lower in depressed patients than in HC (0.38 ± 0.12) after 3 months (0.33 ± 0.09) (95% CI −0.0688 to −0.041, $p < 0.0001$) and 6 months (0.34 ± 0.08) (95% CI −0.0613 to −0.0326, $p < 0.0001$) of antidepressant treatment.

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Table 3. Linear regressions adjusted for age, gender, BMI, smoking status, total cholesterol and glycaemia comparing NOS activity (L-Cit/L-Arg) at baseline between healthy controls and depressed patients (whole sample, drug free and completers subgroups)

	Estimate	Std Error	CI 95%	*p* value
Whole sample				
NOS activity	0.084	0.007	(0.071–0.098)	<0.0001
Age	0.001	0.0002	(0.001–0.002)	<0.0001
Gender	0.005	0.006	(−0.007 to 0.017)	0.419
Smoker	0.006	0.008	(−0.009 to 0.022)	0.426
BMI	−0.0007	0.001	(−0.002 to 0.001)	0.440
Total cholesterol	0.0002	0.003	(−0.005 to 0.006)	0.956
Glycaemia	−0.002	0.004	(−0.010 to 0.007)	0.712
Drug free subgroup				
NOS activity	0.078	0.010	(0.058–0.098)	<0.0001
Age	0.001	0.0002	(0.001–0.002)	<0.0001
Gender	0.006	0.007	(−0.009 to 0.02)	0.455
Smoker	0.012	0.010	(−0.008 to 0.033)	0.232
BMI	−0.002	0.001	(−0.004 to 0.0006)	0.138
Total cholesterol	0.001	0.003	(−0.005 to 0.008)	0.717
Glycaemia	−0.001	0.006	(−0.013 to 0.010)	0.818
Completers subgroup				
NOS activity	0.091	0.01	(0.072–0.11)	<0.0001
Age	0.001	0.0002	(0.001–0.002)	<0.0001
Gender	0.007	0.007	(−0.007 to 0.021)	0.346
Smoker	0.009	0.01	(−0.010 to 0.029)	0.352
BMI	−0.001	0.001	(−0.003 to 0.002)	0.461
Total cholesterol	0.0005	0.003	(−0.006 to 0.007)	0.874
Glycaemia	−0.006	0.006	(−0.016 to 0.005)	0.306

Table 4. Logistic regression adjusted for age, gender, BMI, smoking status, total cholesterol, and glycaemia for NOS activity (L-Cit/L-Arg) at baseline as a predictor of response after 3 months of antidepressant treatment

	OR	CI 95%	*p* value
NOS activity	29.20	(1.58–536.37)	0.023
Age	1.001	(0.97–1.02)	0.931
Gender	1.06	(0.61–1.85)	0.818
Smoker	0.95	(0.64–1.40)	0.813
BMI	1.03	(0.97–1.09)	0.237
Total cholesterol	1.27	(0.7–2.30)	0.432
Glycaemia	2.49	(0.55–11.20)	0.232
HDRS	0.99	(0.94–1.06)	0.975

Mixed-effect models confirmed that NOS activity (L-Cit/L-Arg) significantly increased over time in the whole sample of depressed patients ($p = 0.036$) (Fig. 2) (Table 7), in the depressed patients who completed the study ($p = 0.028$) (Table 7), in depressed patients who were responders at M3 ($p = 0.041$) (Table 7), but not in the drug-free subgroup of depressed patients ($p = 0.41$), and in depressed patients who were non-responders to treatment ($p = 0.56$) (Fig. 2). No antidepressant class effect was found.

In the whole sample, there was no association between HDRS total score changes and NOS activity change (L-Cit/L-Arg) over the time. Moreover, the HDRS items measuring anxiety (psychic anxiety or somatic anxiety) were not correlated with the NOS activity (L-Cit/L-Arg) at baseline, M3 and M6 (online Supplementary Table S3). However, the NOS activity (L-Cit/L-Arg) was significantly associated with the 'psychomotor retardation' item (i.e. slowness of thought and language; decrease in the faculty of concentration; decrease in motor activity) of HDRS (standardized β ($s\beta$) $= -4.72 \times 10^{-2}$; 95% CI −0.09 to −0.003; $p = 0.036$). Finally, in the completers subgroup, the NOS activity (L-Cit/L-Arg idem) changes were significantly associated with six HDRS item score changes: depressed mood ($s\beta = 2.35 \times 10^{-2}$; 95% CI 0.003–0.044; $p = 0.026$), suicide ($s\beta = -3.08 \times 10^{-2}$; 95% CI −0.06 to −0.002; $p = 0.036$), late insomnia ($s\beta = -2.63 \times 10^{-2}$; 95% CI −0.05 to −0.007; $p = 0.007$), gastrointestinal somatic symptoms ($s\beta = -2.92 \times 10^{-2}$; 95% CI −0.05 to −0.008; $p = 0.007$), general somatic symptoms ($s\beta = -2.05 \times 10^{-2}$; 95% CI −0.04 to −0.0006; $p = 0.045$) and genital symptoms ($s\beta = 1.88 \times 10^{-2}$; 95% CI 0.002–0.04; $p = 0.037$).

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Table 5. Demographics, clinical, and biological characteristics over time among drug free and completers subgroups

	Healthy controls	Depressed patients					
		Drug free			Completers		
		M0	M3	M6	M0	M3	M6
Gender (male)	457 (51%)	50 (31.45%)	28 (35%)	19 (33.93%)	78 (31.97%)	63 (34.24%)	61 (33.15%)
Age (years)	39.82 ± 18.61	42.58 ± 13.86	42.23 ± 12.98	42.61 ± 12.96	45.74 ± 12.47	46.45 ± 11.80	46.17 ± 12.09
Non-smoker (*n*, %)	799 (90.1%)	105 (66.04%)	60 (73.17%)	47 (81.03%)	153 (62.70%)	117 (63.93%)	116 (65.17%)
BMI	23.07 ± 2.43	23.7 ± 5.01	24.71 ± 5.17	25.09 ± 4.56	24.25 ± 4.59	24.77 ± 4.71	25.07 ± 4.70
Total cholesterol (mmol/L)	4.95 ± 1.11	5.03 ± 1.09	5.54 ± 1.10	5.51 ± 1.08	5.18 ± 1.16	5.61 ± 1.09	5.56 ± 1.12
Glycaemia (mmol/L)	4.68 ± 0.60	4.92 ± 0.95	5.67 ± 3.37	5.28 ± 1.50	4.88 ± 0.87	5.16 ± 1.2	5.27 ± 1.35
NOS activity	0.38 ± 0.12	0.31 ± 0.1	0.31 ± 0.08	0.32 ± 0.09	0.30 ± 0.08	0.33 ± 0.1	0.34 ± 0.08
HDRS score		23.99 ± 4.43	10.74 ± 7.31	8.02 ± 6.18	24.38 ± 4.92	11.04 ± 6.11	10.03 ± 7.14

Discussion

This metabolomic study compared the NOS activity (L-Cit/L-Arg) of depressed patients to HC and described its outcome after antidepressant treatment. To our knowledge, this is the largest longitudinal case-control study that assessed NOS activity and its outcome in depressed patients.

Firstly, we observed that NOS activity was significantly lower in depressed patients as compared to HC. Our results are in line with those of previous studies, which sample sizes, however, did not exceed 71 patients (Baranyi et al., 2015; Chrapko et al., 2004; García et al., 2011; Selley, 2004). An additional large study in the general population also observed this association between NOS activity and depressive symptoms (Cepeda et al., 2016). But this study did not focus on MDE in a context of MDD. Our large naturalistic sample of 460 patients with a current MDE in a context of MDD and 895 HC, confers a high degree of generalizability to our results. Indeed, there were conflicting results in the literature: some of them reported higher levels of NOx or an increase of NOS activity (Kim et al., 2006; Lu et al., 2014; Suzuki & Colasanti, 2001), others found lower levels of NOx or a decrease of NOS activity (Baranyi et al., 2015; Cepeda et al., 2016; Chrapko et al., 2004, 2006; García et al., 2011; Selley, 2004). Unfortunately, most of these studies were undersized or assessed depressive symptoms instead of MDE. Moreover, these studies mostly included subjects who received psychotropic drugs, had psychiatric comorbidities or focused on a population subtype (such as suicide attempts or melancholic features). These points could raise concerns for the generalizability of results. Of note, antidepressant medications prescribed before inclusion could have biased the described association between depression and NO production by an effect on NO endothelial bioactivity (Chrapko et al., 2006; Ikenouchi-Sugita et al., 2009; Lara et al., 2003). Nevertheless, Cepeda et al. (2016) did not find any difference in fractional exhaled NO levels in subjects treated with antidepressants as compared to controls. To limit these potential biases, we focused not only on the whole sample of 460 depressed patients but also on the homogenous subgroup of 159 antidepressant 'drug free' patients at baseline.

Secondly, we show for the first time that the baseline NOS activity predicted the responder status after antidepressant treatment among depressed patients. Indeed, a lower NOS activity at baseline was associated with a higher response rate after antidepressant treatment. This result remained significant using the QIDS-C but not with the QIDS-SR. Indeed, Rush et al. (2003) reported the QIDS-SR might be slightly less sensitive to residual symptoms than the QIDS-C. Our results go beyond those of Suzuki and Colasanti (2001) and Chrapko et al. (2006), who found no significant difference in pre-treatment nitrite or nitrate levels between patients who were responders and non-responders to antidepressant treatment. Nevertheless, the effect size is moderate.

Thirdly, we showed that the NOS activity increased significantly over time in depressed patients after initiation of antidepressant treatment. These results are consistent with those of previous studies (Baranyi et al., 2015; Lara et al., 2003; Yoshino et al., 2015). In particular, Lara et al. (2003) showed that healthy volunteers treated with paroxetine for 8 weeks have a significant increase in NOx plasma levels. This study is of interest because it shows the specific effects of antidepressant drugs and can exclude the potential confounding effects of major depression and its recovery. Moreover, Chrapko et al. (2006) showed that

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Table 6. Demographics, clinical, and biological characteristics over time among responders and non-responders to antidepressant treatment

		Depressed patients			
		Responders		Non-responders	
	Healthy controls	M3	M6	M3	M6
Gender (male)	457 (51%)	61 (35.47%)	59 (37.11%)	48 (31.17%)	16 (20.25%)
Age (years)	39.82 ± 18.61	45.28 ± 13.12	44.55 ± 13.09	46.33 ± 11.97	47.97 ± 11.21
Non-smoker (*n*, %)	799 (90.1%)	116 (67.84%)		91 (59.48%)	47 (60.26%)
BMI	23.07 ± 2.43	24.45 ± 4.83	25.03 ± 4.60	25.27 ± 4.8	25.93 ± 5.08
Total cholesterol (mmol/L)	4.95 ± 1.11	5.55 ± 1.13	5.51 ± 1.12	5.45 ± 1.16	5.55 ± 1.06
Glycaemia (mmol/L)	4.68 ± 0.60	5.21 ± 2.05	5.23 ± 1.4	5.40 ± 2.14	5.06 ± 0.92
NOS activity	0.38 ± 0.12	0.32 ± 0.09	0.33 ± 0.08	0.34 ± 0.10	0.34 ± 0.09
HDRS score		7.04 ± 3.93	6.32 ± 4.12	19.22 ± 8.55	19.35 ± 5.95

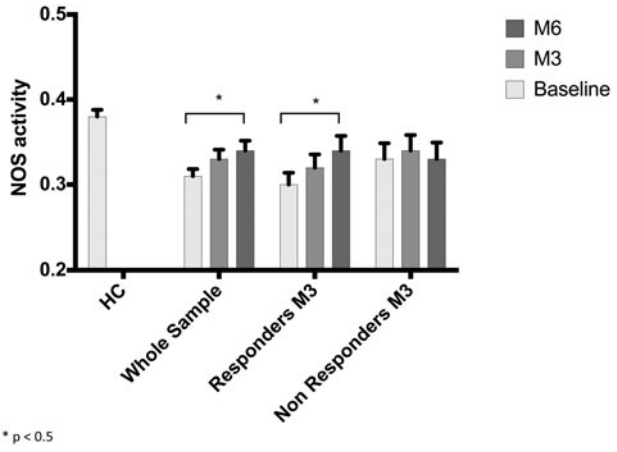

Fig. 2. Interleaved bars of NOS activity change after treatment in patients with MDE (baseline, M3, M6) in different groups (responder M3/non-responder M3) and healthy controls. * $p < 0.5$.

plasma NOx levels increased in both HC and depressed patients after 8 weeks of paroxetine treatment, suggesting specific effects of antidepressant drugs as well. To date, our study is the first naturalistic study with significant results on the NOS plasma activity in a large cohort of patients with a current MDE requiring the beginning of antidepressant drug treatment and followed for 6 months. Moreover, Yoshino et al. (2015) found that fluoxetine significantly increased eNOS mRNA expression in several brain regions. In our study, no antidepressant class effect was evidenced but our study was not designed to show such differences if any. Other studies found the opposite results. Lu et al. (2014) did not show any differences for NOS activity before and after fluoxetine treatment in the follow-up of seven patients with a MDE, probably because of a lack of power. Moreover, Finkel et al. (1996) reported that serum nitrite/nitrate concentrations, indicators of NO production, in depressed patients with ischemic heart disease decreased following paroxetine administration. Similarly, Suzuki and Colasanti (2001) found that nitrate levels significantly decreased after recovery in patients with a MDE.

Fourth, the NOS activity restoration after antidepressant treatment was associated with some HDRS items score improvement. To date, most studies that focused on the NO pathway in depression looked at correlations between nitrite/nitrate levels and the global depression scores in cross-sectional designs (Chrapko et al., 2004; Kim et al., 2006; Lu et al., 2014). These study found results in line with the findings from our study, in which there was no significant correlation between the HDRS total score and NOS activity at baseline and follow-up (M3 and M6). Furthermore, we cannot determine whether the NOS activity restoration is due to the improvement of MDE, to the antidepressant effect, or both, or some other causes. Regarding retardation, several studies suggest that exercise can modulate NO synthesis in various tissues via altering NOS activity (Boo & Jo, 2003; Gielen et al., 2005; Laughlin et al., 2001). Thus, the improvement of NOS activity over time could be associated with the improvement of physical retardation. Moreover, the HDRS items measuring psychic anxiety or somatic anxiety were not correlated with the NOS activity in this sample, this result being coherent with the result of Chrapko et al. (2004) but at odds with results from the study of Suzuki, Yagi, Nakaki, Kanba, and Asai (2001). Furthermore, studies are needed to evaluate the specific impact of anxiety on NOS activity.

Our study has several limitations. The first one was the differences between cases and controls at baseline in terms of sociodemographic features (Table 1). To control for these differences, we performed multivariate models controlling for these differences. However, residual confounders cannot be excluded (Jepsen, Johnsen, Gillman, & Sørensen, 2004). Moreover, the majority of depressed patients were previously treated with antidepressants, even if we had an antidepressant 'drug-free' subgroup of depressed patients. Furthermore, benzodiazepines, as well as psychotherapies, were allowed during the study within the sample for depressed patients. Fernández-Cancio, Fernández-Vitos, Imperial, and Centelles (2001) reported that some benzodiazepines competitively inhibit NOS activity. Nevertheless, in the present study, benzodiazepines were used only at the minimum effective dose and for the minimum duration. We found no significant difference in NOS activity between patients with benzodiazepines (0.3 ± 0.09) and patients without benzodiazepines (0.31 ± 0.10) at baseline ($p = 0.41$). Regarding psychotherapies,

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Table 7. Mixed effect models adjusted for age, gender, BMI, smoking status, total cholesterol, and glycaemia for NOS activity (L-Cit/L-Arg) over the time in the whole sample of depressed patients (whole sample, completers, and responders at M3 subgroups)

	Estimate	CI 95%	*t*(df)	*p* value
Whole sample				
Time	0.003	(0.0002–0.007)	2.101 (714.8)	0.036
Age	0.002	(0.001–0.002)	6.196 (472.9)	<0.0001
Gender	0.001	(−0.013 to 0.016)	0.152 (414.2)	0.880
Smoker	−0.002	(−0.012 to 0.008)	−0.397 (703.9)	0.692
BMI	0.000001	(−0.001 to 0.001)	0.002 (486.9)	0.998
Total cholesterol	0.009	(−0.006 to 0.024)	1.229 (697.9)	0.220
Glycaemia	−0.003	(−0.025 to 0.02)	−0.225 (852.5)	0.822
HDRS	−0.0004	(−0.001 to 0.0005)	−0.851 (857.8)	0.395
Completers				
Time	0.005	(0.0005–0.008)	2.206 (458.4)	0.028
Age	0.002	(0.001–0.003)	4.478 (207.8)	<0.0001
Gender	0.001	(−0.016 to 0.022)	0.305 (190.2)	0.761
Smoker	−0.002	(−0.018 to 0.008)	−0.753 (372.1)	0.452
BMI	0.000001	(−0.002 to 0.002)	−0.278 (222.1)	0.782
Total cholesterol	0.009	(−0.002 to 0.034)	1.746 (395.3)	0.081
Glycaemia	−0.003	(−0.051 to 0.025)	−0.659 (454.7)	0.510
HDRS	−0.0004	(−0.002 to 0.0005)	−1.007 (528.7)	0.314
Responders at M3				
Time	0.005	(0.0003–0.01)	2.052 (292.1)	0.041
Age	0.002	(0.001–0.003)	4.546 (137.7)	<0.0001
Gender	−0.0006	(−0.022 to 0.020)	−0.051 (128.0)	0.960
Smoker	0.0005	(−0.014 to 0.015)	0.070 (256.1)	0.945
BMI	−0.001	(−0.003 to 0.001)	−1.039 (148.7)	0.300
Total cholesterol	0.004	(−0.018 to 0.026)	0.348 (231.3)	0.728
Glycaemia	0.007	(−0.024 to 0.037)	0.426 (357.5)	0.670
HDRS	−0.0004	(−0.002 to 0.001)	−0.602 (323.2)	0.547

all patients could benefit from supportive psychotherapy throughout the study. Besides, the use of the Hamilton Depression Rating Scale-17 in order to measure depressive symptoms in this multicenter investigation could be questioned. Indeed, in their review, Bagby, Ryder, Schuller, and Marshall (2004) found that even if the Hamilton depression scale's internal reliability is adequate, many scale items are poor contributors to the measurement of depression severity. However, the reliability of HDRS-17 was established in a recent meta-analytic review that found good overall levels of internal consistency, inter-rater and test-retest reliability (Trajković et al., 2011). And our results with the HDRS are coherent with those obtained with the QIDS-C. Another limitation is that we did not take into account several factors which can affect NOx levels, such as dietary nitrate intake, physical activity, and renal function (Trajković et al., 2011). Thus, our models were adjusted on confounding factors such as cholesterol levels (Di Massimo et al., 2006; Kelm, 1999), or factors known to reduce NO production (Higman et al., 1996) such as smoking status. Moreover, the attrition rate during the study period was high (44,35% at M3 and 60% at M6), but similar to the attrition rate of other studies. That may explain the loss of significance when the NOS activity changes over time were assessed in the subgroup of antidepressant drug-free patients at baseline. Finally, it has been suggested that NO has an important role in the central nervous system and in the brain pathophysiology of MDD (Chrapko et al., 2004; Feron et al., 1999). But, we assessed peripheral deregulations of NO independently from any central hypotheses.

Our study has a few important strengths. Firstly, it is a naturalistic study of 'real-life' patients, that reduces the gap between research and practice. However, we cannot exclude that antidepressants and their doses might have introduced some variance and caused differences in response rates. However, assessments of MDD were not performed by treating psychiatrists but by independent senior psychiatrists and NOS activity was not known by treating psychiatrists and senior assessing psychiatrists. Secondly, this study provided an assessment until the sixth month of treatment on a large sample of patients with a current MDE requiring antidepressant treatment. And our study is longer than others and

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more powerful (Bernstein et al., 1998; Xing et al., 2002). Thirdly, in our study, the patients had homogenous clinical characteristics of MDE-MDD at baseline, especially the HDRS score. Fourthly, because detectable changes in the concentration of NO and/or its metabolites are highly dependent from timing and method of collection, our measurements were centralized and performed always at the same time under reproducible standardized conditions for depressed patients and HC.

To conclude, patients with a current MDE in a context of MDD have a decreased NOS activity that improves over 6 months period in responders to antidepressant treatment. This activity is correlated with several HDRS-17 items scores. In light with these data, further studies would be necessary to fully understand the changes of the entire metabolites of the nitrergic signaling and their relationship with MDE. For example, a double-blind randomized controlled trial of an antidepressant drug *v.* placebo could be planned in patients with a current MDE, to replicate our results and contribute to validate the NOS activity as a biomarker of antidepressant drug response in major depression.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0033291720001749

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Conflict of interest. None reported.

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