Title: Peripheral tryptophan, serotonin, kynurenine and their metabolites in major depression: a case-control study

Running Head: Tryptophan pathway in major depression

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ABSTRACT:

Aim: Tryptophan is the sole precursor of both peripherally and centrally produced serotonin and kynurenine. In depressed patients, tryptophan, serotonin, kynurenine and their metabolite levels remain unclear. Therefore, peripheral tryptophan and metabolites of serotonin and kynurenine were investigated extensively in 173 patients suffering from a current major depressive episode (MDE) and compared to 214 healthy controls (HC).

Methods: Fasting plasma levels of 11 peripheral metabolites were quantified: tryptophan, serotonin pathway (serotonin, its precursor 5-hydroxy-tryptophan and its metabolite the 5-hydroxy-indole acetic acid), and kynurenine pathway (kynurenine and six of its metabolites including anthranilic acid, kynurenic acid, nicotinamide, picolinic acid, xanthurenic acid and 3-hydroxy-anthranilic acid).

Results: 60 (34.7%) patients were antidepressant drug free. Tryptophan levels did not differ between MDE patients and HC. Serotonin and its precursor (5-hydroxy-tryptophan) levels were lower in MDE patients than HC. Whereas, its metabolite (5-hydroxy-indole acetic acid) levels were within the standard range. Kynurenine and four of its metabolites (kynurenic acid, nicotinamide, picolinic acid and xanthurenic acid) were lower in MDE patients.

Limitations: Whilst, the results of this study demonstrate an association between the metabolites studied and depression, conclusions about causality cannot be made.
Conclusions: This study uses the largest ever sample of MDE patients, with an extensive assessment of peripheral tryptophan metabolism in plasma. These findings provide new insights into the peripheral signature of MDE. The reasons for these changes should be further investigated. These results might suggest new antidepressant therapeutic strategies.

Keywords: 3-hydroxy-anthranilic acid; kynurenic acid; nicotinamide, picolinic acid; xanthurenic acid.

INTRODUCTION

Major depressive disorder (MDD) is the leading cause of disability worldwide and therefore a public health priority 1. However, understanding the pathophysiology of the condition remains challenging. Tryptophan is the sole precursor of peripherally and centrally produced serotonin, kynurenine and their metabolites 2 (figure 1). Serotonin has previously been widely linked to major depression with the classical hypothesis being low serotonin levels in the central nervous system 3. However, previous studies of central serotonin levels 4, 5 failed to show lower concentrations in cerebrospinal fluid (CSF) of patients with major depressive episode (MDE) compared to controls. Peripheral serotonin assessment in MDE could be an important line of study since serotonin is principally stored in the periphery 6. The majority of studies available, which assess peripheral serotonin levels (blood, platelet, serum or plasma) 7-12, have reported lower concentrations in MDE patients compared to controls. The serotonin precursor is tryptophan, which is converted into 5 hydroxy-tryptophan, and then into serotonin. Serotonin is catabolized into 5-hydroxy-indole acetic acid. Whether tryptophan...
Peripheral levels are decreased in MDE is unclear. Therefore, the observed decrease in peripheral serotonin levels in MDE patients could be the result of either a low rate of synthesis and/or a high turnover. Peripheral serotonin synthesis depends on availability of tryptophan and two enzymatic conversions. First, tryptophan hydroxylase 1 catalyzes the conversion of tryptophan to 5-hydroxy-tryptophan. Secondly, 5-hydroxy-tryptophan is converted into serotonin by aromatic amino acid decarboxylase enzymes. In the case of a high turnover rate, an increase in metabolite levels should be observed. Testing these hypotheses requires a complete assessment of the serotonin pathway, including serotonin, its precursors and its metabolite in MDE patients compared with healthy controls (HC).

Tryptophan is also converted into kynurenine (figure 1). Kynurenic acid, a kynurenine metabolite, which interacts with glutamate as an N-methyl-D-aspartate (NMDA) receptor antagonist, has been associated with neuroprotective effects. Lower kynurenic acid levels were associated with cognitive problems in MDE patients. In addition, previous studies have indicated that xanthurenic acid could modulate synaptic transmission in the hippocampus, a structure highly involved in MDE. Two recent meta-analyses report lower kynurenine (1408 MDE patients and 1316 controls) and kynurenic acid levels (501 MDE patients and 527 controls) in MDE patients. But exploration of all steps in the pathway in an MDE sample compared to healthy controls is lacking.

Therefore, the aim of the present study was to extensively investigate plasma tryptophan levels and 10 metabolites of the serotonin and kynurenine pathways. These were assessed in both MDE patients and HC, and associated with the clinical features of MDE patients.

MATERIALS AND METHODS
**Study samples**

Quantification of tryptophan and metabolites of the serotonin and kynurenine pathways was performed in two different samples. First, patients from the METADAP cohort, a multicenter, observational cohort study of 624 patients diagnosed with MDD and current MDE (ClinicalTrials.gov identifier: NCT00526383). This cohort study, recruited from six university psychiatry departments between November 2009 and March 2013, has been previously described. Patients with psychotic symptoms, bipolar disorders, psychotic disorders, eating disorders, current substance abuse or dependence, pregnancy, organic brain syndromes or severe unstable medical conditions were not included. Patients receiving antipsychotics or mood stabilizers before inclusion and/or for a period of four months or more during the last year were also excluded. The Hamilton Depression Rating Scale 17 (HDRS) total score was use to assess severity of MDE symptoms. The analyses described herein were performed on the 173 patients of the cohort who completed the study and for whom fasting plasma samples were available. Second, 214 selected HC were recruited by the clinical research units of 10 French university hospitals between January 2011 and February 2012 as part of the VARIETE cohort (n=800) (ClinicalTrials.gov identifier: NCT01831648). These cohorts were approved by two ethics committees (Paris-Boulogne Ethics Committee and Paris-Sud Ethics committee, France) before studies began. All patients and HC signed a written informed consent for study participation.

HC and MDE patients were matched using a custom automated algorithm to limit discrepancies due to the physiological and biochemical impact of the parameters under study on the measured metabolites. They were therefore similar in terms of gender, age, body mass index, fasting blood glucose, total blood cholesterol, systolic and diastolic blood pressure. First, the algorithm identified the two nearest HC neighbors for each MDE patient.
in the multivariate space built from those seven parameters scaled to the interval [-1,1], using Euclidean distance. The selection of HC in case control studies by the case-based distance measurements has been previously published. To avoid including the same HC several times in the analysis, the list of selected HC neighbors was then filtered for unique values, which resulted in a subset of 214 HC matched with the 173 MDE patients.

**Metabolite measurement**

The following molecules were quantified: tryptophan, serotonin, 5-hydroxy-tryptophan, 5-hydroxy-indole acetic acid, kynurenine, anthranilic acid, kynurenic acid, nicotinamide, picolinic acid, xanthurenic acid and 3-hydroxy-anthranilic acid. For both cohorts, blood was collected on EDTA and immediately centrifuged (10 minutes, 2,000 G at 4°C). Fasting plasma was aliquoted and stored at -80°C. The method used to analyze the plasma tryptophan and metabolites levels was ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS). Metabolite extracts were prepared from plasma samples (50 μL) via protein precipitation with 250 μL of acetonitrile. The samples were centrifuged (21,000 G, 10 minutes, 4°C) and the supernatants were collected, evaporated under nitrogen and reconstituted in 50 μL of water. In addition to the study samples, a reference human plasma sample was prepared three times in all analytical batches to confirm comparable system performance within and between analyses. Sample extracts were analyzed using a Waters Acquity UPLC system coupled to a Thermo Scientific Q Exactive mass spectrometer. Reversed-phase chromatography was carried out at 40°C with a 0.5 mL/min flow rate and an injection volume of 5 μL on a Waters BEH C18 Acquity column (2.1 × 100 mm, 1.7 μm) using a 20-minute gradient of 100% water to 95% B acetonitrile (both with 0.1% formic acid), with the last minute as column re-equilibration. The Q-Exactive was operated in full MS-SIM.
mode with a scan range of 110-300 m/z. All compounds were measured in positive mode (use of positive/negative switching on a two-minute retention time window). Parameters were set as follows: resolution 17,500, capillary temperature 350°C, heater temperature 350°C, sheath gas flow 60, auxiliary gas flow 20, S-lens RF level 50, spray voltage 3.4 kV in positive mode and 2.7kV in negative mode, automatic gain control target 1e6, maximum ion time 80 milliseconds. Metabolites were quantified in TraceFinder software using calibration curves of authentic reference standards. Due to the endogenous presence of the targeted analytes in plasma, the standards were prepared in the sample reconstitution solvent, leading to a possible bias in reported levels related to sample matrix effect for some metabolites.

**Statistical analysis**

Statistical analyses were performed using R 3.4.2 (r-project.org). For some metabolites (anthranilic acid, picolinic acid, serotonin, xanthurenic acid, 5-hydroxy-tryptophan, 3-hydroxy-anthranilic acid, 5-hydroxy-indole acetic acid), values were below the lower limit of quantification (LLOQ), and were imputed as LLOQ/$\sqrt{2}$ for statistical analysis. Furthermore, since the metabolites were not normally distributed (assessment with Shapiro-Wilk normality test), results were expressed as medians and interquartile range (IQR= first quartile - third quartile). Median and IQR are not sensible to imputation methods if values below the LLOQ do not exceed 50% of median (all studied metabolites) and 25% of IQR (all studied metabolites except xanthurenic acid and 5-hydroxy-tryptophan). Non-Parametric tests were used according to imputation strategy and metabolite distributions.

Step 1: Non-parametric Wilcoxon rank-sum tests were used to compare metabolite levels between patients and HC. Step 2: Nonparametric Wilcoxon rank-sum tests were used to analyze associations between metabolite levels and binary MDE patient characteristics (AD-
free status and previous history of MDE). Spearman correlation tests were used to analyze associations between metabolite levels and MDE symptoms severity. The tests were performed for tryptophan and the 10 metabolites. Thus, the usual significance threshold of 0.05 was divided by 11 (=0.0045) to account for multiple testing (Bonferroni corrections). In case of association between a clinical feature and metabolite levels, post-hoc analyses were performed to compare MDE sub-group depending on clinical features and HC.

**RESULTS**

**Socio-demographic characteristics**

A total of 173 MDE patients (65% female, mean age: 48.0 years) and 214 HC (58% females, mean age: 45.5 years) were analyzed. The matched samples were similar in terms of gender, age, body mass index, fasting blood glucose, total blood cholesterol, diastolic blood pressure (Table S1), but a difference was observed for systolic blood pressure (116 vs 119 mmHg respectively and similar IQR). Among MDE patients, 119 (68.8%) had a history of previous MDE, and 60 (34.7%) had not been treated with AD for at least three years (AD-free) and 109 (63.0%) were non AD-free (status non available for 4 patients). The mean (±sd) HDRS scores for the 173 MDE patients were 23.5 (±4.2).

**Tryptophan**

Tryptophan levels did not significantly differ between MDE patients and HC (Table 1). AD-free status, history of previous MDE (data not shown) and HDRS score (Table S2) were not associated with tryptophan levels in MDE patients.
Serotonin pathway

Plasma levels of serotonin and 5-hydroxy-tryptophan were lower in MDE patients. Median serotonin levels were 5 nM (IQR=2-13) in MDE patients and 172 nM (IQR=97-289) in HC, (35-fold higher). MDE AD-free patients had significantly higher serotonin levels than MDE non-AD-free patients (9 nM, IQR=4-33 vs 3 nM, IQR=2-10, p<0.0001) but no other association between AD-free status and metabolites levels was observed (data not shown). Of note, AD-free MDE patients had significantly lower serotonin levels than HC (9 nM, IQR=4-33 vs 172 nM, IQR=97-289, p<0.0001) (20-fold higher). History of previous MDE (data not shown) and HDRS score (Table S2) were not associated with metabolites levels in MDE patients.

Kynurenine pathway

Kynurenine, kynurenic acid, nicotinamide, picolinic acid and xanthurenic acid levels were lower in MDE patients (Table 1). AD-free status, history of previous MDE (data not shown) and HDRS score (Table S2) were not associated with metabolites levels in MDE patients.

DISCUSSION

Compared to HC, MDE patients had normal tryptophan plasma levels, but lower levels of serotonin and kynurenine pathway metabolites. Antidepressant drug-free MDE patients had significantly lower serotonin levels than HC but higher than antidepressant non-drug-free MDE patients.
These results provide a new insight into the peripheral signature of MDE. Indeed, they emphasize that MDE is not only associated with brain abnormalities as described in the classical hypothesis of a low serotonin level in the central nervous system \(^3\), but also with peripheral pathways of serotonin and kynurenine.

Previous studies about central tryptophan \(^{29}\), serotonin \(^4\), \(^5\) and 5-hydroxy-indole acetic acid \(^4\), \(^5\), \(^29\) failed to show lower concentrations in CSF in MDE patients compared to HC. In a postmortem study, prefrontal cortex levels of kynurenine and its metabolites (3-hydroxykynurenine, kynurenic acid and quinolinic acid) did not differ between MDE (n=40) and controls (n=36) \(^{30}\). Thus, plasma fasting serotonin and kynurenine pathway metabolites levels might be more appropriate and convenient for clinical practice than the same central metabolites to discriminate MDE patients from HC.

Regarding tryptophan, our results are in line with those of a recent meta-analysis in 1222 MDE patients and 1181 controls \(^{14}\), indicating the lack of difference in peripheral tryptophan levels between MDE patients and HC \(^{31}\).

Consistently with the majority of previous studies about peripheral serotonin levels (blood, platelet, serum or plasma) \(^7\)-\(^{12}\), we observed a dramatic decrease in fasting serotonin plasma levels in MDE patients as compared to HC. Interestingly, serotonin levels were 20-fold lower in AD-free MDE patients than in HC, which rules out the role of previous AD drug treatment to account for this observation. Since its precursor, 5-hydroxy-tryptophan, was decreased, such lower serotonin levels might be the result of a lower conversion of tryptophan into 5-hydroxy-tryptophan, suggesting a decrease in tryptophan hydroxylase 1 activity. Indeed, genetic polymorphisms of peripheral tryptophan hydroxylase 1 have been associated with MDE \(^{32},^{33}\). Furthermore, tetrahydrobiopterin (BH4), a cofactor necessary for the tryptophan hydroxylase activity could be decreased in MDE patients \(^{34},^{35}\). Since 2003, (classification of
TPH isoform), both academia and the pharmaceutical industry have worked on TPH 1 inhibitors as pharmacological targets, in order to decrease peripheral serotonin synthesis. Benefits on multiple diseases are expected: carcinoid syndrome, pulmonary arterial hypertension, obesity, diabetes and gastro-intestinal disorders. However, the results of our study suggest that peripheral serotonin is involved in MDE pathophysiology. Thus, new drugs which could decrease peripheral serotonin levels may induce MDE. Therefore, we recommend repeated screening of depressive symptoms and MDE in studies assessing TPH inhibitors.

We observed that MDE AD-free patients had higher serotonin levels than MDE non-AD-free patients, confirming the results of previous studies. It has been proposed that this decrease in circulating serotonin in patients treated with AD, is the result of a decrease in serotonin platelet content due to inhibition of serotonin reuptake by AD treatment. Indeed, when serotonin is not taken up by platelets, which along with the gut represent the major peripheral reservoir, it is degraded by the intestinal, liver, and lung cells. Of note this mechanism induced by serotonin reuptake inhibition in platelets is present only in the blood but not in brain cells.

In MDE patients, we observed a decrease in the levels of most metabolites in the kynurenine pathway. Regarding kynurenine, our results are in line with those of a recent meta-analysis in 1408 MDE patients and 1316 controls. Our results are also in line with those of a recent meta-analysis (in 501 MDE patients vs 527 controls for kynurenine acid, and 403 vs 366 controls for 3-hydroxy-anthranilic acid). Our study goes beyond this meta-analysis by assessing the association between metabolite levels with MDE recurrence and symptom severity. To the best of our knowledge, we have shown significant decreases levels of
xanthurenic acid, nicotinamide and picolinic acid in MDE patients for the first time. Overall, our results suggest a global decrease in kynurenine pathway activity in MDE patients. Tryptophan-2,3-dioxygenase (TDO) converts tryptophan to kynurenine. Some authors argue that higher glucocorticoid levels in MDE should induce higher TDO activity and thus lower tryptophan levels and higher kynurenine pathway activation in MDE 43. Paradoxically, we observed the opposite (normal levels of plasma tryptophan and lower levels of plasma kynurenine pathway). This observation can be explained by the increased TDO activity after glucocorticoid treatment, which has only been observed during acute corticoid administration 44. In MDE, the higher glucocorticoid levels is not an acute but a chronic state associated with glucocorticoid resistance 17, 45. Our observations might be the result of a decrease in TDO activity 45 due to glucocorticoid resistance. Indeed, in recurrent MDD, increased evening plasma cortisol was associated with a decreased kynurenine/tryptophan ratio 46. Diet changes in MDE patients could be another mechanism explaining the decrease in serotonin and kynurenine pathway metabolites. However, the observation that the precursor of the pathway, tryptophan, was not decreased does not favor diet interference. Nevertheless, such diet interference cannot be ruled out, since vitamin B6 and iron have been shown to regulate tryptophan conversion into kynurenine and its metabolites 47.

The present study has several limits. First, tryptophan and the serotonin and kynurenine pathway metabolites were not assessed at the central level. Therefore, comparison of brain and plasma levels was not possible. Regarding serotonin, it is uncertain whether it can cross the brain blood barrier from the periphery to the brain; therefore, present data do not allow us to speculate about brain serotonin activity 48. Kynurenine pathway biosynthesis differs at the peripheral and central levels. Tryptophan-2,3-dioxygenase (TDO) converts tryptophan into kynurenine in liver whereas in the brain the conversion depends on indoleamine 2,3-
dioxygenase. However, kynurenine can cross the brain blood barrier\(^4\). Second, quinolinic acid, a metabolite with potentially deleterious neuronal excitotoxicity\(^{17,50}\), was not available in the metabolites quantification panel. Third, potential confounders, such as glucocorticoid levels, immunity, inflammation, glucocorticoid treatments, vitamin and mineral levels were not assessed in this study. Fourth, levels of some metabolites were below the lower limit of quantification and were imputed for the analysis. For xanthurenic acid and 5-hydroxytryptophan, imputation of values below the LLOQ exceeded 25% in some groups. Thus, the IQR of these two metabolites should be considered with caution. Finally, the results of this study provides an association but not causality.

This study has several strengths. It provides the largest sample of MDE patients assessed for plasma fasting tryptophan, serotonin and kynurenine pathways as compared to HC. Furthermore, it offers the first extensive quantification of the main metabolites of the tryptophan, serotonin and kynurenine pathways. They were assessed in the same laboratory with controlled sampling and pre-analytic procedures, providing a reliable picture of the peripheral serotonin and kynurenine pathways in MDE patients.

This study provides new insights into the peripheral signature of MDD that might suggest new AD therapeutic strategies. Indeed, dietary intervention such as probiotics, fecal microbiota transplants to increase kynurenine pathway or drugs targeting kynurenine metabolites\(^{51}\) may provide new avenues for antidepressant treatment in patients. Furthermore, this study suggests potential side effects such as MDE induction of new drugs which decrease peripheral serotonin.

**CONCLUSION**
This study provides an extensive plasma assessment of peripheral tryptophan metabolism, with 10 metabolites of the serotonin and kynurenine pathways, in HC and MDE patients. MDE patients have normal tryptophan plasma levels, but lower levels of the serotonin and kynurenine pathways metabolites. The reasons of these changes should be further investigated.

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DECLARATION OF INTEREST:

Romain Colle, Céline Verstuyft, Bruno Fève, Bruno Boniface and Emmanuelle Corruble have no conflict of interest to disclose.

Denis Joseph David currently receives investigator-initiated research support from Lundbeck and served as a consultant in the areas of target identification and validation and new compound development to Lundbeck Inc., Roche and Servier.

Bruno Falissard has been consultant, expert or has given talks for E. Lilly, BMS, Servier, Sanofi, GlaxoSmithKline, HRA, Roche, Boeringer Ingelheim, Bayer, Almirall, Allergan, Stallergene, Genzyme, Pierre Fabre, Astra Zeneca, Novartis, Janssen, Astellas, Biotronik, Daichi-Sankyo, Gilead, MSD, Lundbeck.

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One or more of the authors are employed by a commercial company: "Technologie Servier", [PM, EW, CBN, BW]. These authors performed Metabolites measurement.

AUTHOR CONTRIBUTIONS

Conception of the work: RC, CV, EC, LB
Data collection: BF, PC, CV, EC, LB
Data management: RC, EC, LB
Metabolites measurement: PM, EW, CBN, BW
Statistical analysis: RC, EC, LB
Interpretation of data: RC, PM, CV, BF, EW, CBN, BW, DJD, BF, PC, EC, LB
Drafting: RC, PM, EC, LB
Preparation of the manuscript: RC, PM, CV, BF, EW, CBN, BW, DJD, BB, BF, PC, EC, LB
Final approval: RC, PM, CV, BF, EW, CBN, BW, DJD, BF, BB, PC, EC, LB

FIGURE LEGENDS:

Figure 1: Tryptophan and serotonin and kynurenine pathways.

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Tryptophan and serotonin and kynurenine pathways.
**Table 1:** Plasma levels of tryptophan and serotonin and kynurenine metabolites pathways in healthy controls and in patients with a current major depressive episode.

<table>
<thead>
<tr>
<th>Metabolite Pathway</th>
<th>Healthy Controls (n=214)</th>
<th>MDE (n=173)</th>
<th>Median relative difference (%)</th>
<th>MDE vs Healthy Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median nM</td>
<td>IQR nM</td>
<td>Median nM</td>
<td>IQR nM</td>
<td></td>
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<tr>
<td>Tryptophan</td>
<td>60894</td>
<td>54217-67456</td>
<td>62217</td>
<td>55822-70474</td>
<td>2</td>
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<tr>
<td>Serotonin pathway</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Serotonin</td>
<td>172</td>
<td>97-289</td>
<td>5</td>
<td>2-13</td>
<td>-97</td>
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<tr>
<td>5-hydroxy-indole-acetic acid</td>
<td>22</td>
<td>25-53</td>
<td>24</td>
<td>17-29</td>
<td>9</td>
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<tr>
<td>5-hydroxy-tryptophan</td>
<td>3.9</td>
<td>0.6-8.9</td>
<td>2.2</td>
<td>1.8-3.1</td>
<td>-44</td>
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<tr>
<td>Kynurenine pathway</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>3.6</td>
<td>2.9-4.6</td>
<td>3.5</td>
<td>1.0-4.5</td>
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<tr>
<td>Kynurenic acid</td>
<td>21</td>
<td>17-26</td>
<td>16</td>
<td>11-20</td>
<td>-24</td>
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<tr>
<td>Kynurenine</td>
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<td>1792-2552</td>
<td>1911</td>
<td>1595-2310</td>
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<td>Nicotinamide</td>
<td>184</td>
<td>136-269</td>
<td>98</td>
<td>71-138</td>
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<td>Picolinic acid</td>
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<td>15-43</td>
<td>19</td>
<td>13-29</td>
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<td>Xanthurenic acid</td>
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<td>4.3</td>
<td>2.9-6.3</td>
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<td>3-hydroxy-anthranilic acid</td>
<td>34</td>
<td>25-52</td>
<td>29</td>
<td>21-47</td>
<td>-15</td>
</tr>
</tbody>
</table>

Legends: IQR: interquartile range, MDE: patients with major depressive episode in major depressive disorder. **Bold p value:** after Bonferroni correction (p <0.0045).