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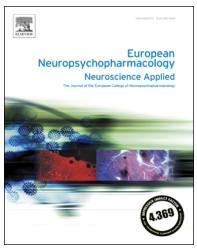

Increased plasma levels of competing amino acids, rather than lowered plasma tryptophan levels, are associated with a non-response to treatment in major depression

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KEYWORDS
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Abstract
Lowered plasma tryptophan (TRP) and TRP/competing amino acid (CAA) ratio may be involved in the pathophysiology of major depression (MDD). Increased cortisol and immune-inflammatory mediators in MDD may affect the availability of TRP to the brain.
We investigated whether baseline or post-treatment TRP, CAAs and TRP/CAA ratio are associated with a treatment response in MDD and whether these effects may be mediated by cortisol or immune biomarkers.
We included 50 medication-free MDD patients with a depressive episode (DSM diagnosis) and assessed symptom severity with the Inventory of Depressive Symptomatology (IDS) before and after treatment as usual for 12 weeks (endpoint). Plasma levels of TRP, CAAs, the ratio, cortisol, CRP and 6 selected cytokines were assayed. The primary outcome was a 50% reduction in the IDS, while the secondary was a remission of the depressive episode.
In IDS non-responders, CAAs increased and the TRP/CAA ratio decreased, while in IDS responders

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CAAs decreased and the TRP/CAA ratio increased from baseline to endpoint. In patients who were still depressed at endpoint TRP and CAAs levels had increased from baseline, while in remitted patients no such effects were found. Increases in CAAs were inversely correlated with changes in interleukin-1 receptor antagonist levels.

The results show that increased CAA levels from baseline to endpoint are associated with a non-response to treatment in MDD patients. This suggests that the mechanism underpinning the CAA-related treatment resistance may be related to changes in immune pathways. CAA levels and amino acid metabolism may be new drug targets in depression.

1. Introduction

Although estimates of lifetime prevalence and course vary considerably across countries, the World Health Organization (WHO) has ranked major depression disorder (MDD) the 4th leading cause of disability worldwide and estimates that by 2020, it will be the second leading cause (Kessler and Bromet, 2013). An estimated lifetime prevalence of 16% has been reported (Andrade et al., 2003; Kessler and Ustun, 2004; Kessler et al., 2010). Treatment includes psychotherapy and pharmacological agents, mainly antidepressants (AD). The tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) were discovered in the late 1950s and early 1960s. Today, practical guidelines recommend treating moderate to severe depressive episode with selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) (Bauer et al., 2007; National Institute for Health and Clinical Excellence, 2009; Ramasubbu et al., 2012). The response to treatment is, however, slow and difficult to predict, and a substantial part of patients will not respond to treatment at all.

The serotonin hypothesis of depression was introduced by Coppen in 1967, and has been a major hypothesis in the pathophysiology in MDD (Coppen, 1967). Tryptophan (TRP), the precursor of serotonin, is an essential amino acid, which is metabolized under constricted control. The availability of plasma TRP to the brain determines the rate of serotonin synthesis in the brain (Moir and Eccleston, 1968). Other amino acids, notably tyrosine (TYR), valine (VAL), phenylalanine (PHE), leucine (LEU) and isoleucine (ILE), compete with TRP for transport across the blood brain barrier (BBB) (referred to as competing amino acids, CAAs) and therefore modulate the availability of TRP to the brain (Fernstrom, 1983). A decreased TRP availability due to increased levels of CAAs may reduce the biosynthesis of serotonin (Fernstrom et al., 1975). TRP homeostasis is generally maintained via the oxidative pathway in which TRP is degraded to several neuroactive compounds in an enzymatic cascade known as the kynurenine (KYN) or the TRP catabolite (TRYCAT) pathway. Quantitatively, this pathway is the major metabolic pathway of TRP; the main exception is the brain, in which almost half of the TRP is used for serotonin synthesis.

In a recent meta-analysis, Ogawa and co-workers concluded that there is convincing evidence for lowered plasma TRP in patients with MDD (Ogawa et al., 2014). These findings support the serotonin hypothesis of MDD (Cowen et al., 1989). While most studies reported significantly lower TRP and TRP/CAA ratio in MDD patients, some studies also found negative results (Myint et al., 2007; Pinto et al., 2012). In our recent study of involvement of the KYN pathway in MDD (Dahl et al., 2015), the levels of TRP and TRP/CAA ratio were not significantly different between depressed MDD patients and controls. Some data has indicated that a lowered TRP/CAA ratio is associated with treatment resistance in MDD (Maes et al., 1997).

Growing evidence has suggested that the lowered availability of TRP to the brain in MDD may be a consequence of increased cortisol production and activated immune-inflammatory pathways (Maes et al., 1991a, 1991b, 1993a., 1994). There is now evidence that activation of immune, e.g. Thelper (Th)-1 and inflammatory pathways (M1) with increased production of interferon-(IFN)γ and increased cortisol production are important in the pathophysiology of MDD (Greden et al., 1980; Maes et al., 1993b; Leonard and Maes, 2012). IDO is activated principally via Th1 responses via IFNγ. The first papers in depression indicating that tryptophan is connected with immune activation and increased IFNγ production were published in 1993b and 1994 (Maes et al., 1994). Previously, relationships between IFNγ and tryptophan catabolism were detected in vivo and in vitro (Yoshida et al., 1981; Werner-Felmayer et al., 1989; Brown et al., 1991).

Th1 cytokines such as IFNγ and hypercortisolemia may activate indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), thereby inducing the production of TRYCATs and lowering plasma TRP (Pemberton et al., 1997; Fujigaki et al., 2001). In MDD inverse associations are detected between lowered availability of plasma TRP to the brain and activated immune-inflammatory pathways and increased glucocorticoid activity (Maes et al., 1991a, 1991b, 1993a, 1994). This is indicative of a close relationship between immune system activity, glucocorticoid activity and the IDO/TDO-induced TRYCAT pathway (Leonard and Maes, 2012). We have recently reported a broader activation of the cytokine network in MDD (Dahl et al., 2014). Most importantly, recovery from depression was associated with normalized cytokines levels. Normalization of the cortisol axis may predict clinical recovery in MDD patients (Greden et al., 1980).

The aim of the present study was to delineate whether baseline or post-treatment TRP, CAAs and TRP/CAA ratio are associated with the response to treatment of acute depressive episode in MDD and whether these putative associations are modulated via changes in glucocorticoid activity or the cytokine network.

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2. Experimental procedures

2.1. Patients

The study population ($n=50$) was recruited from patients with an ongoing depression who were referred to a psychiatric outpatient clinic serving one catchment area in Ringerike Psychiatric Center, Norway (Dahl et al., 2014, 2015). The study was approved by the local Ethical Committee, and the patients provided written consent to participate after receiving verbal and written information regarding the study.

The patients were screened by a psychiatrist before entering the study. The screening involved four steps: (1) a standard psychiatric interview, (2) the Mini-International Neuropsychiatric Interview version 5.0.0 (Norwegian translation), (3) a detailed assessment of previous psychiatric symptoms and treatments from local hospital records, and (4) Young's Mania Rating Scale. The last step was applied to facilitate the ruling out of bipolar spectrum disorders. To be included, the patients had to fulfill the Diagnostic and Statistical Manual of Mental Disorder, 4th edition (DSM-IV) criteria for MDD with ongoing depressive episode, be aged 18–60 years, and have a score of at least 22 on the Inventory of Depressive Symptomatology (IDS) scale.

The duration of the minimum antidepressant washout period allowed for inclusion was 3 weeks, which is in line with previous studies (Brambilla et al., 1997; Pavon et al., 2006; Song et al., 2009; Dowlati et al., 2010). The majority of the patients had a much longer drug-free time period. Patients with present or previous hypomania or mania episodes, or psychotic symptoms were excluded. Additional exclusion criteria were presence of an autoimmune disorder, chronic inflammation, severe metabolic syndrome, body mass index (BMI; $>34\ kg/m^2$), psychotic disorder, and alcohol or drug dependence, receiving anti-inflammatory, antiviral, antibiotic, or immune modulating drugs, and inability to provide informed consent to participate. The procedure for excluding chronic inflammation involved collecting information about chronic inflammatory conditions from the general practitioners who admitted the patients, from interview with the patients, and from the hospital records. In addition, the patients' C-reactive protein (CRP) values at baseline were used. The patients underwent clinical evaluation and blood samples were taken, both as described below, at two time points: at inclusion (referred to as baseline) and after 12 weeks of treatment. A wide-screening blood analysis was used to reveal potential somatic comorbidity. This included liver enzymes, albumin, creatinine, glomerular filtration rate (calculated), alkaline phosphatase, blood lipids, hematologic status, erythrocyte sedimentation rate, CRP, ferritin, vitamin B12, methylmalonic acid, homocysteine, serum folate, thyroid status, cortisol, fasting blood glucoses, and glycated hemoglobin (HbA1c). The screening revealed one patient in need of vitamin B12 therapy, who received vitamin B12 injections and remained in the study.

Of the 50 patients initially enrolled, 43 completed the longitudinal part of the study. The reasons for drop-out were death ($n=1$) and failure to return for the endpoint assessment ($n=6$). The drop-out group did not differ significantly from the completers with regard to both baseline plasma cytokine levels and clinical characteristics.

2.2. Treatment

Treatment was given "as usual", meaning that the choice of treatment was neither influenced by nor an object of this study, and was chosen based on clinical practice.

Out of the 43 patients in the follow-up, 29 did not use any medication. The reason for this relatively high ratio may be explained by a combination of reasons. Since this study had a naturalistic design, the choice of therapy was not standardized but decided by the therapist and patient. To avoid any influence from medication on biological markers on baseline assessments, we included only un-medicated patients. The patients were treated in a specialist unit, offering therapies according to national guidelines. The patient's own preferences were always respected.

The use of antidepressants was recorded. After the medication-free period during baseline assessments, the following medications were administered throughout the entire follow-up phase: escitalopram ($n=6$), venlafaxine ($n=5$), citalopram ($n=1$), mirtazapine ($n=1$), and sertraline ($n=1$). For 3 of these 14 patients, lamotrigine was given in combination with the antidepressant (venlafaxine, citalopram, or mirtazapine), and 1 patient received benzodiazepines (oxazepam and zopiclone) in combination with mirtazapine. In addition, all patients received an average of one psychotherapy session per week; for approximately two-thirds of the sample, this was the only form of treatment (i.e., they did not receive an antidepressant).

2.3. Clinical evaluation

An interview including the Mini-international Neuropsychiatric Interview, version 5.0 (Norwegian translation), was used for diagnostic assessments. The patients were diagnosed with MDD, former depression, or melancholic depression according to DSM-IV criteria. IDS scores were used to assess the severity of depression.

The same psychiatrist (J.D.), who had undergone training and reliability testing in the use of diagnostic and clinical assessments, performed all interviews and evaluations. The achieved intra class correlation coefficient (ICC) and 95% CI for IDS were 0.97 and 0.88–0.99, respectively.

The primary outcome was a 50% reduction in the IDS (clinical improvement, called IDS responsivity), while the secondary outcome was a remission of the depressive episode (clinical remission; DSM definition).

2.4. Blood collection and plasma preparation

All blood samples were collected between 0730 and 0900 h while the participants were lying down and after they had been fasting for a minimum of 8 h. For the cytokine measurements, blood was collected into EDTA tubes, which was turned upside down 10–15 times and then centrifuged at 2000 rpm for 15 min. The plasma was immediately extracted, divided into three Nunc glasses, and then frozen at $-80\ ^{\circ}C$. Blood for the CRP assessment [i.e., high sensitive (hs) CRP] was collected according to the standard procedure performed at the hospital. The procedure has been described in detail previously (Dahl et al., 2014).

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Table 1 Socio-demographic, clinical and biomarker data in patients divided into IDS responders and non-responders to treatment.

Variables	No IDS response	IDS response	F or X^2	df	p
Age (years)	39.8 (12.0)	41.1 (12.6)	0.12	1/41	0.735
Gender (male/female)	3/16	5/19	0.18	1	0.673
BMI (kg/m^2)	25.3 (5.5)	26.6 (5.5)	0.58	1/40	0.452
IDS baseline	37.1 (7.7)	34.4 (7.2)	1.43	1/41	0.238
IDS endpoint	28.7 (9.2)	10.3 (5.3)	67.70	1/41	**<0.001**
ΔIDS	8.6 (6.9)	24.1 (5.4)	67.82	1/41	**<0.001**
Non-remission status (yes/no)	13/6	0/24	23.54	1	**<0.001**
Use of antidepressants (no/yes)	14/5	15/9	0.60	1	0.437
Melancholia (no/yes)	3/16	7/17	1.06	1	0.302
Life time history of MDD (no/yes)	9/10	7/17	1.50	1	0.220
Amino acids					
Tryptophan baseline (μM/l)	54.3 (11.3)	56.4 (11.5)	0.78	1/41	0.543
Tryptophan endpoint (μM/l)	59.6 (13.4)	56.5 (10.3)	0.71	1/40	0.405
Tyrosine baseline (μM/l)	59.9 (14.4)	63.3 (13.4)	0.62	1/41	0.434
Tyrosine endpoint (μM/l)	73.2 (15.0)	61.6 (15.2)	6.03	1/40	0.018
Leucine baseline (μM/l)	110.2 (27.8)	123.7 (21.6)	3.24	1/41	0.079
Leucine endpoint (μM/l)	127.1 (31.6)	118.3 (28.5)	0.89	1/40	0.351
Isoleucine baseline (μM/l)	59.1 (17.9)	67.1 (14.1)	2.68	1/41	0.109
Isoleucine endpoint (μM/l)	69.5 (21.8)	62.5 (15.7)	1.47	1/40	0.232
Valine baseline (μM/l)	206.8 (48.0)	227.4 (38.8)	2.41	1/41	0.128
Valine endpoint (μM/l)	228.9 (49.4)	220.3 (47.5)	0.33	1/40	0.572
Phenylalanine baseline (μM/l)	55.3 (10.6)	59.3 (9.4)	1.68	1/41	0.202
Phenylalanine endpoint (μM/l)	61.1 (7.6)	58.5 (11.0)	0.71	1/40	0.404
CAA baseline (μM/l)	491.3 (112.5)	540.7 (89.3)	2.58	1/41	0.116
CAA endpoint (μM/l)	559.8 (114.0)	521.3 (108.8)	1.24	1/40	0.273
Tryptophan/CAA baseline x 100 (μM/l)	11.14 1.22	10.47 1.40	2.73	1/41	0.106
Tryptophan/CAA endpoint (μM/l)	10.70 (1.54)	11.07 (1.94)	0.45	1/40	0.509
Immune variables					
CRP baseline* (mg/l)	3.0 (4.5)	4.9 (7.0)	1.16	1/41	0.289
CRP endpoint* (mg/l)	2.6 (4.1)	3.4 (4.8)	1.62	1/37	0.211
IL-1RA baseline* (pg/ml)	72.2 (59.1)	112.0 (176.6)	1.30	1/41	0.260
IL-1RA endpoint* (pg/ml)	53.8 (42.0)	58.4 (48.3)	0.44	1/40	0.509
MIP-1 baseline* (pg/ml)	4.2 (3.2)	4.9 (5.9)	0.22	1/39	0.641
MIP-1 endpoint* (pg/ml)	4.7 (6.0)	4.0 (4.1)	0.07	1/38	0.795
IL-6 baseline* (pg/ml)	5.6 3.9	18.6 63.6	1.06	1/41	0.309
IL-6 endpoint* (pg/ml)	4.2 (3.1)	6.4 (14.6)	0.00	1/41	0.983
IL-8 baseline* (pg/ml)	0.139 (0.052)	0.099 (0.047)	5.28	1/41	0.027
IL-8 endpoint* (pg/ml)	1.999 (0.701)	1.605 (0.583)	1.12	1/40	0.295
TNFα baseline* (pg/ml)	17.8 (14.1)	22.4 (16.3)	1.18	1/41	0.195
TNFα endpoint* (pg/ml)	14.0 12.6	15.8 10.0	0.16	1/41	0.730
GCSF baseline* (pg/ml)	22.5 (15.8)	20.4 (12.9)	4.84	1/41	0.034
GCSF endpoint* (pg/ml)	16.5 (12.0)	14.9 (9.8)	0.86	1/41	0.359
Cortisol baseline* (pg/ml)	489 (217)	475 (137)	0.02	1/41	0.903
Cortisol endpoint* (pg/ml)	492 (138)	470 (186)	0.50	1/38	0.486

All results are shown as mean (SD).
IDS=Inventory of Depressive Symptomatology.
ΔIDS (endpoint - baseline values).
Statistically significant p values are in boldface.
*These data are processed in Ln transformation.

2.5. Cytokine and CRP measurements

Measurements of the following cytokines were performed using Bio-Plex xMAP technology (Bio-Rad, Austin, Texas, USA) with a Luminex IS 100 instrument (Bio-Rad, Hercules, California, USA) and Bio-Plex Manager software (version 6.0.1); IL-1RA, IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein 1 alpha (MIP-1a) and TNFα. The exact procedure has been described in detail previously (Dahl et al., 2014).

2.6. High-performance liquid chromatography analysis

The plasma samples from 50 MDD patients were assayed using high-performance liquid chromatography (HPLC) for TRP and for the CAAs tyrosine, valine, phenylalanine, leucine, and isoleucine. The HPLC analyses were carried out at the Laboratory of Medical Bio-chemistry, University of Antwerp, Belgium. The procedure has been described in detail previously (Dahl et al., 2015).

2.7. Statistics

Differences in continuous variables between group means were ascertained with analyzes of variance (ANOVAs). Associations between classification systems were checked using analyzes of contingency tables (χ^2-test). Multivariate repeated measurement (RM) design general linear model (GLM) analyzes were employed to assess the within-subject effects of time and time (baseline and endpoint) X group (responder status) and three-way interactions with use of medication on TRP, CAA and the ratio at baseline and endpoint. We used (automatic stepwise) univariate GLM analysis a) with endpoint TRP, CAAs and TRP/CAA ratio as dependent variables and their baseline values, IDS responsivity, use of antidepressants and their two-way interaction as factors; and b) with the endpoint TRP/CAA ratio as dependent variable and the baseline ratio, IDS responsivity, use of antidepressants and different immune-inflammatory biomarkers and cortisol as explanatory variables. The biomarkers were entered as the baseline values together with the residualized values obtained by the regression of endpoint on baseline values. The residualized values reflect the actual changes in the amino acid levels from baseline to endpoint with all effects of the baseline values partialled out. All tests were two-tailed and a p-value of 0.05 was used for statistical significance. We used IBM SPSS, Windows version 22, to analyze the data.

3. Results

The non-medicated group (those who did not receive antidepressants during therapy) consisted of 24 (83%) female and 5 (17%) male patients. At inclusion, their age was 39±13 (mean±SD) years, BMI 27.0±5.6 kg/m^2, and their IDS score was 35±7. Melancholic depression was diagnosed in 24 patients (83%), 12 patients (41%) had no previous MDD depressive episode, and 17 (59%) had at least 1 previous depressive episode. The medicated group (those who received antidepressants during therapy) consisted of 11 (79%) females and 3 (21%) males. At inclusion, their age was 44±10 years, their BMI was 23.4±4.8 kg/m^2, and their IDS scores was 37±9. Melancholic depression was diagnosed in 9 (64%) patients, 4 (29%) had no previous MDD episode, and 10 (71%) had at least 1 previous depressive episode.

The socio-demographic, clinical and biomarker data in IDS responders and non-responders to treatment are presented in Table 1. There were no significant differences in age, gender, BMI and IDS baseline between the two groups. IDS responders showed significantly lower IDS at endpoint and higher ΔIDS (endpoint – baseline values) than non-responders to treatment. There was a significant association between IDS responder status and remission status at endpoint. Use of antidepressants, melancholia and a life time history of depression were not significantly different between the two groups. Table 1 shows also the amino acid data. There was only one difference between both groups (without p-correction), i.e. lower tyrosine levels in IDS responders to treatment as compared to non-responders. This Table shows also the measurements of cortisol and the immune-inflammatory biomarkers in our study groups. There were no significant differences in the baseline and endpoint biomarker data, except for a small difference in baseline IL-8 and GCSF. We did not use p-corrections to check the many analyzes on clinical, socio-demographic and biochemical data presented in Table 1 because a) some of these variables are strongly intercorrelated (e.g. amino acid levels), and b) these univariate statistical results are used to explore which explanatory variables should be used as determinants of independent associations with the outcome variables in the ultimate multivartiate GLM analyzes presented in Tables 2 and 3. The latter GLM analyzes were interpreted only when there were significant multivariate effects allowing to perform 'protected' univariate tests.

Table 2 shows the results of RM design multivariate GLM analysis with plasma TRP, CAA and the TRP/CAA ratio as dependent variables and time, IDS responsivity and use of antidepressants as factors. We found significant multivariate effects of time X IDS responsivity, time X use of antidepressants and time X IDS responsivity X use of antidepressants. Univariate effects showed that there was a significant effect of time X IDS responsivity on sum CAA and of time X IDS responsivity and time X IDS responsivity X use of antidepressants on the TRP/CAA ratio. This table (see marginal means) shows that the sum of CAAs increased from baseline to endpoint in IDS non-responders and decreased in responders. In IDS non-responders there was a decrease in the TRP/CAA ratio from baseline to endpoint, while in responders an increase was observed. For the ratio TRP/CAA there was an additional effect of antidepressants, that is, in medicated IDS non-responders, but not in responders, there was a decrease in the ratio from baseline to endpoint. We have also checked whether sex may have influenced the results by entering sex as an additional explanatory variable in the analysis. We found that the interaction term time X sex was not significant (F=0.41, df=3/35, p=0.749). There were (as it is well known) significant between subject effects between men and women (F=6.13, df=3/35, p=0.002) with higher levels of tryptophan (F=16.89, df=1/37, $p<0.001$) and CAA (F=13.62, df=1/37, p=0.001) in men while there were no significant sex differences in the ratio (F=0.19, df=1.37, p=0.666). We have also examined possible effects of time on the phenylalanine/tyrosine ratio. RM design GLM analysis did not show significant effects of time X IDS responder status (F=3.51, df=1/38, p=0.069), time X use of antidepressants (F=0.09, df=1/38, p=0.771) and time X IDS responders status X antidepressant use (F=0.00, df=1/38, p=0.968).

Table 2 also shows that there was a multivariate effect of time X endpoint depression diagnosis (remission) on TRP, CAA and the TRP/CAA ratio. Univariate tests showed significant effects of time X endpoint diagnosis on TRP and the sum of CAAs, but not the TRP/CAA ratio. Thus in

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Table 2 Results of repeated measurement design multivariate GLM analysis with plasma tryptophan and sum of competing amino acids (CAA) and the tryptophan/CAA ratio as dependent variables and time, IDS responsivity and use of antidepressants (AD) as factors.

Tests	Dependent variables	Explanatory variables	*F*	Df	*p*
Multivariate	Tryptophan; Sum CAAs; ratio Tryptophan/CAA	Time	2.57	3/36	0.070
		Time X IDS responsivity	6.36	3/36	**0.001**
		Time X use of AD	3.18	3/36	**0.036**
		Time X IDS responsivity X use of AD	3.46	3/36	**0.026**
Univariate	Sum CAAs	Time X IDS responsivity	11.56	1/38	**0.002**
	Trytophan/CAA ratio	Time X IDS responsivity	10.41	1/38	**0.003**
		Time X IDS responsivity X AD	7.00	1/38	**0.012**
Multivariate	Tryptophan; Sum CAAs, ratio Tryptophan/CAA	Time	2.80	3/38	0.053
		Time X remission	4.92	3/38	**0.005**
Univariate	Tryptophan	Time X remission	8.87	1/40	**0.005**
	Sum CAAs	Time X remission	12.19	1/40	**0.001**

Statistically significant *p* values are in boldface.

Marginal means (SE) in IDS responders and non-responders

	Time	IDS non-response ($n=18$)	IDS response ($n=24$)
Sum CAAs	Baseline	490.8 (27.0)	537.4 (21.6)
	Endpoint	561.4 (28.5)	510.1 (22.9)
Tryptophan/CAA ratio	Baseline	11.24 (0.35)	10.15 (0.34)
	Endpoint	10.46 (0.46)	10.50 (0.44)

Marginal means (SE) in patients with and without endpoint remission

	Time	Endpoint non-remission ($n=12$)	Endpoint remission ($n=30$)
Tryptophan	Baseline	54.1 (3.3)	56.3 (2.1)
	Endpoint	63.5 (3.3)	55.6 (2.1)
Sum CAAs	Baseline	495.1 (29.4)	531.9 (18.6)
	Endpoint	583.7 (31.4)	519.5 (19.9)

patients who were still depressed at endpoint we found increases in TRP and sum of CAAs from baseline to endpoint, while in those in remission no such effects were found. The small number of subjects did not allow for analyzes regarding the use of AD.

Table 3 shows the results of RM design multivariate GLM analysis with all 5 CAAs as dependent variables and time, IDS responsivity and use of antidepressants as factors. There was a significant multivariate effect of time X IDS responsivity but not time X use of antidepressants. Univariate tests showed significant effects of time X IDS responsivity on all 5 CAAs. The marginal means show that in IDS non-responders all CAAs increased from baseline to endpoint while they decreased in IDS responders. This table also shows the multivariate and univariate effects of time X diagnosis at endpoint. We found a significant multivariate and univariate interaction effects on all CAAs. A non-response to treatment was associated with an increase in all CAAs from baseline to endpoint and a decrease in CAAs in those who were in remission.

Table 4 shows the results of univariate GLM analysis with endpoint TRP and CAAs values and the TRP/CAA ratio as dependent variables and their baseline values, IDS responsivity and use of antidepressants as factors. Overall the endpoint TRP, all CAAs and ratio values were predicted by their baseline values, and CAA and the ratio, but not TRP, also by IDS responsivity. The use of antidepressants had no significant effect on the endpoint TRP, CAA and ratio data. There were significant interactions between use of antidepressants and IDS responsivity in the case of isoleucine and the ratio, showing that these two variables are modulated by this interaction as described in Tables 2 and 3.

We have also examined the possible effects of cortisol, CRP and the 6 cytokines on the changes in the tryptophan/CAA ratio. Therefore we have performed univariate GLM analyzes (as listed in Table 4) with the endpoint tryptophan/CAA ratio as dependent variables and the baseline ratio values, IDS responsivity and use of antidepressants and their interaction (see Table 4) as factors, with additionally the immune-inflammatory biomarkers and cortisol entered as baseline values and the residualized values obtained by the regression of endpoint on baseline values as explanatory variables. These residualized values thus reflect the actual changes in the variables from baseline to endpoint. The results show that there were no significant associations between the changes in the ratio from baseline to endpoint and the baseline or residualized values of the immune-inflammatory biomarkers or cortisol.

Since this study found that increments in CAA levels were more important predictors of treatment non-responsitivity than the ratio we have also examined the effects of the biomarkers on the endpoint CAA values. Toward this end we

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Table 3 Results of repeated measurement design multivariate GLM analysis with plasma competing amino acids as dependent variables and time, IDS responsivity and use of antidepressants (AD) as factors.

Tests	Dependent variables	Explanatory variables	*F*	Df	*p*
Multivariate	Valine, leucine, isoleucine, phenylalanine, tyrosine	Time	0.76	5/34	0.585
		Time X IDS responsivity	3.68	5/34	**0.009**
		Time X use of AD	0.97	5/34	0.450
Univariate	Valine	Time X IDS responsivity	6.30	1/38	**0.016**
	Leucine	Time X IDS responsivity	12.71	1/38	**0.001**
	Isoleucine	Time X IDS responsivity	15.40	1/38	**<0.001**
	Phenylalanine	Time X IDS responsivity	4.56	1/38	**0.039**
	Tyrosine	Time X IDS responsivity	8.17	1/38	**0.007**
Multivariate	Valine, leucine, isoleucine, phenylalanine, tyrosine	Time	2.00	5/36	0.104
		Time X endpoint remission	2.61	5/36	**0.041**
Univariate	Valine	Time X endpoint remission	8.14	1/40	**0.007**
	Leucine	Time X endpoint remission	10.11	1/40	**0.003**
	Isoleucine	Time X endpoint remission	9.03	1/40	**0.005**
	Phenylalanine	Time X endpoint remission	8.03	1/40	**0.007**
	Tyrosine	Time X endpoint remission	10.88	1/40	**0.002**

Statistically significant *p* values are in boldface.

Marginal means (SE) in IDS responders and non-responders

	RMs	IDS non-response	IDS response
Valine	Baseline	205.8 (11.6)	225.3 (9.3)
	Endpoint	230.4 (12.4)	215.6 (10.0)
Leucine	Baseline	109.8 (6.6)	123.4 (5.3)
	Endpoint	127.2 (7.7)	115.4 (6.2)
Isoleucine	Baseline	58.5 (4.2)	66.6 (3.4)
	Endpoint	70.3 (4.8)	60.7 (3.8)
Phenylalanine	Baseline	56.8 (2.7)	59.2 (2.1)
	Endpoint	61.3 (2.5)	57.7 (2.0)
Tyrosine	Baseline	59.8 (3.7)	62.9 (3.0)
	Endpoint	72.2 (4.0)	60.7 (3.2)

Marginal means (SE) in patients with and without endpoint remission

	RMs	Endpoint non-remission	Endpoint remission
Valine	Baseline	208.9 (12.7)	223.1 (8.1)
	Endpoint	241.2 (13.6)	217.2 (8.6)
Leucine	Baseline	112.3 (7.2)	120.9 (4.6)
	Endpoint	132.9 (8.5)	117.8 (5.4)
Isoleucine	Baseline	60.9 (4.7)	65.3 (3.0)
	Endpoint	72.8 (5.3)	62.6 (3.0)
Phenylalanine	Baseline	53.9 (2.8)	59.4 (1.8)
	Endpoint	61.3 (2.8)	58.9 (1.8)
Tyrosine	Baseline	59.0 (4.0)	63.3 (2.5)
	Endpoint	75.4 (4.4)	63.0 (2.8)

performed automatic stepwise regression analyzes with the endpoint CAA data as dependent variables and the baseline CAA values, IDS responsivity, gender, age and the biomarkers (baseline and residualized) as explanatory variables (see Table 4).

We found that 61.2% of the variance in endpoint CAA was explained by baseline CAA, IDS responsivity and the residualized sIL-1RA levels. Similar association were found when we examined the sum of the BCAAs (valine+leucine +isoleucine) and tyrosine, which were both significantly related to residualized sIL-1RA. We found that 40.3% of the variance in endpoint phenylalanine was predicted by baseline phenylalanine and the residualized CRP as significant explanatory variables.

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Table 4 Results of univariate GLM analysis with endpoint tryptophan, competing amino acids (CAAs) and the tryptophan/CAA ratio as dependent variables and their baseline values, IDS responsivity and use of antidepressants (AD) and their interaction as factors.

Variables	Model *F* statistic (*p* value)*	Baseline values *F* statistic (*p* value)	IDS responsivity *F* statistic (*p* value)	AD use *F* statistic (*p* value)	IDS X AD *F* statistic (*p* value)
Tryptophan	5.92 (0.001)	21.30 (<0.001)	0.92 (0.343)	2.16 (0.150)	0.317 (0.577)
CAA	10.02 (<0.001)	30.79 (<0.001)	9.09 (0.005)	0.78 (0.383)	2.91 (0.096)
Valine	7.04 (<0.001)	21.86 (<0.001)	4.41 (0.043)	0.33 (0.517)	2.46 (0.126)
Leucine	11.62 (<0.001)	37.81 (<0.001)	10.01 (0.003)	0.99 (0.326)	3.71 (0.062)
Isoleucine	11.49 (<0.001)	35.49 (<0.001)	12.34 (0.001)	0.13 (0.721)	5.21 (**0.028**)
Phenylalanine	6.51 (<0.001)	15.43 (<0.001)	4.80 (0.035)	2.39 (0.131)	0.93 (0.341)
Tyrosine	4.86 (0.003)	9.99 (0.003)	8.04 (0.007)	0.94 (0.339)	0.14 (0.712)
Tryptophan/CAA	8.51 (<0.001)	23.56 (<0.001)	8.23 (0.007)	0.59 (0.446)	7.32 (**0.010**)

All tested at df=4/37; all other tests at df=1/37.
Statistically significant *p* values are in boldface.

4. Discussion

The major finding of the current study was that in IDS non-responders the sum of CAAs increased and the ratio decreased from baseline to endpoint, while in IDS responders CAAs decreased and the TRP/CAA ratio increased. In patients who were still depressed (according to DSM V criteria) at endpoint there was an increase in sum of CAAs and TRP from baseline to endpoint, while in those in remission no such effects were found. Thus, increasing CAA levels from baseline to endpoint are associated with a treatment non-response as defined with both the primary (clinical improvement or IDS response) and secondary (clinical remission) outcome criteria. The results also show that the changes in the TRP/CAA ratio in IDS responders versus non-responders can be explained by increased CAA levels and not by lowered TRP levels. The treatment non-response was accompanied by increasing levels in all 5 CAAs from baseline to endpoint.

In the study of Maes and co-workers (Maes et al., 1997) it was suggested that the lowered TRP/CAA ratio in depression may be a marker of treatment resistance, whereas in the current study we found that the sum of CAAs is increased in non-responders to treatment. In the Maes study, however, the availability of TRP was evaluated in patients with TRD and compared to non-TRD patients. As discussed in this paper, one of the weaknesses in that type of study is the assessment of TRD whereby the diagnosis of TRD is made retrospectively and by patients' self-reporting. The accuracy of staging of depression based on prior treatment nonresponse may be less adequate that the clinical delineation of treatment resistance in a prospective study. Clinical trials by Møller and co-workers showed that trials with tricyclic antidepressants, which preferentially act on serotonergic relative to noradrenergic neurons (e.g. amitriptyline and clomipramine), revealed a significant although moderate association between clinical improvement and the baseline plasma ratio TRP/CAA (Moller et al., 1990). These results are not in accordance with those of the present study showing that increments in CAA from baseline to endpoint are associated with a treatment non-response.

Our findings may suggest that increases in plasma CAA concentrations may interact with treatment responsivity in MDD. A first explanation is that increased CAA levels may lower the availability of TRP to the brain by competing for its uptake thereby decreasing the synthesis of serotonin in the brain (see Introduction). This is in part supported by our results that IDS non-responsivity to treatment is accompanied by a lowered ratio as compared to an increased ratio in responders. A second explanation is that increased CAA levels may also contribute to the immune-inflammatory pathophysiology of MDD. Thus, in the presence of high levels of the branched-chain amino acids (BCAAs), valine, leucine and isoleucine, microglial cells have been shown to exhibit an unusual phenotype characterized by a partial skewing toward the M2 state that could result in a less efficient microglial response to local damage and the establishment of a low-grade inflammatory state (De Simone et al., 2013). This could be an explanation for the observed effects of elevated CAAs in our study whereby elevated levels may in fact prolong a central low-grade inflammatory state in MDD thereby interfering with the negative immunoregulatory effects of antidepressants and psychotherapy (Thornton et al., 2009; Maes et al., 2012a). Therefore, increased levels of CAAs during treatment may counteract treatment effects. Thus lowering CAA levels may possibly help to improve treatment response, suggesting that CAA and amino acid metabolism may be new drug targets in depression. Nevertheless, future research should additionally examine free serum tryptophan, which also determines the availability of tryptophan to the brain (Badawy, 2015).

A systematic review of the literature from 1996 to 2000 revealed that tryptophan and 5-hydroxytryptophan or their combination is better than placebo in the treatment of depression although possible publication bias hampered further conclusions on the clinical efficacy of this serotonin precursor (Shaw et al., 2002; Parker and Brotchie, 2011). Long-term studies are needed to evaluate the efficiency of tryptophan supplementation because of its induction of TDO, which is not an issue for 5-hydroxytryptophan. One also has to take into account the possible risk of serotonin syndrome as with all serotonin-enhancing drugs. The occurrence of eosinophilia-

myalgia syndrome in the 1990s led to the withdrawal of tryptophan preparations from the US over-the-counter market. Therefore, future studies should examine the combined effects of including 5-hydroxytryptophan as a diet supplement and lowering CAA levels in the treatment for MDD.

Another finding of our study is that baseline or post-treatment levels of cortisol and immune biomarkers and their actual changes from baseline to endpoint were not related to changes in tryptophan availability to the brain. Nevertheless, in the present study we found significant inverse correlations between the changes in sIL-1RA and CAA levels from baseline to endpoint. More specifically, the actual changes in sIL-1RA levels were inversely associated with those in BCAA and tyrosine levels, while changes in CRP were positively associated with changes in phenylalanine. It is known that the BCAA/tricarboxylic acid cycle is down-regulated by inflammatory signals causing increased BCAA levels (Burrill et al., 2015). Thus, the inverse correlation between the changes in sIL-1RA and CAA/BCAA levels detected here may be a consequence of attenuated anti-inflammatory effects following lowered sIL-1RA, which is known to inhibit IL-1-related inflammatory signaling including in depression (Maes et al., 2012b). Previously, positive correlations between serum levels of tyrosine and inflammatory biomarkers have been described (Mohorko et al., 2015). Inflammatory signals are also known to elevate plasma phenylalanine levels in for example schizophrenia (Okusaga et al., 2014). Old literature exists about amino acid blood levels in immune activation especially sepsis. All amino acids in blood become elevated because of muscle protein break-down and relative liver dysfunction (Freund et al., 1979; Askanazi et al., 1980; Vente et al., 1989; Sprung et al., 1991; Chiarla et al., 2004). Special about phenylalanine is that it accumulates while tyrosine levels are lowered (Neurauter et al., 2008; Ploder et al., 2008; Ormstad et al., 2013). On the other hand Van Gool et al. used the phenylalanine/tyrosine ratio as an estimate of tetrahydrobiopterin deficiency (Van Gool et al., 2003). Therefore, we may suggest that changes in CAA levels are partly modulated by the immune-inflammatory system and that the mechanism underpinning CAA-related treatment resistance may be in part be related to changes in immune-inflammatory biomarkers. Nevertheless, previous studies reported that low tryptophan was associated with higher risk of depression in studies that did not exclude patients with an inflammatory background. Thus, our study population with exclusion criteria including presence of an autoimmune disorder and chronic inflammation, represents a subgroup of depressed patients and, therefore, our results can not be generalized to depression comorbid with immune-inflammatory disorders.

In conclusion, the present study shows that from baseline to endpoint the sum of CAAs increased and the ratio decreased in IDS non-responders. Further, a significant inverse correlation was found between the changes in IL-1RA and CAA levels from baseline to endpoint, indicating that the mechanism underpinning the CAA-related treatment resistance may be related to changes in immune-inflammatory biomarkers. Future studies should examine the combinatorial treatments of a TRP supplement and anti-depressive drugs that lower CAA levels and inflammatory signaling in the treatment of MDD.

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Contributors

Authors Dahl, Ormstad and Andreassen designed the trial. Maes and Ormstad, who also performed interpretation and the main manuscript preparation, designed the current analysis. Maes performed the data analyzes. Dahl collected the clinical data, while Verkerk performed the HPLC analyzes. All authors were involved in manuscript preparation, contributed to, and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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