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RESEARCH ARTICLE

Investigation of the Relationship Among Cortisol, Pro-inflammatory Cytokines, and the Degradation of Tryptophan into Kynurenine in Patients with Major Depression and Suicidal Behavior

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Abstract: ***Background:*** The increased degradation of tryptophan (Trp) along the kynurenine (Kyn) pathway due to inflammation and/or activation of the hypothalamic-pituitary–adrenal (HPA) axis has been reported among the biological factors involved in the pathophysiology of major depressive disorder (MDD) and suicide. However, the interaction among these multiple factors is not yet completely clarified.

Methods: We studied plasma levels of Trp, Kyn, cortisol and proinflammatory cytokines (IL-1, IL-6, IL-12, IL-20) and calculated the ratio Kyn/Trp as an index of the breakdown of Trp into Kyn in 31 suicidal MDD patients and 67 non-suicidal MDD patients.

Result: We confirmed that suicidal MDD patients have reduced plasma Trp, higher Kyn and Kyn/Trp ratio, and no difference in cortisol levels than non-suicidal MDD patients. IL-1 and IL-12 levels were significantly higher in suicidal MDD than in non-suicidal MDD (p=0.034 and p=0.023, respectively), whereas Il-6 and IL-20 levels were equal in the two groups. The Kyn/Trp ratio was positively correlated with a pro-inflammatory cytokines index (r=0.309, p=0.002) and cortisol (r=0.368, p=0.001). Notably, the variance in the Kyn/Trp ratio explained by the model including both cortisol and inflammatory parameters as dependent variables, substantially improved compared with the models in which the two parameters were considered separately.

Conclusion: These findings show that both cortisol and proinflammatory cytokines are involved in the enhanced breakdown of Trp into Kyn occurring in suicidal MDD patients, thus adding new knowledge on the biological mechanisms leading to the activation of the Kyn pathway in MDD and suicide.

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1. INTRODUCTION

Individuals suffering from major depression disorder (MDD) are at a significantly higher risk for suicide compared to the general population [1-3]. The biological underpinnings underlying the co-occurrence of MDD and suicidal behaviour are still a matter of research. Growing evidence is demonstrating the involvement of the tryptophan (Trp) to kynurenine (Kyn) pathway in the pathophysiology of MDD and suicide [4-11]. In particular, increased breakdown of Trp along the Kyn pathway induces a reduction of circulating Trp levels which may lead to a subsequently reduced availability of the amino acid in the brain for the synthesis of the neurotransmitter serotonin (5-HT). A bulk of evidence has pointed to a central role of 5-HT in regulating mood and emotions and consequently to a dysfunction of the 5-HT system in the pathophysiology of both MDD and

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suicide [4, 12-14]. We have recently demonstrated that the plasma Kyn/Trp ratio, an index of the amount of Trp degraded along the Kyn pathway [15], is higher in suicidal MDD than in non-suicidal MDD patients [5]. The degradation of Trp into Kyn is the rate-limiting step of the Kyn pathway and occurs through the action of the tryptophan-2,3-dioxygenase (TDO) and/or the indoleamine 2,3-dioxygenase (IDO) enzymes [16, 17]. While the former enzyme is mostly stimulated by Trp itself or cortisol, the latter is primarily stimulated by pro-inflammatory cytokines that are produced predominantly by activated macrophages in the course of inflammation [16, 17]. In our recent work [5], although we found that the Kyn/Trp ratio was overall positively correlated with circulating levels of cortisol, cortisol levels were not different between suicidal and non-suicidal MDD patients. The activated Kyn pathway could finally lead to an increased formation of downstream metabolites of Kyn, and in particular, to an imbalance in the formation of neurotoxic (*e.g*. quinolinic acid and 3-hydroxykynurenine) and neuroprotective (*e.g*. kynureninc acid) metabolites in favor of the formers [16, 17]. Indeed, several sets of findings show a link between levels of quinolinic acid and suicide risk. Brundin *et al*. [8] showed increased levels of quinolinic acid in suicidal subjects due to reduced activity of amino-β-carboxymuconate-semialdehyde-decarboxylase enzyme. Achtyes *et al*. [18] reported plasma quinolinic acid levels to significantly influence the odds of suicidal ideation in post-partum women with severe and suicidal depression. Bay-Richter *et al*. [19] found higher cerebrospinal fluid (CSF) quinolinic acid and lower kynurenic acid levels in suicidal mentally ill patients than in healthy controls, and Erhardt *et al*. [20] demonstrated higher CSF levels of quinolinic acid and no difference in kynurenic acid in suicide attempters compared with controls. In contrast, Trepci *et al*. [21] found no difference between suicidal and non-suicidal bipolar disorder patients in the CSF levels of quinolinic acid, but this could probably be due to the fact that in this study, the interval between suicide attempt and CSF withdrawal was longer than in previous work [20].

Here, we have further investigated the possible mechanisms mediating the increased conversion of Trp into Kyn in suicidal MDD with respect to non-suicidal MDD patients by focusing on the role of inflammatory cytokines and cortisol. The elucidation of the biological mechanisms underlying the increased metabolism of Trp along the Kyn pathway in suicidal MDD patients may highlight some possible biomarkers predicting suicidal ideation/behavior, and in a longer perspective, targets for developing pharmacological strategies for suicidal prevention in MDD patients.

2. MATERIALS AND METHOD

2.1. Participants

98 MDD patients with active depressive symptoms measured as a Hamilton Depression Scale (HAM-D) [22] score higher than 7 were recruited in the Psychiatry and Emergency Department of Monastir University Hospital, Tunisia, between April 2014 and December 2015. The participants were part of a previous larger study as described in Messaoud *et al.* [5].

67 MDD patients never attempted suicide while 31 had at least one suicide attempt. The diagnosis of MDD was performed following the criteria of the Diagnostic and Statistical Manual of Mental Disorders version IV (DSM-IV) for a major depressive episode [23]. Patients who had comorbidity with an Axis II personality disorder according to the DSM-IV or major neurological disorders were excluded.

Participants underwent a venous blood sample withdrawal between 8:00 and 10:00 a.m. after overnight fasting. The sample was then centrifuged at 4000 g for 10 min and the obtained plasma was stored at -80°C until the analysis.

For suicidal patients who were admitted in the emergency ward of the Monastir University Hospital, the blood withdrawal and the psychiatric assessment were conducted within a day after the admission in order to have the blood sample withdrawal conducted between 8:00 and 10:00 a.m. after overnight fasting as in the non-suicidal group.

All patients gave written and informed consent before participation. The study followed the principles of the Helsinki Declaration and was approved by the Ethical Committees of the Monastir University Hospital and of the San Raffaele Scientific Institute.

2.2. Assessment of Depression Severity

We used the 17-item version of the HAM-D [22] to assess the severity of unipolar MDD patients.

2.3. Plasma Levels of Trp and Kyn

The levels of Trp and Kyn in the plasma of the suicidal and non-suicidal MDD patients were conducted following a well-validated method in our laboratory [5, 24, 25]. The method consists of a high-performance liquid chromatography (HPLC) system, an isocratic gradient of acetonitrile-phosphate buffer 0.004 M, pH 3.5, and an analytical Synergi Fusion-RP 80A column (4μm; 250mm×4.6mm; Phenomenex, Aschaffenburg, Germany). The levels of Trp were quantified using a Shimadzu RF-10 AXL fluorometric detector set at excitation and emission wavelengths of 285 and 345 nm, respectively, and those of Kyn using a Varian ProStar 310 UV–Vis detector set at 360 nm.

2.4. Plasma Levels of Cortisol

Plasma levels of cortisol were measured using an electrochemiluminescent immunoassay (ECLIA) on a COBAS 6000 (e601Modul) (Roche Diagnostics GmbH, Mannheim, Germany).

2.5. Plasma Levels of IL-1, IL-6, IL-12, IL-20

Plasma levels of the pro-inflammatory cytokines IL-1, IL-6, IL-12, IL-20, were quantified by Enzyme-linked immunosorbent assay (ELISA) (CUSABIO BIOTECH CO., LTD). Briefly, the samples and standard solutions were incubated in 96-well microplates coated with the anti-human antibodies for the cytokines of interest for 2 hours at 37°C. Afterward, the liquid of each well was removed and biotinylated antibodies for the cytokine of interest were added and incubated for 1 hour at 37°C. After washing, the third incubation with HRP-avidin was carried

out for 1 hour at 37°C, and then the plates were washed and incubated for 15-30 min with the substrate solution. Finally, the reaction was stopped with the stop solution. The amount of cytokines was determined by measuring the absorbance at 450 nm with correction at 540 nm using a microplate reader. The standard curve demonstrated a direct relationship between the optical density and cytokine concentrations. The detection interval was between 31.25 pg/mL and 2000 pg/mL for IL-1, between 7.8 pg/mL and 500 pg/mL for IL-6, between 4.7 pg/mL and 300 pg/mL for IL-12 and between 7.8 pg/ mL and 500 pg/mL for IL-20. The coefficients of variation for intra- and inter-assay were <8% and <10%, respectively.

2.6. Statistical Analysis

Data analysis was performed using SPSS 24 (Chicago, IL). Continuous variables were indicated as mean ± standard error of the mean (SEM). Differences between suicidal and non-suicidal MDD patients were computed using a Generalized Linear model with age, sex, and BMI as covariates followed by Bonferroni post-hoc correction. A value of $p<0.05$ was considered statistically significant. The levels of cytokines and cortisol were log-transformed since their empirical distribution violated the assumption of normality. The relationship between the plasma levels of Trp, Kyn or Kyn/Trp ratio and the pro-inflammatory cytokines or cortisol was investigated using partial correlation analysis with age, sex and BMI as covariates. For this purpose, we computed z scores for the levels of pro-inflammatory cytokines and cortisol. This procedure allowed us calculating 1) an index of pro-inflammatory cytokines as the sum of the z scores for IL-1, IL-6, IL12 and IL-20, and 2) an index of the factors (pro-inflammatory cytokines and cortisol) known to stimulate the degradation of Trp into Kyn by enhancing the activity of the IDO and TDO enzymes. Finally, we tested whether the levels of Trp, Kyn, and the ratio Kyn/Trp were statistically associated with the levels of pro-inflammatory cytokines and cytokines/cortisol applying a linear regression model with Trp, Kyn and Kyn/Trp ratio as independent variables and cytokines and cytokines/cortisol as dependent variables. The choice of the best fitting model was made through the multiple R^2 and adjusted R^2 parameters, as well as with the Akaike Information Criteria (AIC) and Bayesian Information Criteria (BIC).

3. RESULTS

Suicidal MDD patients were younger than non-suicidal MDD patients ($p<0.001$), and no difference between the two groups was found concerning the distribution males/females (with females being respectively 65% and 68% of the population), and the BMI. The severity of depression measured with the HAM-D scale was higher in suicidal than in non-suicidal MDD patients ($p=0.003$) (Table **1**).

As we previously reported in a larger study population [5], after accounting for the possible confounding effects of age, sex and BMI, plasma levels of Trp were lower in suicidal MDD than in non-suicidal MDD (Fig. **1A**; $F_{1, 94}=8.418$, $p=0.005$), the levels of Kyn (Fig. **1B**; $F_{1, 94}=6.402$, $p=0.013$) and of the Kyn/Trp ratio (Fig. **1C**; $F_{1, 94}=10.264$, $p=0.002$) were higher in suicidal than in non-suicidal MDD patients, and no statistically significant difference in cortisol levels between the two groups was found (Fig. **1D**; $F_{1, 94}=0.311$, $p=0.578$). IL-1 (Fig. **1E**; $F_{1, 94}=4.637$, $p=0.034$) and IL-12 (Fig. **1G**; $F_{1, 94}=5.313$, $p=0.023$) plasma levels were significantly higher in suicidal MDD than in non-suicidal MDD, whereas Il-6 (Fig. **1F**; $F_{1, 94}=0.382$, $p=0.538$) and IL-20 (Fig. **1H**; $F_{1, 94}=0.085$, $p=0.771$) levels were equal in suicidal and non-suicidal MDD patients.

As indicated in Table **2**, after adjusting for the factors age, sex and BMI, plasma levels of Il-1 were positively correlated with the levels of Kyn (r= 0.345, $p= 0.001$) and the Kyn/Trp ratio (r= 0.273, $p= 0.008$), whereas the levels of IL-6 were positively correlated with the levels of Kyn (r= 0.255, $p= 0.013$). Cortisol was significantly and positively correlated with the levels of Kyn (r= 0.386, $p<0.001$) and of the Kyn/Trp ratio (r= 0.368, $p= 0.001$). The pro-inflammatory cytokines index was significantly and positively correlated with the levels of Kyn (r= 0.328, $p= 0.001$) and the Kyn/Trp ratio (r= 0.309, $p= 0.002$). Interestingly, when we studied the correlation between the index given by the sum of pro-inflammatory cytokines and cortisol and the breakdown of Trp into Kyn, we found a positive and significant correlation with Kyn levels (r= 0.431, $p<0.001$) and the Kyn/trp ratio (r= 0.407, $p<0.001$).

Table 1. Demographic and clinical data of the study population.

Study Variable (mean ±SD)	MDD without a History of Suicide Attempt (*n*=67)	MDD with a History of Suicide Attempts (*n*=31)	Statistics	
			t, df or χ^2, df	*p*-value
Age (years)	41.2±11.7	31.9±9.8	t=4.136 df=96	$p<0.001$
Gender (M/F)	24/43	10/21	χ^2 (df=1)	$p=0.730$
BMI (kg/m^2)	26.1±2.3	26.1±2.7	t=0.025 df=96	$p=0.980$
HAM-D	15.3±2.2	16.4±1.5	t=-2.633 df=96	$p=0.003$

BMI: Body mass index; HAM-D: Hamilton Depression Scale; Boldface indicates significant differences at the level $p<0.05$.

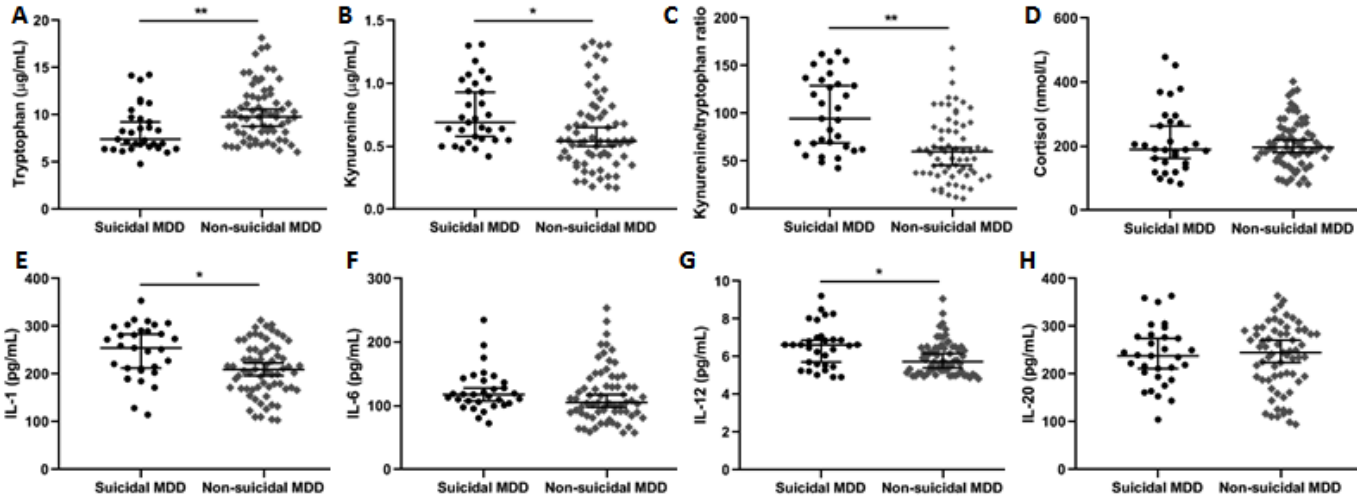

Fig. (1A-H). Plasma levels of tryptophan, kynurenine, kynurenine/tryptophan ratio, cortisol, Interleukin 1 (IL-1), Interleukin 6 (IL-6), Interleukin 12 (IL-12) and Interleukin 20 (IL-20) in suicidal and non-suicidal MDD patients. Data are reported as median ± 95% confidence interval. **p*<0.05, ***p*<0.01 suicidal versus non-suicidal MDD patients by generalized linear model analysis with age, sex and BMI as covariates followed by Bonferroni post-hoc correction.

Table 2. Correlation matrix of Tryptophan to Kynurenine biomarkers, cortisol and pro-inflammatory cytokines and their index after adjusting for the factors age and BMI.

	IL-1 (Z Score)	**IL-6 (Z Score)**	**IL-12 (Z Score)**	**IL-20 (Z Score)**	**Cortisol (Z Score)**	**Index of Proinflmmmatory Cytokines (IL-1 + IL-6 + IL-12 + IL-20) (Z-Scores)**	**Index of Proinflmmmatory Cytokines (IL-1 + IL-6 + IL-12 + IL-20) + Cortisol (Z-Scores)**
Trp	*r*= -0.074 *p*= 0.476	*r*= 0.108 *p*=0.298	*r*= -0.114 *p*= 0.275	*r*= -0.108 *p*= 0.302	*r*= -0.115 *p*= 0.268	*r*= -0.088 *p*=0.399	*r*= -0.120 *p*= 0.248
Kyn	*r*= 0.345 *p*= 0.001	*r*= 0.255 *p*= 0.013	*r*= 0.112 *p*= 0.285	*r*= 0.034 *p*=0.743	*r*= 0.386 *p*< 0.001	*r*= 0.328 *p*= 0.001	*r*= 0.431 *p*< 0.001
Kyn/Trp	*r*= 0.273 *p*= 0.008	*r*= 0.187 *p*= 0.071	*r*= 0.118 *p*= 0.258	*r*= 0.107 *p*= 0.303	*r*= 0.368 *p*< 0.001	*r*= 0.309 *p*= 0.002	*r*= 0.407 *p*< 0.001

Boldface indicates significant association at the level *p*<0.05.

Finally, we investigated the impact of pro-inflammatory cytokines and cortisol on the degradation of Trp into Kyn. The linear regression models indicated in Table **3** showed that the association between the ratio Kyn/Trp, and Kyn and pro-inflammatory cytokines or pro-inflammatory cytokines+cortisol were statistically significant. Fig. (**1**) reports the scatterplot showing the regression line of the models investigating the association between inflammatory cytokines (Fig. **2A**) or inflammatory cytokines+cortisol (Fig. **2B**) as dependent variables and the Kyn/Trp ratio as the independent variable. The variance explained by the model substantially improved when cortisol was included among the inflammatory parameters. Furthermore, the AIC and BIC were lower when cortisol was added, indicating that this model had a better fit compared to the one where cortisol was not present.

4. DISCUSSION

Enhanced degradation of Trp into Kyn has been shown to be among the possible neurobiological mechanisms associated with the development of depression and increased risk of suicide. In this work, we have examined the contribution of the two main factors, inflammatory cytokines and cortisol, influencing the conversion of Trp into Kyn in suicidal and non-suicidal MDD patients. As we have previously shown [5], suicidal MDD patients display greater degradation of Trp into Kyn than non-suicidal MDD patients as measured by the Kyn/Trp ratio, but while the circulating levels of cortisol are comparable in the two groups, pro-inflammatory cytokines, namely IL-1 and IL-12, are higher in suicidal than in non-suicidal MDD patients.

This finding is in agreement with the growing evidence indicating an inflammatory state among the factors linked to the pathophysiology of suicidal behavior [26]. In keeping, other authors have also indicated enhanced degradation of Trp into Kyn, and subsequent imbalance in the formation of the neurotoxic quinolinic acid with respect to the neuroprotective kynureninc acid in suicidal patients [8, 18-20]. Concerning the mechanisms leading to greater degradation of

Table 3. Linear regression model data with Trp, Kyn, and Kyn/Trp ratio as independent variables and cytokines and cytokines+cortisol as dependent variables. Multiple R^2 and adjusted R^2 parameters and the Akaike Information Criteria (AIC) and Bayesian Information Criteria (BIC) are included for the relative best-fitting model.

	Cytokines						Cytokines+cortisol					
Marker	**Beta**	**p-value**	**Multiple R^2**	**Adjusted R^2**	**AIC**	**BIC**	**Beta**	**p-value**	**Multiple R^2**	**Adjusted R^2**	**AIC**	**BIC**
Trp	-0.15	0.31	0.01	0.0001	509.9	517.6	-0.16	0.21	0.016	0.006	504.9	512.7
Kyn	0.04	0.003	0.09	0.079	45.1	52.8	0.048	0.00006	0.16	0.148	37.8	45.5
Kyn/Trp ratio	6.68	0.002	0.09	0.085	1025.8	1033.6	7.3	0.00005	0.16	0.15	1008.9	1016.7

Lower values of the AIC and BIC indicate the best fitting model. Beta indicates the direction of the association and its effect size, p-value shows the strength of the association while R^2 the proportion of variance explained by the model.

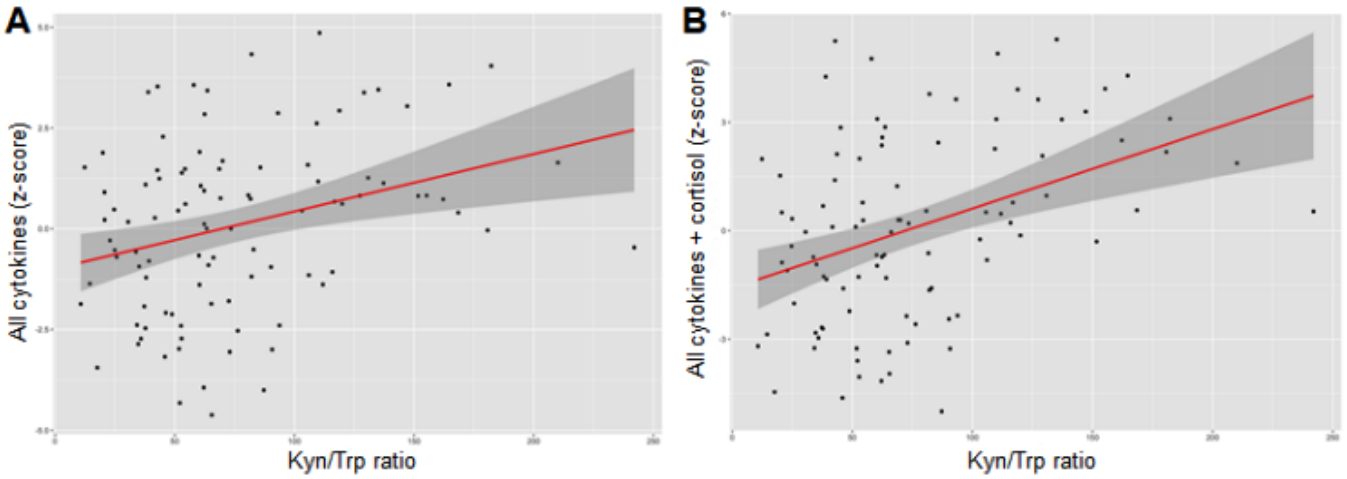

Fig. (2). Scatterplot indicating the regression line (red line) with the 95% interval confidence (gray area) of the model investigating the association between inflammatory cytokines (**A**) or inflammatory cytokines+cortisol (**B**) as dependent variables and the Kyn/Trp ratio as the independent variable. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Trp into Kyn in suicidal MDD patients, we have found that the Kyn/Trp ratio is positively associated with both cortisol and pro-inflammatory cytokine levels. When both factors are considered together, they better explain the variance observed in the ratio.

Two enzymes, TDO and IDO, concur to the conversion of Trp into formyl-Kyn, which is then rapidly transformed into Kyn by a formamidase, an enzyme abundant in most mammalian organs [16]. Although with some limitations, the ratio between Kyn and Trp plasma levels can thus provide a good index of the functioning of the Kyn pathway in a pathological compared with a healthy/control condition [15]. The elucidation of the mechanism/s underlying the dysfunctional Kyn/Trp ratio is not trivial since the activity of the TDO and IDO enzymes is differentially controlled. TDO is primarily induced by Trp itself, glucocorticoid hormones, heme and glucagon, while IDO expression is stimulated by pro-inflammatory cytokines [16]. However, there is evidence showing that immune activation and inflammatory cytokines can also induce the TDO enzyme [27-29]. Indeed, IL-1β was shown to enhance TDO expression and, consequently, Kyn production in endometrioma stromal cells [27]. In mice displaying depressive-like behavior because of a lipopolysaccharide (LPS) injection, increased levels of TDO mRNA were seen in the brain and liver [28] and in the prefrontal cortex [29]. Future work should thus clarify the exact mechanisms controlling the activity of the TDO enzyme in physiological and pathological states. In this context, our data suggest that inflammation plays a significant role in the enhanced degradation of Trp into Kyn in suicidal MDD patients, but the contribution of cortisol cannot be neglected. Notably, a dysregulation of the hypothalamic-pituitary-adrenal axis, which leads to changes in circulating cortisol levels is among the risk factors for suicide [30], and in a meta-analysis on suicidal behavior and cortisol levels, an age-dependent relationship between cortisol levels and suicide was found, with a positive association below the age of 40 and a negative association above 40 years of age [31].

Therefore, it is very likely that both IDO and TDO enzymes are dysfunctional in our suicidal MDD individuals. In keeping, in a post-mortem study examining the ventrolateral prefrontal cortex of depressed patients who died by suicide, Clark *et al*. [32] found a reduced expression of IDO but also of TDO, while in a post-mortem study in the anterior cingulate cortex of depressed individuals, Miller *et al*. [33] showed increased TDO2 protein.

This study has some limitations, including the fact that we did not measure in the plasma other inflammatory cyto-

kines previously linked to depression and suicidal behavior, such as IL-2 [34] or TNF-alpha [35], and our findings may not generalize to all MDD patients since we excluded patients with comorbid personality disorders. In addition, we did not perform multiple testing of cortisol levels throughout the day, a measure that may have provided a better estimation of cortisol secretion and thus its possible link to the degradation of Trp into Kyn. Lastly, the sample size might not have statistical power to detect association signals of moderate/small magnitude.

CONCLUSION

Not with standing these limitations, our findings indicate that both cortisol and proinflammatory cytokines are involved in the enhanced breakdown of Trp into Kyn occurring in suicidal MDD patients, and consequently that when analyzing the involvement of the Kyn pathway in suicide, not only inflammation but also the HPA axis should be concomitantly considered to have a more precise understanding of the underlying neurobiological framework. Indeed, when both proinflammatory cytokines and cortisol were considered together, we were able to predict a greater variance in the Kyn/Trp ratio than when the two factors were singularly examined. Overall, this is a further proof that there are multiple cooperating factors involved in the neurobiology of suicide and in the associated dysfunction of the Kyn pathway.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ethical Committees of the Monastir University Hospital and of the San Raffaele Scientific Institute, Tunisia.

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All humans procedures followed in accordance with the guidelines of the Helsinki Declaration.

CONSENT FOR PUBLICATION

All patients gave written and informed consent before participation.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, [GL], upon reasonable request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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