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IMPACT OF DIFFERENT ANTIPSYCHOTICS ON CYTOKINES AND TRYPTOPHAN METABOLITES IN STIMULATED CULTURES FROM PATIENTS WITH SCHIZOPHRENIA

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SUMMARY

Background: *An imbalance of tryptophan metabolites plays a role in the pathophysiology of schizophrenia. Also cytokines seem to be involved and are able to enhance the tryptophan metabolism. In this study the impact of cytokines, tryptophan metabolites and antipsychotics was evaluated in schizophrenic patients/ healthy controls and correlated with the psychopathology of schizophrenia.*

Subjects and methods: *This study investigated 12 patients with schizophrenia and 24 matched controls. Peripheral blood mononuclear cell cultures were stimulated in vitro with lipopolysaccharide (LPS) or polyinosine-polycytidylic acid (poly I:C) and different antipsychotics (quetiapine, risperidone, haloperidole and clozapine) were added. The cytokines IL-4, IL-10, IFN-γ and tryptophan metabolites were analysed. Symptom severity was assessed using the positive and negative syndrome scale (PANSS).*

Results: *Peripheral mononuclear cells of schizophrenia patients showed a reduced IFN-γ response to LPS ($p=0.008$). When quetiapine and risperidone were added this imbalance between patients and controls disappeared. Tryptophan levels were significantly lower in patients' cells cultures when the cells were stimulated with LPS ($p=0.029$). A group effect for lower levels in the patients' cell culture was evaluated for tryptophan and kynurenine ($p=0.043$; $p=0.05$). In addition, high tryptophan levels correlated with low PANSS negative scores in patients and higher kynurenine levels resulted in higher PANSS positive scores.*

Conclusions: *Only two atypical antipsychotics were identified to reverse the imbalanced cytokine levels in schizophrenia. The low concentrations of tryptophan and kynurenine in these patients could be a sign of a fast degradation of tryptophan – yet tryptophan metabolites could not be changed by any of the investigated antipsychotics.*

Key words: *schizophrenia – tryptophan – risperidone – quetiapine - cytokines*

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INTRODUCTION

In the pathophysiology of schizophrenia several neurotransmitter systems seem to be involved. It has been believed that mainly dopamine is a key player in this disease. The dopamine hypothesis postulates that symptoms of schizophrenia result from an excess of dopamine (Howes & Kapur 2009). However also serotonine was identified to contribute to psychotic states (Geyer & Vollenweider 2008). The serotonine production depends on the availability of tryptophan, which is an essential amino acid. It gets degraded either to serotonine or over the kynurenine pathway to several neuroactive metabolites. The observation that the psychomimetic drug ketamine (an NMDA antagonist) induces schizophrenia-like symptoms in healthy individuals and exacerbates psychotic states in patients with schizophrenia, led to the assumption that NMDA glutamatergic neurotransmission is involved in schizophrenia (Krystal et al. 1994, Lahti et al. 1995). The tryptophan pathway metabolites can specifically act as NMDA receptor antagonists or agonist and therefore resulting in beneficial or detrimental effects for psychiatric diseases. The tryptophan downstream metabolite kynurenine is a precursor for the NMDA glutamatergic receptor antagonist kynurenic acid or for the NMDA receptor agonist quinolinic acid (Guillemin et al. 2001). This NMDA receptor dependent glutamatergic dysfunction has been identified to be part of the pathophysiology of schizophrenia (Javitt 2007). Therefore the precise understanding of the tryptophan breakdown and its actions on the serotonergic as well as the glutamatergic system could help to shed light on the pathophysiology of schizophrenia. Alterations of these tryptophan metabolites are already widely identified in schizophrenia: Linderholm et al. (2012) showed that in patients with schizophrenia kynurenine and kynurenic acid levels in the CSF were increased compared to healthy individuals. In contrast, plasma concentration samples of drug-free schizophrenia patients revealed that kynurenic acid was lower and 3-hydroxykynurenine was higher in patients compared with healthy controls (Myint et al. 2011). However the precise role of tryptophan metabolites in the pathophysiology of schizophrenia remains elusive.

What seems relevant is that the tryptophan metabolism gets induced by pro-inflammatory immune states over the kynurenine arm. In the case of schizophrenia, the pathophysiology has been found to be associated with several immune alterations: Mainly pro-inflammatory cytokines, which mediate inflammatory immune responses, seem to be deregulated in this disease. The enzyme indoleamine 2,3-dioxygenase (IDO) is the first

enzyme of the tryptophan pathway and it gets activated by pro-inflammatory cytokines like interferon (INF)- γ (Yasui et al. 1986). A meta-analysis showed that the cytokine balance in schizophrenia is generally shifted towards an inflammatory response. Levels of interleukin (IL)-1RA, sIL-2R and IL-6 were shown to be significantly higher in patients than in controls. These differences were interpreted as an immune activation in schizophrenia (Potvin et al. 2008). These cytokines were also found to influence the sleep-wake regulation (Weschenfelder et al. 2012).

Another way of activation of the tryptophan metabolism over the kynurenine pathway is by infectious agents. In schizophrenia, there is growing evidence from animal models (Meyer et al. 2009) and clinical studies that an infection or multiple infections are involved in the pathophysiology of the disease (Torrey et al. 2007, Dalman et al. 2008). However, it seems that no specific bacteria, protozoa or virus can be identified that might be clearly responsible for the disease schizophrenia. Discussion continues that the interaction between pathogens and the immune system might be crucial at least in a subgroup of schizophrenia patients (Krause et al. 2010). As infectious agents can activate the tryptophan degrading enzyme IDO over Toll-like receptors (TLR), which are conserved family receptors that recognize pathogen-associated molecular patterns and promote the activation of immune cells (Belladonna et al. 2009), this stimulation method of the tryptophan metabolism was chosen for the present study. Specifically, the TLR 3 recognizes and becomes activated by viral products including Polyinosinic:polycytidylic acid (poly I:C), which mimics a viral activation and the TLR 4 becomes activated by lipopolysaccharides (LPS), proteins from gram negative bacteria (Raetz et al. 1991, Alexopoulou et al. 2001).

Taken this together, the aim of the present study was to shed some light on the pathophysiology of schizophrenia and therefore it seems promising to investigate the tryptophan metabolites and the cytokines that are activated by TLR ligands. As a second step, this study aims to investigate whether antipsychotics are able to influence the cytokine and tryptophan metabolite balances in schizophrenia. Finally, we investigated the association between the psychopathology of the disease and tryptophan metabolites.

SUBJECTS AND METHODS

Subjects

Altogether 36 subjects were recruited. At the department of Psychiatry, Ludwig-Maximilians University, Munich, Germany, 12 patients suffering from schizophrenia, according to the Diagnostic and Statistical Manual (DSM-IV) were included. All patients were inpatients and their symptom severity was measured with the positive and negative syndrome scale (PANSS). In addition, 24 age and gender matched controls that did not meet any criteria for mental illness according to DSM-IV served as healthy controls. Patients and controls did not have acute or chronic inflammatory diseases based on patients medical history and anamnesis. Patients were stable under antipsychotic treatment (monotherapy) or unmedicated. This study was approved by the institutional ethical committee of Ludwig Maximilian University Munich and the study conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000). All participating subjects gave written informed consent and patient anonymity was preserved.

Sample collection and cell culture

Blood was withdrawn from all study participants between 8 to 10 am. $2x10^5$ peripheral blood mononuclear cell (PBMC) in 250µl of RPMI culture medium was cultured with 750 µl RPMI culture medium with and without LPS (5µg/ml) or poly I:C (5µg/ml) and/or different antipsychotic medications. PBMC cultures without immune challenge and with LPS or poly I:C without any medication were performed as controls. To the rest of the cultures different antipsychotics (clozapine 500ng/ml, haloperidol 10 ng/ml, quetiapine 200 ng/ml, risperidone 40 ng/ml) were added. PBMC cultures were incubated at 37°C for 72 hours. Culture supernatants were collected for cytokines and kynurenines analyses.

The cytokines, IL-4, IL-10 and IFN-γ were analyzed using Millipore´s MILLIPLEX map High Sensitivity Human Cytokine Panel (Millipore Corporation, Billerica, MA).

Tryptophan metabolites analyses were performed using ultra performance liquid chromatography (UPLC) with HSST3 column - Tandam quadropole MS from Waters Corporation, MA after solid phase extraction of the culture supernatant with Oasis MCX column. The following tryptophan metabolites were measured: Tryptophan and kynurenine. The detailed analysis method is as follows:

Extraction

For the equilibration process equilibration fluid 1 and equilibration fluid 2 is added to the solid-phase column (Oasis MCX 1cc) and carefully sucked dry. Afterwards the sample is added together with 50µl of internal standard and mixed well. The column is washed subsequently in 1ml washing solution, dried and centrifuged for 5 minutes. Then, the sample is eluted in the elution fluid and evaporated for 15-30 min with nitrogen at 40°. The sample is taken up in 100 µl H2O (MS method: KYN 2350) and this solution is again diluted 1:100 (MS method: KYN 1350). Of both solutions 10µl are injected using the full loop option.

Chromatographic system and conditions

The analysis was carried out on a Water AQUITY UPLC (TM) system with cooling autosampler and

column oven. An ACQUITY UPLC tm HSST3 column (50mm x 150 mm, 1.8 µm (Waters Corp, Milford, MA, USA)) was employed for separation with the column temperature maintained at 45°C. The gradient elution for UPLC analysis consisted of two solvent compositions: Solvent A 0.1% acetic acid in water and solvent B 0.1% acetic acid in methanol. The gradient began with 98% eluent A and changed linearly to 50% A within 10 min, goes to 100% methanol in 20min, stayed for 2 min and changed back to 98% A within 2 min and stayed 5 min. Throughout the UPLC process the flow rate was set at 0.35 ml/min and the run time was 21 min. A Waters Xevo^{tm} tandem quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) interface was used for analytical detection. The ESI source was set in positive ionization mode. Quantification was performed using MRM of the transitions of M7Z 209.1 to 94.1 for kynurenine and m/z 205.1 to 146.1 tryptophan, with scan time of 0.025 per transition. The optimal MS parameters were as follows: capillary voltage 3.5 kV, cone voltage 8 V, source temperature 150°C and desolvation temperature 600°C. Nitrogen was used as the desolvation and cone gas with a flow rate of 800 and 4 L/h, respectively. Argon was used as the collision gas at a pressure of approximately 0.3 Pa. The optimized collision energy for kynurenine was 20 V and for tryptophan 22 V. All data collected in multi-channel analysis (MCA) mode were acquired and processed using Mass Lynx tm V 4.1 software with Target Lynx tm V 4.1 program (Waters Corp., Milford, MA, USA).

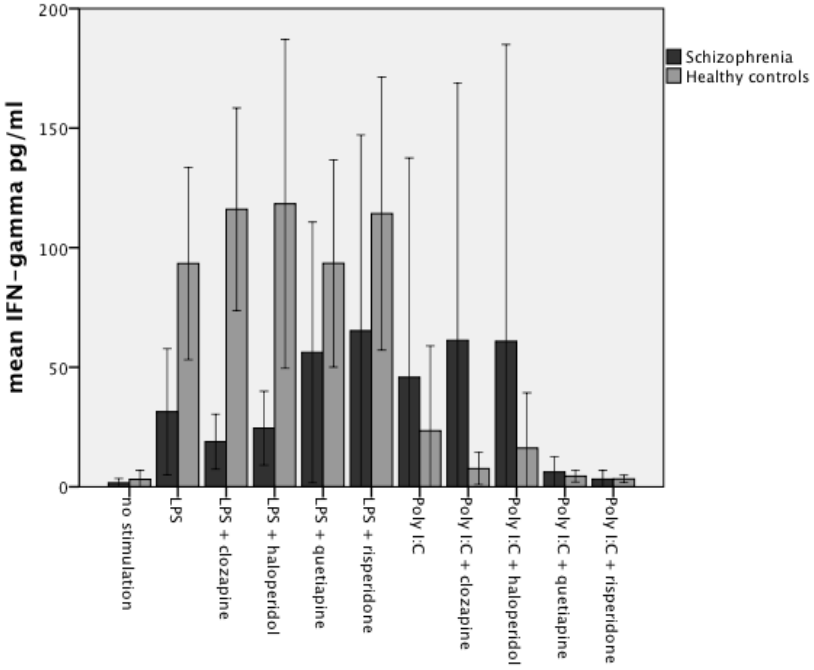
Statistical analyses

The distribution of the data was checked using KSD. For scewed data, nonparametric test was used to compare the groups. For normally distributed data, t-test was used. For calculation of group effects on biochemical data, general linear model was applied. For correlations between clinical parameters and biochemical data, Pearson's correlation of coefficient test was used. The significance was considered for 'p' value 0.05 and below. The SPSS version 18.0 was used.

RESULTS

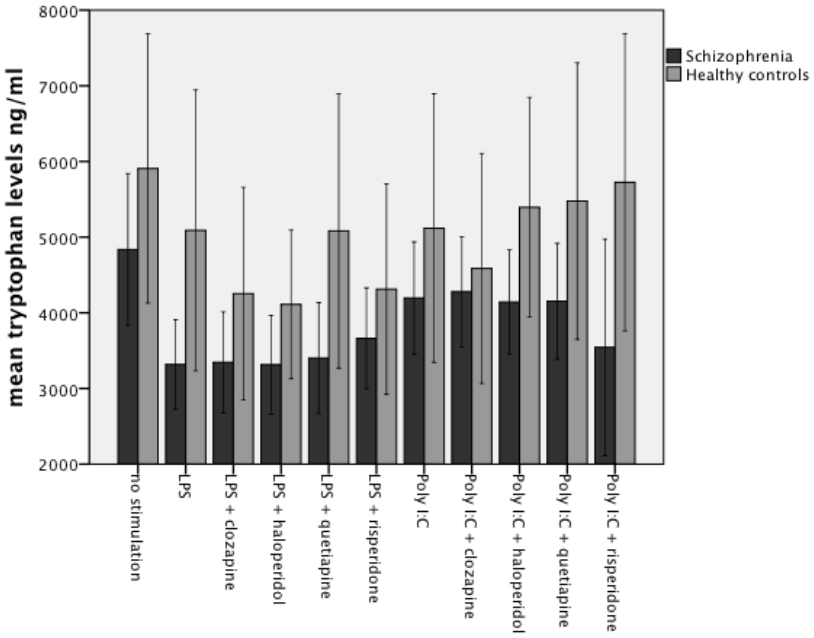
Impact of immune stimulation and antipsychotics on cytokine production

Baseline findings: Without stimulation the levels of IFN-γ (Figure 1) and IL-10 were close to zero in patients and controls. Also for IL-4 no differences between the two groups were found (IL-4 rose to only 0.4 to 0.5 pg/ml). Findings after stimulation and addition of antipsychotics: After LPS stimulation the amount of IFN-γ production was increased in both groups, however, there were lower values in patients compared to controls. The difference reached a significant level for LPS stimulation only (p=0.008), for LPS plus clozapine (p=0.0001) and for LPS plus haloperidol (p=0.048). Interestingly, when quetiapine or risperidone were added, the difference between schizophrenic patients and controls was diminished and therefore the LPS induced low IFN-γ was nearly equalized between the two groups.



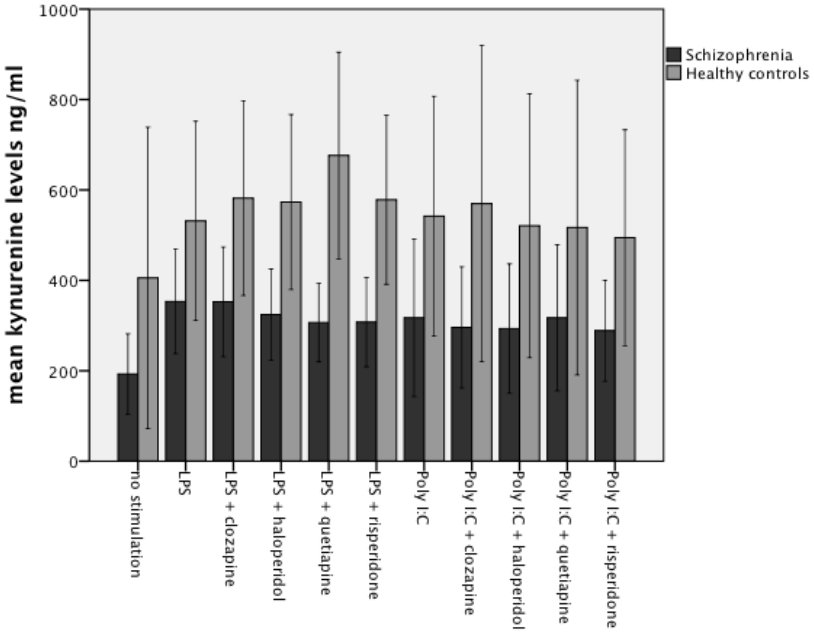
The bars mark the impact of LPS / poly I:C stimulation and antipsychotics on IFN-γ in pg/ml) production in schizophrenia patients and controls. PBMC blood culture was performed in medium alone and in medium stimulated with LPS/ poly I:C. In addition, different antipsychotics were added. After 72 h of incubation, IFN-γ values were measured. Error bars represent a confidence interval of 95%.

Figure 1. IFN-γ levels in schizophrenia and controls



The bars mark the impact of LPS / poly I:C stimulation and antipsychotics on tryptophan in ng/ml) production in schizophrenia patients and controls. PBMC blood culture was performed in medium alone and in medium stimulated with LPS/ poly I:C. In addition, different antipsychotics were added. Error bars represent a confidence interval of 95%.

Figure 2. Tryptophan levels in schizophrenia and controls



The bars mark the impact of LPS / poly I:C stimulation and antipsychotics on kynurenine levels in ng/ml) production in schizophrenia patients and controls. PBMC blood culture was performed in medium alone and in medium stimulated with LPS/ poly I:C. In addition, different antipsychotics were added. Error bars represent a confidence interval of 95%.

Figure 3. Kynurenine levels in schizophrenia and controls

The results for poly I:C stimulation showed in the patient group a tendency towards higher levels of IFN-γ production than controls. Because of the high variation, a significant increase was reached in the patients only for poly I:C plus clozapine (p=0.0001.) and a trend of an increase in patients for poly I:C plus haloperidol (p=0.083). As in the LPS stimulation experiments, the same reversal effect of quetiapine and risperidone was found in poly I:C stimulated cultures. These two antipsychotics were able to reduce the IFN-γ production nearly to a level as low as without stimulation and therefore close to the control level (see Figure 1). In a generalized linear model, a trend of group effect (p=0.074) on IFN-γ secretion was found for LPS stimulation, while for poly I:C, a significant group effect was found in the opposite direction (p=0.02).

Also trends of a within-subject effect of quetiapine and risperidone were observed in generalized linear model calculations (p=0.078 and p=0.074).

For the cytokine IL-4 that was additionally investigated a relatively equal distribution of IL-4 production in both groups could be shown, which was independent of the addition of drugs.

Similar results were found for the cytokine IL-10. The patient and control group reacted with analogous levels to different stimuli and antipsychotics (data not shown).

Impact of stimulation and antipsychotics on tryptophan metabolites

Tryptophan levels were generally lower in patients' cell culture and the difference became significant when stimulated with LPS (p=0.029). All the added antipsychotics showed no clear effect (see Figure 2).

In a generalized linear model, a significant group effect was observed in LPS stimulation (p=0.045), and a trend of group effect was found in poly I:C stimulation (p=0.068). No within subject effect of medication could be demonstrated.

The downstream metabolite of tryptophan is kynurenine, which was also generally lower in the patients' cell culture (p=0.005 to 0.07 in LPS and LPS+antipsychotics). In a generalized linear model, the significant group effect was observed in LPS stimulation (p=0.05), but not in poly I:C stimulation. No within subject effect of medication was observed (see Figure 3).

The kynurenine/tryptophan ratios, which indicate the tryptophan breakdown, were also generally lower in the patients' cell culture; although there was no statistical significance. Neither group effect nor medication effect was found in a generalized linear model (data not shown).

Association of psychopathology and tryptophan metabolites

The tryptophan levels showed a significant inverse correlation with PANSS negative scores (R=-0.451 to -0.726) (see Figure 4).

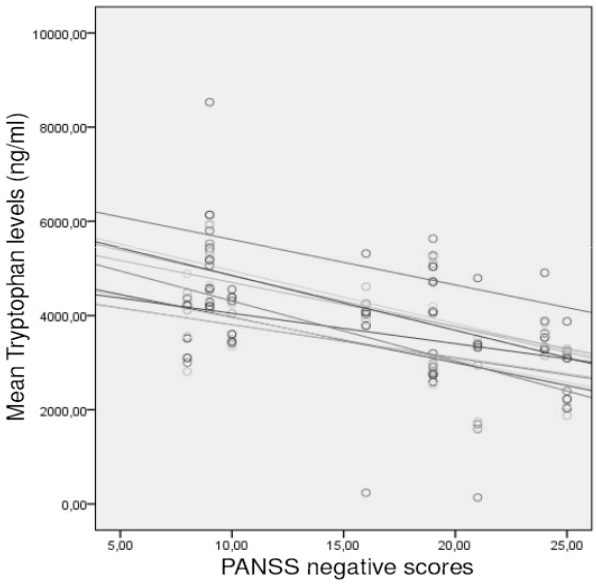


Figure 4. Tryptophan levels were correlated with PANSS negative scores in schizophrenia patients

The basal (unstimulated) kynurenine levels showed a significant positive correlation with PANSS positive scores (R=0.665; p=0.029) (see Figure 5).

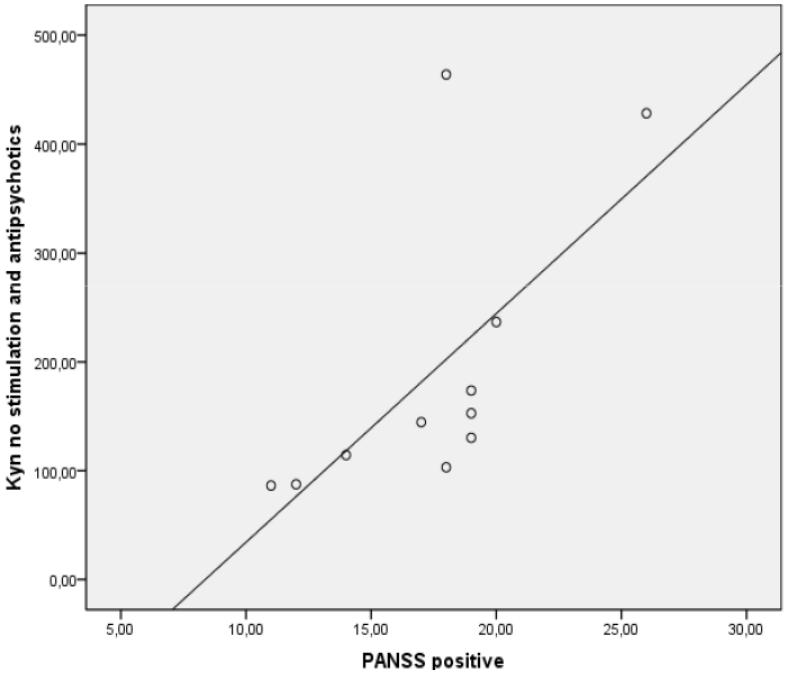


Figure 5. Unstimulated kynurenine levels were correlated with PANSS positive scores in schizophrenia patients

DISCUSSION

The present study revealed that peripheral immune cells of schizophrenia patients showed a reduced IFN-γ response to LPS (TLR 4) stimulation and an increased IFN-γ response to poly I:C (TLR 3) stimulation. The TLRs regulate the activation of immune cells and recognize different microbial pathogens and their products. TLR 3 recognizes and becomes activated by viruses or poly I:C which mimics a viral double stranded DNA; TLR 4 becomes activated by gram negative bacterial toxin such as LPS and heat-shock protein 60. Based on the levels of activation of those receptors, the response measured in terms of cytokines release could be different. The different findings for the levels of IFN-γ after poly I:C and LPS stimulation could be due to prior exposure of immune cells to minuscule amounts of endotoxins that then cause a refractory state to subsequent endotoxin challenge (Biswas & Lopez-Collazo 2009). It seems therefore possible that in the study patients prior TLR 3 expression was higher in response to poly I:C stimulation whereas prior TLR 4 expression was lower in response to LPS stimulation. Müller and co-workers found that TLR 3 is significantly overexpressed in monocytes from schizophrenic patients after stimulation with poly I:C whereas stimulation with LPS in these patients resulted in lower expression of TLR 4 (Muller et al. 2012). These findings could also be interpreted that a pre-existing TLR activation induces stimulation-refractory states resulting e.g. in a lower production of IFN-γ in patients after TLR 4 stimulation, which was similar to the condition 'endotoxin tolerance' (Biswas & Lopez-Collazo 2009). Comparable responses to LPS were also observed in the whole blood culture study in major depression (Krause et al. 2012).

Regarding findings of antipsychotic drugs on IFN-γ production, it could be stated that while clozapine and haloperidol could enhance the difference in IFN-γ response to stimulation between schizophrenia patients and controls, this difference to stimulation was abolished when quetiapine or risperidone were added. Interestingly, this finding was true for LPS and poly I:C stimulation for risperidone and quetiapine only. An explanation why haloperidol and clozapine did not show the same effect could be that haloperidol is a typical antipsychotic that stimulates different receptors and clozapine is the antipsychotic that stimulates a huge variety of receptors, therefore showing blurred effects. Especially quetiapine and risperidone have been identified in literature to exert immunomodulatory effects: For risperidone a normalizing effect of inflammatory parameters and a restoration of anti-inflammatory pathways has already been proven: risperidone prevented increased inflammatory parameters induced by LPS in brain cortex and restored anti-inflammatory pathways decreased by LPS challenge (Macdowell et al. 2011). The influence of risperidone on cytokines has also been explored by Maes et al. (1996) and the authors found that the soluble IL-2 receptor was increased after treatment with risperidone in schizophrenic patients. In addition, risperidone, but not haloperidol was shown to inhibit IFN-γ-induced microglial activation in vitro and may therefore have an anti-inflammatory effect (Kato et al. 2007). Also quetiapine may have an anti-inflammatory effect via the inhibition of microglial activation over the inhibition of TNF-α after stimulation with IFN-γ (Bian et al. 2008). The precise mechanism of how quetiapine and risperidone can reverse the significant difference of IFN-γ levels between schizophrenia patients and controls remains elusive. Further studies are needed to address these differences between antipsychotics and investigate their possible mechanisms. However, our present findings could potentially serve as the basis for future biomarker studies that predict treatment response to quetiapine and risperidone.

The other two investigated cytokines of the present study, IL-4 and IL-10 did not show any difference between schizophrenia patients and controls. Even though these cytokines were identified to play a role in the pathophysiology of schizophrenia in literature. In patients plasma levels of IL-4 were found to be decreased compared to controls (Kim et al. 2004) and also in the CSF IL-4 levels differed between patients and controls (Mittleman et al. 1997). Findings on cytokines in schizophrenia remain controversial. Just recently IL-4 genes and schizophrenia were investigated and no associations were observed between the clinical course and psychopathology of the disease and the genotypes of the polymorphisms for IL-4 (Fila-Danilow et al. 2012). Also for IL-10, an increased mRNA expression was detected in schizophrenia patients (Chang et al. 2011), while no differences were observed in IL-10 gene expression in patients and controls (Freudenreich et al. 2010)

For the investigated tryptophan metabolites differences between patients and healthy controls were observed in the present study. In schizophrenia patients we saw lower tryptophan levels after stimulation with LPS compared to controls. In addition, a group effect was found with lower tryptophan in patients after adding different antipsychotics. For the downstream metabolite of tryptophan, kynurenine, the results were similar to tryptophan. Also lower kynurenine levels were measured in the patient population as a group effect in LPS-stimulated samples when antipsychotics were added. These low concentrations of tryptophan and kynurenine in schizophrenia patients could be most probably due to the fast degradation of tryptophan over the kynurenine branch of its metabolism. Tryptophan pathway abnormalities have been already widely identified (see Schwarcz et al. 2012) and specifically, abnormalities of kynurenines were stated in psychiatric disorders (see Myint 2012). Our results are in line with previous studies showing that serum tryptophan concentrations in drug-naïve schizophrenic patients are decreased. Also kynurenine levels were decreased, but

did not reach statistical significance (Kim et al. 2009). Interestingly we demonstrated in the present study that the stimulation with LPS and further addition of antipsychotic drugs also led to lower tryptophan and kynurenine levels in schizophrenia. This indicates that in schizophrenia the investigated drugs were not able to reverse this existing imbalance of tryptophan metabolites. A question here is whether the other metabolites from the further downstream kynurenine pathway are also imbalanced. A previous study that analyzed both branches of the kynurenine metabolism showed an imbalance between kynurenic adic and 3-hydroxykynurenine (Myint et al. 2011). As kynurenine metabolites have been identified to alter the glutamatergic and acetylcholinergic neurotransmissions, which are also part of the disease pathology (Erhardt et al. 2009) targeting selective tryptophan metabolites with specific drugs other than antipsychotics could help to restore the tryptophan pathway balance and therefore expand the treatment options for schizophrenia. Currently there are already different enzyme inhibitors of the tryptophan pathway available: The inhibition of the kynurenine catabolising enzyme kynurenine 3-monooxygenase (KMO) seems to be most promising (Zwilling et al. 2011). Future studies are needed to clarify whether the activity of tryptophan pathway enzyme e.g. KMO are altered in schizophrenic patients. So far, a study demonstrated an increase in 3-hydroxykynurenine which is the product of KMO. This supports the use of KMO in the treatment of schizophrenia (Myint et al. 2011). However one post mortem study already found KMO expression and activity to be decreased in certain areas of the brains from schizophrenia patients (frontal eye field, Brodman area 6) (Wonodi et al. 2011). Another way of therapeutical influence on the tryptophan breakdown is over pro-inflammatory cytokines that regulate the IDO tryptophan pathway enzyme. Therefore, anti-inflammatory drugs have been tested in the treatment of schizophrenia (see Muller 2013).

Furthermore the levels of tryptophan metabolites seem to influence the symptoms of schizophrenia. When these metabolites were correlated with the psychopathology of the disease, our study revealed that the higher the tryptophan levels in the culture the lower the PANSS negative scores. And the higher the kynurenine levels in the culture were, the higher the PANSS positive scores. Some tryptophan downstream metabolites have already been associated with cognitive deficits in schizophrenia. Alexander et al. injected kynurenine into rats and found their cognitive flexibility impaired compared to controls (Alexander et al. 2012). Also in schizophrenia patients higher initial plasma kynurenic acid levels on admission or increased kynurenic acid/kynurenine ratio after treatment were found to be associated with a reduction of clinical symptoms scores upon discharge (Myint et al. 2011).

The present study has some limitations: The measurement of cytokines and tryptophan metabolites was performed in the periphery on blood mononuclear cells culture. Therefore the results can only give hints regarding the changes in the brain. However, It is well documented that tryptophan and kynurenine are able to cross the blood-brain barrier (Fukui et al. 1991). Another aspect is that this study measured acute in vitro effects of LPS and poly I:C and antipsychotics. In vivo there can be a delay in the onset of the therapeutic response to antipsychotics which is related to complex changes in neurotransmitter, endocrine and immune dynamics and the differential effects of the active metabolites of the drugs. The acute effects or lack of combined biological reaction in the present study on the in-vitro blood cells in culture may therefore have limited transferability to the therapeutic effects in vivo. In addition, the cytokine IL-17 has not been investigated, even though it was shown to be associated with schizophrenia (Borovcanin et al. 2012), and it also seems to be influenced by antipsychotics (Himmerich et al. 2011).

Nevertheless, the results of this study showed the imbalances in immune response and tryptophan metabolites in schizophrenia patients.

CONCLUSIONS

Immune dysbalance is present in the peripheral immune cells of some patients with schizophrenia. The immune dysbalance can be reversed by quetiapine and risperidone only. Therefore these two antipsychotics seem to have immunomodulatory effects. Also tryptophan pathway abnormalities are present in schizophrenia patients and are associated with the psychopathology. However, as antipsychotics cannot influence these tryptophan dysbalances it seems promising to investigate drugs that target specifically the tryptophan pathway in order to get new treatment options for schizophrenia.

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Conflict of interest: None to declare.

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