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I. Dedication

My sincerest thanks for the valuable support in preparing my thesis go to my direct supervisor, Dr. Aye-Mu Myint, and my doctoral supervisor, PD Dr. Markus Schwarz, as well as to the staff at the PNI laboratory of the University of Munich.

After a project is finished it is easy to forget the inconveniences it caused and just be glad that it is achieved successfully. Therefore, I want to always remember that my project would never have been possible without the support (financial, moral and physical) of my parents. My thanks go also to Jörg Casparis: you put up with my ups and downs and constantly motivated me with your reminders that the end was in sight.

Of course, as a doctoral student one always carries the sole responsibility for one's thesis. However, such a project could never be completed if one was not surrounded by many, many people who help, be it in large or small ways: Cornelia Maier, Thomas Görlitz, Ursula Nägele, Angela Amorin-Nink, Karl Moser, Juliane Mayerhofer, Maximilian Moser, Edith Moser, Michaela Wallner, Jacquie Klesing, Malgorzata Roos and many more.

All's well that ends well!

Stephanie Moser

Contents

1 Introduction

1.1 Definition of the term „depression“

When originally used in psychiatry, the term depression (Latin: deprimere = press or push down) had a much less specific meaning than today, i.e. it referred to a general prostration and an impairment of mental functions. When the term was adopted into psychiatric nomenclature in the first half of the 19th century, it was initially used as a class heading for groups characterised by sub-average mental functioning. Kraepelin (1913) used this term to denote “melancholic or depressive conditions with sad or anxious mood and an impediment of thinking and acting” [author’s translation]. Since that time further attempts have been made to define the term, e.g. by Jaspers (1913, 1959) and Bleuler (1916) (Möller H-J. et al., 2005a). In the 20th century the term manic-depressive psychosis increasingly gained acceptance and recently, the term affective psychoses. With the introduction of the new diagnosis and classification systems ICD-10 and DSM-III-R (1987, 1991), the previous aetiopathogenetically oriented triadic classification system of organic and endogenous psychoses versus “neuroses, personality disorders and other non-mental disorders” (ICD 9, DSM III, 1980) was done away with. The atheoretical, purely descriptive term “depressive episode” in ICD-10 or “major depression” in DSM-IV took the place of the classical differentiation between “endogenous” and “neurotic” depressions. The term “affective disorders” is used to refer to the whole group of these disorders. The ICD-10 classification of affective disorders is subdivided into manic episodes, bipolar affective disorder, depressive disorder (“major depression”), persistent affective disorder, recurrent brief depressive disorder (“minor depression”), other affective disorders and the unspecified affective disorders.

The depressive disorders are by far the most important of the affective disorders. Today, they are among the most common mental disorders. Frequency rates de-

pend on the sample and diagnosis criteria and differ between countries and cultures. About 5%-10% of the German population (approx. 4 million people) are afflicted with depressions requiring treatment (point prevalence). Between 10% and 20% of people (8%-12% of men, 10%-25% of women) suffer from a depression at some time in their lives. A large German study found that the lifetime prevalence of a depressive disorder was 16.4% (men: 10.4%; women: 20.4%) (Statistisches Bundesamt, 1998). It is noteworthy that about 50% of people with depression do not consult a physician and about 50% of depressions are not recognised by general practitioners. The average age at onset is between 30 and 45 years for unipolar depressions and 20 to 35 years for bipolar depressions. Late-life depression is the most common mental disorder among people over the age of 65. The prevalence is estimated to be at least 10% (Statistisches Bundesamt, 2010).

1.2 Symptoms

The World Health Organization (WHO) study "Global Burden of Disease 1990" found that depressions have a more negative effect on people's quality of life than cardiovascular diseases and are predicted to be the second most important cause of disability in 2030 (Murray et al., 1997).

The clinical picture of depression can take many forms. The leading symptoms are depressive mood, inhibited drive and thinking and sleep disorders. The degree of depression can range from slightly depressed mood to melancholic, seemingly hopeless and persistent inability to feel anything ("feeling of lack of feeling"). Other symptoms include loss of interest, inability to make decisions, anxiety, inner restlessness, brooding and physical symptoms. Depressive patients have a pronounced suicide risk. Fifteen per cent of patients with a severe depressive disorder commit suicide, 20%-60% have suicide attempts in their medical history

and 40%-80% have suicidal thoughts during a depression. Depressed people have a negative view of themselves and the surrounding world, often become socially withdrawn and sometimes become strangers to themselves (self-alienation).
Some patients can be recognised relatively easily on the basis of their outer appearance and quiet, hesitant voice. Such cases are differentiated into different subtypes: inhibited depression, agitated depression, masked (somatic) depression and Sisi syndrome.

1.3 Aetiology

1.3.1 Genetics

Adoption, twin and family studies have proven that genetic factors play a role in the occurrence of depressive disorders. Twin studies found a concordance rate of 60% for recurrent depressive disorders. In adoption studies, the adopted offspring of depressive parents developed depression more frequently than the offspring of healthy parents. However, studies conducted in recent years were unable to clarify the type of inheritance. If the cause was a larger gene defect, a so-called “major gene effect”, with high penetrance, the concordance rate in identical twins should be a lot higher. For this reason, it is assumed that several genes are most likely involved, whose effects summate and, together with other factors, predispose to depression (Wehling, 2005).

1.3.2 Biological rhythms

Clinical observations indicated already early on that chronobiological factors play an important role. Some depressions are seasonal and occur more frequently in

spring or autumn: the existence of a special type of depression, so-called seasonal depression, was first recognised in the mid-1980s. This kind of depression occurs only in autumn or winter or both and is characterised by special, “atypical” symptoms (including increased appetite and need for sleep).

The daily fluctuations and the nocturnal and early morning awakenings typical of “endogenous” depressions are signs of a disturbance of circadian rhythm. Experimental sleep research has shown that depressive patients show more light sleep stages and fewer deep sleep stages than healthy controls. They have a longer sleep onset latency, REM latency is reduced and various biological rhythms are desynchronised. The antidepressive efficacy of sleep deprivation is assumed to be based on a resynchronisation (Möller H-J. et al., 2005b).

1.3.3 Neuroendocrinology

1.3.3.1 Hypothalamus-pituitary-adrenal axis

A large percentage of depressive patients show abnormalities in tests such as the dexamethasone suppression test or dexamethasone/cortisol-releasing-hormone (CRH) test or show decreased adrenocorticotropin (ACTH) release, all of which reflect an overstimulation of the hypothalamus-pituitary-adrenal (HPA) axis. In contrast to the hypercortisolism caused by an adrenal tumour, in depression the overstimulation is caused by disturbances in CNS control. Dysfunctions in central glucocorticoid receptors are assumed to be responsible. Furthermore, research reports show that depressive patients have lower thyroid stimulating hormone (TSH) plasma concentrations and that the TSH response to thyrotropin releasing hormone (TRH) stimulation is decreased or TRH levels in the CNS are increased (Müller et al., 2005).

The neuroendocrine reaction to stress is regulated by the HPA axis. The cascade starts in the CNS with increased CRH release by the hypothalamus. CRH stimulates the pituitary to release ACTH, which in turn stimulates the synthesis of corti-

sol in the adrenal cortex. In addition to this mechanism, arginine vasopressin (AVP), which is also released during stressful situations, is co-secreted (Scott et al., 1998). AVP also acts on e the pituitary and increases the release of ACTH through a synergistic effect with CRH.

1.3.3.2 Hypothalamus-pituitary-thyroid axis

Hypothyroidism was shown to cause major depression, which remitted after treatment with thyroxine (Bartalena et al., 1990). There is some evidence for a dysfunction of the hypothalamus-pituitary-thyroid (HPT) axis in depression. Thyroxine (T4) levels are increased in some depressive patients (Wahby et al., 1988) as a result of the effects of TSH on TRH (Wahby et al., 1988, Hein et al., 1990). T4 levels and the concentration of free T4 have been reported to decrease after treatment with antidepressants (Gendall et al., 2003).

1.3.4 Amine deficiency hypothesis

For more than 20 years hypotheses have existed which suggest that depressive disorders are related to reductions in the neurotransmitters noradrenaline and serotonin. Studies have shown that concentrations of noradrenaline and serotonin in particular are lower in depressive patients than in healthy controls. The main support for this hypothesis came from the clarification of the mode of action of antidepressants, which increase the concentration of amines in the synaptic cleft. Particularly noteworthy in this context are the tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) (Bunney et al., 1965, Schildkraut et al.,

1965). However, this hypothesis does not explain either why 2 to 3 weeks of treatment are necessary to reduce depressive symptoms, although monoamines are replaced within one to two days, or why other substances such as cocaine or amphetamines (which promote serotonergic and noradrenergic transmission) are not effective in treating depressive patients (Sakash et al., 2002). Furthermore, the hypothesis cannot explain why antidepressants are effective in other dysfunctions such as social phobias (Sheehan et al., 1993) and why other drugs such as tianeptine are effective even though they are assumed to increase serotonin reuptake, i.e. they have the opposite effect to the antidepressant selective serotonin reuptake inhibitors (SSRI) (Loo et al., 1999, Pineyro et al., 1999, Wilde et al., 1995). There is also no explanation as to why the density of serotonin receptors increases after longer-term electroconvulsive therapy, one of the most effective treatments for depression (Butler et al., 1993).

Despite these limitations, the amine deficiency hypothesis drove the development of safe antidepressants such as the SSRIs, which include citalopram, fluoxetine, fluvoxamine, paroxetine and setraline, the selective noradrenaline reuptake inhibitors and the dual antidepressants such as venlafaxine and milnacipran, which modify both the noradrenergic and the serotonergic systems (Charney et al., 1981).

1.3.5 The role of inflammatory changes

The macrophage theory is the first theory to address the role of the immune system and inflammatory changes in the development of depression. In this hypothesis, interleukin (IL) 1β, which is secreted by macrophages, directly stimulates the secretion of CRH by the hypothalamus and induces hyperactivity of the HPA axis. This is a link between the immune system, neuroendocrinology and neurotransmitter replacement in depressions. Acute infections in humans and animals have

been shown to be accompanied by a group of unspecific symptoms, i.e. fever, loss of appetite, hyperalgesia, hypersomnia, anhedonia and depressive mood (Hart, 1988). The release of proinflammatory cytokines such as IL-1β, IL-6 and tumour necrosis factor (TNF) α is an integrated part of the host response to infections. These cytokines play a central role in the interplay of neurotransmitters and the neuroendocrinological system. Stimulation of the HPA axis by IL-1 increases the secretion of ACTH by the pituitary and of glucocorticoids by the adrenal cortex (Tsagarakis et al., 1989). Evidence also exists that IL-1 increases the in vivo turnover rate of serotonin in some regions of the rat brain (Gemma et al., 1994). Furthermore, IL-1 activates the central noradrenergic system. For this reason, in rat models there is a measurable increase in the concentration of 3-methoxy-4-hdroxyphenylglycol, a metabolite of noradrenaline (Dunn, 1988).

Experiments have shown that a systemic injection of lipopolysaccharides (LPS), a component of the cell walls of gram-negative bacteria, not only promotes the release of proinflammatory cytokines but also causes depressions and "sickness behaviour" (Bluthe et al., 1999). Other studies have shown that interferon (IFN), a cytokine used in the treatment of cancer and virus infections, can also cause depressions. When high doses of i.v. IFN-α were administered in short intervals, 20%-60% of patients developed mental disorders. The symptoms remitted after discontinuation of the medication and a brief latency period of a few days (Iivanainen et al., 1985, Meyers et al., 1991, Poutiainen et al., 1994, Mattson et al., 1983, Rohatiner et al., 1983). In contrast to these severe side effects resulting from administration of high doses, less severe neuropsychiatric effects are reported during treatment with lower doses. Also, the latency period is longer and only 4%-16% of patients experience side effects (Schaefer et al., 2002).

Depressions have been proven to be associated with a defective regulation of immune system mediators. In vitro studies have shown that when human monocytes are incubated with various antidepressants and LPS, noticeably less IL-1, IL-6 and TNF-α is released (Xia et al., 1996). Other studies have shown an increase in the anti-inflammatory cytokine IL-10 and a decrease in IFN-γ (Kubera et al., 2000a,

Kubera et al., 2000b). However, there is some controversy concerning the anti-inflammatory effects of various antidepressants. In animal experiments, rats were infected with LPS; the subsequent symptoms such as weight loss, refusal to eat and decreased consumption of a saccharin solution could be treated by chronic treatment with tricyclic antidepressants, but not with venlafaxine or SSRIs (Shen et al., 1999). This finding is supported by another in vivo study in which desipramine reduced the secretion of IL-1 and TNF-α after infection with LPS but no effects were seen with venlafaxine or paroxetine (Connor et al., 2000). In a rat model of olfactory bulbectomy, the increase of acute phase protein was found to be reduced by tricyclic antidepressants and SSRIs (Leonard et al., 2002). Furthermore, SSRIs decreased the release of IL-6 and acute phase proteins in patients with depressions (Sluzewska et al., 1995).

IL-1 indirectly increases the synthesis of prostaglandin E2 (PGE2), an inflammatory mediator and cofactor in the expression of indoleamine 2,3-dioxygenase (IDO). An increased PGE2 concentration was measured in the saliva, blood and cerebrospinal fluid of depressive patients (Nishino et al., 1989, Calabrese et al., 1986, Gerner et al., 1983). On the basis of these findings, the inhibitory function of antidepressants on cyclooxygenase (COX), which is responsible for the synthesis of PGE2, is also being considered as a mechanism for their antidepressant effect, as inflammatory reactions are thus reduced (Leonard, 2001).

Furthermore, in recent years interest has grown in the positive effect of proinflammatory cytokines on the enzyme IDO, which catabolises tryptophan and results in reduced synthesis of serotonin (Carlin et al., 1987, Carlin JM, 1987, Taylor et al., 1991, Yasui et al., 1986). The involvement of proinflammatory cytokines in the pathophysiology of depressions has been clearly proven. Several mechanisms allow the cytokines to reach the target receptors in the brain from the peripheral blood, including active transport, transport via the circumventricular organs, transport by binding to receptors in the blood vessels that supply the brain and retrograde transport of cytokines along the vagus nerve (Maier et al., 2003). These proinflammatory cytokines have a protective and a degenerative effect on

neurones and glial cells, depending on the concentration and the duration of exposure (Allan et al., 2005).

These observations give rise to the question whether depression is a neurodegenerative disorder that is induced by chronic inflammations.

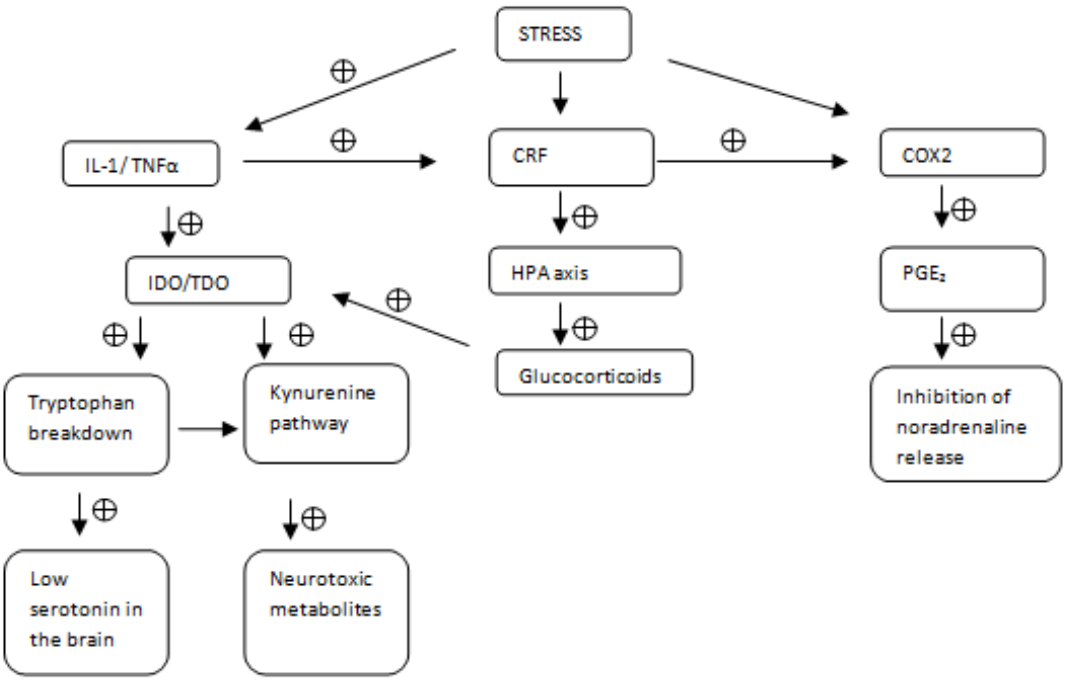


Figure 1: Stress-induced immune activation and the association with neuroendocrine and neurotransmitter changes (adapted from (Myint et al., 2007))

1.4 Immune hypothesis of depression

1.4.1 Psychoneuroimmunology

Psychoneuroimmunology is concerned with the interaction between the nervous, endocrine and immune systems. The interfaces are the pituitary, adrenal glands and immune cells. The focus is on the effect of the psyche on the immune system, for example how stress can have a negative effect on immune factors. Chronic

stress causes a decreased concentration of secretory IgA in saliva and an increased release of glucocorticoids. Corticosteroids inhibit cytokine production and reduce the reactivity of B and T lymphocytes and the activity of natural killer cells.

1.4.2 Basic immunological principles

The cells of the immune system are defined by their surface marker molecules and by the cytokines they secrete. CD3 markers are present on all T lymphocytes (CD3+). These lymphocytes are divided into several subpopulations, which are defined with the help of antibodies and have different functions. They include the T helper cells (CD4+), which induce the immune response, and the cytotoxic T cells (CD8+), which regulate an existing immune response. They also have toxic and lysing effects. Antigen-presenting cells, such as monocytes (the precursor cells of macrophages) and certain types of lymphocytes, release activating cytokines, which activate B and T lymphocytes. The T helper 1 system, which consists of parts of the cellular system, becomes active in acute inflammations. Cytokines characteristic for this process are INFγ, IL-2 and IL-12. These cytokines are produced not only by T helper cells, but also by monocytes/macrophages and other cells. They are referred to as the type 1 immune response. The humoral system, called the type 2 immune response, becomes active in chronic inflammation. Pro-inflammatory cytokines such as TNF-α, IL-1, IL-2 and IL-6 are released mainly by monocytes/macrophages (Muller et al., 2007). In small amounts these cytokines cause activation of the adhesion molecules and chemotaxis, with the aim of initiating an inflammation and recruiting further leucocytes. If they are present in large amounts and if they enter the blood, these cytokines cause the body to react with fever, hepatocytes to produce acute phase protein and more leucocytes to be released by the bone marrow. TNF-α primarily activates the type 1 immune re-

sponse, whereas IL-6 activates the type 2 immune response and promotes antibody production. The type 1 and 2 immune responses are normally in equilibrium. In the CNS, microglial cells and astrocytes are the carriers of the immune response. They also release cytokines: microglial cells mainly release type 1 cytokines, astrocytes primarily type 2 cytokines. These two cell types are thus also in a functional immunological equilibrium (Aloisi et al., 2000). In recent years studies have shown that the proinflammatory and type 1 immune systems are activated in depression.

1.4.3 Tryptophan metabolism

In the CNS, tryptophan-kynurenine metabolism is mainly localised in microglial cells and astrocytes. Proinflammatory cytokines have been found to have a profound effect on the metabolism of serotonin, dopamine and noradrenaline in mouse and rats (Dunn et al., 1999). Clinical studies found significantly increased tryptophan concentrations in patient serum after administration of IL-2 or IFN-α (Brown et al., 1991). The rate-determining step in tryptophan metabolism to kynurenine is the activity of the almost ubiquitously expressed enzyme IDO. The further conversion of kynurenine to either kynurenic acid or 3-hydroxykynurenine, the precursor of quinolinic acid, is regulated by the enzymes kynurenine aminotransferase and kynurenine hydroxylase. Kynurenic acid is an NMDA receptor antagonist, the only endogenous one known so far, and thus interacts with glutamatergic neurotransmission. Quinolinic acid acts as an endogenous NMDA receptor agonist and thus has the opposite effect to kynurenic acid on glutamatergic neurotransmission. The activity of IDO and kynurenine hydroxylase is regulated by cytokines. Type 1 cytokines such as IFN-γ and TNF-α are potent inductors of IDO and kynurenine hydroxylase, while type 2 cytokines

such as IL-4 and IL-10 inhibit them (Weiss et al., 1999). Apart from IFN-γ and TNF-α, also other mediators of inflammation such as PGE2 induce increased IDO activity (Braun et al., 2005, Robinson et al., 2005). The close association between the immune system and kynurenine metabolism is reflected in their important functions in inflammatory diseases. The degradation of tryptophan to quinolinic acid in the CNS can take place in microglial cells and infiltrated monocytes/macrophages but not in astrocytes, because they lack the degrading enzyme 3-hydroxykynurenine (Saito et al., 1993, Alberati-Giani et al., 1996). In humans, the greatest concentrations of quinolinic acid are found in the cortex, not in subcortical regions, which is why it is not surprising that high levels of quinolinic acid are associated with disorders of cortical functions (Heyes et al., 1998). However, quinolinic acid levels in the blood and CNS are not related to each other: during a local inflammatory process in the CNS, quinolinic acid production increases in the CNS but the blood levels remain unchanged. However, an immune activation in the blood can result in an increase of quinolinic acid levels in the CNS (Saito et al., 1993). Tryptophan availability is the limiting factor in serotonin synthesis. A type-1–induced (e.g. IFN-γ), IDO-mediated decrease in the availability of tryptophan in the CNS results in a serotonergic deficit (Grohmann et al., 2003). A positron emission tomography study in depressive patients accordingly found a lack of tryptophan in the limbic and paralimbic cortex (Leyton et al., 2006, Rosa-Neto et al., 2004). In 2003, the possible role of the imbalance between neurotoxic and neuroprotective tryptophan metabolites was proposed as a pathophysiological link between immune activation and neurochemical changes which leads to chronic psychiatric disorders such as depression (Myint et al., 2003).

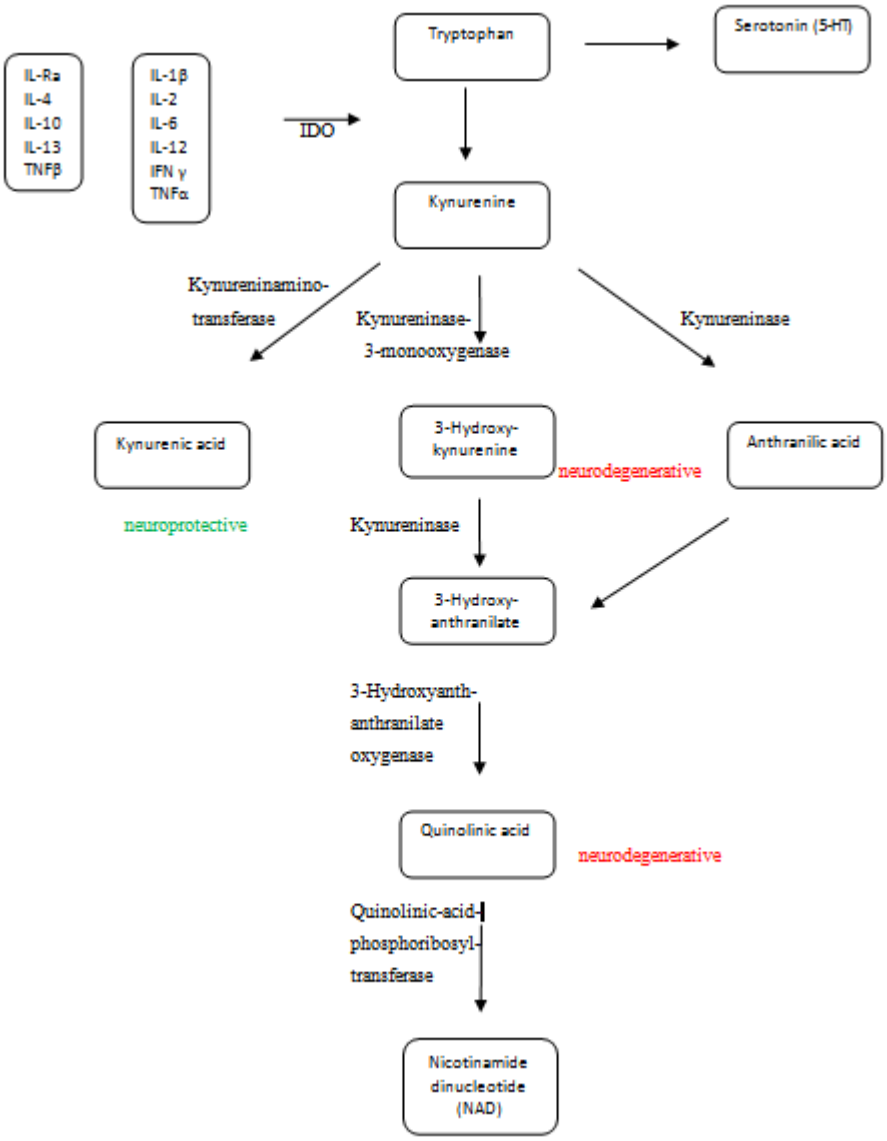


Figure 2: Tryptophan-metabolism (adapted from (Myint et al., 2007))

1.4.3 Kynurenine metabolites and cytokines as possible biomarkers in depression

The term “biomarker” was first introduced in 1989 and defined as a “substance used as an indicator of a biologic state”(Zhang et al., 2009). A biomarker can be the concentration of a certain enzyme or hormone, the presence or absence of a specific biological molecule or a particular genotype that is only present in a population displaying a certain phenotype or endophenotype. Different types of biomarkers exist, providing different information. They either allow an objective assessment of physiological states and their underlying pathogenesis or give information about response to pharmacological treatment. Some markers predict risk or monitor clinical progress, although they are not causally related to the pathophysiology (Zhang et al., 2009).

Biomarkers can be very useful tools in psychiatry. As mentioned above, one of the key goals in psychiatry is to identify possible aetiological factors for disorders (Keshavan et al., 2008). However, biomarkers also have great advantages in the clinical setting. So far, a diagnosis of depression is made only on the basis of clinical features. There is clearly no biological diagnostic test able to supersede a clinical diagnosis based on symptoms, but biomarkers can be very useful in monitoring and predicting treatment response, compliance, better distinguishing between subtypes and providing an additional objective diagnostic tool. Only very recently has evidence accumulated indicating a strong reciprocal connection between the neuronal and immune system. Both appear to communicate via neurotransmitters and cytokines and can even regulate each other (Petrovsky, 2001, Masek et al., 2003, Reyes-Garcia et al., 2009). Cytokines are small proteins that regulate infection in inflammation (Kronfol et al., 2000). Cytokines thus represent possible immunological peripheral markers of physiological states in the brain. Cytokines from the periphery could either enter the brain directly and affect neurons, astrocytes and microglia (Quan, 2008, Monji et al., 2009, Watanabe et

al., 2010) or activate and modulate certain metabolic pathways whose metabolites have harmful effects on the neuronal system. The kynurenine pathway is one of the pathways that have been consistently correlated with major psychiatric disorders (Myint et al., 2007a, Myint et al., 2007b, Kim et al., 2009). The studies by Myint and co-workers suggested that tryptophan breakdown and kynurenine metabolites play a role in all three major psychiatric disorders, namely major depression, bipolar disorder and schizophrenia. Although these disorders have different clinical symptoms, the changes in the interaction between the immune system and the tryptophan pathway show some similarities. Kynurenine metabolites and certain cytokines could thus serve as biomarkers, since specific metabolic and immunological changes might only appear in depression.

1.5 Drugs for the treatment of depression

1.5.1 Antidepressants

Antidepressants are a heterogeneous group of active substances that treat depressive disorders of different causes and with different characteristics by improving mood or increasing drive, or both. They act across disease classifications, i.e. in depressive disorders of different causes. Clinical studies show that antidepressants are effective in the acute treatment of depressive disorders of all severities. Their efficacy is mainly based on their effects on one or more neurotransmitter systems (serotonin, noradrenaline, dopamine) in the CNS. Many antidepressants also affect other neurotransmitter systems (e.g. acetylcholinergic or histamniergic), which can result in side effects. Treatment with antidepressants is divided into acute treatment, maintenance treatment for up to 12 months and relapse prophylaxis that can last years.

The best known antidepressants can be separated into the following groups:

- Tricyclic antidepressants

The tricyclic antidepressants were the most frequently prescribed until the advent of the serotonin reuptake inhibitors. One of their main disadvantages is the relatively long latency of antidepressant effect of 4 to 6 weeks. All tricyclic antidepressants inhibit to varying degrees the reuptake of serotonin and noradrenaline from the synaptic cleft. Furthermore, depending on the type of binding they act as antagonists of different strengths at central receptors. These receptors include histaminergic, muscarinergic, alpha-adrenergic, dopaminergic and serotonergic receptors. The effects at these receptors determine the side-effect profile of the different substances, which is relevant for treatment.

The tricyclics are divided into two main classes: tertiary amines (e.g. imipramine), which act as dual reuptake inhibitors, are metabolised to secondary amines and have particularly pronounced anticholinergic side effects; and secondary amines (e.g. desipramine), which inhibit noradrenaline reuptake somewhat more selectively and have fewer anticholinergic effects. These vegetative side effects are noticeable mainly in the regulation of blood pressure, the digestive system, cardiac rhythm and in the sleep-wake cycle.

- Selective serotonin reuptake inhibitors

These days, selective serotonin reuptake inhibitors (SSRIs) are the most commonly used drugs in depression. In up to 50% of patients they take effect already after 2 to 3 weeks. SSRIs selectively inhibit the reuptake pump for serotonin at the synaptic cleft and thus increase the “relative” concentration of the transmitter. Serotonin can thus bind more to all 5-HT receptor subtypes. The 5-HT$_{1A}$ autoreceptors and presynaptic 5-HT$_{1B/D}$ receptors, which have a control function via a negative feedback mechanism, are also more strongly activated. The continual

stimulation of these receptors results in their destabilisation and down-regulation, which shows a very close temporal correlation with the delayed onset of the anti-depressive effect.

The family of SSRIs includes fluoxetine, fluvoxamine, paroxetine, citalopram, escitalopram and sertraline. They are characterised by a generally mild side-effect profile, but frequent sexual dysfunctions, strong hepatic metabolization and relatively low affinity for histaminergic, dopaminergic, alpha-adrenergic and cholinergic receptors. They alter REM sleep and are safer than tricyclics in case of overdose.

- Selective noradrenaline reuptake inhibitors

Reboxetine is the only selective noradrenaline reuptake inhibitor (SNRI). The reuptake of noradrenaline from the synaptic cleft results in a down-regulation of α_2 and β receptors and in an increased sensitivity of post-synaptic α_1 receptors. The main side effects are sympathetic vegetative effects such as tachycardia, tremor and sweating, but also problems with micturation.

- Selective serotonin-noradrenaline reuptake inhibitors

Like tricyclics, selective serotonin-noradrenaline reuptake inhibitors (SSNRIs) affect the reuptake of both serotonin and noradrenaline. However, they do not block any monoaminergic receptors. Venlafaxine is the only drug of this class currently on the market in Germany.

- Monoamine oxidase inhibitors

Monoamine oxidase inhibitors (MAO inhibitors) act by blocking the enzyme monoamine oxidase. This enzyme cleaves amines such as serotonin and noradrenaline, i.e. transmitters in the synaptic cleft, and thus decreases their availability for transmitting signals in the CNS. Thus, blocking the enzyme results in a "relative" increase of the neurotransmitters in the synaptic cleft. MAO-A metabolises dopamine and phenylethylamine and MAO-B degrades serotonin and noradrenaline.
MAO inhibitors are divided into selective and non-selective MAO inhibitors. Selective reversible inhibitors of MAO-A (e.g. moclobemide) inhibit only type A monoamine oxidase; MAO-B–inhibiting active substances (e.g. selegiline) are primarily used to treat Parkinson's. Non-selective, irreversible MAO inhibitors (e.g. isocarboxazid, tranylcypromine) inhibit MAO-A and MAO-B.

1.5.2 Immune effects of antidepressants

Some antidepressants seem to shift the balance between the type1 and type2 immune response from a proinflammatory to an anti-inflammatory immune response. An in vitro study showed that substances such as sertraline, clomipramine or trazodone significantly reduce the ratio of INF-γ to IL-10 (i.e. the ratio between pro- and anti-inflammatory cytokines). These antidepressants reduce the production of INF-γ. Sertraline and clomipramine, on the other hand, cause a significant increase in IL-10 (Maes et al., 1999). The relevance of cytokines for the pathogenesis of depressive disorders is also confirmed by the fact that when hepatitis C or malignant melanoma are treated with INF-γ, a large proportion of patients develop a depression requiring treatment. Studies have shown a decrease in proinflammatory cytokines after treatment of major depression (MD). This is not a sec-

ondary effect but a direct effect of antidepressants on the immune cells. The exact mechanism has yet to be determined. However, the fact that 5-HT_1 and 5-HT_2 receptors as well as high affinity 5-HT transporters have been demonstrated on the cells suggests involvement of the serotonergic system. This process is supported by the anti-inflammatory and peripherally analgesic effects of 5-HT_2 antagonists (Sasaki et al., 2006). Serotonin antagonist and reuptake inhibitors (SARI) such as trazodone also have this mode of action, which could explain their good efficacy in treating exacerbations of pain in depression, fibromyalgia and somatoform disorders. In vitro studies also suggest that, by binding to the 5-HT_{2C} receptor, trazodone also indirectly inhibits an increase of the second messenger cGMP caused by agonists at the NMDA receptor (Marcoli et al., 1998). Trazodone may also have neuroprotective effects through the inhibition of the glutamatergic effects at the second messenger level.

1.5.3 New treatment approach: COX-2 inhibitors as antidepressants

Prostaglandin E2 (PGE2) is an important mediator of inflammation. Signs of inflammation, increased proinflammatory cytokines and an increased level of PGE2 have been described in MD. An in vitro study reports higher PGE2 secretion from lymphocytes of depressed patients than from those of healthy controls (Song et al., 1998). Also, some animal studies show that COX-2 inhibition can lower the increase of proinflammatory cytokines such as IL-1β, TNF-α and PGE2 and can also decrease clinical symptoms like anxiety and clinical decline. Because of the involvement of PGE2 in the pathophysiology of MD, Müller and coworkers performed a clinical trial using the COX-2 inhibitor celecoxib: the group of patients treated with reboxetine alone showed a 49% improvement of symptoms. In addition to this therapeutic effect, the data clearly showed an advantage and trend towards significance of the combination of reboxetine and celecoxib (Muller et al.,

2006). The clinical antidepressant effect of COX-2 inhibitors can be explained by their anti-inflammatory effects in the CNS. Treatment with a COX-2 inhibitor prevents the dysregulation of the HPA axis and the increase of cortisol, which has a key function in MD (Hu et al., 2005). In an animal model the functional effect of IL-1 (e.g. sickness behaviour) and other proinflammatory cytokines in the CNS were found to be antagonized by treatment with a selective COX-2 inhibitor (Cao et al., 1999). A possible antidepressant effect of the COX-2 inhibitor rofecoxib has already been observed in osteoarthritis patients with comorbid depression (Collantes-Estevez et al., 2003).

1.6 Aim of the Study

The aim of this case-control study was to evaluate the in vitro effects of certain psychopharmaceutical drugs such as reboxetine, venlafaxine, fluoxetine, imipramine and celecoxib on peripheral monocytes and their kynurenine metabolism and cytokine release. Also, the effect of the mitogen LPS on kynurenine metabolism and cytokine release was investigated. Our hypothesis was that inflammatory processes give rise to greater concentrations of the products of this metabolism in the body and that these influence processes in the brain, so that they may be valid biomarkers for depression. Whole blood cultures from depressive patients were compared with those of healthy, age-matched controls. The pro- and anti-inflammatory cytokines were measured with the ELISA analysis technique and the results with and without medication compared. At the same time, the concentrations of the tryptophan metabolites formed with and without psychopharmaceuticals were measured by means of high pressure liquid chromatography (HPLC).

2 Material and Methods

21 patients (9 male and 12 female) displaying major depression were recruited from the psychiatric hospital of the Ludwig Maximilians University (LMU) in Munich. Psychiatric diagnosis was confirmed via clinical interview using DSM-IV criteria. The inclusion criteria also include age between 18-60 years and both gender. Exclusion criteria included acute and chronic infections, chronic inflammatory and autoimmune diseases and anti-inflammatory and immunosuppressive medication. 5 of the patients were “drug-free” patients who did not take any antidepressive medication. The remaining 16 patients took different drugs, including levothyroxine sodium, glimepiride, enalapril, escitalopram, seratraline, zopiclone, lorazepam, paroxetine, thyroxine, amitryptiline, citalopram, levothyroxine sodium and potassium iodide, mirtazapine, venlafaxine and bupropion. History of smoking and drug and alcohol use and other medications were recorded. 38 control persons were matched for age and gender and exclusion criteria are the same as in depressive patients. For both, patients and controls, the same procedure, consisting of questionnaires and blood collection, was applied. Except for HAMD and MRDS interview, this was only done in patients. The study was approved by the responsible Institutional Ethical Committee of the LMU, and all patients and controls provided written, informed consent.

2.1 Psychological Parameter

Personal data of all patients and controls were collected with a short anamnesis. All possible confounding factors, such as weight, height, smoking, drug- or alcohol abusus, acute infection diseases and medication were documented.

For the psychological data following interviews were arranged:

- The M.I.N.I is a short and structured interview for diagnosting psychiatric diseases in DSM-IV and ICD-10. It was developed for not professional interviewers. The interview takes about 15 min, consists of 17 modules and each of them contains one diagnostic criteria. The questions should be answered only with yes or no. Studies have shown that the reliability and validity according to the CIDI were really high but can be arranged in a shorter term (Amorim et al., 1998).

- The Perceived Stress Scale (PSS) is one of the most frequently used psychiological instruments for measuring the perception of stress with a five point Lickert Scale. Response alternatives are: 1. Never, 2. Almost never, 3. Sometimes, 4. Fairly often, 5. Very often. Its main purpose is to provide a measurement to which degree certain situations in one`s life are perceived as particular stressful. Items comprise questions about their lives. It also includes queries about the current level of experienced stress. The items are easy to understand and the response alternatives are simple to grasp (Cohen et al., 1983). There are three different kinds of the scale, either consisting of four (PSS-4), ten (PSS-10) or fourteen (PSS-14) items. In this study, the PSS-14 was used.

- The Life event questionnaire is an inventory-type questionnaire in which subjects mark the life events or changes which have occurred during the past year. It indicate whether the event was considered “good” or “bad” and rates the impact of the event on a 4-point scale.

- The attitude towards life was screened with an interview of eight questions which are related to the daily routine. The possibilities for answering were “yes”, “no” or “unclear”.

- The Hamilton Depression Scale (HAMD) is a depression test measuring the severity of depression symptoms. The scale is basically quantitative. It was constructed for the sole purpose of rating the actual clinical picture, and it is not to be considered a diagnostic tool (Hamilton, 1960).

- The Montgomery-Asberg Depression Rating Scale (MADRS) is a 10-item diagnostic questionnaire which is used to measure the severity of depressive episodes in patients with disorders.

2.2 Biochemical parameters

8 x 5.5 ml of venous blood were withdrawn with a heparinised blood tube (Sigma) between 8-10 am. During a period of 1-2 hours two identical Cell Star 24-well plates (Greiner Bio One) were prepared with stimulant and antidepressants. Peripheral blood was added. After 72 hours of incubation at 37° C, 600µl of supernatant was removed. Cytokines were determined by Millipore`s MILLIPLEX map High Sensitivity Human Cytokine Panel (Millipore Corporation, Billerica, MA). For the different concentrations of kynurenine metabolites Ultra Performance Liquid Chromatography and Mass Spectrometry (UPLC-MS/MS) method was used.

2.2.1 Study design

Each well of the Cell Star 24 well plate contained a total volume of 1ml which was composed out of different antidepressants, RPMI medium with stimulant

(750 µl when no antidepressant was added) and whole blood (250µl). Lipopolysaccharide (LPS) (from Salmonella; Sigma) was used as a stimulant. LPS concentration in each well was 4.3 µg/ml. The antidepressants which were used in this study, were fluoxetine, reboxetine, venlafaxine, imipramine and celecoxib. Antidepressant concentrations in the appropriate wells were 50ng/ml, 200ng/ml, 250ng/ml, 300 ng/ml and 350ng/ml respectively. Afterwards, the plate was incubated for 72 h at 37 degree in humidified chamber with 5 % CO2.

2.2.2 In vitro LPS stimulation

LPS is released from Gram-negative bacteria and is a strong inducer of the innate immunity (Bernardi et al., 2009, Wang et al., 2006). It acts through the toll-like receptor 4 (TLR-4), amember of the toll-like family that play a central role in the recognition of infectious pathogens and are expressed on immune cells, including macrophages, dendrites, B and some Tcells (Wang et al., 2006). LPS binds TLR-4 receptor via an leucine-rich extracellular domain and signaling involves the recruitment of a number of intracellular adaptor protein that activate transcription factors and protein kinases that induce production of inflammatory agents (Wang et al., 2006). In experimental biology, LPS is used to mimic a bacterial infection.

2.3 Sample analysis

2.3.1 Cytokine concentrations

The supernatant concentrations of IL-4, IL-10, IFNγ and TNF-α were determined using Millipore´s MILLIPLEX map High Sensitivity Human Cytokine Panel

(Millipore Corporation, Billerica, MA). Detection range was 0,13 pg/ml to 10000pg/ml. Acquired fluorescence data were analysed using Bio-Plex software (version 4.1; Bio-Rad Laboraties). Preparation assay was performed according to the manufacturer`s protocol by using customized reagents and solutions.

2.3.1.1 Milliplex™ map kit principle

Multianalyte profiling for IL-4, IL-10, IFN- γ, TNF- α in whole blood supernatant was performed on the Bio-Plex Luminex system (Bio-Rad Laboratories, INC., Hercules, California), which is based on the Luminex® x MAP® technology. Luminex ® uses probrietary techniques to internally colourcode microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly colored bead sets can be created. Each of which is coated with a specific capture antibody. After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. The microspheres are allowed to pass rapidly through a laser which excites the internal dyes marking the microsphere set. A second laser excites PE, the fluorescent dye on the reporter molecule. Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter signal. This capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample. Open-architecture x MAP r technology enables multiplexing of many types of bioassays reducing time, labor and costs over traditional methods. This Bio-Plex suspension arry system is a flow-based dual-laser system for simultaneously identifying and quantitating up to 100 different analytes in a single biomolecular assay.

Reagents supplied by the kit:	**Materials required but not provided:**
High Sensitivity Human Cytokine Standard High Sensitivity Human Cytokine Quality Controls 1 and 2 Set of one 96- Well Filter Plate with 2 Sealers Assay Buffer 10x Wash Buffer High Sensitivity Human Cytokine Detection Antibodies Streptavidin- Phycoerythrin Mixing bottle Premixed 13 plex Beads for IL-10, IL-4, IFNγ, TNFα	Luminex Sheath Fluid Adjustable pipets with Tips capable of delivering 25 µl to 200µl Multichannel Pipets capable of delivering 5 µl to 50µl Reagent reservoirs Polypropylene Microfuge Tubes Rubber Bands Absorbent Pads Laboratory Vortex Mixer Sonicator Titer Plate Shaker Vacuum Filtration Unit Luminex 100 tm IS, 200 tm or HTS by Luminex Corporation Plate Stand

Table 1: Reagents and materials for cytokine analysing

2.3.1.2 Preparation of reagents

Quality Controls

Before use, Quality Control 1 and Quality Control 2 were reconstituted with 250 µl deionized water. The vial was inverted several times to mix and let rest for 5-10 minutes.

High Sensitivity Human Cytokine Standard

Prior to use, High Sensitivity Human Cytokines Standard was reconstituted with 100 µl deionized water to give a 10000 pg/ml concentration of standard for all analytes. The vial was inverted several times to mix and vortexed for 10 seconds. Then it was let rest for 5-10 minutes.

Working Standards

The standard working solution ranged from 0.13 to 10000pg/ml. 130 µl of deionized water was added to one tube and 200 µl of assay buffer to six other tubes. 30 µl of the 10000pg/ml was added to the first tube with the deionized water, to get 2000 pg/ml concentration. Then 50µl of the 2000 pg/ml is transferred to the next tube to create 400pg/ml concentration. Always 50 µl of the former dilution is added to the next tube. This gave the concentrations of 80 µl, 16µl, 3.2µl, 0.64µl and 0.13µl, respectively. The 0 pg/ml standard (Background) was Assy Buffer.

2.3.1.3 Immunoassay procedure

The filter plate was pre-wet by adding 200µl wash buffer to each well. Then it was sealed and shook on plate shaker for 10 minutes at room temperature. Washing buffer was removed by vacuum and 25µl of premixed beads were added to each well. The fluid was removed by vacuum again. 50µl of standard or control were added in the appropriate wells. 50µl of assay buffer was added to the background and sample wells and 50 µl of RPMI medium was added to the control and standard wells. Then after centrifuging the samples, 50 µl of each was added to the appropriate wells. The plate was incubated for 17 hours at 4°C with shaking. After 17 hours, the fluid was gently removed by vacuum and the plate was washed twice by vacuum filtration between each wash. Subsequently 50µl of Streptavidin-Phycoerythrin was added to each plate and incubated again for 30 minutes at room-temperature for 30 minutes. In the next step, all contents were removed by vacuum and washed twice with 200µl washing buffer with vacuum filtration between each wash. In the last step, 100µl of Sheath Fluid was added to all wells and shaked for 5 minutes to suspend beads. The plate was run on Luminex 100 Tm and acquired fluorescence data were analysed using Bio-Plex software (version 4.1; Bio-Rad Laboraties).

2.3.2 Tryptophan and Kynurenine concentration

2.3.2.1 Solid-phase extraction

Solutions	Ingredients
Internal standard	7.5 µl N-TYR stock solution (1mg/ml) dissolved in 1 ml 9 M H_3PO_4 (phosphoric acid)
Equilibration fluid 1	Methanol
Equilibration fluid 2	H_2O
Washing solution	0.1 M citric acid
Elution fluid	200 ml MTBE (ter-butyl methylether) with 400 ml acetonitril and 5% NH_4OH (Ammoniumhydroxid)

Table 2: Solutions for solid-phase extraction

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 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°. In the following 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.

2.3.2.2 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 withon 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 quadropole 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).

2.4 Statistical analysis

The Kolmogorov-Smirnov test was used to check the normality of the data in patients and control groups. Descriptive statistics together with plots (error bars correspond to 95% confidence intervals) were provided. To compare skewed data between groups a non-parametrical test Mann Whitney U test was applied. For normally-distributed data, the two sample Student´s t-test was applied. To control for the different confounding factors multiple univariate analysis was used. These tests were made with SPSS 18.0 (SPSS Inc., Chicago, Illinois).

The null hypothesis was rejected at $P < 0.05$. For faster illustration in the graphs, the following categories for P-values were used: < 0.001 = ***, from 0.001 until 0.01 =**, from 0.01 until 0.05 =* and from 0.05 until 0.1 = # (tendency).

For the summary, statistical results were summarized in an Excel spreadsheet (Microsoft Office) and the mean values were integrated as a graphical presentation in the text.

3 Results

3.1 Demographic data

Altogether we included 59 Caucasian study participants - 21 patients suffering from depression and 38 healthy control individuals – who fulfilled the inclusion criteria as described in chapter 2 “Material and Methods”. Several psychopathological scores like PSS, life event - and attitude towards life questionnaire, M.I.N.I, HAMD and MADRS were carried out. The median age for patients was 43.19 and for healthy controls 45.61. The percentage of men was similar in the patient group (75%) and in the control group (73%), so the male:female ratio did not differ significantly between the two groups . The same was true for body mass index (BMI; $p = 0.460$) and the age ($p = 0.201$). The parameters medication ($p = 0.356$), nicotine abusus ($p = 0.754$) and race ($p = 1.0$) showed no difference between the groups. A significantly higher number of patients than healthy controls had a history of alcohol consumption ($p = 0.04$) and a family history of depression ($p< 0.001$). Concerning the medication status, only five patients did not receive any medication before the diagnosis MD was confirmed. So only these people were drug naïve with a recent onset of MD.

Tabel 3 is presenting the epidemiological and clinical data of the 59 participants who were included into our study (Tab.3).

	Patients with major depression (n=21)	Healthy controls (n=38)
Sex (male/female)	9/12	16/22
Age (years)	43.19	45.61
BMI (kg/m^2)	24.14	25.58
Family History (yes/no)	8/13	0/38
Alcohol	4/17	0/38
Drug	1/21	0/38
Nicotine	4/21	9/38
Antidepressant medication status		
Medication-free	5/21	
Psychopathology scores:		
PSS	45.57	42.18
Life Events		
Attitude towards life	54.07	42.18
HAMD	13.93	
MADRS	18.86	

Table 3: Demographic data of study participants

3.2 Immunological findings: cytokines

Whole blood cultures were stimulated with LPS and different antidepressant medications were added. After incubation for 72 h, the supernatant was removed. The concentrations of the cytokines IFN-γ, IL-4 and IL-10 were determined by Milli-

pore´s MILLIPLEX map High Sensitivity Human Cytokine Panel. IFN-γ levels, which characterize the Th1 immune response, and IL-4 and Il-10, which represent the Th2 immune response are presented separately below.

3.2.1 Th1/proinflammatory cytokines: IFN-γ

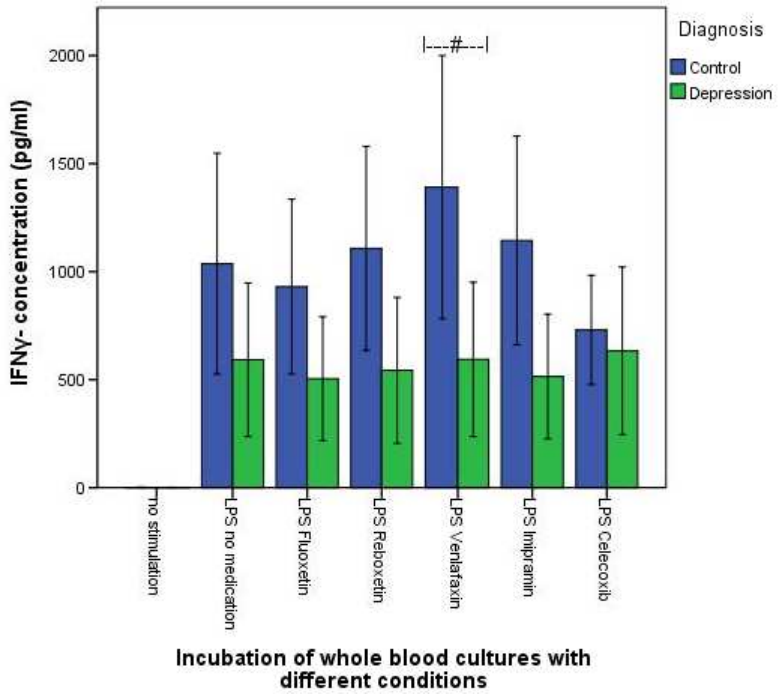


Figure 3: Mean in vitro IFNγ concentrations (pg/ml) in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups, error bars correspond to 95% confidence intervals, and ´#` indicates a p-value in the range 0.05 to 0.1 (exceptionally Mann-Whitney U test was used here).

The mean IFN-γ production before and after LPS stimulation was compared between patients and controls. The controls` mean values were numerically higher across the different culture conditions, however, none of the differences was sta-

tistically significant, although the culture condition treated with LPS and venlafaxine showed a trend towards statistical difference ($p = 0.071$; Mann-Whitney U test) (Fig. 3).

In the culture from healthy controls, which contained celecoxib as a antiinflammatory drug, the mean value of the IFN-γ concentration reached the lowest level compared to other medications after LPS stimultation. This numerical decline of IFN-γ was only detectable in the blood from healthy controls but not in the blood from patients.

Group also had no effect (either depressed or control) on the level of IFN-γ when the parameters family history and alcohol were controlled in a univariate analysis of variance.

3.2.2 Th2/antiinflammatory cytokines: IL-4, IL-10

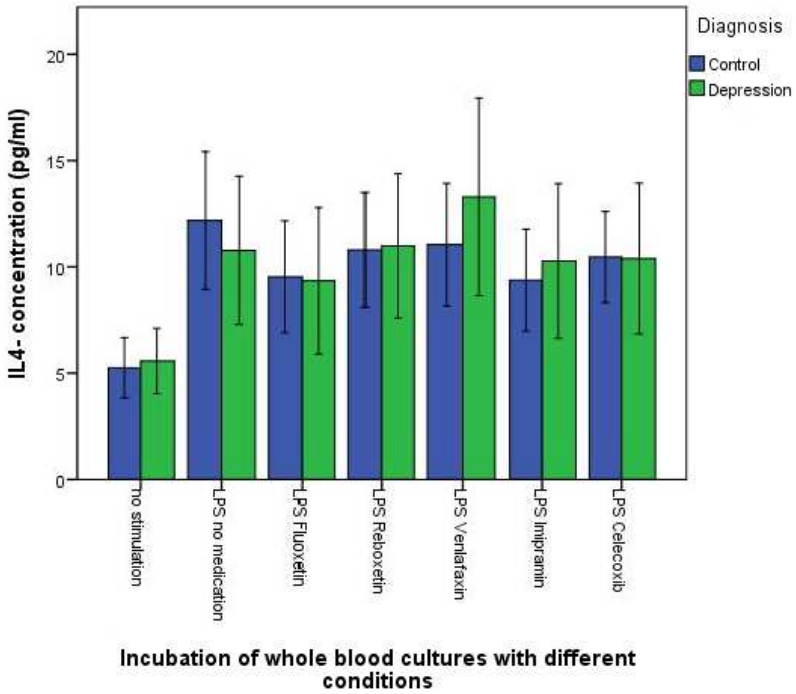


Figure 4: Mean in vitro IL4 concentrations (pg/ml) in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups and error bars correspond to 95% confidence intervals.

After incubation under different conditions, no significant differences were found between IL-4 levels in the depressed and control groups, although the absolute values increased (Fig.4). However, in a univariate analysis of variance, alcohol consumption showed an effect on IL-4 when blood cultures were stimulated with LPS ($p< 0.001$), and treated with fluoxetine ($p = 0.048$), reboxetine ($p = 0.005$), venlafaxine ($p = 0.002$) or imipramine ($p = 0.015$), but not with celecoxib. Celecoxib had no significant effect on IL-4 production. Family history also correlated significantly with the IL-4 level after treatment with LPS and reboxetine ($p = 0.008$).

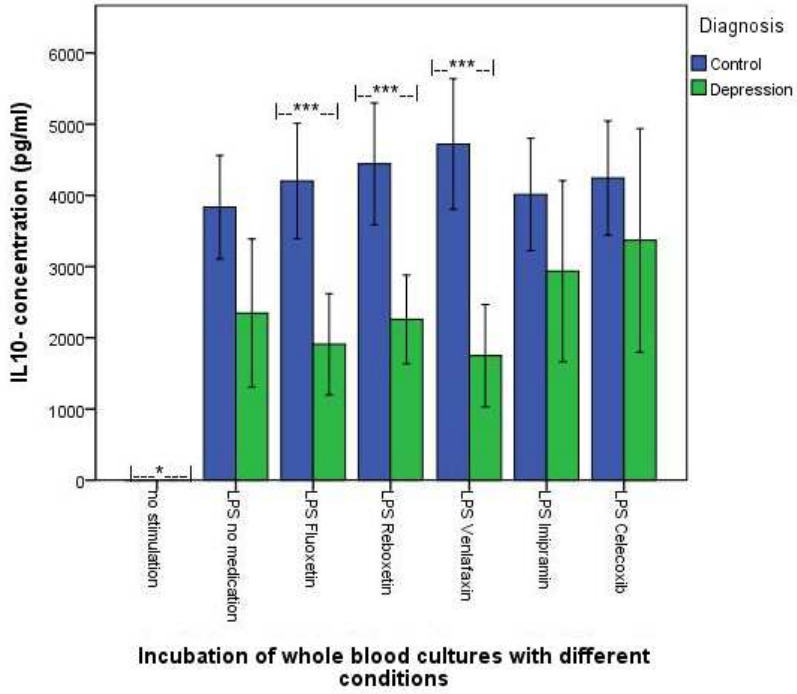


Figure 5: Mean in vitro IL10 concentrations (pg/ml) in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups, error bars correspond to 95% confidence intervals, ´*` indicates a p-value in the range 0.01 to 0.05 (exceptionally Mann-Whitney U test was used here) and ´***`indicates a p-value < 0.001.

The mean IL-10 values of the control group were higher than those of the patient group across all conditions. However, the differences only reached statistical significance in the unstimulated culture ($p = 0.039$; Mann-Whitney U test). Significant differences were observed for the cultures with LPS and fluoxetine ($p< 0.001$), reboxetine ($p< 0.001$) and venlafaxine ($p< 0.001$). The significant difference between both groups was abolished in the cultures which were treated with LPS and imipramine or celecoxib mainly due to a numerical increase of the IL-10 concentration in the patient group (Fig.5).

Group (either depressed or control) had no effect on the level of IL-10 when the parameters family history and alcohol were controlled in a univariate analysis of variance.

3.3 Metabolites of the Tryptophan pathway

One of the main questions was the effect of antidepressant drugs and celecoxib on the kynurenine pathway. This section presents the concentrations and ratios of the TRP catabolites in the order of the biochemical pathway. The mean concentrations are presented and compared and the influence of confounding factors like family history, alcohol consumption and diagnosis are controlled.

3.3.1 Metabolites

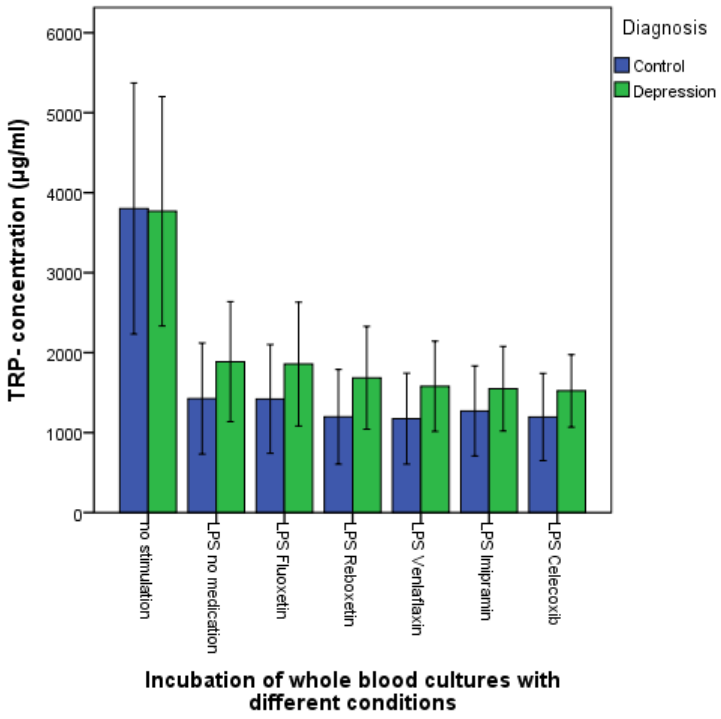


Figure 6: Mean in vitro TRP concentrations (µg/ml) in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups and error bars correspond to 95% confidence intervals.

Stimulation with LPS induced a reduction in TRP levels. Remarkably, in every condition mean TRP concentration, after adding LPS, were higher in patients than in controls (Fig.6). However, the differences did not reach statistical significance in any of the conditions in either the t-test or when confounding factors were considered in a univariate analysis of variance.

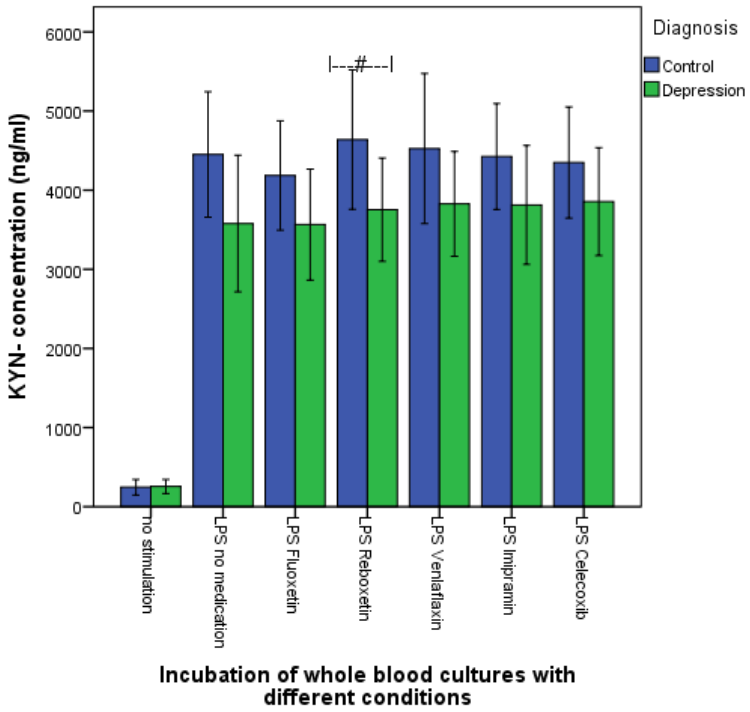


Figure 7: Mean in vitro KYN concentrations (ng/ml) in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups, error bars correspond to 95% confidence intervals and ´#` indicates a p-value in the range 0.05 to 0.1.

The means of KYN concentrations were numerically higher in controls than in patients. However, the differences were not significant and only the difference in the culture treated with LPS and reboxetine showed a trend towards statistical

difference (p = 0.084) (Fig.7). When tested for confounding factors in a univariate analysis of variance, no significant differences were observed.

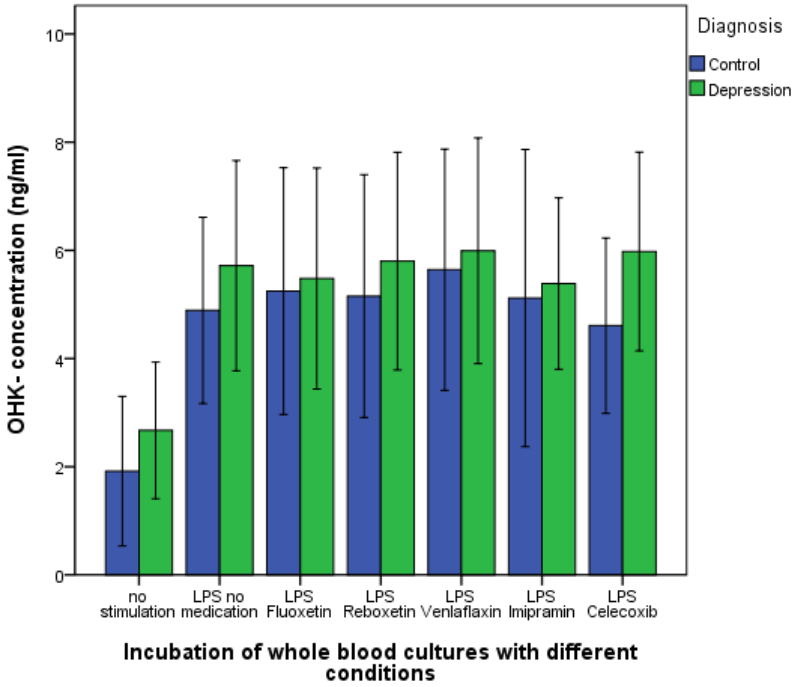


Figure 8: Mean in vitro OHK concentrations (ng/ml) in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups and error bars correspond to 95% confidence intervals.

In every condition, the mean OHK concentrations were slightly higher in the depressed group than in the controls; however, none of the differences was significant (Fig.8). Also, a univariate analysis of variance showed no statistically significant findings related to alcohol consumption or family history.

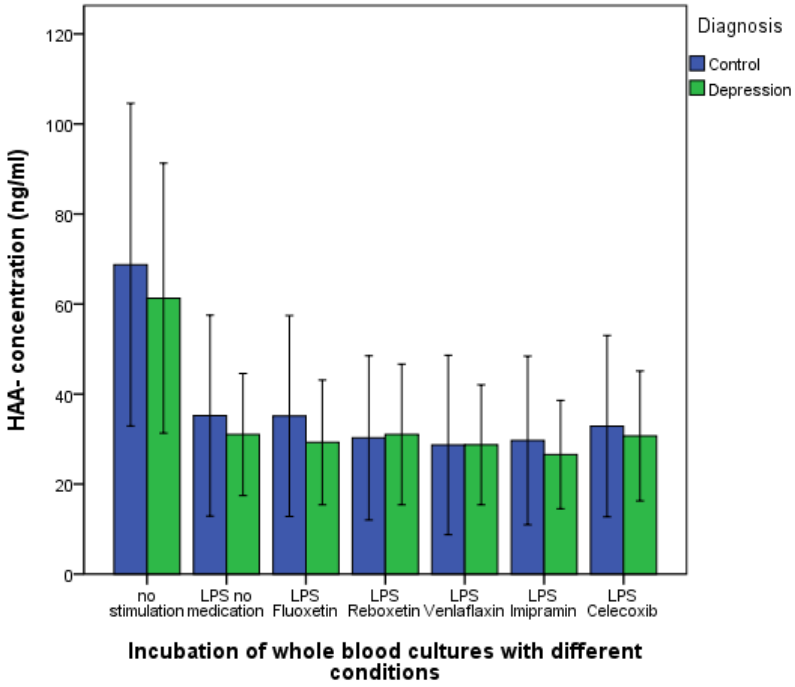


Figure 9: Mean in vitro HAA concentrations (ng/ml) in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups and error bars correspond to 95% confidence intervals.

AA showed no significant differences in the mean concentration between the two groups. However, it is noteworthy that HAA is the only parameter in the catabolic pathway of TRP, that decreased after LPS induction (Fig.9). A univariate analysis of variance showed no statistically significant findings relating to alcohol consumption or family history.

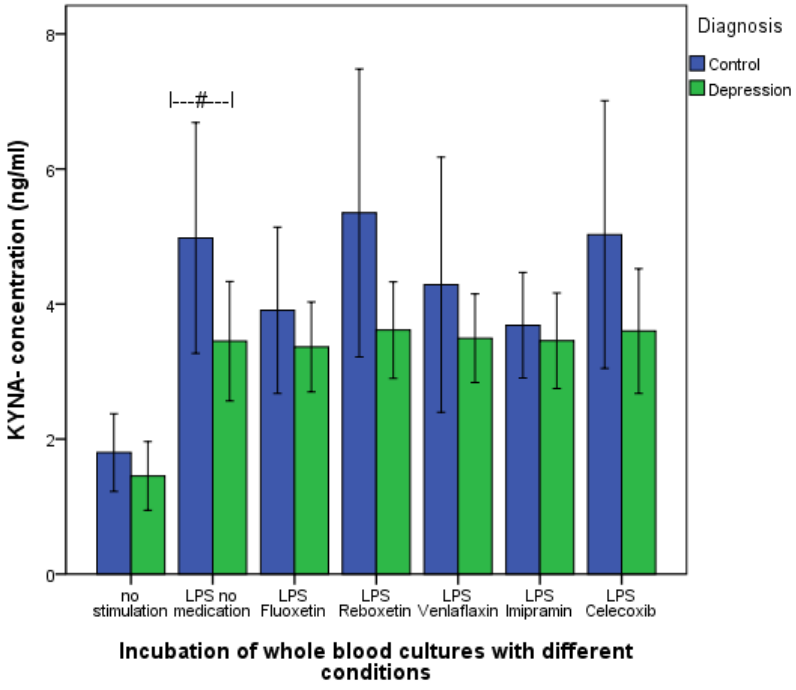


Figure 10: Mean in vitro KYNA concentrations (ng/ml) in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups, error bars correspond to 95% confidence intervals and ´#` indicates a p-value in the range 0.05 to 0.1.

The mean concentrations of the neuroprotective metabolite KYNA increased after LPS stimulation in both groups as assumed. Values were higher in the control group than in the patient group, but differences were not significant. Only the stimulation with LPS showed a trend towards a plunted increase in patients ($p = 0.084$) (Fig.10). The addition of medications did not result in a difference between patients and controls.

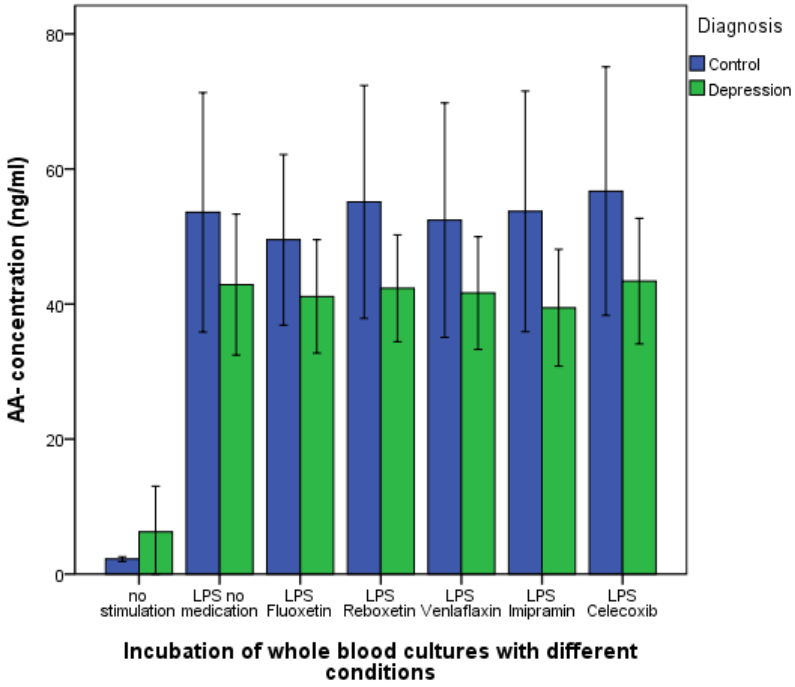


Figure 11: Mean in vitro AA concentrations (ng/ml) in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups and error bars correspond to 95% confidence intervals.

In vitro production of the metabolite AA increased with LPS stimulation and was higher in the control group than in the patients group across all culture conditions (Fig.11). However, differences were not significant in either the t-test or when influencing parameters were considered in a univariate analysis of variance.

3.3.2 Ratios between the metabolites

For analysing biochemical pathways, it is important to investigate not only the levels of the single intermediates but also to focus on the ratios between the metabolites. Out of that we can get informations about the conversion rates of the distinct enzymatic steps. Before going into details, it can be summarized that nearly all ratios, except OHK/KYN, were higher in controls than in patients.

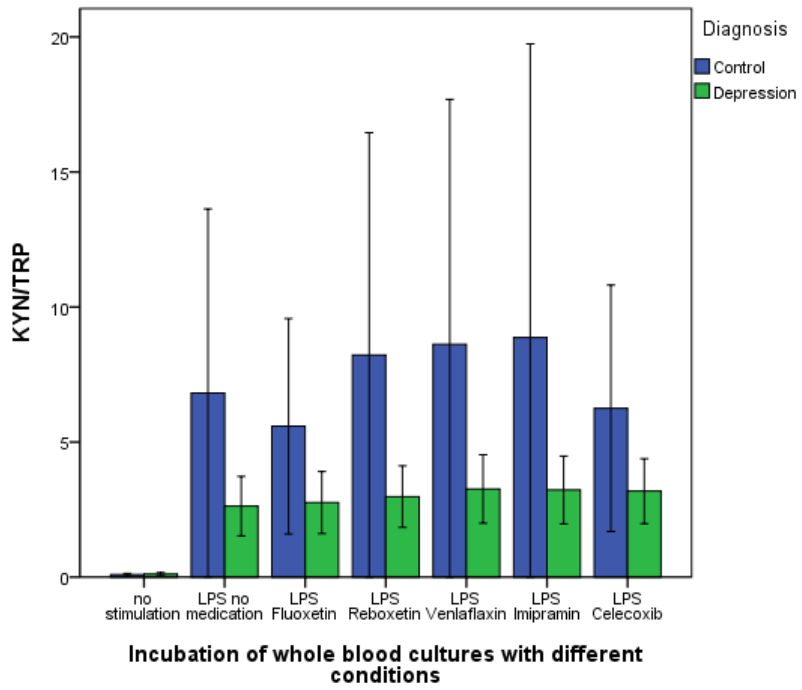


Figure 12: Ratio of mean concentrations of KYN/TRP in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups and error bars correspond to 95% confidence intervals.

The ratio of KYN/TRP describes the first enzymatic step in the degradation pathway and is referred to as the “tryptophan breakdown index”. It is calculated by: kynurenin/tryptophan. The index indicates indirectly the sum of activities of TDO

and IDO. Generally, the ratios of different conditions were numerically higher in the control group, but none of the differences between groups was significant (Fig.12).

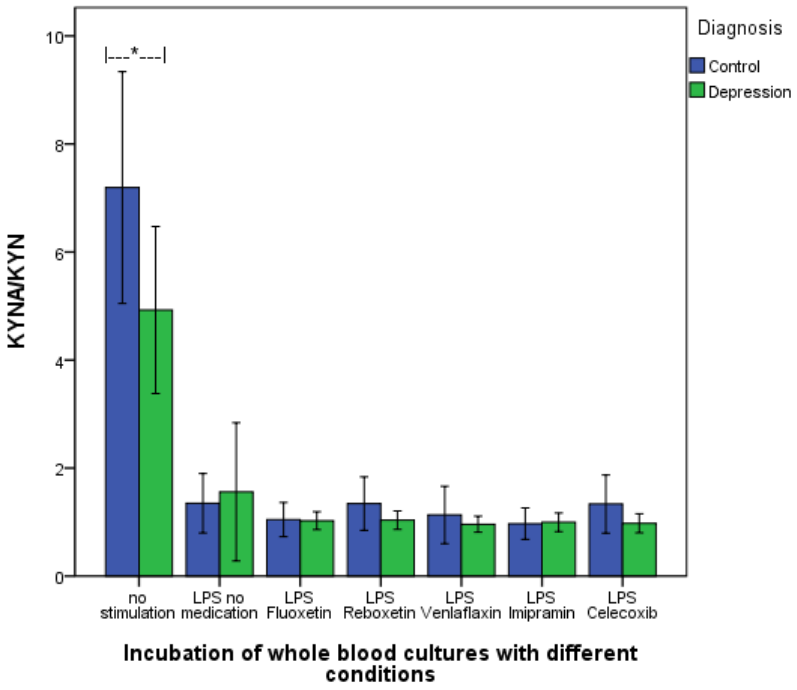


Figure 13: Ratio of mean concentrations of KYNA/KYN in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups, error bars correspond to 95% confidence intervals and ´*` indicates a p-value in the range 0.01 to 0.05.

The ratio KYNA/KYN allows a statement to be made regarding the neuroprotective and neurodegenerative distribution of the metabolites, because both KYNA and QUIN are formed from KYN. Therefore, this ratio is also called as the “neuroprotective ratio”. In the unstimulated condition, the ratio was significantly higher ($p = 0.045$) in the controls than in the patients. This difference was no longer present when the blood was stimulated. Generally, the ratio decreased after stimulating the whole blood cultures (Fig.13).

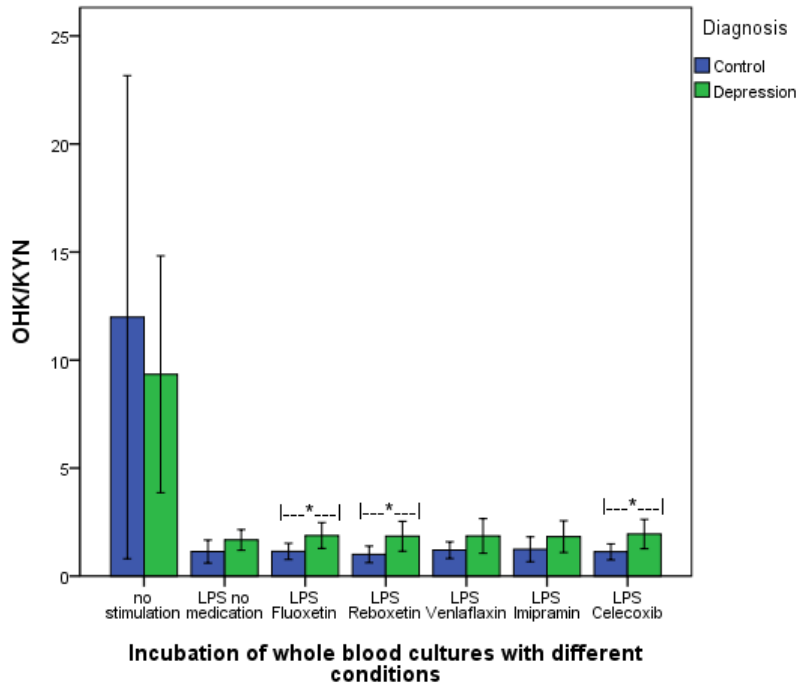


Figure 14: Ratio of mean concentrations of OHK/KYN in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups, error bars correspond to 95% confidence intervals and ´*` indicates a p-value in the range 0.01 to 0.05.

The OHK/KYN ratio decreased in both patients and controls after treatment with LPS alone or with antidepressant drugs. This part of the catabolism represents the first step towards the neurodegenerative metabolite QUIN. In the unstimulated condition, the mean ratio of OHK to KYN was higher in controls than in patients, but this relation was reversed after stimulation, i.e. it was higher in the patient group than in the control group. The mean OHK/KYN ratio was significantly higher in the patient group than in the control group in stimulated cultures treated with fluoxetine ($p = 0.033$), reboxetine ($p = 0.03$) and celecoxib ($p = 0.03$) although there were no significant differences in the individual metabolites OHK and KYN (Fig.14).

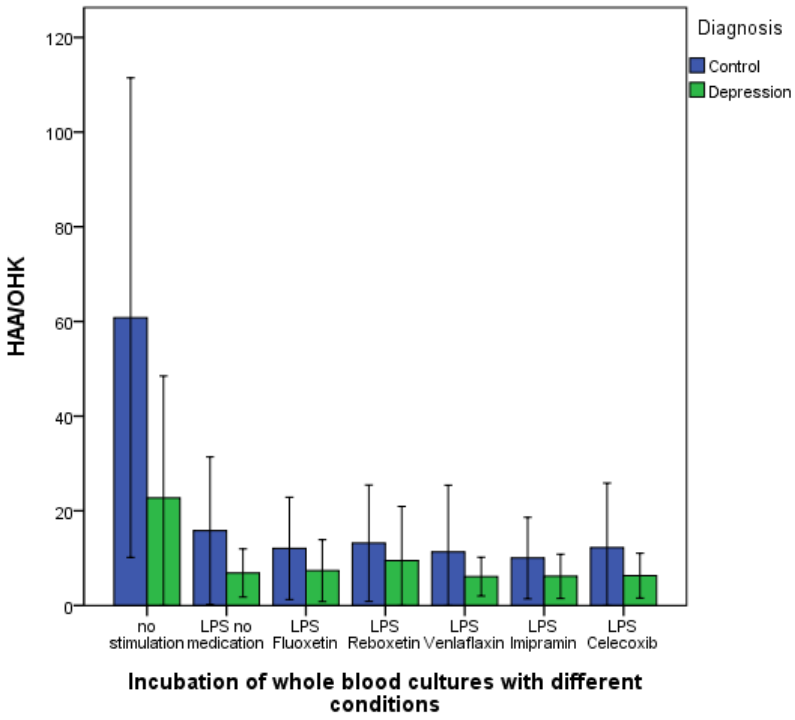


Figure 15: Ratio of mean concentrations of HAA/OHK in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups and error bars correspond to 95% confidence intervals.

The next step in the enzymatic conversion towards QUIN is expressed by the HAA/OHK ratio. In this step, the mean ratio was also higher in the controls. No significant differences in the HAA/OHK ratio were found in any of the conditions with either with the t-test or in the univariate analysis of variance (Fig.15).

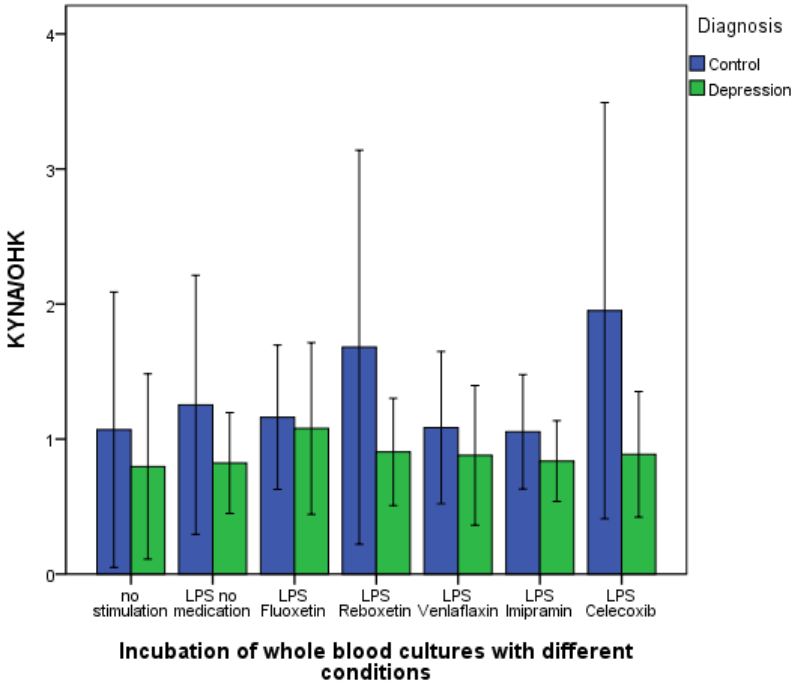
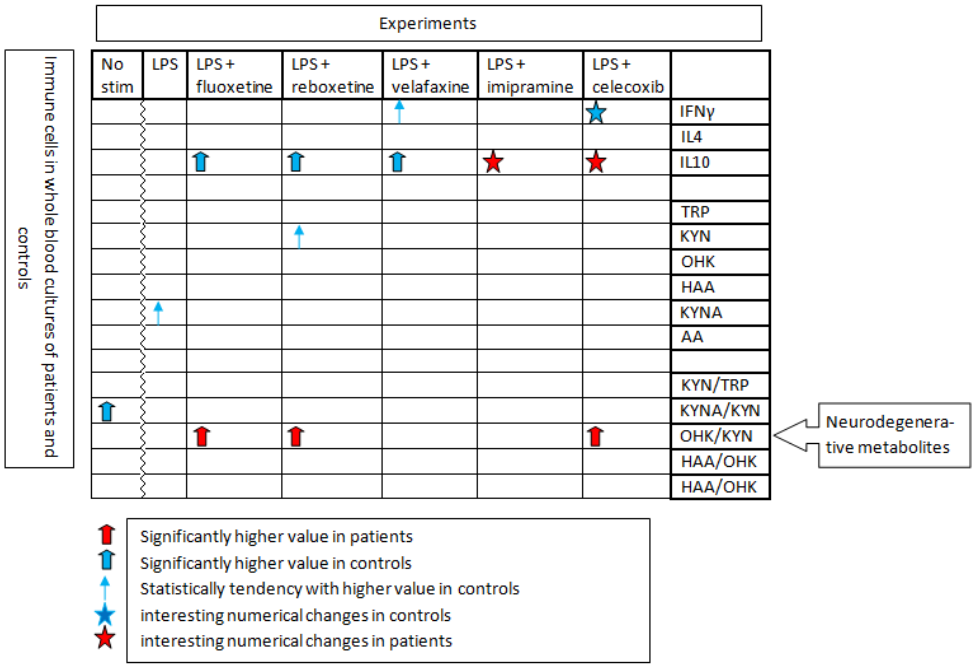


Figure 16: Ratio of mean concentrations of KYNA/OHK in whole blood cultures from patients with major depression and healthy controls after incubation of cultures under different conditions. Bars (Control, Depression) show the concentrations in the two study groups and error bars correspond to 95% confidence intervals.

We used the ratio KYNA/OHK to examine the ratio between the neuroprotective metabolite KYNA and the neurotoxic metabolite OHK. The mean ratios of KYNA to OHK were higher in the control than in the patient group across all culture conditions. The data showed no significant differences in either the t-test or in the univariate analysis of variance (Fig.16).

3.4 Summary of the results



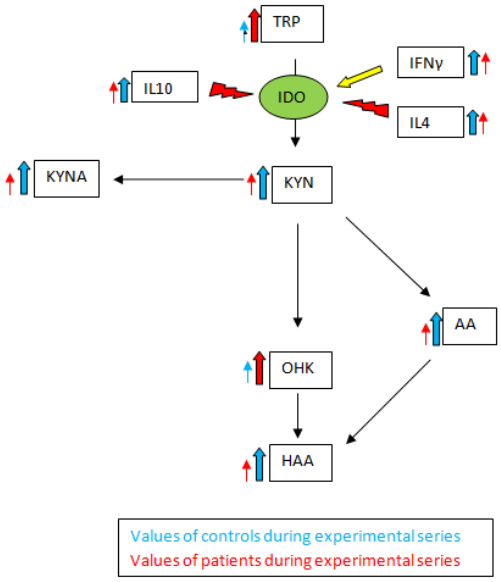


Figure 17: Graphical summary. Whole blood cultures from depressed patients and controls were first treated in vitro either without stimulation or with LPS and then different antidepressants or celecoxib were added. Cytokines and tryptophan pathway metabolites were measured. Up arrow indicates elevated levels either in controls (blue) or in patients (red).

4 Discussion

Several lines of evidence point out the important role of the pro-inflammatory immune process in the pathophysiology of depression. Moreover, several in vivo and in vitro studies have demonstrated a modulating effect of antidepressant drugs on the immune system. On the other hand, clinical trials have shown the antidepressant effect of anti-inflammatory drugs like celecoxib. The missing link in understanding the mechanism was the proposed translation of immune signals into neurotransmitter changes as the physiological basis of altered mood states. The kynurenine pathway provides several functional links between pro- and anti-inflammatory cytokines on the one hand and the neuroactive tryptophan pathway intermediates including serotonin and some kynurenine metabolites on the other hand. Using in vitro stimulated peripheral immune cells, the aim of this study was to investigate the effect of antidepressants and celecoxib on the production of pro- and anti-inflammatory cytokines and on the formation of kynurenine pathway intermediates.

4.1 Cytokines

As part of our research project we investigated changes in cytokines by examining whole blood cultures from depressed patients and healthy controls before and after stimulation with LPS and antidepressants. Some antidepressants influence the proinflammatory immune state in depressive disorders. The enzyme IDO metabolizes TRP to KYN, and KYN is then converted to 3HK by the enzyme kynurenine hydroxylase, through which the metabolite QUIN is formed later. Both enzymes are induced by the T-helper type 1 cytokine IFNγ and inhibited by type 2 cytokines such as IL-4.

In our study, the production of the proinflammatory cytokine IFNγ in response to immune challenge increased more in controls than in patients. Kim and co-workers made the same observation in an in vitro study (Kim et al., 2007). IFNγ response was lowered only by celecoxib and only in cultures from healthy controls while this reversal was not observed in the patients. This lower production of cytokines in response to LPS stimulation indicates immune cell exhaustion, which means that patients´ cells might have been in an LPS-refractory phase induced by a pro-inflammatory state, as described for endotoxin tolerance (Biswas et al., 2009). We also found that IFNγ was increasing with venlafaxine in the cultures from control´s group and did not change in the patients` blood. In contrast to other studies (De Berardis et al., 2010) we couln´t support the notion that venlafaxine influences the proinflammatory cytokine secretion in patients.

Stimulation with LPS enhanced production not only of IFNγ but also of the anti-inflammatory cytokines IL-4 and IL-10. Production increased significantly more in controls than in patients. However, this significant difference no longer existed when the cells were treated with imipramine and celecoxib. This indicates that the medications imipramine and celecoxib could reverse the abnormal response in the blood cells of the patients. Strong evidence of an immunosuppressive effect of imipramine was also demonstrated by Szuster-Ciesielka, who found that imipramine decreased the production of proinflammatory cytokines (IL-2, IL-4, IFNγ, and IL-12) while it stimulated anti-inflammatory cytokines (IL-10 and TGF-β) (Szuster-Ciesielska et al., 2003). Also Kubera et al. found that several antidepressant compounds – including imipramine, venlafaxine, 1-5-hydroxytryptophan and fluoxetine – increased production of the anti-inflammatory cytokine IL-10 and reduced the INF-γ/IL-10 ratio significantly (Kubera et al., 2000a). Taken together, we can expect that patients were rather in a proinflammatory state, which was mainly due to a reduction in anti-inflammatory signals and not to enhanced proinflammatory signals.

Several mechanisms have been proposed for the depressogenic action of cytokines, including the induction of extrahypothalamic CRF and vasopressin, the development of glucocorticoid resistance, the activation of IDO, and the increased expression of the serotonin transporter (Miller, 2009, Miller, 2008). Many studies show that treatment with cytokines results in depressive mood changes. Another hypothesis as to why a pro-inflammatory state could result in depression is the neurodegeneration hypothesis of depression, which proposes a cytokine-induced imbalance in kynurenine metabolites as part of the pathophysiological process (Myint et al. 2003).

In the publication of their study, Kim et al. (2007) wrote that many research projects try to find biomarkers that predict the formation, development and, in the best case, remission of a depressive illness. This was also the aim of our study. Numerous studies have been published on the association between depression and immune and cytokine function. However, fewer have investigated the function of cytokines in the response of depression to antidepressant medications. After treatment, we would logically expect cytokine levels to normalize and the depressive episode to resolve. However, a recent meta-analysis by Hannestad and coworkers contradicted this idea (Hannestad et al., 2011). Although on the one hand they confirmed elevated levels of circulating proinflammatory cytokines, on the other they showed that antidepressant drugs do not have a significant effect on serum levels of cytokines. Their results are not fully comparable to ours because they analysed TNFα, IL-1β and IL-6, which were chosen because they are elevated in depression (Dowlati et al., 2010, Howren et al., 2009). Thus, these authors concluded that proinflammatory cytokines contribute to depressive symptoms and that normalization of cytokine levels is not associated with remission. Maes et al. (2011) may have an explanation for this phenomenon. In their study, they showed that this missing effect of the drugs might be associated with autoimmune responses directed against 5-HT. The incidence of anti-5-HT-antibody positivity is significantly higher in depressed patients, regardless of whether or not

the patients were medicated. Contradictory to the statement made by Hannestad et al. (2011), Janssen et al. (2010), who reviewed the scientific literature from the past 20 years, found that antidepressants appear to normalize serum levels of cytokines, especially TNFα, INF-γ, IL-6 and IL-1β.

Generally, it is important to note that a multiplicity of medications act differently on a multiplicity and heterogeneity of cytokines. Many facts need to be studied in more detail. For example, inflammatory genes and cytokine genes represent a new target for research because of different genetic variants. A study investigated the association between genetic variants of the IL-1β gene and amygdala and anterior cingulate cortex responsiveness to emotional stimuli and response to antidepressant treatment (Baune et al., 2010). It is also established that there is a link between genetic variants and response to medical treatment. Yu et al. (2003) for example found that patients with MD who were homozygous for the -511T allele of the IL-1β gene had a trend towards less severity of depressive symptoms and were more favorable for therapeutic response to fluoxetine than -511C carriers. Such research may also enhance the understanding of the pathogenesis of MD. Intracellular signalling pathways are another focus for future research because they are responsible for cytokine activity. Specific receptors and enzymes are important for the complex chain reaction in cytokines and modulate their actions. Not only the variety and function of cytokines need to be studied further but also the mechanism of antidepressant effect on cytokine function. Some in vitro studies indicate that antidepressants may inhibit proinflammatory cytokine activity through their effects on intracellular cyclic adenosine monophosphate (cAMP). An increase in cAMP levels in different peripheral blood mononuclear cells (PBMC) leads to a decrease in proinflammatory cytokine levels (Hashioka et al., 2007). Moreover, anti-inflammatory cytokines are up-regulated through the same mechanism. For example, higher cAMP levels increase the expression of IL-10 mRNA and intracellular IL-10 in monocytes (Maes, 2001). Another mechanism for the effects of antidepressant drugs may be through influencing 5-HT levels. Peripheral 5-HT is not only present in brain and gut, but is also stored in platelets

and immune cells like T-cells, monocytes or mast cells. T lymphocytes express 5-HT receptors as well as high affinity 5-HT transporters (Aune et al., 1994). Recent studies have revealed effects of 5-HT on innate immune cells: Kushnir-Sukhov et al. (2006) described that 5-HT induces adhesion and chemotaxis in mast cells. Boehme et al. (2004) revealed for the first time an important role in eosinophil migration to the lung. Nakamura et al. (2008) showed that 5-HT enhanced phagocytosis in murine macrophages. Further there is evidence that 5-HT alters the cytokine profile of dentritic cells, increasing IL-1β and IL-8 and decreasing IL12 and TNF-α (Muller et al., 2009). So, generally these authors revealed diverse roles for 5-HT in immune functions. This may be another target for antidepressant medications, that has to be explored in further studies.

As explained in the introduction, the HPA axis is well known to be more active in depressed patients. The normal task of glucocorticoids is to inhibit the production of inflammatory cytokines. This inhibition seems to be disturbed in both acute depressive episodes and chronic depression. Studies show that cortisol and proinflammatory cytokine levels are increased in depression; this can be interpreted as a dysregulation of the HPA-axis feedback mechanism, i.e. higher levels of peripheral corticosteroids appear unable to stop the production of proinflammatory cytokines in MD (Pace et al., 2007, Fitzgerald et al., 2006).

4.2 Tryptophan metabolites

As described in the introduction, tryptophan catabolites may have detrimental effects. Different mechanisms may explain how the metabolites work in the brain. First, metabolites like 3HK induce the production of radical oxygen species, which can cause mitochondrial dysfunction and influence energy metabolism.

Second, QUIN is a NMDA-receptor agonist with a potency to exert neurotoxic effects through induction of excitotoxicity (Stone et al., 1981). Effects of the catabolites include the destruction of postsynaptic elements, degeneration of nerve cells – such as hippocampal cell death – and a reduction in cerebral cholinergic circuits (Maes et al., 2011, Maes et al., 2010). Because of these detrimental effects, the catabolites may be possible biomarkers for depression and for changes in depression during medical treatment. Many studies have shown a connection between MD and changes in tryptophan metabolism.

In our study, TRP levels decreased after LPS stimulation. This could be due to a higher level of IFNγ, which increases IDO activity (IDO degrades TRP). We also found an increased level of KYN after inflammatory stimulation. KYN is again metabolized either via the toxic quinolinic pathway, in which 3HK, 3HAA and finally QUIN are produced, or via the kynurenic acid pathway, in which neuroprotective KYNA is the final metabolite. In accordance with the fact that IFNγ also activates KMO (Yasui et al., 1986), which degrades KYN to 3HK, we also found increased levels of 3HK.

HAA levels decreased in patients and controls after stimulation with LPS, although changes were not significant. There are two possible explanations for the reduction in HAA levels:

a) KYNase is suppressed by the proinflammatory state, thus less 3HK is converted to HAA, or
b) proinflammatory states activate the enzyme HAAO, which converts more HAA into QUIN, so that HAA levels decrease.

AA levels also increased after LPS stimulation. This means that kynurinase enzyme activity cannot have been suppressed by a proinflammatory state. Therefore,

the low HAA level after LPS stimulation must have been due to increased activity of HAAO, resulting in an increased transformation of HAA into QUIN.

Although cytokine changes are well documented in depression, the role of tryptophan metabolism in terms of the balance between neuroprotection and neurodegeneration in MD has not yet been fully explored. Studies have provided evidence that 3HK causes neuronal apoptosis (Okuda et al., 1998) and that QUIN causes excitotoxic neurodegenerative changes (Schwarcz et al., 1983). In contrast, KYN can also be metabolized into KYNA, an NMDA receptor antagonist (Perkins et al., 1982) that acts protectively against the excitotoxic action of QUIN (Stone et al., 2002). However, the importance of the tryptophan catabolites in vivo may be diminished by the finding that the concentrations achieved were significantly lower than those that would be needed to impair the viability of neurons (Stone, 1993, Moroni, 1999).

Since more KYN is formed after LPS stimulation, KYNA levels also increased. Across all the culture conditions, KYNA levels were numerically lower in patients than in the healthy controls, although none of the differences was statistically significant. In line with our findings, Myint et al. (2007b) reported significantly lower plasma KYNA levels in depressed patients. Thus, it is suggested that in depression, the metabolism of KYN is preferentially directed into the quinolinic pathway. In contrast to our study, the patients in Dr. Myint`s study were medication naïve or medication free whereas our patients were receiving medication, and Myint et al. investigated plasma levels whereas we used whole blood culture supernatant. Moreover, that study had a higher statistical power due to a higher number of patients and controls. Because of the relatively small sample size, our study may have been under-powered, which might explain the missing significance of our findings.

4.3 The ratios of tryptophan metabolites

The tryptophan breakdown index was generally numerically higher in the stimulated condition, although the difference was not statistically significant. This effect can be explained by induction of IDO activity by proinflammatory cytokines.

In the basal, unstimulated condition, the healthy controls showed higher KYNA levels in terms of the ratio to KYN. This finding is in accordance with the findings of Myint et al. (2007a) who also found a higher ratio in the plasma of healthy controls than in unmedicated depressed patients. After LPS stimulation, this difference in the ratio was no longer present and the values of the ratio decreased. This indicates that even though both KYNA and KYN levels are increased after LPS stimulation, less KYN is transformed into KYNA. The KYNA/KYN ratio is interpreted as an index of neuroprotection. Thus, the reduced conversion into KYNA, which is a neuroprotective metabolite, may contribute to an imbalance in the neuroprotective and neurodegenerative pathways. Wichers and coworkers were the first to examine the increases in the neurotoxic potential of tryptophan catabolites. The development of depressive symptoms was significantly associated over time with the KYN/KYNA ratio, which reflects an increase in neurotoxic potential (Wichers et al., 2005). This finding is also in agreement with the findings of a study in hepatitis C patients in which the neurotoxic challenge was higher when patients were treated with IFNα and subsequently developed of depressive symptoms (Wichers et al., 2005). In contrast, Van Gool et al. (2008) did not find that an increased production of neurotoxic metabolites was associated with the development of depressive symptoms during IFNα therapy.

We also found that the ratio 3HK/KYN decreased after LPS stimulation, although both 3HK and KYN levels increased. This ratio was higher in the patients. Moreover, the ratio of HAA/3HK was also reduced after LPS stimulation.

Similar to the findings of other studies, our data demonstrate that the further catabolization of 3HK beyond HAA into QUIN may be significantly induced in the inflammatory state. Our finding thus supports the neurodegenerative hypothesis of depression of Myint et al. (2003), which proposes that an accumulation of neurotoxic QUIN might be involved in the physiology of depression. Unfortunately, in our series of experiments QUIN was not quantified. So we can´t confirm wether there would have been significant results. This needs to be included in further projects.

We also found that the KYNA/3HK ratio was lower in the unstimulated cultures of patients across all culture conditions, which indirectly indicates that there is an imbalance between KYN metabolites in depressed patients.

4.4 Anti-inflammatory effects of medications and new drug targets

Our findings of altered IL-10 production may help to explain the beneficial therapeutic effect of celecoxib in depressed patients as reported by Müller and colleagues who found that celecoxib add-on therapy to standard antidepressants resulted in better treatment response in depressed patients (Muller et al., 2006). In our experiments, celecoxib enhanced the production of the anti-inflammatory cytokine IL-10 and abolished the statistically significant difference from the healthy controls. According to our findings, this beneficial clinical effect of the COX2 inhibitor celecoxib might be mediated through enhanced production of the anti-inflammatory cytokine IL-10. Since prostaglandin E2, a product of COX2, is involved in cytokine production, inhibition of the COX2 enzyme might have a beneficial effect on the inflammatory status in depressed patients. In addition to the effect on IL-10, celecoxib antagonized the numerical LPS-induced increase of

IFNγ production in the blood cultures of healthy controls. This modulating process was not seen in the culture derived from patients. Therefore, in healthy subjects celecoxib appears to have additional beneficial effects via suppression of a proinflammatory reaction, while this effect is blocked in depressed patients. For understanding, why the cells of depressed patients didn´t react in the same way like the controls, the intracellular signaling pathways need to be analysed more and may serve as new targets for future research.

Regarding the findings of IL-10, imipramine showed similar effects to celecoxib. There was an numerical increase of the IL-10 concentration in depressed patients and could therefore be beneficial in terms of re-balancing the immune function and eventually reversing the inflammatory response seen in depression. This findings may be supported by other authors, like Kubera et al. (2001), who found significantly increased production of IL-10 incubation with imipramine. The antidepressant-induced changes were detectable in IL-10 and IFNγ for both groups without difference between patients and controls, which may be due to methodological differences such as incubation time and medication status of the patients. Also Himmerich et al. (2010) identified tricyclic antidepressants like imipramine to suppress proinflammatory cytokines in in vitro experiments with blood from patients suffering from MD. Therefore, as in this study, imipramine could lead to a dominance of anti-inflammatory cytokines. Although on the basis of our data we cannot explain the mechanism of action of imipramine on IL-10, our results underline the evidence for imipramine having beneficial effects on the inflammatory status in depression.

Celecoxib did not affect LPS-induced changes in tryptophan metabolites. Since whole blood culture contains different types of cells, the culture of specific immune cells may give a clearer answer.

Apart from medications that influence the inflammatory state in depressed patients, several possible pharmacological targets remain as topics for further re-

search. For example, inhibitors of the activities of the enzymes along the KYN pathway may be able to counteract the detrimental effects. Another strategy may be to antagonize the possible neurotoxic effects by increasing the systemic protective effects of KYNA through blockade of the organic acid transporter by probenecid (Carrillo-Mora et al., 2010). Wang et al. (2009) found that LPS may induce IDO via IFNγ-independent mechanisms. Thus, the blockade of LPS-induced IDO expression may be an interesting topic for future research. Other studies found a glia-depressing factor, which might have a significant impact not only on the regulation of KYNA metabolism but also on the regulation of glia/astroglia activity and glia proliferation (Baran et al., 2010). The synthesis of this factor is increased by the inflammation-induced activation of microglia. This suggests that microglia activity and the associated increased synthesis of glia-depressing factor are novel drug targets that may dampen neuroinflammation in depressed patients. Specific antioxidants may also be possible pharmacological targets. Epigallocatechin-3 gallate is a component of green tea that may attenuate the activation of inflammatory, oxidative and nitrosative stress pathways in mice brains (Sachdeva et al., 2010) and that has neuroprotective effects against QUIN-induced excitotoxicity (Jang et al., 2010). Other projects concentrate on direct inhibitors of IDO, like norharman, which counteracts IDO activation and attenuates the neurotoxic consequences (Eggers et al., 2004). However, this finding should be interpreted carefully because these treatments could also abrogate the antiproliferative and antioxidative effects of IDO activation, possibly resulting in negative feedback. As explained, many different pathways are involved in depression, so the development of novel antidepressants should include not only inflammation and serotonin but also the catabolism of tryptophan and neurogenesis.

4.5 General and methodological limitations

This study has some general and methodological limitations. The general limitation is the fact that the in vitro data may not reflect the in vivo situation since cells isolated outside the body are detached from the internal influences of homeostasis mechanisms. The use of cell cultures and the stimulation and subsequent measurement of cytokines or metabolites are very common, according to the multifarious protocols for cell culture experiments. Many studies have been performed with purified cells or cell lines but fewer with whole blood (Yaqoob et al., 1999). Cell isolation processes, which are needed for PBMCs, may not only damage cells but also require conditions that are less like physiological conditions. On the other hand, the conditions in whole blood cultures are also not equivalent to those in vivo and the number of cells cultured is neither known nor controlled (Yaqoob et al., 1999). The variability and levels of cytokines in isolated PBMC are larger than in whole blood cultures. Therefore, it is more difficult to reach significant levels and the variation may be increased. Theoretically, cell cultures can be further isolated. For example, culturing monocytes for 24 hours results in the generation of a population of veiled accessory cells (Ruwhof et al., 2002), which are further dissociated from the in vivo condition.

Another factor that may explain why only a few results were significant is the concentration of our antidepressant medications. We wanted to simulate the in vivo situation as realistically as possible and therefore we used concentrations in a therapeutic range. If the concentrations had been higher, the measurements might have been clearer. However, to our knowledge there is no evidence in the literature for different results concerning the application of different concentrations.

Regarding the method for determination of cytokine levels, we chose Luminex® x MAP technology®, a capture/detection sandwich type immunoassay, for the quan-

titative analysis of cytokines. An earlier study compared three different available multiplex kits with each other and with enzyme-linked immunosorbent assay (ELISA), the "gold standard" of protein quantification (Djoba Siawaya et al., 2008). This study also took the supernatants of stimulated whole blood cultures. The great advantage of the Luminex system is the possibility to analyse up to 100 different microsphere sets in a single 50 µl sample. Siawaya et al. concluded that the Luminex technology is a good screening tool for the selection of markers but that promising candidates can then be validated using ELISA with higher accuracy and proven reliability (Djoba Siawaya et al., 2008). In principle, this methodical procedure corresponds to our study design.

Another possible confounder of our results may be the subjects' cortisol levels. Cortisol can influence the immune system and affects the KYN pathway through activation of tryptophan 2,3-dioxygenase (TDO), resulting in enhanced TRP breakdown to KYN (Young, 1981). Several studies have shown that depressive patients have increased cortisol secretion, even throughout the day. This finding was confirmed by Piwowarska et al. (2009); however, other studies did not obtain the same results (Posener et al., 2000, Young et al., 2001). In our study, blood was always taken before ten in the morning to reduce cortisol influences. The actual cortisol level of each participant was not measured, so that we cannot make a statement about how cortisol concentrations may have affected our data.

Another limitation was the relatively small sample size. A high heterogeneity in the small population of patients might have contributed to the high degree of variation in the data. The small sample size, in addition to the confounding factors, might also explain why we found only numerical differences that did not reach statistical significance. In their systematic review, Janssen et al. (2010) found that a strong immunosuppressive effect is more pronounced when PBMCs are used rather than whole blood samples, although the whole blood cultures used in our study design reproduced the in vivo situation more realistically. However,

for statistical analysis the data become less significant and gain variance. However, neither of these methods simulates true biological processes. Although both methods are established standards in immunological research, care needs to be taken when extrapolating from study conditions to those in humans. Moreover, the patients were being treated with antidepressant medications and were in a stable state at the time of sample collection.

Nevertheless, this study demonstrated imbalances in the immune system and tryptophan metabolism in stable depressed patients receiving antidepressant monotherapy.

5 Summary

Numerous studies have described the influence of the immune system to the pathophysiology of depressive disorders and shown that there is an activation of the pro-inflammatory immune system in depressive state. Characteristics of this immune activation are increased synthesis of proinflammatory cytokines and an increased numbers of lymphocytes and phagocytic cells. These cytokines change in tryptophan metabolism, the activity of the key-enzyme indoleamine 2,3-dioxygenase (IDO). Tryptophan (TRP), the precursor of serotonin is catabolized by IDO to kynurenine (KYN), which releases in the further degradation cascade at least three neuroactive intermediates. The close functional relationship between the effects of cytokines and the TRP-KYN metabolism is the basis for the central role of kynurenine in depressive disorders. Since IDO is ubiquitously present in the human body, the peripheral blood mononuclear cells (PBMC) form a representative model, which allows inferences about intracerebral processes. The hypothesis of this dissertation is that inflammatory processes result in elevated concentrations of the metabolites of TRP-KYN metabolism and thereby affect cerebral processes. These metabolites may represent biomarkers of depressive disorders. Another aspect is the observation that various antidepressants can change a present pro-inflammatory immune status into an anti-inflammatory immune status.

This study examines the effect of different antidepressants (reboxetine, fluoxetine, venlafaxine, imipramine) and the COX-2 inhibitor celecoxib on the immune system and the metabolism of tryptophan. This was done in mitogen-stimulated and unstimulated whole blood cultures of 21 depressive patients and 80 healthy control subjects. The whole blood was cultured in 24-well plates and stimulated with lipopolysaccharide (LPS). The proinflammatory cytokine IFNγ, the anti-inflammatory cytokines IL-4 and IL-10 and tryptophan metabolites were analyzed

in the supernatants of stimulated and unstimulated cultures. These measurements were made using Luminex and HPLC methods.

The results show that the immune response through the synthesis of proinflammatory IFNγ - and anti-inflammatory IL-4 and IL-10 in the control group was significantly higher compared to the group of patients. This significant difference was repealed by treatment with imipramine and celecoxib. This allows the conclusion that these drugs may antagonize the abnormal immune response of mitogen-stimulated cells in depressive patients. In all patients` blood cultures, the metabolites of tryptophan metabolism showed decreased TRP levels, increased KYN levels and reduced concentrations of 3-hydroxy-anthranilic acid (HAA). The concentration of KYNA tended to be higher in all cultures of the control group compared to the group of patients, but this difference was not significant. In the unstimulated conditions the controls showed higher KYNA values in relation to the ratio of KYN. After LPS stimulation this difference in the ratio of KYNA/KYN (index for neuroprotection) has been repealed. After stimulation, the ratio 3HK/KYN decreased despite the increase in 3HK and KYN levels. This ratio showed increased values in all patient cultures. The study also showed that the ratio of KYNA/OHK in patients with unstimulated samples was lower in comparison to all other culture conditions. This observation indicates indirectly the inter-individual imbalance of KYN metabolites in depressed patients. The observation of increased expression of antiinflammatory IL-10 under antidepressant therapy emphasizes the positive effect of celecoxib. For imipramine a similar effect was observed.

In summary, the results of this study give new approaches for future research projects, which analyse the interaction of antidepressant therapy with the immune system and the metabolism of tryptophan in depressive illness.

6 Zusammenfassung

Zahlreiche Studien haben den Einfluss des Immunsystems auf die Pathophysiologie depressiver Erkrankungen beschrieben und gezeigt, dass bei depressiven Störungen ein proinflammatorischer Immunstatus vorliegt. Charakteristisch für diese Immunaktivierung sind eine vermehrte Ausschüttung proinflammatorischer Zytokine sowie eine vermehrte Synthese von Lymphozyten und phagozytierenden Zellen. Diese Zytokine verändern im Tryptophanstoffwechsel die Aktivität des Schlüsselenzyms Indolamin 2,3-dioxygenase (IDO). Tryptophan (TRP), der Vorläufer des Serotonins wird durch IDO zu Kynurenin (KYN) katabolisiert, welches in der weiteren Abbaukaskade mindestens drei neuroaktive Zwischenprodukte freisetzt. Der enge funktionelle Zusammenhang zwischen den Zytokineffekten und dem TRP-KYN-Metabolismus ist die Basis für die zentrale Rolle des Kynurenins bei depressiven Störungen. Da die IDO im menschlichen Körper ubiquitär vorhanden ist, bilden mononukleäre Zellen des peripheren Blutes (PBMC) ein repräsentatives Modell, welches Rückschlüsse auf intracerebrale Prozesse erlaubt. Die Hypothese dieser Arbeit ist, dass Metabolite des TRP-KYN-Stoffwechsels aufgrund inflammatorischer Prozesse in erhöhter Konzentration entstehen und hierdurch cerebrale Vorgänge beeinflussen. Diese Metabolite können Biomarker depressiver Störungen darstellen. Ein weiterer Aspekt ist die Beobachtung, dass verschiedene Antidepressiva einen vorliegenden proinflammatorischen Immunstatus in eine antiinflammatorischen umwandeln können.

Diese Studie untersucht die Wirkung verschiedener Antidepressiva (Reboxetin, Fluoxetin, Venlafaxin, Imipramin) sowie des COX-2-Inhibitors Celecoxib auf das Immunsystem und den Tryptophan-Stoffwechsel. Dies erfolgt in mitogenstimulierten und unstimulierten Vollblut-Kulturen von 21 depressiven Patienten und 80 gesunden Kontrollpersonen. Das Vollblut wurde in 24-Well-Platten kultiviert und mit Lipopolysaccharid (LPS) stimuliert. Das proinflammatorische

Zytokin IFNγ, die antiinflammatorischen Zytokine IL-4 und IL-10 und die Tryptophan-Metabolite wurden in den Überständen von stimulierten und unstimulierten Kulturen analysiert. Diese Messungen erfolgten mittels Luminex- und HPLC-Verfahren.

Die Ergebnisse zeigen, dass die Immunantwort durch die Synthese von proinflammatorischem IFNγ - und antiinflammatorischer IL-4 und IL-10 bei der Kontrollgruppe signifikant höher im Vergleich zur Patientengruppe war. Dieser signifikante Unterschied wurde durch die Behandlung mit Imipramin und Celecoxib aufgehoben. Dies ermöglicht die Schlussfolgerung, dass diese Medikamente die abnorme Immunantwort mitogen-stimulierter Zellen depressiver Patienten antagonisieren können. Die Metabolite des Tryptophan-Stoffwechsels zeigten in allen Blutkulturen der Patientengruppe verminderte TRP-Level, erhöhte KYN-Level und verringerte Konzentrationen der 3-Hydroxy-Anthranilsäure (HAA). Die KYNA Konzentration war in allen Kulturen der Kontrollgruppe tendenziell höher im Vergleich zur Patientengruppe; dieser Unterschied war jedoch nicht signifikant. In den unstimulierten Konditionen zeigten die Kontrollen höhere KYNA-Werte in Bezug auf das Verhältnis zu KYN. Nach der LPS-Stimulierung wurde der Gruppenunterschied des Quotienten KYNA/KYN (Index für Neuroprotektion) aufgehoben. Nach der Stimulation verminderte sich trotz der Erhöhung der 3HK- und KYN- Level das Verhältnis 3HK/KYN. Dieses Verhältnis zeigte in allen Patientenkulturen erhöhte Werte. Die Studie zeigte auch, dass der KYNA/OHK Quotient bei Patienten mit unstimulierten Proben niedriger im Vergleich zu allen anderen Kulturbedingungen war. Diese Beobachtung zeigt indirekt das interindividuelle Ungleichgewicht der KYN- Metaboliten bei depressiven Patienten.

Die Beobachtung der gesteigerten Exprimierung des antiinflammatorsischen IL-10 unter Antidepressiva-Therapie unterstreicht die positive Wirkung von Celecoxib. Auch für Imipramin wurde ein ähnlicher Effekt beobachtet.

Zusammenfassend liefern die Ergebnisse dieser Studie neue Ansätze für zukünftige Forschungsprojekte, welche die Interaktion antidepressiver Therapien mit dem

Immunsystem und dem Tryptophan-Metabolismus depressiver Erkrankungen anaylsieren.

II. Abbreviations

AA	Anthranilic acid
ACTH	adrenocorticotropin
cAMP	cyclic adenosine monophosphate
BMI	body mass index
COX	cyclooxygenase
CNS	central cervous system
CRH	cortisol-releasing-hormone
DSM	diagnostic and statistical manual of mental disorders
ELISA	enzyme-linked immunosorbent assay
cGMP	cyclic guanosine monophosphate
HAA	3-hydroxy- anthranilate
HAAO	3-hydroxy anthranilate oxygenase
HAMD	Hamilton Depression Scale
HPA axis	hypothalamus-pituitary-adrenal axis
HPLC	high pressure liquid chromatography
HPT axis	hypothalamus-pituitary-thyroid axis
5-HT	serotonin
ICD	international statistical classification of diseases and related health problems
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin

KYN	kynurenine
KYNA	kynurenic acid
LMU	Ludwig-Maximilians Universität
LPS	lipopolysaccharide
MARDS	Montgomery-Asberg Depression Rating Scale
MAOI	monoamine oxidase inhibitor
MD	major depression
M.I.N.I.	mini international neuropsychiatric interview
NAD	nicotinamide andenine dinucleotide
NMDA	N-methyl-D-aspartate
OHK= 3HK	3-hydroxy-kynurenine
PBMC	peripheral blood mononuclear cells
PGE2	prostaglandin E2
PSS	perceived stress scal
QUIN	quinolinic acid
SNRI	selective noradrenaline reuptake inhibitor
SSNRI	selective serotonin-noradrenaline reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant
TDO	tryptophan 2,3-dioxygenase
TNF	tumour necrosis factor
TRH	thyrotropin releasing hormone
TRP	tryptophan
TSH	thyroid stimulating hormone

UPLC-MS	ultra performance liquid chromatography and mass spectrometry
WHO	world health organisation

III. Figures

IV. Tables

V. References

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