THE ROLE OF INFLAMMATION AND THE KYNURENINE PATHWAY IN MOOD DISORDERS AND PREGNANCY

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ABSTRACT

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Inflammation and the kynurenine pathway are involved in multiple physiological and pathophysiological states, however their role in depression during and after pregnancy, suicidality, and pre-eclampsia remain to be understood. Here, we sought to understand how the kynurenine pathway and its metabolites as well as their interactions with inflammation may influence these conditions.

First, we analyzed *suicide warning* in women with mood and anxiety disorders. We identified a distinct immunobiological profile linked to cross-diagnostic suicide risk in women with mood disorders, attending a psychiatric outpatient clinic. This consisted of a strong proinflammatory profile, containing white blood cell count and polymononuclear leukocyte cell count which may be associated with the underlying pathobiology of *suicide warning*.

Next, we analyzed inflammation and the kynurenine pathway in peripartum depression and postpartum depression and suicidality to understand how they could influence psychiatric health. We found plasma IL-6 predicted depression scores throughout the first, second, and third trimester. In the third trimester we found increased neurotoxic kynurenine metabolite quinolinic acid in the plasma of women with depression compared to health controls. Additionally, we found plasma IL-1β and IL-6 correlated with placental tissue expression of indolamine 2,3-dioxygenase 1 (IDO) connecting the

placenta with peripheral inflammation in the plasma. There was a different inflammatory profile in postpartum depression and suicidality, with increased IL-8 and decreased IL-2, indicating the mechanisms causing peripartum depression and postpartum depression may be different.

Finally, we looked at placentas from women with pre-eclampsia compared to healthy controls and found they had dysregulated tryptophan metabolism. There was a decrease of IDO, a compensatory increase in expression of tryptophan-2,3-dioxygenase, and this was associated with a decrease of serum amyloid A.

Collectively, this dissertation highlights the importance of inflammation and the kynurenine pathway in the pathophysiology of psychiatric disorders and pregnancy states in females. Further research of inflammation and the kynurenine pathway may lead to screening panels and treatments for suicide, peripartum depression, postpartum depression and suicidality, and pre-eclampsia.

To my family, dog, mentors, and coworkers

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PREFACE

At the time of writing this dissertation, three chapters describing my research are in preparation or have been accepted for publication. Chapter 3 has been accepted for publication to the *Journal of Affective Disorders*. Chapter 4 will be analyzed in greater detail and be compiled into three separate manuscripts and submitted in the near future. Chapter 5 has been accepted for publication to the *International Journal of Tryptophan Research*.

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KEY TO ABBREVIATIONS

ACMSD	Aminocarboxymuconate-Semialdehyde Decarboxylase
AIDS	Acquired Immune Deficiency Syndrome
BBB	Blood-Brain Barrier
BMI	Body Mass Index
C-	Caesarean
CAT-MH™	Computer Adaptive Test- Mental Health™
cDNA	Complementary DNA
CNS	Central Nervous System
CRP	C- Reactive Protein
CSF	Cerebrospinal Fluid
C-SSRS	Columbia Suicide Severity Rating Scale
Ct	Comparative Threshold Cycle
DSM	Diagnostic and Statistical Manual
EOS	Eosinophil Count
EPDS	Edinburgh Perinatal Depression Rating Scale
GC-MS	Gas Chromatography–Mass Spectrometry
HPA	Hypothalamic-Pituitary-Adrenal Axis
HPLC	High Performance Liquid Chromatography
IDO	Indolamine 2,3-Dioxygenase 1
IDO2	Indolamine 2,3-Dioxygenase 2
IFN-γ	Interferon-γ

IL-10	Interleukin-10
IL-12p70	Interleukin-12p70
IL-13	Interleukin-13
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IRB	Internal Review Board
KMO	Kynurenine-3-Mono-Oxygenase
KYN	Kynurenine
KYNA	Kynurenic Acid
ln	Natural Logarithm
LPS	Lipopolysaccharide
LR	Linear Regression Corrected for Age
LYMPH	Lymphocyte Count
MONO	Monocyte Count
mRNA	Messenger RNA
n	Number
NMDA	N-methyl-D aspartate
PE	Pre-eclampsia
PIC	Picolinic Acid
PMN	Polymorphonuclear Leukocyte Count

PPD	Peripartum Depression
qPCR	Quantitative Polymerase Chain Reaction
QPRT	Quinolinate Phosphoribosyltransferase
QUIN	Quinolinic Acid
RiR	Ridge Regression
RNA	Ribonucleic Acid
SD	Standard Deviation
SDHA	Succinate Dehydrogenase
SE	Standard Error
SAA	Serum Amyloid A
SSRI	Selective Serotonin Reuptake Inhibitors
Sβ	Standardized Beta
TDO	Tryptophan-2,3-Dioxygenase
TNF-α	Tumor Necrosis Factor-α
TRP	Tryptophan
UPLC-MS/MS	Ultra-High Performance Liquid Chromatography, Coupled to Tandem Mass Spectrometry
WBC	White Blood Cell Count
WGCNA	Weighted Co-expression Network Analysis
WR	Weighted Linear Regression

Chapter 1: Introduction

Inflammation

I. Discovery of inflammation

Inflammation was first documented by the description of edema in the *Corpus Hippocraticum* attributed to Hippocrates (371 BC to 350 BC) (Touwaide & De Santo, 1999), and later by Roman writer Aulus Celsus (30 BC to 45 AD) who provided the earliest description of the four signs of inflammation: pain, redness, swelling, and warmth (Schmidt-Bleek, Kwee, Mooney, & Duda, 2015). Cytokines in particular were first discovered in a human model by the assessment of pus in the laboratory (Dinarello, 2007). Cytokines are small proteins secreted by cells (J.-M. Zhang & An, 2007) that direct and amplify immune responses (Holdsworth & Gan, 2015) in both an anti-inflammatory or pro-inflammatory manner depending on the context. The ancient world documented that when people would become ill, they would have pus coupled with local area swelling, fever, and pain. In the mid-1940's, pus was examined further and "soluble factors", cytokines as they are called today, were first characterized (Dinarello, 2015).

In 1972, Gery *et al* analyzed human, mouse, rabbit, and rat tissue cells and established cytokines are present at baseline in all animals in their bone marrow, spleen, and thymus cells. Upon stimulation of the cell suspension with lipopolysaccharide (LPS), an increase in the levels of cytokines was observed which was further induced by the addition of macrophages. Once he purified the human peripheral leukocytes and removed the macrophages and other adherent cells, it destroyed the suspensions ability to produce cytokines (Gery & Waksman, 1972). Further, in 1980, Mier *et al* established

cytokines were an important factor for T cells longevity, and once cytokines were removed by purification, the serum was incapable of stimulating peripheral blood lymphocytes (Mier & Gallo, 1980).

Both experiments offer important insights as they established: cells must be present in the fluid to produce cytokines; specific cell types responsible for cytokine production; and that the removal of cytokines inhibits peripheral lymphocytes' ability to stimulate an inflammatory response. Since then, cytokines have been studied, and great advances have been made in the realm of diagnostics, prognostics, and therapeutic agents in human and animal diseases.

II. Innate and adaptive immune response

Inflammation is a defense mechanism to assaults on the host and is involved in normal wound healing. The innate immune system is activated immediately after an injury and is the first line defense system. The initial adaptive immune response can take 4-7 days before it takes effect. Both systems work harmoniously and synergistically in normal physiology.

The innate immune system, upon encounter with pathogenic microorganisms, drives local immune cells into action and inflammatory cells are recruited from peripheral circulation directly to the impacted areas. Macrophages secrete chemokines to attract more immune cells, such as neutrophils and monocytes, from the peripheral circulation and cytokines have an array of mechanisms of action upon activation. Macrophages also bind to common patterns on bacteria and engulf the bacterium by phagocytosis.

While the innate system is important for the control of common bacterial infections, it is not always able to eliminate pathogens. Therefore, a more targeted and efficient removal of pathogens by the adaptive immune system is required. The key initiating factors of the adaptive immune system response are immature dendritic cells, which are highly efficient antigen presenting cells due to their ability to acquire pathogens (i.e. viral particles and bacteria) by different methods, such as receptor-mediated endocytosis, phagocytosis, and macropinocytosis. The acquired pathogens are broken down into peptides that are then loaded onto major histocompatibility complex class I and II molecules. This leads to activation of the immature dendritic cells into mature dendritic cells which carry the pathogens antigens as antigen-presenting cells to peripheral lymph nodes and present them to T lymphocytes thus initiating an antigen-specific immune response. Therefore, the immature dendritic cells function as a link between the innate and adaptive immune response instead of just facilitating the clearing of pathogens with no further role (Savina & Amigorena, 2007).

The activated dendritic cells also secrete cytokines similarly to macrophages. The innate and adaptive immune system have similarities such as the secretion of cytokines (Ito, Connett, Kunkel, & Matsukawa, 2013). Yet, there are some key differences in their mechanism of action. The innate immune system is intrinsic, rapid, and involves macrophages. The adaptive immune system is highly specific to pattern recognition and takes time to recognize and present pathogens to T cell lymphocytes. Both innate and adaptive immune systems are important in protection of the host organism against bacteria, tissue damage, and viruses.

III. Dysregulation of inflammatory response

Inflammation must be tightly regulated in order to be beneficial to the host. Without proper regulation, cytokines can cause significant damage to body tissues and organs. During an infection, cytokines are modulated, and pro-inflammatory cytokines are increased drastically to eliminate the invader. However, if after clearance or control of the infection the pro-inflammatory cytokine expression is not decreased, the chronically raised cytokine levels cause chronically activated immune cells which affect the hosts' homeostasis. On the other hand, when immunity is compromised, patients are more susceptible to a wide array of secondary infections that are life threatening. Two examples that illustrate the serious and detrimental effects of cytokine dysregulation are acquired immune deficiency syndrome (AIDS) and autoimmune disease.

AIDS is the advanced disease state of human immunodeficiency virus, a lentivirus which over time suppresses the immune system. However, AIDS itself does not typically kill the patient as it is a secondary disease, which is acquired during the time the patient has AIDS and takes advantage of the deteriorated immune system that ultimately causes death (Armstrong-James, Meintjes, & Brown, 2014).

In contrast, autoimmune diseases are characterized by one's immune system attacking itself due to the inability to recognize itself. The National Institute of Health estimates that 23.5 million of Americans (78% of which are women) (Fairweather, Frisancho-Kiss, & Rose, 2008) suffer from autoimmune diseases (Campbell, 2014), and the prevalence is rising. The immune system components involved in this self-attack can range from lymphocytes, macrophages, and dendritic cells. Interestingly, the study of autoimmune disease sheds light on how complex modulation by cytokines can be. Cytokines

traditionally thought to promote inflammatory immune responses, may additionally display immunosuppressive actions.

One key cytokine that has been studied in the field is interleukin-2 (IL-2) (O'Shea, Ma, & Lipsky, 2002), which illustrates the significance of tight regulation of the immune system to maintain homeostasis, as both increases and decreases have pathogenic results. IL-2 is essential for activating lymphoid proliferation. It would be anticipated that a decrease in IL-2 production would have an immunosuppressive effect. Conversely, IL-2 knock-out in mice produce higher levels of multiple autoantibodies and roughly half of the mice die by autoimmune haemolytic anaemia before 2 months of age while the surviving animals later develop inflammatory bowel disease (Horak, Lohler, Ma, & Smith, 1995). This demonstrates that a largely pro-inflammatory cytokine, such as IL-2, can also possess a negative regulatory immune response function which is important in maintaining self-tolerance.

There are multiple disease states that are characterized by the dysregulation of the immune system, whether it be an increased cytokine, a decreased cytokine, or improper lymphocyte cell function. With further advances in understanding of how these disease states take place, it must be recognized that correct modulation of these factors must be balanced and monitored. It appears there is an optimal concentration for physiological homeostasis and a deviation in either direction could be catastrophic.

IV. Inflammation and the brain

Inflammation in the brain can arise from peripherally or locally produced immune factors. Some widely accepted methods by which peripheral inflammation can reach the

central nervous system (CNS) include passive or active transport across the blood-brain barrier (BBB) and the stimulation of change in the CNS by the vagal nerve.

The BBB is essential to health as it prevents toxic pathogenic material from crossing over from peripheral circulation into the brain and is regarded as the primary defense mechanism in keeping the brain healthy. The BBB is a capillary barrier with multiple tight, adherent gap junctions between vascular endothelial cells and regulates the flow of cells and molecules in and out of the brain. While immune components can cross the BBB, the traffic is tightly monitored to ensure a stable environment required for the optimal functioning of the CNS. Its key components are microvascular endothelial cells, basement membranes, pericytes, and astrocytes (Takeshita & Ransohoff, 2012). These components change the expression levels of cell adhesion molecules, ligands, integrins, chemokines, and chemokine receptors to help orchestrate the passing of immune components from the periphery into the brain (Lee & Imhof, 2008).

As mentioned above, pro-inflammatory cytokines can pass the BBB either by active transporters, for example, IL-1β (W. A. Banks, Kastin, & Durham, 1989) and TNF-α (Osburg et al., 2002), or through passive diffusion across more permeable regions, such as the circumventricular organs (W. A. Banks, 2005). In addition, injury and inflammatory conditions will make the specialized tight junctions of the BBB more permeable to peripherally derived cytokines (Quagliarello, Wispelwey, Long, & Scheld, 1991). Once the pro-inflammatory cytokines reach the CNS they cause microglial activation which leads to increased production of cytokines and nitric oxide, which affect neuronal signaling (Quan & Banks, 2007).

Another method of how peripheral inflammation can reach the brain is by vagal nerve-mediated signaling. The vagal nerve is the longest nerve in the human body and mediates the parasympathetic nervous system response (Bonaz, Sinniger, & Pellissier, 2017), as well as the innate immune response (Valentin A. Pavlov & Kevin J. Tracey, 2012). The afferent vagal nerve fibers express IL-1β receptors which are involved in detecting peripheral inflammation and conveying the signals to the brain (V. A. Pavlov & K. J. Tracey, 2012). Recently it has been proposed that dysregulation of inflammation in the gut, a parasympathetic organ controlled by the vagal nerve, causes a chronic pro-inflammatory immune state which can lead to neurodegenerative disorders. The proposed mechanism is inflammation from the gut travels to the brain via the vagal nerve (Houser & Tansey, 2017).

Lymphocytes can infiltrate into the brain by transmigration from the periphery and trigger or regulate inflammation (Takeshita & Ransohoff, 2012). Lymphocytes transmigrate into the brain by binding to the endothelial adhesion molecules, which are upregulated in inflammatory states, to assist in the crossing of the BBB. The steps include rolling, activation, arrest, crawling, and finally transmigration (Takeshita & Ransohoff, 2012). In pathologic conditions, such as multiple sclerosis, the BBB is compromised and leukocytes are able to enter the brain with ease (Larochelle, Alvarez, & Prat, 2011). Administration of natalizumab, which is an antibody directed against integrin VLA-4, prevents lymphocytes from being able to transmigrate through the endothelial adhesion molecule (Lee & Imhof, 2008). The drug modulates integrins and prevents the proper steps of entry from taking place.

Psychological stress is a major influence that contributes to the induction of inflammation in the periphery. One of the downstream effects of inflammatory mediators, such as cytokines, is activation of the hypothalamic-pituitary-adrenal axis (HPA) which results in an increase of adrenal glucocorticoids that bind to glucocorticoid receptors distributed throughout the CNS and peripherally (Dunn, 2000; Smith & Vale, 2006). Glucocorticoids have also been reported to regulate other genes such as cytokines and can suppress their action (Brattsand & Linden, 1996; Coutinho & Chapman, 2011; Fantuzzi & Ghezzi, 1993; Patricia A. Zunszain, Anacker, Cattaneo, Carvalho, & Pariante, 2011) causing decreases in the inflammatory response. Thus, inflammatory mediators, such as cytokines, closely and reciprocally interact with the HPA axis and its dysregulation has been consistently detected in psychiatric disorders and inflammatory states (Juruena, Bocharova, Agustini, & Young, 2017).

Another effect of psychological stress is recruitment of monocytes to the brain. Previous studies have found that psychological stress in humans can cause peripheral monocytes to have increased pro-inflammatory gene expression (A. H. Miller, Maletic, & Raison, 2009) which causes endothelial cells to express chemokine CC2 that facilitates monocyte recruitment to the brain (Sawicki et al., 2015). Also, microglia activation and neuro-inflammatory signaling occurs following prolonged stress exposures in a rat model (Johnson et al., 2005). Microglia activation refers to alterations in morphology that also correspond to increased expression levels of inflammatory mediators such as cytokines and chemokines. By inhibiting microglial activation in multiple models of stress induction via anti-inflammatory intervention, there is a decrease of depressive-like behavior in animal models (Hinwood et al., 2013).

Inflammatory monocytes can traffic through the body and have the enhanced capacity to release pro-inflammatory cytokines upon entering a tissue. Stress-induced trafficking of inflammatory monocytes contribute to mental health conditions such as recurrent anxiety and depressive behavior as well as somatic disorders (Wohleb & Delpech, 2016). Trafficking of monocytes in the presence of stress can influence neuro-inflammatory signaling and behavior (Beumer et al., 2012). In an animal model it was shown that physical stress, such as peripheral surgery, can disrupt the BBB by TNF-α release, facilitating the migration of macrophages to the hippocampus (Terrando et al., 2011). In another animal model, it was demonstrated that liver inflammation and drug induced colitis increased monocyte adhesion along cerebral endothelial cells, and was associated with microglial activation, decreased central neural excitability, and the development of sickness behavior (D'Mello et al., 2013). Thus, there is a link between peripheral organ inflammation and cerebral changes that impact behavior.

V. Receptors for cytokines in the brain

Cytokines can exert multiple, and profound effects on the CNS. Some of the effects are exerted by a direct effect on neurotransmission. There are neuronal receptors for certain cytokines, including IL-6 and IL-1β in, for example, the pyramidal cells of the hippocampus and neurons of the ventromedial hypothalamus (Aniszewska et al., 2015; O'Leime, Cryan, & Nolan, 2017; Schobitz, de Kloet, Sutanto, & Holsboer, 1993; Yabuuchi, Minami, Katsumata, & Satoh, 1994). Binding of the cytokine to the neuronal receptors directly affects neurotransmission (Dunn, Wang, & Ando, 1999). In addition to such direct and specific effects, cytokines impact other neurotransmitter systems by effects on production, metabolism and recycling of neurotransmitters (Andrew H. Miller,

Haroon, Raison, & Felger, 2013). For example, TNF-α can potentiate glutamate neurotoxicity by specifically inhibiting glutamate uptake (Chao & Hu, 1994; Zou & Crews, 2005). Altered glutamate neurotransmission might be a key biological mechanism underlying depressive and suicidal symptoms (Brundin, Erhardt, Bryleva, Achtyes, & Postolache, 2015; Steiner et al., 2011).

VI. Inflammatory hypothesis of depression and suicide

Depression is a serious mental illness and is considered a factor contributing to suicide. 1 in 20 Americans age 12 or older were currently depressed between 2009-2012 (Pratt LA, 2014). This is a startling statistic, and causes a large economic burden costing $210.5 billion in 2010 when accounting for workplace costs, direct costs, and suicide-related costs (Greenberg PE, 2015). Almost 43% of people with severe depression had serious difficulties in multiple aspects of their lives including work, home, and social activities (Pratt LA, 2014). Depression is diagnosed by a physician and the current gold standard for the diagnosis relies on a list of symptoms derived from the Diagnostic and Statistical Manual of Mental Disorders (Association, 2013). Even though there is no biological criteria, depression can be diagnosed and diagnosis coded for the patient in annual visits to the general practice doctor (Clinic, 2017). There are currently no biomarkers or neurobiologic tests to diagnose depression or suicide, though a large body of evidence has accumulated showing that inflammation might contribute to the pathophysiology of depression and suicide.

The inflammatory hypothesis of depression was first recognized by farmers when they observed sick animals behaved differently. Holmes et al confirmed in 1963 that rats exposed to toxins had a profound change in food consumption and body temperature as

well as behavioral abnormalities such as decreased interest in reward induced by this challenge (Holmes & Miller, 1963). It was discovered that animals produce cytokines that act upon the brain, which caused this sickness behavior. Sickness behavior is the subsequent uncomfortable feeling such as malaise, lassitude, fatigue, numbness, coldness, muscle and joint aches, and reduced appetite that results from viral or bacterial infections (Dantzer, 2009). These cause the animal to be behaviorally and psychologically unwell.

The causal evidence that inflammation can induce depression in humans originates from clinical trials with interleukins, as well as experimental studies in which participants had received injections of inflammatory agents and developed depressive symptoms (Belvederi Murri et al., 2017; Bonaccorso et al., 2002; Cooper et al., 2017; Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; O'Connor et al., 2009). One of the most referenced incidences of this causal relationship was when hepatitis C patients were treated with interferon. In one study, 30 patients were treated for the hepatitis C virus for 3 months using interferon-α, and after 3 months of treatment 40.7% of patients developed major depression (Bonaccorso et al., 2002). Interferon-α treatment does not only cause depression but is linked to the development of treatment-resistant depression (Chiu, Su, Su, & Chen, 2017).

Several studies have also confirmed the connection between IL-2 immunotherapy and development of the depressive symptoms (Buter, de Vries, Sleijfer, Willemse, & Mulder, 1993; Capuron, Ravaud, Miller, & Dantzer, 2004). For example, in one study 48 cancer patients were assessed for depressive symptomology after receiving subcutaneous IL-2. After assessment, it was found that patients who received the immunotherapy of IL-2

had significantly increased levels on the depression scale administered (MADRS) compared to those who had not received the cytokine therapy (Capuron, Ravaud, & Dantzer, 2000).

In a double blinded study, twenty healthy male volunteers were injected with an endotoxin (Salmonella abortus equi) or saline in two experimental sessions. Psychological tests were administered one, three, and nine hours after. While the endotoxin had no effect on physical sickness, there was a transient increase in anxiety and depressed mood in the volunteers (Reichenberg et al., 2001). This decrease in mood was demonstrated moreover in thirty healthy male volunteers who received Salmonella typhi vaccine compared to a placebo (Wright, Strike, Brydon, & Steptoe, 2005).

Laboratory induced systemic inflammation can lead to depressive-like symptoms. In animal models, LPS (Robert Dantzer et al., 2008) as well as POLY I:C (Gibney, McGuinness, Prendergast, Harkin, & Connor, 2013) injection is used to induce a depressive phenotype by increasing the immune system response. Following injection, mice begin to act helpless as demonstrated by the forced-swim test and tail suspension test (O'Connor et al., 2009). Mice have this response to both stress-induced and LPS induced models of depression (Guan, Lin, & Tang, 2015). Stress-induced depression models increase cytokine expression centrally (Guan et al., 2015), as well as peripherally (Cheng, Jope, & Beurel, 2015).

Inflammatory cytokines can trigger depression in humans (Moreira et al., 2015) and animal models (Fischer, Elfving, Lund, & Wegener, 2015; Rossetti et al., 2015). Data from primary psychiatric patients with depression show that inflammation is increased in

their blood and CNS (Dowlati et al., 2010). Further, the increase in inflammatory mediators might be particularly pronounced in suicidal patients (Gananca et al., 2015). Successful treatment of depression is associated with decreased inflammatory cytokines in circulation (Dahl et al., 2014), and in the CNS (Kranaster et al., 2017). Depressed individuals who commit suicide have increased perivascular Iba-1 immunolabeling compared to non-depressed patients, illustrating increased presence of vascular-associated macrophages in the brain has an association with depression (Torres-Platas, Cruceanu, Chen, Turecki, & Mechawar, 2014). Trafficking of macrophages to the brain represents a cellular pathway in which the immune system communicates back to the brain to regulate behavior. Macrophages reside within the perivascular space, meninges, and choroid plexus and are functionally different from brain microglia. These are a small selection of immune cells, but their proximity to vascular and parenchymal cells in the brain make them important transducers of peripheral immune signaling to the brain (Serrats et al., 2010). Brain macrophages are more efficient at antigen presentation and evoke a more robust pro-inflammatory response relative to microglia (Galea, Bechmann, & Perry, 2007).

Inflammation has been linked to suicide as well. A meta-analysis of literature found there were seven articles reporting increased suicide risk and case reports of attempted suicides when they used interferon therapies to treat chronic hepatitis C (Laura A. Lucaciu & Dan L. Dumitrascu, 2015). Moreover, when patients with multiple sclerosis were treated with interferon-β there were eleven cases of severe depression with suicidal ideation or attempts, all of these cases were people who had never been diagnosed with psychiatric disorders previously (Fragoso et al., 2010). This link between

suicidality and inflammation is found in both the periphery as well as the CNS. A meta-analysis found IL-1β and IL-6 to be significantly increased in both the blood and postmortem brain samples in patients with suicidality compared to controls and psychiatric patients without suicidality (Black & Miller, 2015).

In the periphery, IL-6 and TNF-α is increased, and IL-2 is decreased in suicide attempters plasma compared to non-suicidal depressed patients and healthy controls (Janelidze, Mattei, Westrin, Traskman-Bendz, & Brundin, 2011). Another study analyzed recently attempted suicidal depressed patients versus non-suicidal depressed patients and controls, and found IL-2 is significantly decreased in suicidal depressed patients compared to controls and non-suicidal depressed patients (Kim et al., 2008). Inflammatory indexes (summation of score based off TNF-α, IL-6, IL-10, and CRP) of plasma protein from people with major depressive disorder with high suicidal ideation had significantly higher inflammatory index scores compared to both patients with only major depressive disorder and normal controls (O'Donovan et al., 2013).

In the CNS, there is increased microgliosis in the dorsolateral prefrontal cortex, anterior cingulate cortex, and mediodorsal thalamus in the postmortem brains of completed suicides compared to the controls, depressed, or schizophrenic brain (Steiner et al., 2008). In the CSF, IL-6 protein is significantly elevated in suicide attempters compared to controls, with violent suicide attempters having the highest IL-6 concentrations (Lindqvist et al., 2009). In teenage suicide completers messenger RNA (mRNA) of pro-inflammatory IL-1β, IL-6, and TNF-α were significantly increased in the prefrontal cortex compared to healthy controls (Pandey et al., 2012). These studies demonstrate that there is increased inflammation in certain brain regions when someone is suicidal.

The kynurenine pathway

I. Kynurenine pathway biology

Cytokines also affect the catabolism of tryptophan (TRP), an essential amino acid, through the increased activation of the enzymatic kynurenine pathway. The first enzyme and rate-limiting step of pathway activation is the enzyme indoleamine 2,3-dioxygenase (IDO), which breaks down TRP into kynurenine (KYN) (Ban, Chang, Dong, Kong, & Qu, 2013) and away from serotonin synthesis (Davis & Liu, 2015). KYN and metabolites produced downstream in the pathway exert inflammatory effects and specifically alter glutamate neurotransmission (Steiner et al., 2011). Quinolinic acid (QUIN) is a neurotoxic N-methyl-D aspartate (NMDA) receptor agonist, while picolinic acid (PIC) is an antagonist of QUIN neurotoxicity and potential neurotrophic substance (Jhamandas, Boegman, Beninger, Miranda, & Lipic, 2000). Aminocarboxymuconate-semialdehyde decarboxylase (ACMSD) is a key enzyme at a divergent point in the kynurenine pathway, and generates PIC from the same precursor as QUIN, which is derived spontaneously. See Figure 1.1 for more detail.

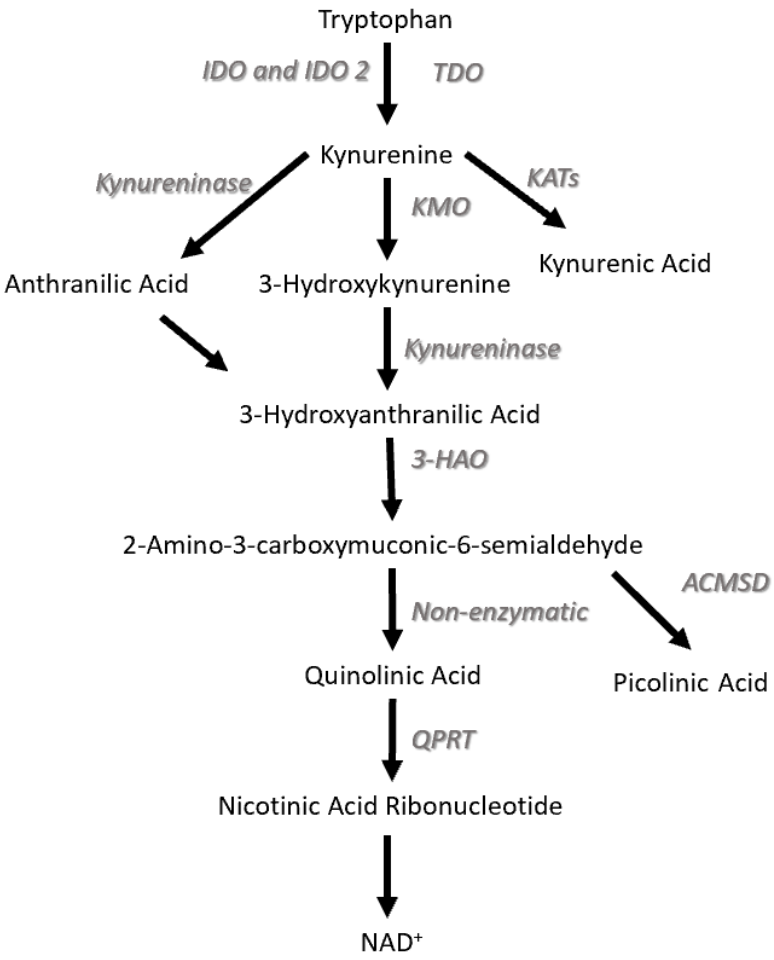

Figure 1.1 Schematic overview of the kynurenine pathway Shows the main enzymes and the metabolites they produce. Enzymes are listed in *italic font* in the figure. Abbreviations: ACMSD, Aminocarboxymuconate-Semialdehyde Decarboxylase; IDO, Indoleamine 2,3-dioxygenase; IDO2, Indoleamine 2,3-dioxygenase 2; TDO, tryptophan 2,3-dioxygenase; KATs, kynurenine aminotransferases; KMO, Kynurenine 3-Monooxygenase; 3-HAO, 3-Hydroxyanthranilate 3,4-dioxygenase; NAD+, Nicotinamide adenine dinucleotide; QPRT, Quinolinate-phosphoribosyltransferase.

II. The kynurenine pathways role in depression and suicide

Altered glutamate neurotransmission may be a key biological mechanism underlying depressive and suicidal symptoms (Brundin et al., 2015; Steiner et al., 2011). When inflammation occurs, QUIN is produced in excessive levels through the kynurenine pathway. This leads to over excitation of the NMDA receptor, which results in an influx

of calcium into the neuron. The high levels of calcium trigger an activation of destructive enzymatic pathways including nitric oxide synthesis and proteases (Dong, Wang, & Qin, 2009). These enzymes will degrade crucial proteins in the cell and increase nitric oxide levels, leading to an apoptotic response. LPS, a bacterial endotoxin and inducer of the innate immune response, can further this detrimental effect, and has been shown to upregulate enzymes within the kynurenine pathway, causing increased QUIN production (Chiarugi, Calvani, Meli, Traggiai, & Moroni, 2001). This finding has been replicated in rodents receiving a systemic LPS injection (Heyes & Morrison, 1997).

A previous study utilized postmortem tissue and demonstrated an upregulation of microglial QUIN in the subgenual anterior cingulate cortex and the anterior midcingulate cortex, regions previously reported to be responsive to infusion of the antidepressant and NMDA receptor antagonist ketamine (Steiner et al., 2011). Ketamine exhibits rapid and long lasting anti-suicidal effects, as shown in several clinical trials (DiazGranados et al., 2010; Price, Nock, Charney, & Mathew, 2009). These findings suggest NMDA-receptor signaling is an integral part of the pathophysiology of suicidal ideation.

Increased QUIN in the CSF is associated with suicide attempts and intent as well. There are increased levels of neurotoxic QUIN in the CSF of suicidal attempters, with QUIN levels twice as high as in healthy controls (Erhardt et al., 2013). Further, QUIN levels correlated with the degree of suicidal intent (Erhardt et al., 2013).

IDO may be pertinent to the degree of suicidal ideation. Depressed patients with a history of suicide attempts have increased plasma KYN and KYN/TRP ratio compared to those who suffer solely from depression and controls (Sublette et al., 2011). The KYN/TRP ratio is also increased in suicidal depressed patients who actively expressed

suicidal intent and who had previously attempted (Bradley et al., 2015). The increased KYN and KYN/TPR ratio indicates an increase in IDO activity and implies an increased flux down the kynurenine pathway.

Pregnancy

I. Inflammation in pregnancy

Inflammation is an important and highly controlled process during pregnancy. Pregnancy is a continually changing immunological state that is tightly regulated. The placenta develops as a unique hybrid organ consisting of cells from both the mother and the fetus (Wang & Zhao, 2010). It plays several pivotal roles that are key to maintain a healthy pregnancy; acting as an immunological barrier to infection, inhibiting fetal rejection from the maternal immune system, providing nourishment and oxygen to the growing fetus, as well as secreting hormones that regulate fetal growth and progression of pregnancy. The placenta contains many immunologically active components, such as specialized macrophages (Hyde & Schust, 2016).

Excessive and inadequate inflammation are potentially hazardous to the health of the pregnancy and the fetus (Shelton, Schminkey, & Groer, 2015). To promote and maximize the reproductive success of the mother and fetus it is hypothesized that pregnancy mimics the innate immune systems response to stress, causing local inflammation with implantation requiring high immune activation, followed by decreased immune reactivity in the second trimester allowing for growth, then a sharp increase in inflammation during labor (Schminkey & Groer, 2014). Immune system dysregulation during pregnancy is linked with adverse outcomes for the fetus including preterm birth, and hindered cognitive development in the offspring (Lisa M. Christian, 2015).

II. The kynurenine pathway in placenta

While the immune-suppressive state during the second trimester increases the mother's risk for infection, these modifications are vital for the preservation of pregnancy. T cell activation has been reported to result in early pregnancy loss (Sharma, 2014). One central component to suppression of T cell activation in the mother involves expression of IDO by the placenta (Honig et al., 2004). The placenta has the highest expression of IDO of all mammalian tissue (Dharane Nee Ligam, Manuelpillai, Wallace, & Walker, 2010). Activation of IDO initiates the kynurenine pathway and has been proposed to protect the fetus by starving T cells of TRP (Munn et al., 1998), thereby preventing their proliferation and promoting apoptosis. Thus, IDO activation is critical in maintaining a normal pregnancy (Bonney & Matzinger, 1998); however, aberrant IDO activity in response to maternal immune activation could lead to pathological events in the mother and offspring.

Stress during pregnancy compromises the maternal physiology, causing increases in pro-inflammatory cytokines (Parker & Douglas, 2010). Pregnant women may be especially sensitive to develop psychiatric symptoms in response to stress or infection because of increased IDO activity in the placenta. The human placenta not only expresses IDO, it also expresses ACMSD, quinolinate phosphoribosyl transferase (QPRT), and several other kynurenine pathway enzymes. Kynurenine metabolites PIC and QUIN have been detected in blood and cytotrophoblasts cell cultures (Manuelpillai et al., 2005; Manuelpillai et al., 2003), with the production of these metabolites and the expression of IDO increasing with LPS stimulation.

These same metabolites in the placenta may also be involved in peripartum depression (PPD) and suicidality. These metabolites can cross the BBB via the mechanisms listed above, can directly impact CNS physiology, and could result in depression and suicidality in the mother.

III. Peripartum depression and suicidality

Up to 20% of pregnant women in the United States are affected by depression during the peripartum period (O'Hara & Wisner, 2014). Severe perinatal depression has unique characteristics including agitation, anxiety, suicidal ideation, rumination, and fear of causing harm to the infant (Meltzer-Brody, Boschloo, Jones, Sullivan, & Penninx, 2013). Milder forms are also associated with a considerable psychiatric burden to the mother, and may lead to short- and long-term consequences for the child (Mian, 2005). Such outcomes for the offspring include growth delays, prematurity, low birth weight, and decreased responsiveness to stimuli in newborns (Davalos, Yadon, & Tregellas, 2012). Suicidal ideation is elevated in the peripartum period with prevalence as high as 14% (Paris, Bolton, & Weinberg, 2009), producing severe psychological suffering, even if the act is not followed to completion. Likewise, during the postpartum period suicidal ideation is reported in 8-14% of all women (L. M. Howard, Flach, Mehay, Sharp, & Tylee, 2011; Lindahl, Pearson, & Colpe, 2005; Pinheiro, da Silva, Magalhaes, Horta, & Pinheiro, 2008). The National Violent Death Reporting System from 2003 to 2008 (Gold, Singh, Marcus, & Palladino, 2012) reported that 113 women committed suicide in the 17 states included in the report, with 45 completed suicides during pregnancy and 68 in the postpartum period. The highest occurrence of suicide was during the first two months after delivery, and suicide persists as a leading cause of maternal death in the

peripartum period in the industrial world (Gissler, Hemminki, & Lonnqvist, 1996; Grigoriadis et al., 2017; Oates, 2003). It should be noted that overall suicidality is considered to be a rare event in the peripartum period, but one can imagine how each ended life can impact multiple individuals (Orsolini et al., 2016).

Patients exhibiting PPD and suicidality are treated with SSRIs. Disappointingly, this is only effective in approximately 50% of patients and adverse effects are possible. SSRIs administered during pregnancy pose risks including the newborn exhibiting SSRI discontinuation syndrome (Boucher, Bairam, & Beaulac-Baillargeon, 2008), and the mother experiencing postpartum hemorrhage (K. Palmsten et al., 2013). Therefore, both physicians and mothers are reluctant to use SSRIs, and depression often goes untreated. This can lead to substantial suffering for the mother and could affect the bonding between the mother and the infant (Reay, Matthey, Ellwood, & Scott, 2011). However, not treating a mother with depression has been shown to have detrimental outcomes including alcohol and illicit substance use, poor nutrition and prenatal medical care, smoking, and increased risk of suicide which hinders not only the mother but her fetus as well (Pearlstein, 2008).

Increases in pro-inflammatory cytokines in the blood of pregnant women are associated with depressive symptoms (L. M. Christian, Franco, Glaser, & Iams, 2009). It has been demonstrated that at different time points of pregnancy there is an increase in IL-6,TNF-α (Azar & Mercer, 2013; Haeri, Baker, & Ruano, 2013) and IL-1β (Corwin, Bozoky, Pugh, & Johnston, 2003) in depressed patients versus healthy controls. The hypothesis of maternal immune dysregulation stemming from psychosocial stress is becoming increasingly accepted (Lisa M. Christian, 2015).

During the postpartum period, there is an increased KYN/TRP ratio for at least six weeks (Schrocksnadel et al., 2003), strongly suggesting the changes induced by pregnancy remain after delivery of the placenta. Maternal monocytes are important mediators of immune response and are known to interact with decidual cells (connective tissue cells developed during pregnancy) in the placenta (Tang, Hu, Liu, Kwak-Kim, & Liao, 2015). Pregnancy prompts expression of IDO in maternal peripheral blood monocytes (Miwa et al., 2005). It has been demonstrated that expression of numerous immune-associated markers on peripheral monocytes are altered in pregnant women (Koldehoff, Cierna, Steckel, Beelen, & Elmaagacli, 2013). Thus, it is plausible that immunological alterations in the placenta induce IDO expression in circulating maternal blood cells. Circulating monocytes can potentially migrate to the CNS and initiate neuroinflammation, in addition to the direct entry of kynurenine metabolites that can occur across the BBB (Reynolds & Morton, 1998; St'astny, Skultetyova, Pliss, & Jezova, 2000; Steiner et al., 2012).

IV. Inflammation and kynurenine pathway dysregulation in pre-eclampsia

Another pregnancy complication that involves inflammation is pre-eclampsia (PE). PE is characterized by hypertension, proteinuria, maternal systemic inflammation, and has the potential to be life threatening to both the mother and the fetus, with major risk for preterm birth (Hashemi et al., 2017). PE account for 10-15% of maternal deaths worldwide (Sahai, Saraswathy, Yadav, Arora, & Krishnan, 2017). While the cause of PE is currently unknown, it is thought to be linked to the inflammatory changes in the placenta and plasma. Studies show women with PE have increased cytokine protein levels (Bueno-Sanchez et al., 2013) including a large increase in plasma IL-8 in women

with PE compared to controls (Leik & Walsh, 2004). There is also a dysregulation in the kynurenine pathway in PE, and IDO is significantly decreased in women with PE compared to controls (Iwahashi et al., 2017). Unfortunately, there is still no clinically validated biomarker for PE.

Overlap between disease states in pregnancy

Inflammation and kynurenine pathway dysregulation overlap between a variety of pathological conditions in pregnancy. Women with PE are more likely to suffer from comorbid depression (Qiu, Sanchez, Lam, Garcia, & Williams, 2007). When women take antidepressants during the duration of their pregnancy they have increased risk of developing PE (Kristin Palmsten, Setoguchi, Margulis, Patrick, & Hernández-Díaz, 2012), with a larger increased risk for women taking serotonin-norepinephrine reuptake inhibitors and tricyclic antidepressants. In a study of 623 women, depression and anxiety in early pregnancy were associated with increased risk of developing PE (Kurki, Hiilesmaa, Raitasalo, Mattila, & Ylikorkala, 2000).

The goal of this dissertation is to unveil the roles of the kynurenine pathway and inflammation in women with mood disorders, also in relation to pregnancy and in a common disease of pregnancy, namely PE. While some work has been done in the topic, as seen earlier in this chapter, a lot is still to be discovered. This dissertation specifically aims to uncover the physiological relevance of the kynurenine pathway in relation to suicidality, depression, peripartum depression, and pre-eclampsia. The following chapters will address the similarities and differences between these different pregnancy pathologies along with psychiatric disorders.

Chapter 2: Statistical Analysis of Human Subjects

Introduction

Human subjects have more variability between each subject compared to animal models as the human condition cannot be controlled under strict scrutiny. For example, animal models such as mice or rats are controlled for age, background genetics, sex, and medications. Humans, on the other hand, have comorbid conditions such as different medications taken, different dosages, different genetic backgrounds, and different ages. Therefore, there are more stringent and corrective statistics performed on human subject models compared to their animal model counterparts. It is important to note that these factors, referred to as confounding factors when they influence the statistical analysis, can be corrected for in some models of statistical analysis such as regressions, and are corrected for in analysis of some sort in all chapters of this dissertation. Two validated and popular software programs, R and IBM SPSS, were used. Below will highlight some of the statistical methods used in the following dissertation.

Statistical Methods

I. Student's t-test

In human models, as previously stated, there is heteroscedasticity between different subjects. In a classic human study, one is looking for differences between two or more groups. For example, Group A receives a medication, and Group B receives a placebo. It is important to see if variables between these two groups exists that could be

contributing to the output result. One example of this are demographics, such as body mass index (BMI), age, how many previous pregnancies a woman has had etc. A way to analyze if there is a statically significant difference between the two groups demographics is by utilizing a method known as the Student's t-test. Student's t-tests are used to determine if there is a significant difference between the means of two groups which are related (Kenton, 2019). In the following chapters Student's t-tests are used to verify there was not a significant difference between the demographics of the cohorts used.

II. Regression analysis

Regressions are a type of analysis that are used to understand the relationship between independent variables and dependent variables. It is a mathematical way of finding which variables have an impact (Gallo, 2015). A regression coefficient indicates the response of a variable for one unit of change in the predictor variable while holding other predictors in the model constant, isolating the role of one variable (Editor, 2013). The p-value is as normally understood in biological analysis, with a p-value less than or equal to 0.05 indicating a statistically significant relationship between the model and the variable being tested.

There are subsets of regressions with both linear regression and logistic regressions being a common type of regression used to estimate the relationships in a linear model (Unknown, 2018). These models differ as linear regressions are used when a variable is continuous in nature and exhibits a linear regression line, while logistic regression is when the dependent variable is binary in nature (example: Yes/No answers). Both can have confounding factors which can be corrected for. For instance, in Chapter 3 linear

regressions were corrected for age. The purpose for this, is age is a confounding factor, and can influence the levels of certain cytokines being analyzed in that chapter. By correcting for this relationship, one can analyze the relationship between cytokines and the outcome, without the influencing factor of age.

Ridge regression (RiR) is a special regression model of statistical analysis that is used to account for multicollinearity. Multicollinearity is an issue because it can inflate the standard errors of the regression coefficients, resulting in a false nonsignificant p-value and it reduces the predictability of the regression model. The source of our multicollinearity originates from using independent variables that interact as a set of variables (NCSS). For example, as discussed in the previous chapter, some cytokines and the kynurenine pathway interact closely, therefore they can contribute to the multicollinearity of the model.

III. Correlation analysis

Correlation analysis is performed to measure the strength and direction of the relationship between two numerical continuous variables (research). This is important in human studies as one wants to know if two variables tend to change in a coupled fashion, which may establish a possible connection. However, this cannot be confirmed by solely correlation analysis as correlation does not equal causation. This is typically a downfall seen in human subject studies, as it is primarily driven by correlative analysis, but cannot establish the causation of the correlation. Both Pearson's and Spearman correlations were used in the following chapters. Pearson's correlations evaluate the linear relationship between continuous variables, whereas Spearman correlation evaluates the monotonic relationship between continuous or ordinal variables (Minitab,

2019). For both, the closer the correlation coefficient is to +1 or -1 the stronger the relationship is, where a value of zero means the relationship is random and non-existent.

IV. Weighted correlation network analysis

The body's physiology is interconnected. The analytes studied in this dissertation, cytokines and the kynurenine pathway metabolites and enzymes particularly, are all interrelated, and one can influence the others levels. This allows for some very interesting analysis. Weighted correlation network analysis (WGCNA) is popular among analyses for studying biological networks based on pairwise correlations between variables and assesses how subsets of analytes can be related and influence a certain state (example: suicidality). To model a WGCNA analysis one constructs a co-expression network of markers that express similarly, then modules are identified via hierarchical clustering. The modules first principal component is then calculated which is referred to as "the eigengene". The eigengenes are the most representative immunobiological profiles of the respective modules (consisting of the cumulative signal of the analytes in the module). Two sets of Spearman correlation analyses are conducted with the eigengenes. In the first, eigengenes association with the subject's characteristics (example: CAT-MH depression and suicide risk) is established. The results show whether a module (and therefore the analytes within it) is significantly associated with the subject's characteristics. The association of the eigengenes with the individual analytes within the module is established. The resulting coefficients are denoted "module membership" values. The closer these values are to +1 or -1, the more of an influence that analyte has within the module to the subject's characteristic

assessed. Researchers are then able to study inter-module and intra-module relationships and find the key drivers in the modules of interest.

Conclusion

Statistical analysis and methodology are critical to robust and accurate statistics in any model being used. Human subject research is typically accompanied by a more complex analysis to ensure the authenticity of the research and to validate that the correlations and possible drivers are accurate and do not have interfering noise of confounding factors. Above summarizes the statistical analysis used throughout this dissertation and explains why it is of relevance to this research. The following chapters will look deeper into these statistical practices in the Methods sections.

Chapter 3: An Inflammatory Profile Linked to Increased Suicide Risk

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Abstract

Background: Suicide risk assessments are often challenging for clinicians, and therefore, biological markers are warranted as guiding tools in these assessments. Suicidal patients display increased cytokine levels in peripheral blood, although the composite inflammatory profile in the subjects is still unknown. It is also not yet established whether certain inflammatory changes are specific to suicidal subjects. To address this, we measured forty-five immunobiological factors in peripheral blood and identified the biological profiles associated with cross-diagnostic suicide risk and depression, respectively.

Methods: Sixty-six women with mood and anxiety disorders underwent computerized adaptive testing for mental health, assessing depression and suicide risk. Weighted correlation network analysis was used to uncover system level associations between suicide risk, depression, and the immunobiological factors in plasma. Secondary regression models were used to establish the sensitivity of the results to potential confounders, including age, BMI, treatment and symptoms of depression and anxiety.

Results: The biological profile of patients assessed to be at increased suicide risk differed from that associated with depression. At the system level, a biological cluster containing increased levels of interleukin-6, lymphocytes, monocytes, white blood cell count (WBC) and polymorphonuclear leukocyte (PMN) count significantly impacted suicide risk, with the latter two inferring the strongest influence. The cytokine interleukin-8 was independently and negatively associated with increased suicide risk. The results remained after adjusting for confounders.

Limitations: This study is cross-sectional and not designed to prove causality.

Discussion: A unique immunobiological profile was linked to increased suicide risk. The profile was different from that observed in patients with depressive symptoms and indicates that granulocyte mediated biological mechanisms could be activated in patients at risk for suicide.

Introduction

Inflammation first emerged as a potential trigger of depression in the late 1980s, when treatment with interferons for diseases such as cancer and hepatitis was found to cause depression and suicidal behavior (Capuron et al., 2000; Capuron et al., 2004; Dieperink et al., 2004). It is now known that what we consider to be primary depression is also associated with peripheral inflammatory changes (Dowlati et al., 2010; Howren, Lamkin, & Suls, 2009; Liu, Ho, & Mak, 2012; Valkanova, Ebmeier, & Allan, 2013). Moreover, there is mounting evidence that patients with suicidal ideation and behavior have pronounced inflammatory changes in both blood and CSF (Black & Miller, 2015; Janelidze et al., 2011; O'Donovan et al., 2013). Inflammatory factors in the blood can

reach the CNS via several pathways, including passive or active transport across the BBB, immune cell transmigration and vagal nerve signaling (W. Banks, 2014; Hosoi, Okuma, & Nomura, 2002). Within the brain, they may play a role in the progression of psychiatric diseases, triggering symptoms by acting on neural structure and function (Udina et al., 2012). As such, inflammatory factors may be targets for future therapies and interventions. They might also serve as biomarkers, reflecting the severity of symptoms and indicating suicide risk (Falcone et al., 2010; Sudol & Mann, 2017).

The purpose of this study was to identify immunobiological factors associated with increased suicide risk in a psychiatric outpatient setting. We focused on a patient sample with mood and anxiety disorders in different stages, as this represents one of the most common populations encountered in an outpatient clinic. The critical question addressed here was whether we could detect a biological profile indicative of cross-diagnostic suicide risk among these women. The cross-diagnostic design was adapted from the Diagnostic and Statistical Manual-5 (DSM-5) criteria, which recognizes suicidality as a cross-diagnostic entity (American Psychiatric Association, 2013). Thus, the sample, women with mood and anxiety disorders, was chosen for clinical relevance. They were assessed for suicide risk, the unifying factor, for which we aimed to identify biological risk-indicators.

Previous studies, by us and others, have often focused on the association of a limited number of inflammatory factors with depressive symptoms and suicide risk (Adhikari et al., 2018; Yang et al., 2018). However, inflammatory factors interact in complex networks, and it is still not known what specific inflammatory factor, or groups of inflammatory factors, might best reflect depressive and suicidal symptoms. To address

this knowledge gap, we designed this study to measure 45 immunobiological factors in peripheral blood from women seeking care in an outpatient psychiatric clinic. The complete list of immunobiological analytes is found in Table 3.1. Some of the biological analytes, including cytokines, kynurenine pathway metabolites, vascular endothelial growth factor (VEGF) and antibody titers, have previously been implicated in depression and suicidal behavior (Arling et al., 2009; Ganança et al., 2016; Isung et al., 2012; Ling, Lester, Mortensen, Langenberg, & Postolache, 2011; T. B. Meier et al., 2016; Okusaga et al., 2011; Simanek et al., 2014). A well-replicated finding is that the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are elevated in suicidal patients compared to healthy controls, which was confirmed in a recent meta-analysis (Black & Miller, 2015). However, other immunobiological factors may be decreased in suicidality. As one example, we and others have found that levels of interleukin-8 (IL-8), a cytokine with neuroprotective functions, might be lower in suicidal patients compared to healthy controls (Isung et al., 2012; Janelidze et al., 2015).

Table 3.1 Complete list of the immunobiological measures

Immunobiological Analyte	Abbreviation
Picolinic Acid	PIC
Quinolinic Acid	QUIN
Picolinic Acid/ Quinolinic Acid Ratio	PIC/QUIN ratio
Tryptophan	TRP
Kynurenine	KYN
Kynurenine/ Tryptophan Ratio	KYN/TRP ratio
C-reactive Protein	CRP
Serum Amyloid A	SAA

Table 3.1 (cont'd)

Immunobiological Analyte	Abbreviation
Intercellular Adhesion Molecule 1	ICAM-1
Vascular Cell Adhesion Molecule 1	VCAM-1
Placental Growth Factor	PIGF
Vascular Endothelial Growth Factor	VEGF
Basic Fibroblastic Growth Factor	bFGF
Soluble Fms-like Tyrosine Kinase 1	sFLT-1
Interferon- γ	IFN-γ
Interleukin-10	IL-10
Interleukin-12p70	IL-12p70
Interleukin-13	IL-13
Interleukin-1β	IL-1β
Interleukin-2	IL-2
Interleukin-4	IL-4
Interleukin-6	IL-6
Interleukin-8	IL-8
Tumor Necrosis Factor- α	TNF-α
Herpes Simplex Virus Type 1 IgG titer	HSV1 IgG t
Herpes Simples Virus Type 1 Avidity	HSV1 Av.t
Herpes Simplex Virus Type 1 Positivity	HSV1 pos.
Herpes Simplex Virus Type 2 Positivity	HSV2 pos.
Cytomegalovirus IgG Titer	CMV IgG t
Cytomegalovirus Avidity	CMV Av.t
Cytomegalovirus Positivity	CMV pos.
Toxoplasma Gondii IgG Positivity	*T. Gondii* IgG pos.
White Blood Cell Count	WBC
Red Blood Cell Count	RBC
Hemoglobin	HGB

Table 3.1 (cont'd)

Immunobiological Analyte	Abbreviation
Hematocrit	HCT
Mean Cell Volume	MCV
Mean Cell Hemoglobin Concentration	MCHC
Red Blood Cell Distribution Width	RDW
Platelet Count	PLT
Polymorphonuclear Leukocyte Count	PMN
Lymphocyte Count	LYMPH
Monocyte Count	MONO
Eosinophil Count	EOS
Basophil Count	BASO

Here, we used network analysis to identify the composite immunobiological profiles associated with depression and suicide risk. Our hypothesis was that we would find a distinct biological profile associated with suicide risk. Our primary outcomes were the immunobiological networks associated with suicide risk and depression, respectively, as calculated using weighted co-expression network analysis (WGCNA). Next, we performed sensitivity analyses to establish whether the outcomes were affected by potential confounders including age, treatment and the degree of current symptoms of anxiety and depression. As a result of this systems level approach, we observed a unique proinflammatory cell profile in women with increased suicide risk, which was different from our findings in patients with depressive symptoms.

Materials and Methods

I. Participants

The study was approved by the Mercy Health Saint Mary's Institutional Review Board. A total of 66 women, age 18-67, with mood and anxiety disorders, active or in remission to cover a full range of symptom severity, were included in this study (see Table 3.2). Study participants were recruited by advertisement in the outpatient clinics of Pine Rest Christian Mental Health Services (Pine Rest) located in Grand Rapids, Michigan, USA. The past/current psychiatric diagnoses of the subjects were established using the Structured Clinical Interviews for DSM-IV-TR (First, Spitzer, Gibbon, & Williams, 2002). The psychiatric and somatic diagnoses of the patients as well as ongoing medication are listed in Tables 3.2 and 3.3. No patients in this study had signs of liver disease or signs of an on-going infection. Blood samples were collected at Pine Rest, between July 9, 2012 and March 29, 2013. The exclusion criteria included a history of psychotic disorders; drug or alcohol dependence in the three months prior to study enrollment; organic mood disorder due to a general medical condition or substance use; current inpatient treatment; or a diagnosis of major/minor neurocognitive disorders. Additionally, no patients with borderline personality disorder/emotionally unstable personality disorder were included. This study is an extension of the analysis of subjects described in Bryleva et al. (Bryleva et al., 2017) which looked at single markers in relation to traditional diagnostic tools (Table 3.4).

Table 3.2 Primary DSM-IV-TR diagnoses of the study participants

Psychiatric Diagnosis	Current *PTE*	Remiss-ion *PTE*	Somatic conditions								
			Allergy or Asthma *PDC*	Cardio-vasc. *PDC*	Digestive *PDC*	Endo-crine *PDC*	MSN *PDC*	Pain *PDC*	RE *PDC*	Repr *PDC*	Sleep *PDC*
MDD	25 *(37.9%)*	8 *(12.1%)*	4 *(12.1%)*	6 *(18.2%)*	4 *(12.1%)*	2 *(6.1%)*	8 *(24.2%)*	3 *(9.1%)*	2 *(6.1%)*	2 *(6.1%)*	2 *(6.1%)*
Depressive Disorder NOS	3 *(4.5%)*	2 *(3.0%)*	0 *(0.0%)*	0 *(0.0%)*	0 *(0.0%)*	0 *(0.0%)*	1 *(20.0%)*	0 *(0.0%)*	0 *(0.0%)*	0 *(0.0%)*	0 *(0.0%)*
Dysthymic Disorder	10 *(15.2%)*	0 *(0.0%)*	2 *(20.0%)*	2 *(20.0%)*	2 *(20.0%)*	0 *(0.0%)*	4 *(40.0%)*	1 *(10.0%)*	0 *(0.0%)*	2 *(20.0%)*	1 *(10.0%)*
Anxiety	23 *(34.9%)*	0 *(0.0%)*	3 *(13.0%)*	5 *(21.7%)*	2 *(8.7%)*	5 *(21.7%)*	6 *(26.1%)*	3 *(13.0%)*	0 *(0.0%)*	0 *(0.0%)*	3 *(13.0%)*
Bipolar I	8 *(12.1%)*	2 *(3.0%)*	2 *(20.0%)*	2 *(20.0%)*	0 *(0.0%)*	2 *(20.0%)*	1 *(10.0%)*	0 *(0.0%)*	0 *(0.0%)*	0 *(0.0%)*	1 *(10.0%)*
Bipolar II	6 *(9.1%)*	7 *(10.6%)*	2 *(15.4%)*	2 *(15.4%)*	0 *(0.0%)*	1 *(7.7%)*	3 *(23.1%)*	2 *(15.4%)*	0 *(0.0%)*	2 *(15.4%)*	1 *(7.7%)*
Bipolar NOS	2 *(3.03%)*	0 *(0.0%)*	0 *(0.0%)*	1 *(50.0%)*	0 *(0.0%)*	1 *(50.0%)*	0 *(0.0%)*	0 *(0.0%)*	0 *(0.0%)*	0 *(0.0%)*	0 *(0.0%)*

MDD, major depressive disorders; NOS, not otherwise specified. PTE, Percentage of total enrolled; PDC, Percentage of people in diagnostic category. The major comorbid somatic disorders, grouped per system are shown. MSN, Musculoskeletal and nervous system disorders; RE, respiratory disorders; Repr, reproductive disorders

Table 3.3 Types of medication used by study participants

Medication	N
Anti-inflammatory	25
Lithium	3
Antiepileptic	27
Antipsychotic	17
Psychostimulants	12
Benzodiazepines	30
SNRI	28
SSRI	25
TCA	5

N, number of patients; SNRI, serotonin–norepinephrine reuptake inhibitors; SSRI, selective serotonin re-uptake inhibitors; TCA, tricyclic antidepressant

Table 3.4 Study participant demographics

Diagnosis	Total Subjects	Age	HAMD 17 Total Score	HAMD 25 Total Score	CES-D Total Score	PHQ-9 Total Score
Suicide Risk	*(Count)*	*Median (IQR)*	*Median (IQR)*	*Median (IQR)*	*Median (IQR)*	*Median (IQR)*
No	51	45.0 (39.0-55.0)	12.0 (7.0-18.0)	15.0 (8.0-21.0)	25.0 (13.0-30.0)	6.5 (4.0-14.0)
Yes	15	43.0 (33.0-52.0)	18.0 (12.0-24.0)	25.0 (13.0-26.0)	38.0 (21.0-42.0)	18.0 (9.0-22.0)

IQR, interquartile range

II. Assessment of psychiatric symptoms

CAT-MH™ is a computerized assessment of psychiatric symptoms that draws questions related to signs and symptoms of mood and anxiety disorders as well as suicidality (Gibbons et al., 2012). Convergent validity has been confirmed against the Patient Health Questionaire-9 (PHQ-9), Hamilton Depression Rating Scale (HAMD), and Center for Epidemiologic Studies Depression Scale (CES-D) (Gibbons et al., 2012). Adaptive scale score significantly predicts current diagnosis of major depressive disorder, based on Structured Clinical Interview for DSM-IV-TR (Achtyes et al., 2015; Gibbons et al., 2012). There are four mandatory suicide-risk screening questions, which are derived from the Columbia Suicide Severity Rating Scale (C-SSRS). The questions are the following: I) In the past month, have you actually had any thoughts of killing yourself? II) Have you had any intention of acting on these thoughts of killing yourself? As opposed to you have the thoughts, but you definitely would not act on them? III) Have you started to work out, or actually worked out, the specific details of how to kill yourself and did you actually intend to carry out the details of your plan? IV) In the past 3 months, have you done anything, started to do anything, or prepared to do anything to end your life? Examples: Collected pills, obtained a gun, gave away valuables, wrote a will or suicide note, took out pills but didn't swallow any, held a gun but changed your mind about hurting yourself or it was grabbed from your hand, went to the roof to jump but didn't; or actually took pills, tried to shoot yourself, cut yourself, tried to hang yourself. A binary yes/no *suicide warning* (referred to here as “suicide risk”) is generated if the patient responds yes to one or several of these four questions.

III. Blood draws

Blood draws for all participants were done between 8-10 AM, after fasting, within 5 days of completing the psychiatric evaluations. Plasma was separated by centrifugation at 700x*g* at 4°C, aliquoted, and immediately placed in a -80°C freezer until time of analysis. A complete blood count (CBC) was performed using a Beckman Coulter LH755 automated analyzer (BC, Southfield, MI, USA).

IV. Inflammatory- and growth factors

Pro- and anti-inflammatory factors, acute-phase reactants and growth factors in plasma were analyzed on the Meso Scale Discovery Sector 6000 imager according to the manufacturer's protocol (MESO SCALE DIAGNOSTICS, LLC, Rockville, Maryland). All samples were run in duplicate and the mean values were used for statistical analysis. IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IFN-γ and TNF-α were analyzed using the Pro-inflammatory I multiplex panel. Inter-assay coefficients of variation (CV): IL-1β (7.6%), IL-2 (8.9%), IL-4 (9.5%), IL-6 (3.7%), IL-8 (5.3%), IL-10 (2.9%), IL-12p70 (5.9%), IL-13 (3.4%), IFN-γ (3.5%) and TNF-α (6.3%). The Vascular Injury Panel 2 Kit was utilized for the detection of SAA, CRP, VCAM-1, and ICAM-1. Inter-assay CV: SAA (10.9%), CRP (9.3%), VCAM-1 (6.2%), and ICAM-1 (4.8%). The Human Growth Factor I Kit was utilized to detect bFGF, sFLT-1, PIGF, and VEGF. Inter-assay CV: bFGF (10.3%), sFLT-1 (7.3%), PIGF (7.8%), and VEGF (7.6%).

V. Measures of immunity toward pathogen and pathogen activity

HSV1 and HSV2 immunoglobulin G (IgG) titer and IgG avidity were analyzed by the manufacturer's instructions using the SERION ELISA classic Herpes Simplex Virus IgG

kits (Institut Virion\Serion GmbH, Würzburg, Germany). All kits were run on an Infinite 200 PRO plate reader (Tecan Group Ltd., Männedorf, Switzerland) using the Tecan i-control application. To measure antibody avidity, a 6 M urea wash step was included to remove low avidity antibodies from the plate prior to titer quantification as described previously (Hashido, Inouye, & Kawana, 1997). Avidity index was calculated from the ratio of high-avidity IgG to total IgG, with an avidity index (AI) higher than 0.6 indicating recurrent infection (Blackburn, Besselaar, Schoub, & O'Connell, 1991; Hashido et al., 1997). HSV 1 & 2 IgM, *T. gondii* IgM and IgG, and cytomegalovirus CMV IgM, IgG, and IgG avidity were analyzed by the manufacturer's recommendations using IBL Internationals kits (IBL International Corp GmbH, Hamburg, and Germany). All plates were read on a Synergy HT plate reader (BioTek, Winooski, VT) using software version 2.04.11. A 6 M urea wash step was included to measure CMV IgG avidity. All samples were run in duplicate and the mean values of the duplicates were used for analysis.

VI. Measures of kynurenine pathway metabolites

Plasma from the patients was analyzed using gas chromatography-mass spectrometry (GC-MS) to determine PIC and QUIN concentrations. Plasma proteins were precipitated out using 10% trichloroacetic acid and centrifuged. 50 µL of the solution were added to a glass tube along with deuterated internal standard and the previously published protocol was performed (Smythe et al., 2002). The sample was injected into a Thermo Trace GC Ultra gas chromatograph interfaced to a Thermo DSQ II mass spectrometer. The inter-assay CV's were 1.9% for PIC and 4.5% for QUIN.

KYN and TRP were analyzed by high-performance liquid chromatography (HPLC). Plasma proteins were precipitated out using the method stated above. The supernatant

was then filtered through a 0.22 µm PTFE filter into an HPLC polypropylene vial, and 20 µL was injected into the Thermo Scientific Dionex UltiMate 3000 (Timothy B. Meier et al., 2016). The inter-assay CV's were 3.2% (KYN) and 0.8% (TRP).

VII. Statistical analysis

All analyses were performed using R v 3.4.3 (https://cran.r-project.org/). The systems level analyses were assessed using WGCNA. We used the WGCNA R software package to carry out module construction, hub analyte selection and network statistics. The method WCGNA is a data reduction method that serves to reduce large amounts of biological information into modules; forming a network. The network preserves the continuous nature of the underlying correlation information and is highly robust compared to alternative methods. Standard practice in WGCNA is to calculate the first principal component of the modules (called "the eigengene" in WGCNA). The eigengenes are the most representative immunobiological profiles of the respective modules (consisting of the cumulative signal of the analytes in the module). Next, two sets of Spearman correlation analyses are conducted with the eigengenes. In the first, eigengenes association with the patient characteristics (CAT-MH depression and suicide risk) is established. The results are visualized in Figure 3.2 and shows whether a module (and therefore the analytes within it) is significantly associated with the patient characteristics. In the next step, the association of the eigengenes with the individual analytes within the module is established. The resulting coefficients are denoted module membership values. Analytes with high module membership indicate high connectivity to the other analytes contained within the module and are identified as hub markers. These hubs are generally good candidates for biomarkers and focus for

inferring pathology, as they are linked to the most representative expression profile of the module. The soft threshold for the scale-free topology of WGCNA was assessed by fitting over an array of powers from 1-30 for a signed-hybrid network. Good scale-free topology (minimum correlation of 80%) was achieved using a power of 6. The minimum number of markers needed to create a module was set to 1. No modules were merged. All p-values in the results section are reported after treatment and demographic correction unless otherwise stated.

We next performed sensitivity analyses to test whether the relationship between the immunobiological markers and suicide risk or depression were impacted by potential confounders, including treatment, demographics and comorbid depressive/anxiety symptoms by ridge regression (RiR) models. The models were adjusted for BMI, age, anti-inflammatory medications, antiepileptics, antipsychotic, psychostimulants, benzodiazepines, serotonin-norepinephrine reuptake inhibitors, selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressant treatment, anxiety scores derived from HAMD (sum of item 10 and 11) and HAMD17 total scores for depression. RiR was chosen specifically to address multicollinearity in the covariates (de Vlaming & Groenen, 2015). P-values were calculated using a p-value trace, a plot of the negative logarithm of the p-values of RiR coefficients with increasing shrinkage parameters, developed by Cule and colleagues (Cule, Vineis, & De Iorio, 2011) to reduce computational cost. Linear RiR was used for analysis of depression severity, while logistic RiR was used for the binary suicide warning outcome. RiR with only treatment adjustment and standard regression with only the cytokine and outcome were also fit to assess the sensitivity of the results to the exclusion of demographic and treatment adjustments. Measured

analytes were log transformed as necessary. N-fold cross validation was used to establish the value of the tuning parameter, which was set as one standard deviation (SD) from the value that minimized the mean-squared error.

Results

I. System level analysis and module identification

Demographics of the cohort, based on “suicide risk”, is shown in Table 3.4. Using WGCNA, we identified three separate modules, i.e. clusters of highly interconnected biological analytes, among the 45 immunobiological factors. The first module was comprised primarily of red blood cell factors, the second module consisted of kynurenine pathway analytes, and the third module was comprised of white blood cell-related factors (Figure 3.1).

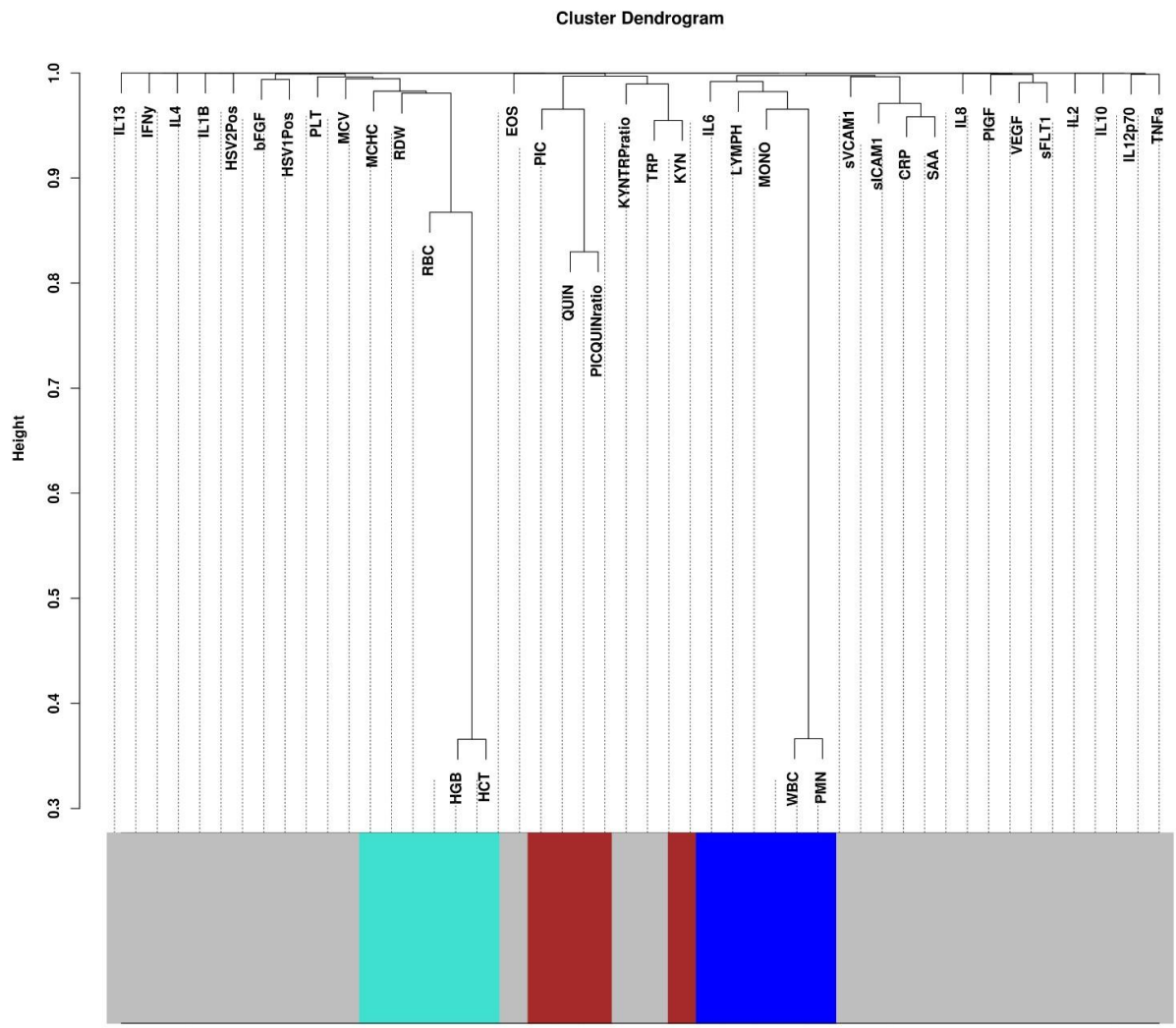

Figure 3.1 Dendrogram of WGCNA biological outcome measures Three biological analyte clusters (modules) were identified, coded here by; turquoise, red blood cell related factors; brown, kynurenine pathway metabolites; and blue, white blood cell- related factors. Analytes with a lower height number have a greater impact on their respective module. The color gray denotes no association of an analyte with a specific module.

II. A biological profile associated with suicide risk

At the network level, WGCNA identified “suicide risk” to be positively associated with the blue module, containing IL-6, LYMPH, MONO, WBC and PMN (WGCNA, r=0.27, p<0.05) (Figure 3.2). WBC (Module membership (MM)=0.94) and PMN (MM=0.86) constituted hubs (a hub consists of analytes inside a module that have high

connectivity, typically of pathophysiological relevance) and had the largest influence on "suicide risk". Module membership information can be found in data table (Table 3.5). Module membership numbers range from +1 (influencing the module in a positive relationship) to -1 (influencing the module in a negative relationship). The closer the module membership number is to +1 or -1 the more influential the marker is on the associated module. There was no system level significant association between "depression" and any of the identified immunobiological modules (WGCNA, Figure 3.2).

Table 3.5 Module membership values

Marker	Blue Module	Brown Module	Turquoise Module
White Blood Cell Count	0.94	-0.09	-0.06
Polymorphonuclear Leukocyte Count	0.86	-0.08	-0.06
Monocyte Count	0.70	0.01	-0.04
Lymphocyte Count	0.66	-0.10	0.00
Interleukin-6	0.59	-0.02	-0.06
C-reactive Protein	0.37	0.09	0.06
Intercellular Adhesion Molecule 1	0.33	0.07	-0.01
Vascular Endothelial Growth Factor	0.30	-0.10	0.08
Eosinophil Count	0.29	0.14	0.13
Platelet Count	0.28	-0.13	-0.35
Mean Cell Hemoglobin Concentration	-0.24	0.12	0.49
Serum Amyloid A	0.23	0.14	-0.10
Herpes Simplex Virus Type 1 Positivity	0.23	-0.26	0.03
Interleukin-1β	-0.20	0.12	0.01
Herpes Simplex Virus Type 2 Positivity	0.18	-0.10	-0.10
Tryptophan	-0.18	0.28	0.18
Kynurenine	-0.18	0.60	0.18
Picolinic Acid/ Quinolinic Acid Ratio	0.17	-0.95	-0.12

Table 3.5 (Cont'd)

Marker	Blue Module	Brown Module	Turquoise Module
Quinolinic Acid	-0.17	0.85	0.13
Basic Fibroblastic Growth Factor	0.15	0.09	0.07
Red Blood Cell Distribution Width	0.12	-0.01	-0.56
Interleukin-13	-0.11	-0.01	0.10
Interferon- γ	0.10	0.14	0.00
Interleukin-12p70	-0.10	0.17	0.19
Interleukin-10	0.10	0.08	0.03
Soluble Fms-like Tyrosine Kinase 1	0.08	-0.01	0.07
Hemoglobin	-0.08	0.16	0.99
Picolinic Acid	0.06	-0.42	-0.02
Placental Growth Factor	-0.04	-0.11	0.27
Interleukin-2	-0.03	0.11	0.12
Red Blood Cell Count	0.02	0.19	0.69
Tumor Necrosis Factor- α	0.02	0.24	0.12
Interleukin-4	-0.01	0.08	0.13
Kynurenine/ Tryptophan Ratio	-0.01	0.36	0.00
Hematocrit	0.01	0.13	0.97
Mean Cell Volume	-0.01	-0.06	0.34
Vascular Cell Adhesion Molecule 1	-0.01	0.03	0.07
Interleukin-8	0.00	0.03	-0.09

The closer to +1 or -1, the more influential the analyte is within that module. Markers listed by decreasing influence on the blue module.

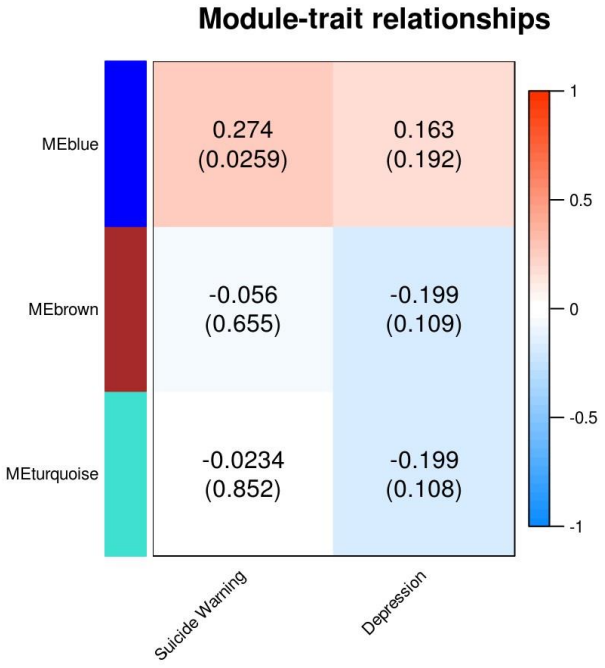

Figure 3.2 Heat map of WGCNA biological outcome measures Module-trait relationships are listed for suicide risk and depression. Module significance was determined by the average of absolute analyte significance for all analytes in the module. The blue module, comprising white blood cell-related factors, was significantly associated with increased suicide risk (R=0.27, p<0.05)

III. Impact of potential confounders

In order to determine if the associations between the immunobiological factors and outcome “suicide risk” and “depression” were influenced by potential confounders, we performed sensitivity analyses where we introduced age, BMI, treatment, depression and anxiety scores into regression models (Table 3.6). The results show that the effect on “suicide risk” by the immunobiological markers was not influenced by symptoms of depression and anxiety. Similarly, the immunobiological profile associated with “depression” was not influenced by symptoms of anxiety. Moreover, to control for

potential confounding effects of medications, we included treatment with anti-inflammatory medications, antiepileptics, antipsychotics, psychostimulants, benzodiazepines, serotonin-norepinephrine reuptake inhibitors, SSRIs, and tricyclic antidepressants as covariates in regression models. The results were not sensitive to the treatment covariates, as illustrated by the regression models (Table 3.6). The independent exploratory analyses also revealed that the IL-8 levels were negatively associated with increased suicide risk (RiR, β=-0.03±0.02, $p<0.05$), and there were significant positive associations between “depression” and the acute phase reactant SAA (RiR, β=0.05±0.02 SE, $p=0.01$) and platelet count (RiR, Beta=0.05±0.02 SE, $p<0.01$) (Table 3.6).

Table 3.6 Sensitivity analyses

Dependent: Depression								
Blood Analyte	***1. Treatment, Demographics, and Anxiety Included***		***2. Treatment and Demographics Included***		***3. Treatment Included***		***4. Unadjusted Regression***	
	Regression Coefficient (±SEM)	p	Regression Coefficient (±SEM)	p	Regression Coefficient (±SEM)	p	Regression Coefficient (±SEM)	p
PLT	0.132 (±0.05)	0.01	0.05 (±0.02)	0.008	0.05 (±0.02)	0.008	0.005 (±0.002)	0.007
SAA	0.035 (±0.01)	0.01	0.05 (±0.02)	0.01	0.05 (±0.02)	0.01	0.30 (±0.11)	0.01
Dependent: Suicide Risk								
Blood Analyte	***1. Treatment, Demographics, Depression, and Anxiety Included***		***2. Treatment and Demographics Included***		***3. Treatment Included***		***4. Unadjusted Regression***	
	Regression Coefficient (SEM)	p	Regression Coefficient (SEM)	p	Regression Coefficient (±SEM)	p	Regression Coefficient (±SEM)	p
CRP	0.03 (±0.01)	0.007	0.03 (±0.01)	0.007	0.03 (±0.01)	0.006	0.60 (±0.24)	0.01
EOS	0.03 (±0.01)	0.01	0.03 (±0.01)	0.01	0.03 (±0.01)	0.01	1.47 (±0.64)	0.02
IL-8	-0.03 (±0.01)	0.04	-0.03 (±0.02)	0.04	-0.03 (±0.02)	0.04	-2.00 (±1.03)	0.05
LYMPH	0.03 (±0.01)	0.03	0.03 (±0.01)	0.03	0.03 (±0.01)	0.03	1.13 (±0.52)	0.03
PMN	0.03 (±0.01)	0.04	0.03 (±0.01)	0.04	0.03 (±0.01)	0.04	1.58 (±0.78)	0.04

Checking the influence of potential confounders on the effect of the biological factors on suicide risk and depression. Significant biological predictors for outcome depression (top rows) and suicide risk (bottom rows) analyzed by four separate regression models. Column (model) 1 shows RiR adjusted for anxiety levels, treatment and demographics for depression, and adjusted for both depression and anxiety scores, treatment and demographics for suicide risk. Column 2 shows RiR adjusted for treatment and demographics only. Column 3 shows the RiR adjusted only for treatments. Column 4 shows the unadjusted regression model, only measuring the impact of the biological factors on the outcomes of depression or suicide risk without any adjustment.

Discussion

In this study, we measured 45 immunobiological markers in peripheral blood and analyzed their associations with cross-diagnostic suicide risk in a population of 66 women with mood and anxiety disorders. We discovered a distinct immunobiological profile in subjects with increased suicide risk, composed of IL-6 and white blood cells, in particular granulocytes. There was also a module-independent negative association between IL-8 and suicide risk. We did not identify any composite network of immunobiological factors linked to depression severity, but independent positive associations between depression and the acute phase mediator SAA as well as increased platelet count. Our results add support to the mounting number of studies demonstrating a significant dysregulation of the immune system in patients with suicidal ideation and behavior. The results are novel in several ways. First, we used an unbiased way to generate the suicide risk assessment (the CAT-MH™ generated *suicide warning*) which was next matched with the biological data. Second, we subjected a large number of immunobiological factors to robust statistical methods, the network analysis WGCNA and sensitivity analyses using RiR models. Our significant results indicate that depression and suicide risk are associated with distinct immunobiological profiles in peripheral blood, confirming our hypothesis. Third, our data analysis show that granulocyte mediated immune reactions could potentially be central among the biological mechanisms responsible for triggering suicidal ideation and behavior.

As strengths of our study, we analyzed a high number of biological analytes in clinically well-characterized patients and subjected the data to robust network analysis and

showed that the data was not impacted by important potential confounders in sensitivity analyses. Our analysis showed that the data was not sensitive to pharmacological treatment, BMI, age or the severity of symptoms of anxiety or depression. However, as a limitation; even if the statistical methods we used are not sensitive to sample size or data heterogeneity, the biological features are cohort specific and need to be confirmed in future studies. Since the population in this study was limited to women with mood and anxiety disorders, there is a need to assess the biological networks connected to suicide risk in additional cohorts before generalizing the results. Other limitations of this study include a lack of data regarding the number of past episodes or disease duration. Such data may provide further insight into specific immunobiological factors that are important during different stages of mood disorders (Brietzke et al., 2009).

We found that suicide risk was associated with increased pro-inflammatory factors and WBCs. CRP is a marker of acute inflammation that has previously been associated with suicidal ideation and behavior (Gibbs et al., 2016; Loas, Dalleau, Lecointe, & Yon, 2016). During the acute-phase response, an elevation of CRP is often accompanied by increased numbers of lymphocytes (Barzilay et al., 2016; Gans et al., 2015). At the network level, we found that suicide risk was linked to a module containing IL-6, total WBC, lymphocytes, monocytes and in particular polymorphonuclear cells (granulocytes). There are some previous reports indicating that suicidality (ideation and behavior) in depressive patients correlates with increased WBCs (Endres et al., 2016). Additionally, impulsivity, which is linked to suicide (Mann, Waternaux, Haas, & Malone, 1999), is associated with increased WBCs (Sutin et al., 2012). In a recent study which evaluated over 300,000 women of which 1,000 later died by suicide, higher levels of

WBCs were predictive of later suicide (Batty, Jung, Lee, Back, & Jee, 2018). Interestingly, our network analysis indicated granulocytes to be the cell type with strongest influence on suicide risk. Granulocytes are cells of the innate immune system that produce a large variety of proinflammatory cytokines, both constitutively, and in increased amounts after stimulation. They fulfill several functions in the immune response including responses towards pathogens, such as parasitic infections, and mediate allergic reactions. Allergies and certain parasitic infections have been linked with increased suicide risk in previous epidemiological studies, and could thus be hypothesized to be triggers of the involved immunobiological mechanisms (Lund-Sorensen et al., 2016; Pedersen, Mortensen, Norgaard-Pedersen, & Postolache, 2012; Y. Zhang et al., 2012).

In addition to increased WBCs, we observed a reduction of IL-8 in women with increased suicide risk. IL-8 is a chemoattractant cytokine that has strong target specificity for neutrophils (Bickel, 1993). The cytokine serves pleotropic and tissue specific functions, including a role in the development and establishment of synaptic plasticity in the CNS (Willette et al., 2013). Isung and colleagues reported lower levels of IL-8 in the cerebral spinal fluid (CSF) of suicide attempters compared to healthy controls (Isung et al., 2012). Additionally, our group has previously found low levels of IL-8 in the plasma and CSF to be associated with increased anxiety levels in suicide attempters (Janelidze et al., 2015). Our current study thus provides additional support, in a third clinical cohort, that low levels of the cytokine IL-8 are associated with suicidal ideation and behavior. The potential biological mechanism(s) impacted by IL-8 in suicidal patients appears to be separate from the module identified by our network

analysis, as IL-8 was not a member of the immunobiological network associated with suicide risk. Instead, we identified IL-6, a pro-inflammatory cytokine that has previously been linked to suicidality in multiple studies (Isung et al., 2014; Janelidze et al., 2011; Lindqvist et al., 2009; Mina et al., 2015), to be part of the immunobiological network linked to suicide risk. IL-6 is a pro-inflammatory and regulatory cytokine that can be secreted by many cells of the immune system, in particular monocytes and granulocytes of the innate immune system (Ericson et al., 1998; Oishi & Machida, 1997; Zimmermann et al., 2016). IL-6 can also be secreted by adipose tissue and in response to exercise (Hallberg et al., 2010; Kern, Ranganathan, Li, Wood, & Ranganathan, 2001; Lyngso, Simonsen, & Bulow, 2002; Makki, Froguel, & Wolowczuk, 2013). However, we did not find any impact of BMI on our data, and the close connection between the IL-6 levels and immune cells in the WGCNA module demonstrates that the cytokine is likely being secreted by immune cells rather than by other cell types in the body in individuals with suicide risk. In addition to being a mediator of immune reactions, IL-6 can bind directly to receptors on neurons in the CNS and has been shown to directly impact the generation of action potentials, which may influence changes in emotion and behavior, including increased risk for suicidal intent and behavior (Gruol, 2015; Xia et al., 2015).

Different immunobiological mediators were implicated in depression. While the network analysis did not find any biological network associated with depression, we detected independent associations for the acute phase mediator SAA and platelet counts in our exploratory sensitivity analyses. SAA is an acute phase mediator that can activate macrophages and glial cells, and is increased in patients with multiple, chronic inflammatory diseases (Muhlebach et al., 2016; Scarpioni, Ricardi, & Albertazzi, 2016;

Ye & Sun, 2015). SAA induces the production of pro-inflammatory cytokines from microglia, including those associated with depression (Eklund, Niemi, & Kovanen, 2012), and we previously found that elevated levels of SAA were associated with depressive symptoms as measured by HAMD (Bryleva et al., 2017). Platelets are also known to increase in acute inflammatory conditions, and play a role in the inflammatory cascade by increasing vascular permeability (Gros, Ollivier, & Ho-Tin-Noe, 2014; Kapur, Zufferey, Boilard, & Semple, 2015; Williams, 2012), detecting and responding to vascular injury. A “platelet hypothesis of depression” has been proposed, as depression is a risk factor for cardiovascular mortality and morbidity, and studies have demonstrated that increased platelet reactivity and aggregation are associated with increased risk for depression (Lederbogen et al., 2001; Musselman et al., 1996; Williams, 2012).

A network analysis approach to finding associated blood markers may be more insightful than analyses focusing on only one or a few markers, as there are often synergistic and distinct effects, depending on what type of immunobiological network is active; for example, the cytokine IL-6 can be elevated as part of a pro-inflammatory cascade, as part of a cytokine release from muscular tissue in response to exercise, or as part of a regulatory anti-inflammatory response. Whether the biological mechanisms implicated here are specific for depressive and suicidal individuals need to be confirmed in additional populations. Causative effects of the proposed mechanisms can be tested in experimental models. Suicidal symptoms and behavior can be dissected into components such as impulsivity, hopelessness and aggression that can be tested

further in relation to the distinct immunobiological profiles, in both experimental models and in human populations.

Conclusion

In this study, we integrated several innovative approaches to assess the immunobiological profiles of women with mood disorders, to identify a unique biological profile in subjects with increased suicide risk. Our study highlights a number of immunological mediators that may be of specific importance in the pathophysiology of suicidal behavior, including the cytokines IL-6 and IL-8 and granulocyte mediated mechanisms. Replication studies in independent cohorts will be necessary to further the development of clinical biomarkers of suicide risk. Additional studies are warranted using network analysis in larger populations of carefully clinically characterized patients, in order to advance our understanding of the complex immunobiological networks underlying depressive illness and suicide risk.

Chapter 4: Inflammation in Peripartum and Postpartum Depression

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This chapter has been modified from three manuscripts that will be submitted separately.

Abstract

Background: The biological mechanisms contributing to PPD and postpartum depression are uncharacterized, and therefore specific clinical treatments are unavailable. Increased inflammation can lead to depression, however the exact biological similarities and differences between PPD and postpartum depression are unclear. To address this, we measured kynurenine pathway metabolites and inflammatory proteins in the plasma from women during the first, second, and third trimester, and postpartum period. Additionally, placenta, an immunologic organ, was analyzed for expression of kynurenine pathway mRNA expression to assess its role in peripartum and postpartum depression.

Methods: 207 women were enrolled during the first trimester of pregnancy and/or the postpartum period to assess depression and suicidality. Linear regression models were age corrected to analyze plasma protein levels, kynurenine metabolites, and placental mRNA expression.

Results: The biological profile of patients who developed depression during pregnancy, as well as women who developed postpartum depression and suicidality were established. Plasma IL-6 correlated positively with Edinburgh Perinatal Depression Scale (EPDS) scores and predicted EPDS scores during the first, second, and third trimester but not postpartum. Placenta IDO correlated with IL-1β at every time point, and with IL-6 at the second and third trimester. IL-2 levels were decreased, and IL-8 levels were increased in postpartum depressed and suicidal women compared to health controls.

Discussion: Unique inflammatory profiles were linked to PPD, postpartum depression, and suicidality. IL-6 levels in plasma predicted the development of depressive symptoms throughout pregnancy. Our data suggests that the kynurenine pathway and inflammation may be associated with PPD, and these changes may stem from the placental tissue. Postpartum depression and suicidality had a different inflammatory profile, with increased IL-8 and decreased IL-2, which may be due to the absence of the placenta. The establishment of plasma biomarkers which predict PPD, postpartum depression and suicidality could lead to a better targeted therapeutic strategy for at-risk patients.

Introduction

Depression is common during and after pregnancy, affecting up to 20% of pregnant women in the Unites States. The prevalence of PPD is estimated to be around 10%, and postpartum depression around 12-15% (M. M. Howard, Mehta, & Powrie, 2017; Marcus, 2009). Frequently, symptoms start during pregnancy although the disease is not recognized and diagnosed until in the postpartum. It is estimated that a proportion of

depressed women are never diagnosed, so the actual percentage of women with PPD and postpartum is likely even higher. PPD and postpartum depression have specific characteristics in addition to depressed mood including agitation, anxiety, suicidal ideation, rumination and fear of hurting the child (Meltzer-Brody, 2011). In the most severe cases, psychotic symptoms develop and can lead to suicides.

Although 10% of pregnant women are affected by depression in the peripartum period, the biological causes are not well understood, and no specific treatment is available. Peripartum and postpartum suicidality and depression treatment is usually with SSRIs, which are effective in only about 50% of all depressed patients (L. M. Howard et al., 2011). When given during pregnancy, SSRIs have been associated with serious risks (Boucher et al., 2008; K. Palmsten et al., 2013). Therefore, physicians and mothers are often reluctant to use SSRIs, and depression in pregnancy is often left untreated.

There is a significant need for improved and specific treatments for PPD. An understanding of the biological mechanisms responsible for the symptoms would make the development of novel, targeted treatments for PPD possible. One potential mechanism of PPD is inflammation, which is known to cause depressive symptoms. An example of this is “Interferon-induced depression”, which occurs when patients who receive interferon-based immunotherapy develop depression and suicidal behavior (L. A. Lucaciu & D. L. Dumitrascu, 2015; Meyers, Scheibel, & Forman, 1991; Miyaoka et al., 1999). Experimental injection of LPS (a bacterial endotoxin) in patients also leads to depressive symptoms (Yirmiya et al., 2000). Further, a wide range of inflammatory challenges in animal models can generate depressive- like behavior (Bay-Richter, Janelidze, Hallberg, & Brundin, 2011; O'Connor et al., 2009; Walker et al., 2013;

Yirmiya, 1996). Several mechanisms connect inflammation with depression and suicidal behavior in the CNS. Figure 4.1 shows a conceptual model of the pathways and mediators we propose are of importance for the generation of PPD.

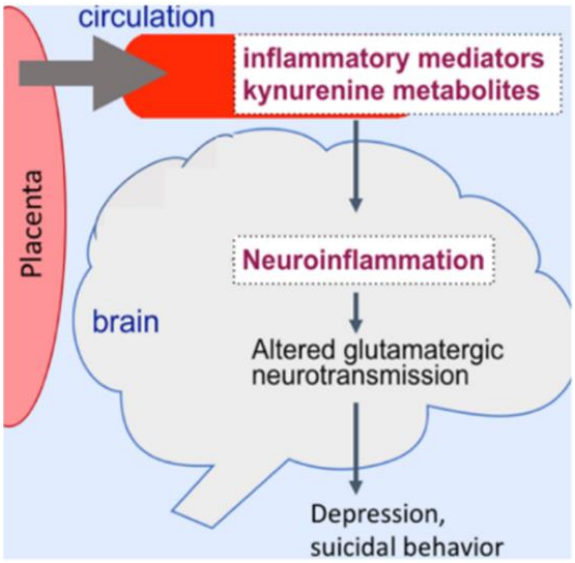

Figure 4.1 Conceptual Model of PPD A proposed mechanism to how the symptoms of PPD and suicidal behavior are generated. The placenta is generating inflammatory mediators and kynurenine metabolites that are secreted into the blood stream, and able to impact the brain causing neuroinflammation. This leads to altered glutamatergic neurotransmission, depression, and suicidal behavior.

Peripheral cytokines reach the CNS through active transport and regions with increased BBB permeability, e.g. circumventricular organs and choroid plexus (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). We and others also found increased BBB permeability in suicidal individuals, which implies that certain patient groups may have an increased sensitivity to peripheral inflammation (Bayard-Burfield, Alling, Blennow, Jonsson, & Traskman-Bendz, 1996; Falcone et al., 2010; Ventorp et al., 2016). Cytokines induce symptoms collectively known as “sickness behavior”, including depressed mood, fatigue, concentration difficulties, and changes in sleep and appetite (Dantzer & Kelley, 1989; R. Dantzer et al., 2008; B. L. Hart, 1988). Moreover, inflammation is coupled to impulsivity and aggression, traits linked to suicidal behavior (Coccaro, 2006;

Coccaro, Lee, & Coussons-Read, 2014; Mommersteeg, Vermetten, Kavelaars, Geuze, & Heijnen, 2008; Suarez, Lewis, & Kuhn, 2002; Swift & Trueblood, 1973). Inflammation induces down-stream pathways leading to decreased levels of several monoamines (e.g. serotonin, dopamine) and an increased production of kynurenine metabolites. Thus, inflammation induces emotions of sadness, fatigue and hopelessness, and affects multiple neural pathways, including those regulating aggression and impulsivity.

Inflammatory cytokines are also potent activators of the kynurenine pathway (Schwieler et al., 2015; Urata et al., 2014), a series of enzymes that metabolize the essential amino acid TRP. The initial step converts TRP into KYN, which is then broken down into neuroactive metabolites (Figure 4.2). The branch of the pathway generating QUIN is often denoted the “neurotoxic branch” while the KYNA producing branch is called the “neuroprotective branch”, even if this is a simplification of the roles of the pathway arms. QUIN, produced by macrophages and microglia, is an agonist of the NMDA receptor and QUIN production is increased during states of inflammation (Stone, 1993; Tavares et al., 2002). We have shown CSF QUIN correlates significantly with inflammation in the plasma (Brundin et al., 2016). Thus, peripheral inflammation correlates with the activation state of the kynurenine pathway in the CNS.

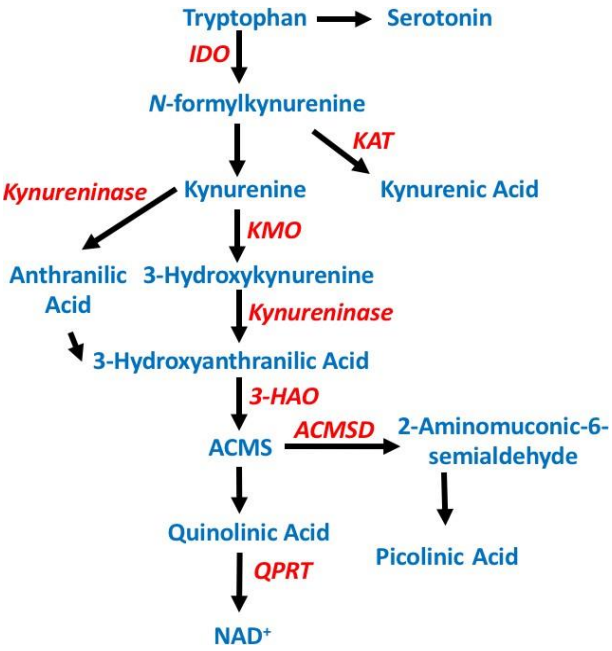

Figure 4.2 Simplified kynurenine pathway Metabolites blue, enzymes red. ACMSD, amino-carboxymuconate-semialdehydecarboxlase; IDO, indoleamine 2,3-dioxygenase; KAT, kynurenine aminotransferases; KMO, kynurenine-3-mono-oxygenase; 3-HAO, 3-hydoxyanthranilate, 3,4-dioxygenase; QPRT, Quinolinate-phospho-ribosyl transferase.

Placental tissue expresses all the enzymes of the kynurenine pathway (Dharane Nee Ligam et al., 2010; Manuelpillai et al., 2003; P. Sedlmayr et al., 2002). IDO is highly expressed in the human placenta and involved in maternal-fetal tolerance mechanisms (Honig et al., 2004). Research suggests maternal-fetal tolerance is established by IDO metabolizing TRP at the placental interface, leading to a localized state of TRP depletion and consequent decreased reactivity of T cells (Munn et al., 1998; Zhu, 2010). Metabolites of the kynurenine pathway, including KYN and QUIN, also regulate the immune system in placenta through highly specific mechanisms (Fallarino et al., 2002; Mezrich et al., 2010; Nguyen et al., 2010). We and others found a reduction of placental IDO in patients with pathological conditions of pregnancy, including pre-eclampsia (manuscript accepted, *International Journal of Tryptophan Research*) and intrauterine

growth restriction (Chang, Li, & Li, 2018; Iwahashi et al., 2017; Murthi, Wallace, & Walker, 2017; Santillan et al., 2015).

The prominent expression and activity of kynurenine enzymes in human placenta has the potential to cause increased amounts of metabolites secreted into the blood stream. This might constitute an important mechanistic pathway which contributes to the generation of depressive symptoms in pregnancy and the postpartum period. Here we enrolled a clinical cohort of over 200 women, sampling plasma samples during the first, second, and third trimesters and/or postpartum as well as the highly immunologic organ, the placenta, upon delivery to analyze the kynurenine pathway and inflammatory factors to further our understanding of the biological mechanisms involved PPD and postpartum depression.

Methods

I. Clinical design

A total of 207 women were enrolled during their first trimester of pregnancy or the postpartum period at two sites: Spectrum Health in Grand Rapids, MI and Pine Rest Christian Mental Health in Grand Rapids, MI. Study design was approved by Michigan State University's Internal Review Board (IRB) approved (IRB # 14-458M). The women were 18-44 years of age with an average age of 26.54. 118 women were recruited from the Spectrum Health site and were followed over four time points during and after pregnancy: first (visit 1), second (visit 2), third (visit 3) trimester and postpartum (visit 4). 89 women were enrolled in the postpartum period from a partial hospitalization program focusing on severe postpartum depression, the Pine Rest Mother and Baby program, at Pine Rest Christian Mental Health. All participants completed an informed consent form

at visit 1 during screening and baseline. A self-reported EPDS was completed looking at the past 7 days for all visits. Significant/severe depressive symptom cut-off score was set to an EPDS score of 13 and above. Below a score of 13 was considered not depressed and a healthy control. Active suicidal behavior consisted of making active preparations and actual suicide attempts which could be completed or interrupted, as defined by the C-SSRS. A somatic health report, as well as vital signs (weight, height, pulse, blood pressure, temperature), and blood draws were completed at all visits. Blood from both locations were immediately transported to the laboratory on ice. Blood was centrifuged, and plasma aliquoted and frozen at -80°C.

II. Plasma protein analysis

IL-1β, IL-2, IL-6, IL-8, IL-10, and TNF-α protein levels were measured in previously unthawed plasma aliquots using the Meso Scale Discovery platform in accordance to manufacturer's instructions (Meso Scale Diagnostics, Rockville, Maryland). The samples were run in duplicate and the mean values of the duplicates were used for final statistical analysis. Inter-assay coefficients of variation (% CV): IL-1β (3.5%), IL-2 (3.0%), IL-6 (2.2%), IL-8 (2.4%), IL-10 (1.8%), and TNF-α (2.8%). Intra-assay coefficients of variation (% CV): IL-1β (18.3%), IL-2 (21.0%), IL-6 (5.5%), IL-8 (3.6%), IL-10 (6.2%), and TNF-α (4.1%). Lower detection limits: IL-1β (0.02 pg/ml), IL-2 (0.12 pg/ml), IL-6 (0.07 pg/ml), IL-8 (0.05 pg/ml), IL-10 (0.02 pg/ml), and TNF-α (0.09 pg/ml).

III. Placenta sample collection and dissection

Placental tissues were put in containers filled with 1L of ice cold sterile GIBCO® Hank's Balanced Salt Solution (ThermoFisher Scientific, Waltham, MA) immediately after delivery and agitated to rinse off blood. Prior to dissection, placental tissues were

weighed. For dissection, the placenta was placed with the maternal side facing downward. A 1 cm x 1 cm x 1 cm cuboidal section of villus was removed, not including the chorionic plate or basal plate. This was repeated four times in a circumferential manner, approximately 3-4 cm from the cord insertion site. Villus samples were frozen at -80°C after being washed in sterile phosphate buffered saline to remove remaining blood for quantitative polymerase chain reaction (qPCR) analysis. In total, placental tissue from 69 patients was processed. The number of placentas was lower than the number of subjects followed by blood and clinical assessment, as the conditions of some deliveries prevented our staff from retrieving/processing the placental tissue.

IV. Placenta qPCR analysis

Ribonucleic acid (RNA) was isolated from flash frozen placenta villus tissue of 4 quadrants using the Quick-RNA™ MiniPrep Plus (Zymo Research, Irvine, CA) according to manufacturers recommended protocol. Multiplex qPCR was performed to quantify mRNA expression of ACMSD, IDO, QPRT, and Succinate Dehydrogenase (SDHA) using the TaqMan multiplex real-time PCR assay (ThermoFisher, Waltham, MA) with primers tagged with ABY™, FAM™, JUN™, and VIC™ dyes (C. Hart, 2014). Mustang Purple™ was used as the reference dye. This assay was run on an Applied Biosystems 7500 Real-Time PCR System (ThermoFisher, Waltham, MA). SDHA was used as the internal control to normalize samples as recommended by Meller et al. for placental tissue (Meller, Vadachkoria, Luthy, & Williams, 2005). qPCR samples were run in triplicate and averaged for final statistical analysis.

V. Measurement of kynurenine metabolites

GC-MS was used to determine QUIN and PIC levels. Plasma proteins were precipitated using 10% trichloroacetic acid and centrifuged. 50 µL of solution was added to a glass tube along with deuterated internal standards as previously published (Smythe et al., 2002), next injected into a Thermo Trace GC Ultra GC interfaced to a Thermo DSQ II MS. High Performance-Liquid Chromatography (HPLC) was used to determine TRP and KYN concentrations. Samples were initially processed by the same method stated above. After centrifugation, the supernatant was filtered through a 0.22 µm PTFE filter and 20 µL was injected into the Thermo Scientific Dionex UltiMate® 3000 (Thermo Scientific, Waltham, MA, USA). The chromatograph separation was achieved on a reversed phase 150x3 mm BDS Hypersil C18 column (Thermo Scientific™) with 3µm particle size. Column and pre-column tubing was maintained at 35°C with isocratic elution (0.8mL/min) of analytes using a mobile phase consisting of 5% methanol in milliQ water containing 50 mM ammonium acetate (pH 4.65). Results were analyzed using the Chromeleon™ 7.2 Chromatography Data System (Thermo Scientific™ Dionex™). Ultra-high performance liquid chromatography, coupled to tandem mass spectrometry (UPLC-MS/MS) was used to determine plasma KYNA concentrations. Plasma proteins were precipitated by adding three volumes of acetonitrile to one volume of plasma. Following centrifugation, the supernatant was transferred to a new tube and evaporated to dryness in a speedvac. The sample was reconstituted in a diluent containing 200nM internal standard in 10mM ammonium formate and 0.1% formic acid in MilliQ water. Following reconstitution, the sample was filtered through a 0.22 µm PTFE filter and 10uL was injected onto a Waters Acquity BEH C18 UPLC

column (100 x 1mm, 1.7 µm particle size) interfaced with a Waters Acquity® UPLC and a Waters Acquity TQ-D mass spectrometer. Analytical conditions were followed according to a previously published protocol (Cellar et. al 2016). The intra-assay % CVs were: 1.1% for TRP, 1.2% for KYN, 6.1% for KYNA, 1.2% for picolinic aid, and 1.2% for QUIN. The lower detection limits were: 33 nM for TRP and KYN, 3 nM for QUIN and PIC, and 0.3 nM for KYNA.

VI. Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics v.24 program. Bivariable comparisons in demographic and clinical characteristics were made using Student's t-test. To adjust for potential confounding factor of age, all predictions were age corrected and skewed data natural logarithm (ln) transformed in linear regression models. Spearman and Pearson correlations were performed for correlative models.

Results

I. Patient characteristics

Patients had a mean age of 26.54 (+/- 5.59 SD), BMI 30.6 (+/- 7.83 SD), and total EPDS score of 12.70 (+/- 8.01 SD). Medications used during pregnancy are listed in Table 4.1. There were no significant differences in mean age, BMI, or current smoking status between women with and without depression. All group-wise comparisons were adjusted for gestational age by weighted regression models.

Table 4.1 Medications used during pregnancy

Medications	*Total*
Antiepileptics	9
Albuterol	12
Anti-inflammatory	9
Prenatal vitamins	134
Antivirals	3
Tricyclics	2
Antibiotics	12
Thyroid Hormone	8
Insulin	3
Antihistamine	24
SSRIs	62
SNRIs	13
Neuroleptics	20
Benzodiazepines	21
Wellbutrin	13
Lithium	5
Hormonal Birth Control	15

II. Longitudinal peripartum depression results

In our longitudinal arm, we found that the severity of depressive symptoms was similar during all time-points, providing support for the notion that depression frequently begins during pregnancy (not primarily in the postpartum) (Figure 4.3, Table 4.2, Not Significant). IL-6 plasma levels significantly predicted depression scores on the EPDS at

all time-points during pregnancy, but not in the post-partum (Figure 4.4 and Table 4.3).

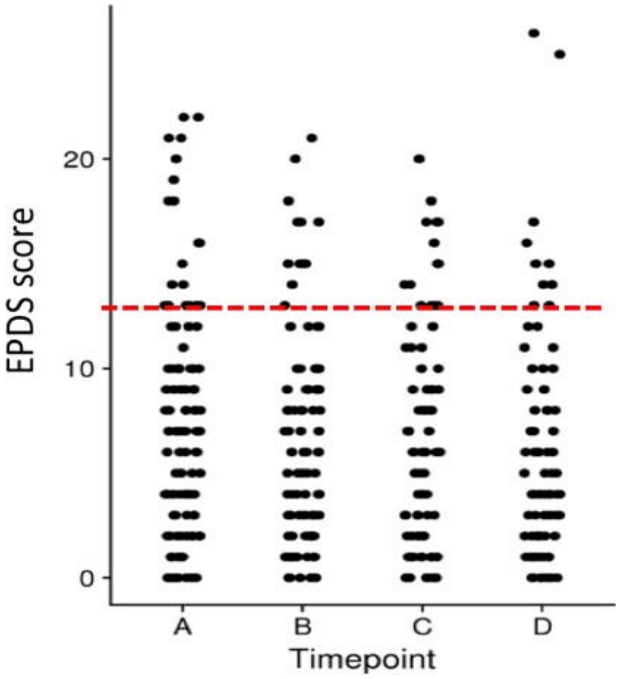

Figure 4.3 The total score on the EPDS at each of the four time-points A score at 13 and above indicates a likelihood of ongoing depression. Total subjects followed in the longitudinal study; *n*=118.

Table 4.2 Significant/severe depression symptoms at each time-point

Time-point	***Total Depressed Individuals***
First Trimester	22
Second Trimester	12
Third Trimester	14
Postpartum	12

The number of women out of 118 enrolled in the longitudinal arm who had significant/severe depressive symptoms (EPDS at 13 and above).

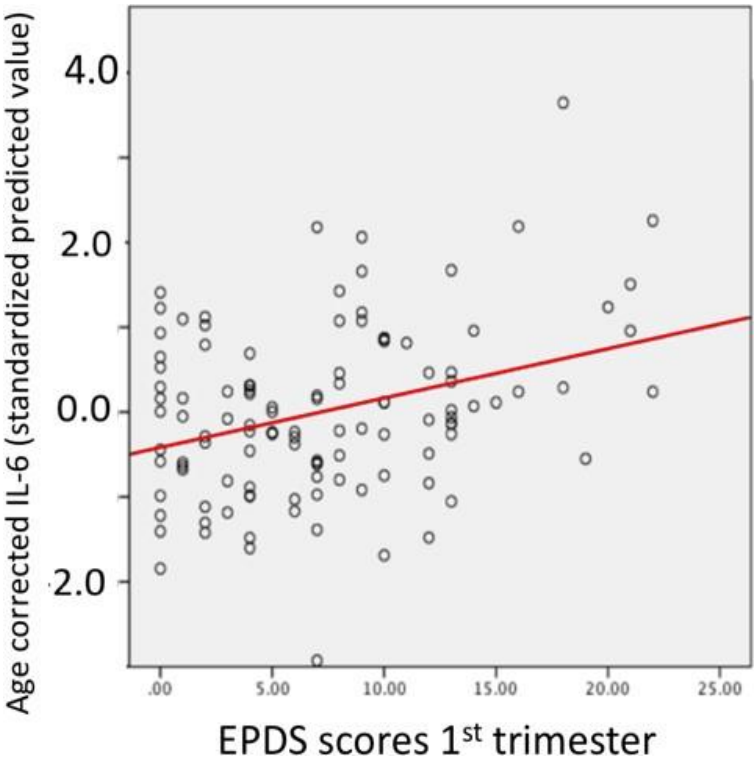

Figure 4.4 Correlation between plasma IL-6 (age corrected) and the EPDS score in the first trimester Age corrected IL-6 correlated with EPDS scores in the first trimester **(**Pearson's Correlation, r=0.23, p=0.003)**.**

Table 4.3 Prediction of depression score by the plasma levels of IL-6 at each time-point

Predictor of EPDS Score	***Time-point***	***Beta***	***p-value***
Plasma IL-6	First Trimester	0.25	**0.007**
Plasma IL-6	Second Trimester	0.24	**0.023**
Plasma IL-6	Third Trimester	0.27	**0.01**
Plasma IL-6	Postpartum	0.13	0.23

Linear regression models corrected for age. Significant values are in bold.

Plasma levels of QUIN (Figure 4.5A, Linear Regression corrected for age (LR), β=0.32, p<0.005), and IL-6 (Figure 4.5B, LR, β=0.28, p<0.01) were significantly elevated in

depressed pregnant women during the third trimester, when dichotomized based on the EPDS score of 13 and above.

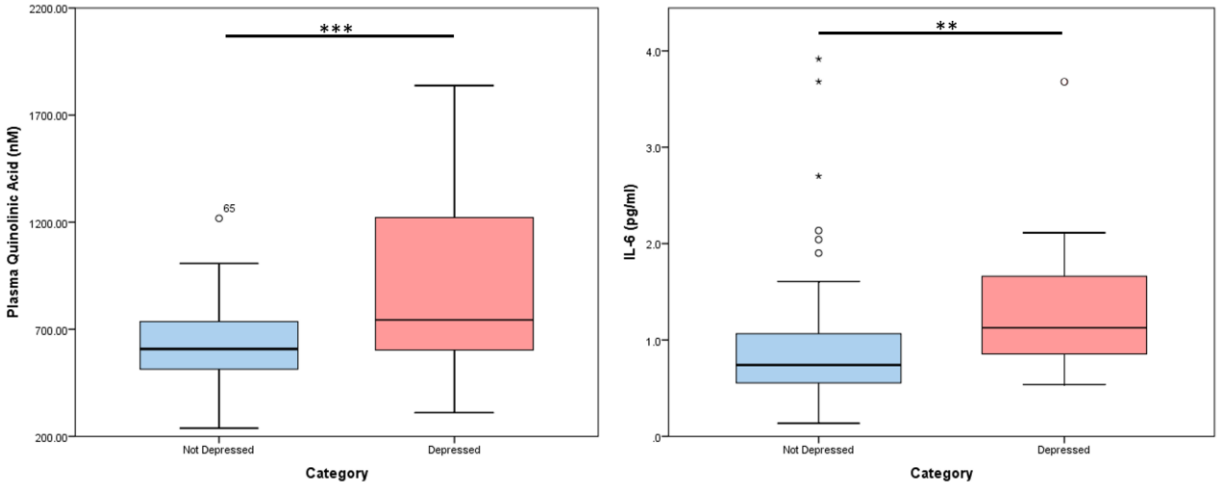

Figure 4.5 Quinolinic acid and IL-6 levels in plasma at the third trimester A) QUIN analyzed by linear regression (β=0.32, p<0.005) and B) IL-6 analyzed by linear regression (β=0.28, p<0.01) were significantly elevated in plasma from women with depression (n=14) compared to non-depressed women (n=70), in the third trimester of pregnancy (linear regression corrected for age). Unadjusted data shown in the figure.

We next analyzed the expression of the kynurenine pathway enzymes mRNA in the placenta from this cohort. The expression of IDO correlated closely with the levels of the pro-inflammatory cytokine IL-1β in plasma already at the first trimester (Spearman's, Rho=0.54, p<0.000) and up until the postpartum visit (Spearman's, Rho=0.39, p<0.003), which occurred a mean of 6 weeks post-delivery (Table 4.4). The expression of IDO correlated with the levels of IL-6 in plasma during the second (Spearman's, Rho=0.39, p=0.002) and third trimester (Spearman's, Rho=0.36, p=0.005) (Table 4.4). This implies that the expression of immunoregulatory kynurenine pathway enzymes in the placenta might impact systemic inflammation, starting early in the pregnancy.

Table 4.4 Spearman's correlation of IDO mRNA in placental tissue with plasma cytokine levels throughout and after pregnancy

Analyte	*Time-point*	*Correlation with placenta IDO mRNA*
Plasma IL-6	First Trimester	Rho 0.; p NS
Plasma IL-1β	First Trimester	**Rho 0.54; p=0.00005**
Plasma IL-6	Second Trimester	**Rho 0.39; p=0.002**
Plasma IL-1β	Second Trimester	**Rho 0.52; p=0.00007**
Plasma IL-6	Third Trimester	**Rho 0.36; p=0.005**
Plasma IL-1β	Third Trimester	**Rho 0.40; p=0.001**
Plasma IL-6	Postpartum	Rho 0.23; p NS
Plasma IL-1β	Postpartum	**Rho 0.39; p<0.003**

2^delta CT normalized to the housekeeping gene. Significant values are in bold.

Interestingly, the increase of QUIN was steeper in the women that developed depression (EPDS>13) as visualized in Figure 4.6. After delivery, the levels decreased back to those observed in non-depressed women. The longitudinal design will also enable us to investigate whether the cytokine and metabolite levels in plasma at the early time-points in pregnancy could predict the onset of depressive symptoms at later time-points.

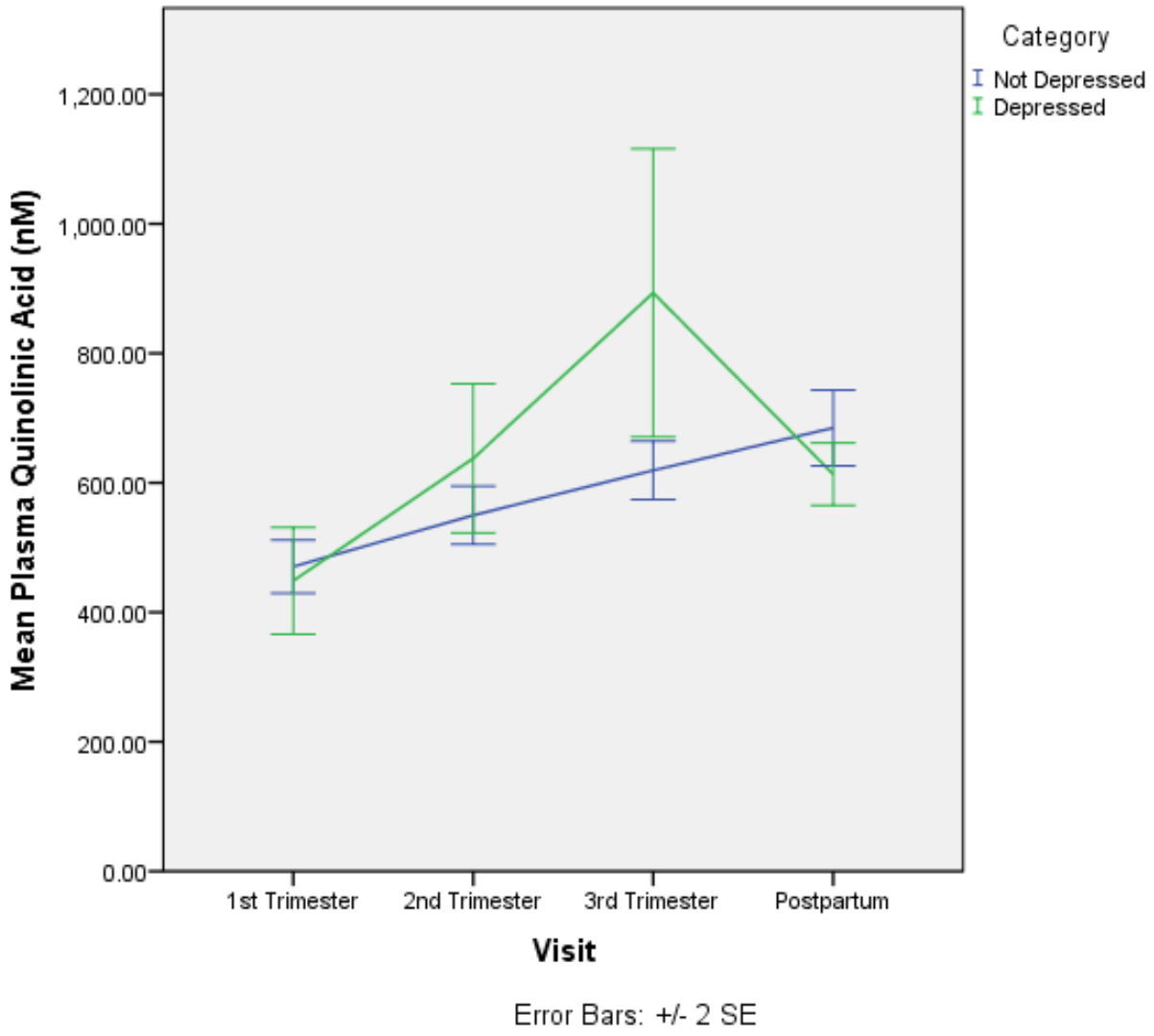

Figure 4.6 Mean quinolinic acid levels in plasma from depressed and non-depressed women in the longitudinal arm Total *n* at each timepoint 71-118, depending on number of missed visits per timepoint.

III. Postpartum depression and suicidality results

There was a significant increase of the cytokine IL-8 (LR, β=0.30, p<0.001) and a decrease of IL-2 (LR, β=-0.31, p<0.001) in the peripheral blood of women with depression in the postpartum (Figure 4.7). The postpartum cohort consisted of both the 118 women that we recruited in the first trimester, and 89 subjects with PPD recruited in the postpartum. A substantial number of the women enrolled in the postpartum arm had

current suicidal ideation (*n*=43), and a total of 14 women displayed suicidal behavior during pregnancy, or in the postpartum as determined by CCRS. Women with depression and suicidal ideation and behavior had similar inflammatory changes as women without suicidal ideation, i.e. low levels of IL-2 (LR, β=-0.31, p<0.001) and high IL-8 (LR, β=0.30, p<0.001) compared to controls, as shown in Figure 4.7.

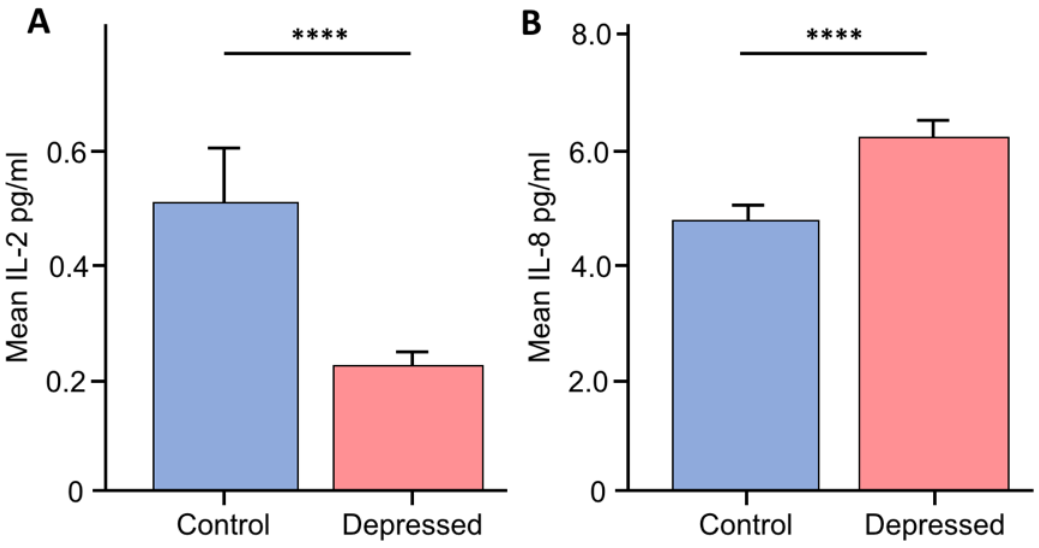

Figure 4.7 Mean IL-2 and IL-8 in plasma from depressed and non-depressed women in the postpartum period A) Plasma IL-2 and B) IL-8 in women with depression (*n*=70) and without depression (*n*=69) in the postpartum period. Mean ±SEM. Linear regression, β=0.31, p<0.001 for IL-2; β=0.30, p<0.001 for IL-8. Controls *n*=72; Depressed *n*=95 (includes patients with suicidal ideation).

Discussion

In this study, we detected increased levels of inflammation in women who were depressed during pregnancy and the postpartum period compared to controls. During pregnancy there was also an increased levels of kynurenine pathway metabolism. There were elevated levels of IL-6 in plasma throughout pregnancy in women with

depression which correlated with increased EPDS scores. This suggesting a more pro-inflammatory environment in the periphery which may be indicative of increased inflammation in the placental microenvironment. Interestingly, we found a different inflammatory profile in the plasma of women postpartum. Postpartum depressed and suicidal women had decreased IL-2 and increased IL-8. The novel observation of a shift from an increased proinflammatory environment throughout pregnancy which correlated to placental IDO, to an expression of different cytokines and no kynurenine pathway change demonstrates the placenta might underlie the biological mechanism of depression in the peripartum period while other mechanisms may be responsible for postpartum depression.

A longitudinal study of PPD of this magnitude has never been performed. We found QUIN was elevated in depressed women at the third trimester compared to healthy controls. Our results give rise to the question of what the biological differences between peripartum and postpartum depression are.

Our data from term placentas imply that the placental production of cytokines and metabolites may contribute to the plasma inflammatory profiles in pregnant women, and potentially influence the development of PPD later. During pregnancy, IL-1β correlated with placental IDO at all time points. Proinflammatory IL-1β is secreted by macrophages and has been shown to contribute to sickness behavior by causing hyperthermia, lethargy, sleep and appetite disturbances, and reduced grooming (Farooq, Asghar, Kanwal, & Zulqernain, 2017). Further, increased IL-1β increases the amount of a catabolite KYN which is generated from the enzymatic activity of IDO (P. A. Zunszain et al., 2012). IL-1β is typically expressed during pregnancy in the plasma of pregnant

mothers, however elevated IL-1β has been associated with negative effects in the mother throughout pregnancy (Brien et al., 2017; Nadeau-Vallee et al., 2017). This is an important finding in PPD as we and others have established an increase of IL-1β is linked to depression itself (Bay-Richter, Hallberg, Ventorp, Janelidze, & Brundin, 2012; Ellul, Boyer, Groc, Leboyer, & Fond, 2016; Li et al., 2017), however, this is the first time IL-1β has been associated with PPD.

Pro-inflammatory IL-6 correlated positively with EPDS scores and predicted EPDS scores during the first, second , and third trimester, and is a potential biomarker for PPD. IL-6 has been linked to stress-related disorders such as depression and anxiety (Erta, Giralt, Esposito, Fernandez-Gayol, & Hidalgo, 2015; Howren et al., 2009). Several previous studies have found an increase of IL-6 in the plasma of suicidal and depressed patients both in the peripheral and central levels (Isung et al., 2014; Lindqvist et al., 2009). Further, Simpson et al. have reported an association between increased IL-6 levels during late pregnancy and postpartum depressive symptoms (Simpson, Steiner, Coote, & Frey, 2016). However, we are the first to report that throughout all trimesters IL-6 was elevated in PPD.

IL-2 is decreased and IL-8 is increased in postpartum depression, and neither cytokine was statistically significant in the peripartum period. Thus, there appears to be differences in the pro-inflammatory profile related to depression in the postpartum period compared to what we observed during pregnancy. We for the first-time report IL-2 is decreased compared to healthy controls in postpartum depression. IL-2 regulates immunity via influencing white blood cell activity (Boyman, Kolios, & Raeber, 2015) and KYN itself can decrease IL-2 levels under immunocompromised conditions (Dagenais-

Lussier et al., 2016). Interestingly, we have previously observed decreased plasma IL-2 in suicidal non-pregnant patients (Janelidze et al., 2011) which seems to also be true during the postpartum period.

IL-8 was increased postpartum and is a chemokine produced by macrophages and endothelial cells (Waugh & Wilson, 2008), such as the BBB. An increase of IL-8 release from the BBB has previously been associated with BBB dysfunction (Kossmann et al., 1997) and may contribute to a leaky BBB which has been linked to depressed and suicidal patients (Najjar, Pearlman, Devinsky, Najjar, & Zagzag, 2013; Steiner et al., 2012; Ventorp et al., 2016). We are the first to identify elevated IL-8 levels in postpartum depression.

When the tightly regulated immunological state of pregnancy is disrupted, the inflammatory profile of pregnancy may shift. According to our model (Figure 4.1) an altered inflammatory profile would induce depression through the actions of cytokines and kynurenine metabolites on neurons. Our data provides compelling evidence that levels of cytokines and kynurenine metabolites could predict risk for PPD and postpartum depression and are potential therapeutic targets.

We propose that PPD is a type of depression with specific underlying pathobiology, characterized by an immunobiological profile similar to that of cytokine-induced depression. The data generated from this cohort is promising but needs to be complemented with a confirmatory study from a wider demographic, gathering samples from a larger cohort of women during pregnancy to support the relationship of these factors to the development of depression. While the scientific premise is compelling, there is still an urgent need to validate the molecular mechanisms in experimental

models as well.

This study has several strengths and limitations. As a strength, we measured the same subjects during a longitudinal duration, so we knew what their first trimester baseline was and what inflammatory changes were associated with the development of depression later. To our knowledge, this is the first-time placental kynurenine pathway activity has been correlated with plasma inflammation in depression. It is challenging to determine the exact underpinning of the biological cause of PPD, especially since the placenta is inaccessible during pregnancy. In this study we only included women with low suicidal tendencies. It would of interest to study an inpatient setting and analyze if the changes we see here are heightened in a more severe population. A limitation to our study is the comparatively low number of subjects which came back for the postpartum visit in our longitudinal arm, as well as being a clinical correlative study that is unable to establish an exact causal relationship. In future studies it will imperative to maintain high numbers of subjects that return for all visits throughout the whole study. Further studies of interest include collecting blood before subjects become pregnant as a general baseline, and not solely in the pregnant and postpartum period.

Conclusion

In summary, we detected IL-6 levels in plasma predicted the development of depressive symptoms throughout pregnancy. Our data suggests that the kynurenine pathway and inflammation may be associated with PPD, and these changes may stem from the placental tissue (IL-1β and IL-6 in the plasma correlated with IDO expression in the placenta). Postpartum depression had a different inflammatory profile, with increased IL-8 and decreased IL-2, which may be due to the absence of the placenta. A better

understanding of the biological role of placental TRP pathways, the kynurenine pathway enzymes, and their interaction with plasma inflammation could lead to better targeted therapeutic strategies for at-risk women for PPD, postpartum depression and suicidality.

Chapter 5: Altered Tryptophan Catabolism in Placentas from Women with Pre-eclampsia

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This chapter is a modified version of a manuscript accepted to the International Journal of Tryptophan Research.

Abstract

Background: The kynurenine pathway enzymes, breaking down tryptophan (TRP), are abundant in placental tissue. These metabolites are involved in immunoregulatory mechanisms, although the role of this pathway in pre-eclampsia (PE) has only begun to be characterized. Here, we determined TRP and metabolite levels together with expression of kynurenine pathway enzymes and inflammatory factors in placentas from women with and without PE.

Methods: 36 placentas (18 PE and 18 controls) were analyzed for expression of kynurenine pathway enzymes IDO and Indolamine 2,3-Dioxygenase 2 (IDO2), tryptophan-2,3-dioxygenase (TDO), *kynurenine*-3-mono-oxygenase (KMO) and QPRT as well as IL-1β, IL-6 and SAA. We measured TRP and KYN content using high-pressure liquid chromatography (HPLC) and QUIN using GC-MS.

Conclusions: TRP content was reduced in placentas from women with PE. There was an increased breakdown of TRP, evidenced by an increased KYN/TRP ratio in

placentas from women with PE. We confirmed a reduced IDO expression in PE and found a compensatory increase in TDO expression. SAA was reduced in PE placentas compared to controls and correlated with IDO expression in healthy, but not in PE placentas. Our data show that TRP content and the inflammatory mediator SAA are both compromised in placentas in PE. TRP might be decreased due to an increased kynurenine pathway activity in PE, with a shift from IDO to TDO expression. Further studies on the role of TRP catabolism and mediators of inflammation in sustaining healthy immunobiological pathways in the placenta are warranted.

Background

PE is one of the most common disorders of pregnancy, affecting around 3-8% of pregnant women in Western countries (Uzan, Carbonnel, Piconne, Asmar, & Ayoubi, 2011). Characterized by pregnancy-onset hypertension, proteinuria and in severe cases organ dysfunction, PE is a leading cause of maternal death and perinatal morbidity around the world (World Health Organization, 2011). While the exact cause of PE is unknown, evidence suggests that it is connected to aberrant placental implantation (Espino et al., 2017; James-Allan, Whitley, Leslie, Wallace, & Cartwright, 2018). Inadequate implantation may lead to poor placental perfusion, which can cause hypoxia and inflammatory changes in placental tissue (Ahn, Park, Gilman-Sachs, & Kwak-Kim, 2011; Al-Jameil, Aziz Khan, Fareed Khan, & Tabassum, 2014; Redman, 1991). Although there is consensus that PE is linked to inflammatory changes in both plasma and placenta, the precise nature and role of these changes in the disease process are not fully understood. It has been proposed that PE is a disorder featuring deficient immunoregulation (Ahmed & Ramma, 2015), and that normal immunoregulatory

negative feedback systems (i.e. “brakes” of the inflammatory cascade) might not function properly.

Catabolism of the essential amino acid TRP is involved in immunoregulation, and within the placenta one of its functions is the regulation of maternal-fetal tolerance. In the placenta, TRP is degraded into nicotinamide adenine dinucleotide (NAD+) by a series of enzymatic reactions, collectively called the kynurenine pathway (Manuelpillai et al., 2003). The activity of the kynurenine pathway increases under inflammatory conditions (Schwieler et al., 2015; Urata et al., 2014), and the different metabolites can exert potent immunoregulatory functions. The first step of the pathway can be carried out by any of the following three enzymes, IDO, IDO2, or tryptophan 2,3-dioxygenase (TDO). Of these, IDO is highly expressed in the human placenta and can regulate maternal-fetal tolerance (Manuelpillai et al., 2003; P. Sedlmayr et al., 2002). Early studies suggest that maternal-fetal tolerance is established by IDO mediated TRP metabolism at the placental interface, which leads to a localized state of TRP depletion and subsequent anergy of reactive T cells (Badawy, Namboodiri, & Moffett, 2016; Munn et al., 1998; P. Sedlmayr et al., 2002). However, recent studies have shown that the metabolite of this first step, KYN, can also regulate the immune system through highly specific mechanisms involving binding to the aryl hydrocarbon receptor (Badawy et al., 2016; Fallarino et al., 2002; Mezrich et al., 2010; Yeung, Terentis, King, & Thomas, 2015). QUIN, a down-stream kynurenine metabolite, can induce apoptosis in T cells and also stimulate cytokine production and activation of the innate immune system (Lugo-Huitron et al., 2013).

IDO expression has been shown to be reduced in placentas from women with PE (Iwahashi et al., 2017), and in a mouse model, pregnant dams deficient in IDO exhibit phenotypes typical of PE, such as proteinuria, endothelial dysfunction and elevated blood pressure (Santillan et al., 2015). Zardoya-Laguardia and colleagues found that reduced levels of IDO in PE were associated with changes in the placental vascular tone (Zardoya-Laguardia et al., 2018). Reduced expression of IDO might be coupled to immune dysregulation in PE, but the enzyme may also be involved in the regulation of oxidative stress in PE (Nishizawa et al., 2011). To date, this reduction of IDO in PE has hitherto not been assessed in the context of other the enzymes of the kynurenine pathway, nor been analyzed in relation to the tissue content of TRP and the immunoregulatory metabolites, KYN and QUIN. Thus, to better understand the importance of the kynurenine pathway in PE, we determined TRP and metabolite content as well as the degree of expression and activity of the kynurenine pathway in placentas from women with and without PE. We included only placentas that were delivered within the gestational window considered term (37-42 weeks), and further corrected all analyses for exact gestational age within this period. Additionally, we investigated the association between the kynurenine pathway and key mediators of inflammation in placental tissue, as inflammatory factors are known to induce the activity of kynurenine pathway enzymes. We measured the inflammatory cytokines interleukin (IL)-1 β, IL-6, and SAA in placental tissue. SAA is a mediator of several central aspects of the immune response (Eklund et al., 2012) and has a critical role within placental tissue, regulating placental formation and homeostasis by modulating metalloprotease activity and invasion by trophoblasts (Sandri et al., 2014). In this study,

our primary hypothesis was that we would find evidence of a dysregulated TRP catabolism, associated with inflammatory changes, in placentas from women with a diagnosis of PE.

Methods

I. Clinical study design

This study was approved by the Lund University Institutional Review Board (IRB), Lund, Sweden. Placenta samples and clinical information were obtained from a total of 42 women enrolled in our Swedish cohort study between 2003-2011.

Women were excluded from the study if they had chronic hypertension. Four of the women gave birth after 42 weeks and were removed from the analysis as they were post-term, and two women were excluded from the analysis due to pre-term birth, defined as birth prior to 37 gestational weeks.

The remaining 36 term placenta samples were analyzed, out of which 18 of the mothers had been diagnosed with late-onset PE and 18 were healthy controls. Late-onset PE was defined as de novo hypertension and proteinuria from 34 weeks of gestation, with blood pressure ≥ 140/90 mmHg and proteinuria ≥ 300 mg/L according to International Society of Hypertension in Pregnancy (ISSHP) definition (Brown, Lindheimer, de Swiet, Van Assche, & Moutquin, 2001). Proteinuria diagnosed by dipstick analysis was accepted as quantification if no other method had been used. All of the women in our cohort had singleton pregnancies. The prevalence of caesarean (C-) section was the same between PE patients and controls. The detailed clinical characteristics of our patient cohort are shown in Table 4.1. The placentas were collected at the delivery ward

of Lund University Hospital, Sweden. Placenta was collected upon delivery, immediately frozen on dry ice and then stored at -80°C.

II. qPCR analysis

Placenta samples were homogenized by automatic homogenizing pestle. RNA was extracted by RNeasy kit (Qiagen, Germantown, MD) according to manufacturer's protocol and stored at -80° C. Complementary DNA (cDNA) was synthesized using 1.5 µg of placental total RNA using the Superscript VILO cDNA Synthesis Kit (Thermofisher, Kalamazoo, MI) according to manufacturer's recommended protocol. Quantitative real-time PCR was performed to analyze mRNA expression levels of IDO, IDO2, IL-1β, IL-6, TDO, KMO, QPRT and SAA - using the Taqman Gene Expression assay (Thermofisher, Kalamazoo, MI) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). All qPCR samples were run in triplicate. One control placenta sample was excluded due to insufficient mRNA yield for all targets. Data was analyzed via the comparative threshold cycle (Ct) method as previously described (Livak & Schmittgen, 2001).

III. Protein analysis

To analyze placental protein levels, each placenta sample was homogenized in RIPA lysis buffer (Cell Signaling Technology, Danvers, Massachusetts) at a final concentration of 350 µg/ml, using double the concentration of protease and phosphatase inhibitors as suggested by Meso Scale Discovery (Rockville, Maryland). IL-1β, IL-6 and SAA were measured using the Meso Scale Discovery platform and run on a Sector 600 in accordance to manufacturer's instructions. Inter-assay coefficients of

variation (CV): IL-1β (1.5%), IL-6 (2.7%) and SAA (2.5%). Intra-assay CV: IL-1β (3.0%), IL-6 (3.0%) and SAA (2.9%).

IV. Detection of tryptophan, kynurenine and quinolinic acid

Plasma samples were analyzed using GC-MS to determine QUIN concentrations. Samples were weighed and homogenized using Bertin Minilys® bead tubes containing 10% trichloroacetic acid. Following subsequent centrifugation, 50 µL of the resulting supernatant were added to a glass tube along with deuterated internal standard and processed in accordance with the previously published protocol (Smythe et al., 2002). Subsequently, each sample was injected into a Thermo Trace GC Ultra gas chromatograph interfaced to a Thermo DSQ II mass spectrometer. The inter/intra-assay % CV was 1.41%/1.36% for QUIN. The lower limit of detection was 3 nM.

TRP and KYN were analyzed by HPLC. Samples were initially processed by the same method stated above. After centrifugation, the supernatant was filtered through a 0.22 µm PTFE filter and 20 µL was injected into the Thermo Scientific Dionex UltiMate® 3000 (Thermo Scientific, Waltham, MA, USA). The chromatograph separation was achieved on a reversed phase 150x3 mm BDS Hypersil C18 column (Thermo Scientific™) with 3µm particle size. Column and pre-column tubing was maintained at 35°C with isocratic elution (0.8mL/min) of analytes using a mobile phase consisting of 5% methanol in milliQ water containing 50 mM ammonium acetate (pH 4.65). TRP and KYN were detected based on comparison with standards, retention times, and fluorescent detection at 254 nm/404 nm (ex/em) for TRP and UV emission spectra at 365 nm for KYN. Results were analyzed using the Chromeleon™ 7.2 Chromatography Data System (Thermo Scientific™ Dionex™). The inter/intra-assay % CVs were

2.1%/0.5% for TRP and 2.6%/1.3% for KYN, respectively. Lower limit of detection was 33 nM for both analytes.

V. Statistical analysis

Statistical analyses were performed using R v3.4.3 (https://cran.r-project.org/) and IBM SPSS Statistics v.24 program. Bivariable comparisons in demographic and clinical characteristics were made using Student's t-tests for continuous variables or Fisher's exact tests for categorical variables, as appropriate. Multivariable regression analyses were performed to test if each biomarker was independently associated with PE. To adjust for potential confounding of gestational age, PE and healthy patients were matched based on gestational age using the genetic algorithm in the R package 'Matchit' (http://gking.harvard.edu/matchit) and then analyzed via a weighted linear regression (WR), with gestational age as a covariate (Ho, Imai, King, & Stuart, 2007). P-values were then calculated using a likelihood ratio test. Levene's test was used to test for heteroscedasticity and normality of residuals was verified visually using qq-plots. Analytes were transformed using Box-Cox as needed, based on these regression diagnostics (Box & Cox, 1964).

Results

I. Patient characteristics

Patient demographics, somatic comorbidities and medications used during pregnancy are listed in Table 5.1. There were no significant differences in mean age, BMI, current smoking status, placental weight, newborn birth weight, gestational age at the time of delivery, or in the mode of delivery between women with and without PE. All group-wise comparisons were adjusted for gestational age by weighted regression models.

Table 5.1 Demographic data for the study participants

	Pre-eclampsia (n=18)	Controls (n=18)	*P*-value
Age [mean (SD)]			
	29.2 (6.5)	29.2 (4.0)	1.0
Body mass index, kg/m^2 [mean (SD)]			
	28.2 (7.7)	26.1 (5.1)	0.3
Complications/symptoms of PE			
HELLP syndrome [n]	2	0	
Intrauterine growth restriction [n]	2	0	
Proteinuria [n]	18	3*	
De novo Hypertension [n]	18	1**	
Gravidity [mean (SD)]			
	2.5 (0.7)	1.6 (0.5)	0.2
Parity [mean (SD)]			
	0.3 (0.6)	0.2 (0.4)	0.3
Smoking [n]			
Never	11	15	
Quit before pregnancy	5	2	
Quit during pregnancy	1	0	
Current	1	1	
Co-morbidities [n]			
Psoriasis	2	0	
Cutaneous Lupus	0	1	
Asthma	1	1	
Medications [n]			
Anti-hypertensives	2	0	
Antibiotics at labor	1	1	
Corticosteroids	3	0	
Gestational age at delivery [mean (SD)]			
	39.1 (1.2)	39.9 (1.3)	0.05
Mode of Delivery [n (%)]			
Vaginal	14 (78%)	14 (78%)	
Cesarean	4 (12%)	4 (12%)	
Male Fetal Sex [n (%)]			
	7 (39%)	9 (50%)	
Placental Weight, g [mean (SD)]			
	617.8 (152.1)	590.9 (100.0)	0.5
Newborn Birth Weight, g [mean (SD)]			
	3405.5 (732.6)	3434.8 (369.3)	0.9

*Out of the three controls with proteinuria, one had a urinary tract infection at the time of testing and two had 1+ for proteinuria with no other symptoms. **The control with elevated blood pressure did not have any other symptoms.

II. Tryptophan and metabolite levels in placenta

Women with PE exhibited significantly lower levels of placental TRP (WR, Standardized Beta (Sß) -0.42, p=0.01) compared to the controls (Figure 5.1A). There was no difference in the levels of KYN between placenta samples from women with PE and controls (NS, WR). However, the activity of the first step of the kynurenine pathway was increased as evidenced by the KYN/TRP ratio which was elevated in women with PE compared to controls (WR, Sß =0.40, p<0.01). The levels of QUIN, a down-stream metabolite regulated by the enzymes KMO and QPRT, were not different between controls and PE placentas (NS, WR).

III. Inflammatory cytokines in placental tissue

To test if inflammatory markers are upregulated in PE placental tissue, we quantified the expression levels of the pro-inflammatory cytokines IL-1β, IL-6, and SAA by qPCR. SAA mRNA was significantly decreased in the placenta tissue from women with PE compared to controls (WR, Sß=-0.44, p<0.005) (Figure 5.1B). There was no observable difference in the expression of IL-1β or IL-6 mRNA in placentas from women with and without PE (WR, NS). We confirmed the SAA mRNA data by measuring protein of SAA in the same samples. There was a significant decrease in SAA protein in placentas from women with PE (WR, Sß=-0.51, p <0.005).

IV. Kynurenine enzyme levels in the placental tissue

We analyzed the mRNA expression of the kynurenine pathway enzymes IDO, IDO2, TDO, KMO and QPRT (see Figure 1.1) in the placental tissue. Placental IDO mRNA levels were significantly lower in women with PE compared to controls (WR, Sß =-0.33, p<0.05) (Figure 5.1C). Correspondingly, there was an increased expression of TDO

mRNA in placenta tissue from the women with PE (WR, Sß=0.40, p<0.05) (Figure 5.1D). There were no significant differences in the expression level of IDO2, KMO and QPRT in placenta tissue of women with and without PE (WR, NS).

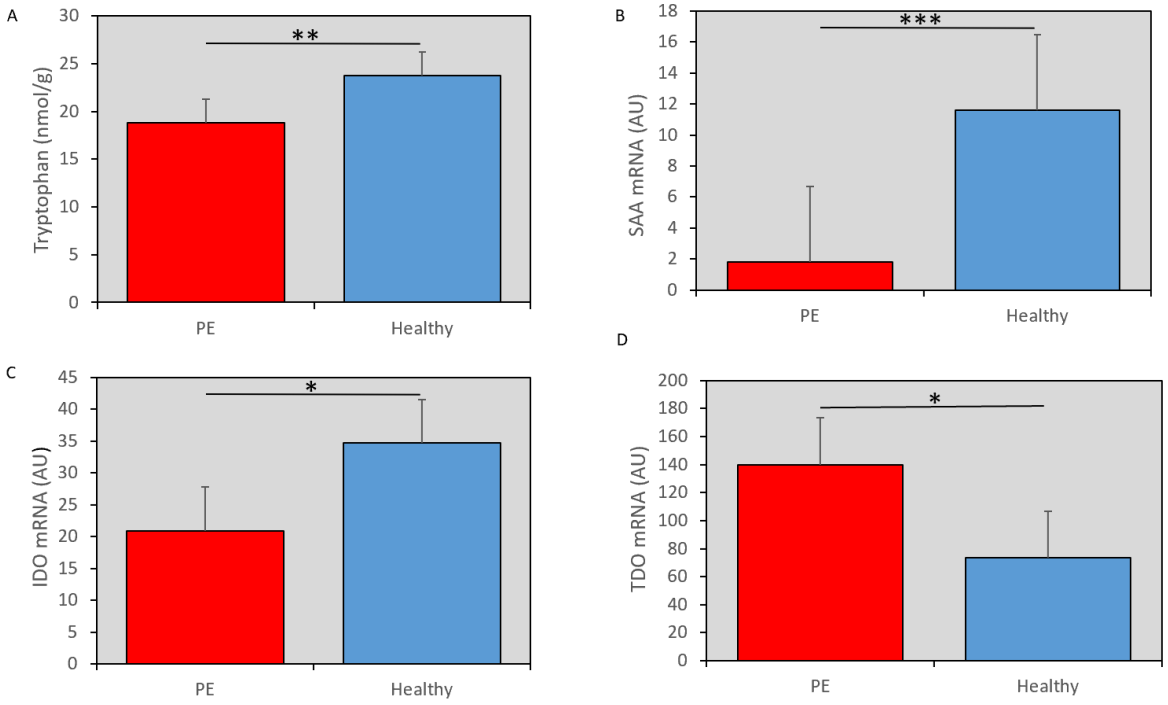

Figure 5.1 Tryptophan content and mRNA expression in PE women compared to healthy controls A) TRP content was reduced in placentas from women with PE compared to healthy controls (nmol/gram tissue, mean ± 2SEM). B) mRNA expression of SAA was significantly decreased in placentas from women with PE compared to controls (AU, as defined below, median ± confidence interval). C) The mRNA expression of IDO was reduced in placentas from women with PE compared to controls (AU, mean ± 2SEM). D) TDO expression was increased in the placentas from women with PE versus the healthy controls (AU, mean ± 2SEM). All mRNA data was analyzed via the Ct method as previously described (see methods); where the relative mRNA expression in arbitrary units (AU) was obtained by normalization against the expression of the housekeeping gene (GAPDH) in each sample. All data shown in figures A-D are raw values and the significance level indicated in the figures is based on the statistical analysis, weighted regression as described in methods. *p<0.05, **p<0.01, ***p<0.005.

V. Associations between IDO and inflammatory factors in placenta

SAA mRNA correlated positively with activity of the first step of the kynurenine pathway, as measured by the KYN/TRP ratio in healthy placental tissue (Pearson's R=0.63, p=0.005, Figure 5.2A), while there was no such correlation in the PE placenta tissue (Pearson's R=-0.3, NS, Figure 5.2B).

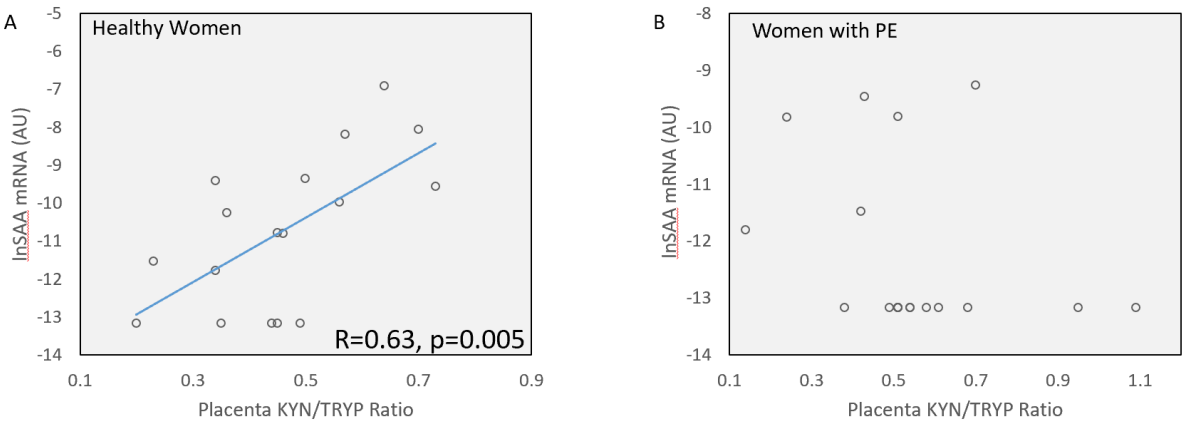

Figure 5.2 mRNA Expression of SAA in healthy placental tissue compared to placentas from women with PE A) The mRNA expression of SAA in healthy placental tissue correlates significantly with the activity of the first step of the kynurenine pathway, as indicated by the KYN/TRP ratio (Pearson's R, 0.63, p<0.005). B) There was no association between the mRNA expression of SAA and the kynurenine pathway activity in placentas from women with PE. Thus, showing a disorganized inflammatory state in women with PE.

Discussion

In this study, we detected significantly reduced levels of the essential amino acid TRP together with decreased levels of the acute-phase mediator SAA in placentas from women with PE. There was also a significantly increased KYN/TRP ratio in PE placentas, indicative of elevated TRP breakdown by increased activity of the first enzymatic step of the kynurenine pathway. The first step of the kynurenine pathway, degrading TRP into KYN, can be performed by one of the three enzymes IDO, IDO2 or

TDO. Using qPCR, we confirmed lower mRNA expression of IDO in PE placentas, in agreement with previously published data (Iwahashi et al., 2017; Santillan et al., 2015). Interestingly, we found a corresponding increase in the expression of TDO in women with PE, whereas IDO2 expression was unaltered. The novel observation of a shift from IDO to TDO expression in PE might underlie the increased breakdown of TRP we observed in PE placentas, despite reduced IDO expression.

To our knowledge, TRP content in the placenta has not previously been determined in PE. We found that TRP content was significantly reduced in PE placentas. A potential explanation for the increased breakdown of TRP, despite reduced IDO, is thus the shift to an increased expression of TDO in the PE placentas. Although less abundant than IDO, the two other enzymes breaking down TRP, IDO2 and TDO, have previously been detected in term placenta (Peter Sedlmayr, Blaschitz, & Stocker, 2014). Compensatory mechanisms within the regulation of kynurenine pathway enzymes are observed in other settings (Braidy, Guillemin, Mansour, Chan-Ling, & Grant, 2011; Lim et al., 2017).

Our results give rise to the question of what the biological differences between IDO and TDO are, and whether these could have any relevance in the pathobiological mechanisms of PE. IDO and TDO both catalyze the breakdown of TRP into KYN, but the enzymes are different in terms of genomic location, tissue expression, structure and mode of activation (Rafice, Chauhan, Efimov, Basran, & Raven, 2009). IFN-γ and several cytokines can induce IDO, whereas TDO can be induced by TRP and the cofactor heme, as well as by glucocorticoids (Badawy, 2017). Increased TDO expression in human placenta has also been observed under infectious conditions (Myatt, 2010). Both enzymes involve oxidation of the substrate TRP, although the exact

catalytic process is not yet fully established. The reaction may differ in the formation of catalytic ferrous-oxy complexes (Nienhaus & Nienhaus, 2017). IDO has anti-oxidant properties, as the superoxide anion is used as the oxygen source (Dang, Dale, & Brown, 2000; Thomas & Stocker, 1999a, 1999b). A previous study found decreased levels of IDO in placentas from women with PE and suggested that the protective function of IDO in PE is not related to immunoregulation, but to its anti-oxidant properties (Nishizawa et al., 2011). It is previously established that oxidative stress is involved in the pathogenesis of PE (Jauniaux, Poston, & Burton, 2006; Myatt, 2010; Perkins, 2006; Thomas & Stocker, 1999b), and one can speculate that an upregulation of TDO cannot completely compensate for a reduction of IDO in this respect, given their different biological properties. Future studies should address the differential regulation of IDO and TDO in placental tissue and determine their mechanistic roles in pathological conditions, such as PE.

Interestingly, we detected significantly reduced levels of SAA mRNA expression in placental tissue from women with PE, and observed a reduction in SAA protein. SAA is an acute-phase protein that increases in response to tissue damage and activates toll-like receptor 2 and toll-like receptor 4, both of which are present in the placenta (Hayati, Mohamed, & Tan, 2010). Binding of these receptors by SAA stimulates the production of several pro- and anti-inflammatory cytokines, such as IL-6 and IL-10 respectively (Eklund et al., 2012). Several previous studies have found a reduction of IL-10 in placentas from women with PE (Hennessy, Pilmore, Simmons, & Painter, 1999; Makris, Xu, Yu, Thornton, & Hennessy, 2006; Rein et al., 2003). To our knowledge, this is the first time that SAA has been measured in placenta samples from PE patients. In

addition to being an inflammatory regulator, SAA also plays an important role in the placental microenvironment through its ability to induce trophoblast invasion and metalloprotease activity, two essential processes in placental formation and homeostasis (Sandri et al., 2014). Since PE is linked to poor placental implantation and development, our findings of decreased SAA in placentas from women with PE are concordant with the findings by Sandri et al, and implicate that SAA could be a pathognomonic factor in the development of PE (Sandri et al., 2014). Interestingly, the levels of SAA correlated significantly with the activity of the kynurenine pathway (KYN/TRP ratio) in healthy placentas, but not those diagnosed with PE. Therefore, it is possible that SAA is a novel regulator of IDO activity, capable of inducing IDO in placental tissue, although this remains to be causally determined in experimental model systems.

This study has several strengths and limitations. As a strength, we measured a range of kynurenine pathway enzymes, including three separate enzymes capable of degrading TRP, as well as both the substrate TRP and metabolite KYN in order to understand the complex nature of changes involved in the first step of the kynurenine pathway in placentas from women with PE. Importantly, the expression of IDO and the other kynurenine pathway enzymes change over pregnancy (Badawy, 2015), and it is therefore difficult to establish group differences unless there is a careful matching for gestational age. This can be challenging, especially since the diagnosis of PE is often associated with pre-term delivery. In this study we only included women with term pregnancies, and we further corrected all statistical analyses for exact gestational age. These adjustments are important in order to establish associations between biological

factors and diseases of the placenta, as the placental environment undergoes profound changes throughout pregnancy, particularly in the weeks leading up to birth. A limitation to our study is the comparatively low number of subjects (n=36), as well as being a clinical correlative study, that is unable to establish causal relationships. In future studies of TRP catabolism in placental tissue, it will be of importance to continue to measure both substrate and metabolites, as well as multiple involved enzymes in order to help interpretation of the observed changes in PE.

Conclusion

In summary, we detected reductions in TRP content and SAA in placental tissue from women with PE compared to controls. While IDO expression was reduced, TDO expression was increased, and the KYN/TRP ratio, indicative of pathway activity, was increased in PE placental tissue. There were no changes in the expression of the down-stream kynurenine pathway enzymes KMO and QPRT, suggesting that the changes in TRP catabolism in PE involve only the first enzymatic step of the pathway. The biological role of placental TRP, the kynurenine pathway enzymes and their interaction with inflammatory factors in placental tissue in PE warrant further investigation.

Chapter 6. Summary, Translational Implications, and Future Directions

Summary of Dissertation

The goal of this dissertation was to understand how dysregulated inflammation can be detrimental to both mental and pregnancy states. In Chapter 3, we established women had heightened proinflammatory profiles in *suicide warning* compared to depression severity as assessed by CAT-MH™. We also discovered *suicide warning* had a strong interconnected white blood cell profile analyzed by WGCNA which was primarily driven by WBC and PMN. This paired with their strong positive associations via RiR suggests these two cell types may be significant in the underlying pathobiology of *suicide warning*. Altogether, our results indicate that there may be different blood analyte profiles related to depression and *suicide warning* with *suicide warning* having a stronger proinflammatory profile, which is connected by a white blood cell network.

Next, in Chapter 4, we defined how dysregulated inflammation in the peripartum period and postpartum can contribute to psychiatric health. Proinflammatory IL-6 levels in plasma may be a biomarker to predicted EPDS depression scores throughout the peripartum period. Plasma IL-1β and IL-6 correlated with placental expression of IDO, linking the kynurenine pathway in the placenta with peripheral inflammation. Interestingly, we found a different inflammatory profile associated with postpartum depression. Possible postpartum markers of depression and suicidality would be decreased IL-2 and increased IL-8 levels. By having a better understanding of the biological changes that occur during PPD, postpartum depression and suicidality we hope to advance the field in the development of better screening tools.

In Chapter 5, we found placentas from women diagnosed with PE had dysregulated TRP catabolism, associated with inflammatory changes. We for the first time measured IDO, IDO2 and TDO mRNA (the enzymes carrying out the first step of the pathway) as well as the expression of several key immunological factors in placentas from PE patients and controls, carefully matched for gestational age. We found that the TRP content in placental tissue from women with PE was significantly reduced, and this was due to an increased breakdown of TRP through the kynurenine pathway. We also discovered that the reduction of IDO in PE was paralleled by a compensatory increase in the expression of TDO. A key immunoregulatory factor, SAA, was significantly reduced in placentas from PE patients. Results indicate that the kynurenine pathway enzymes and tissue levels of SAA may play a role in the development of PE.

The Kynurenine Pathway and Inflammation

The research presented in this dissertation illustrates how the kynurenine pathway and inflammation is dysregulated in women with different pathophysiological conditions (summarized in Figure 6.1). This research further supports the notion that the kynurenine pathway and inflammation are not independent and should be thought of as an interconnected pathway with changes in inflammation impacting levels of the kynurenine pathway and visa versa. Further investigation should take place into the relationship between the marker SAA and the kynurenine pathway, as this relationship has not been established yet, however we found that there seems to be a change in SAA when kynurenine pathway markers change. This could be a novel and important cell culture and animal model study which would shed light into the human model.

Further, in pre-eclampsia we saw a compensatory increase of IDO2 in the placenta and a decrease of IDO1, but both IDO2 and TDO were not measured in the two other cohorts. It could be insightful to analyze if IDO2 expression can be triggered as a compensatory mechanism in other tissues and other physiological and pathophysiological states or is solely a compensatory mechanism in the placenta. Insights into the first rate limiting step of the kynurenine pathway in female human subjects, and the interaction of these enzymes with inflammation is necessary. While Figure 6.1 below summarizes our working model based on this dissertation's novel discoveries, exactly how SAA, IDO2, and TDO interact with the model is still to be discovered.

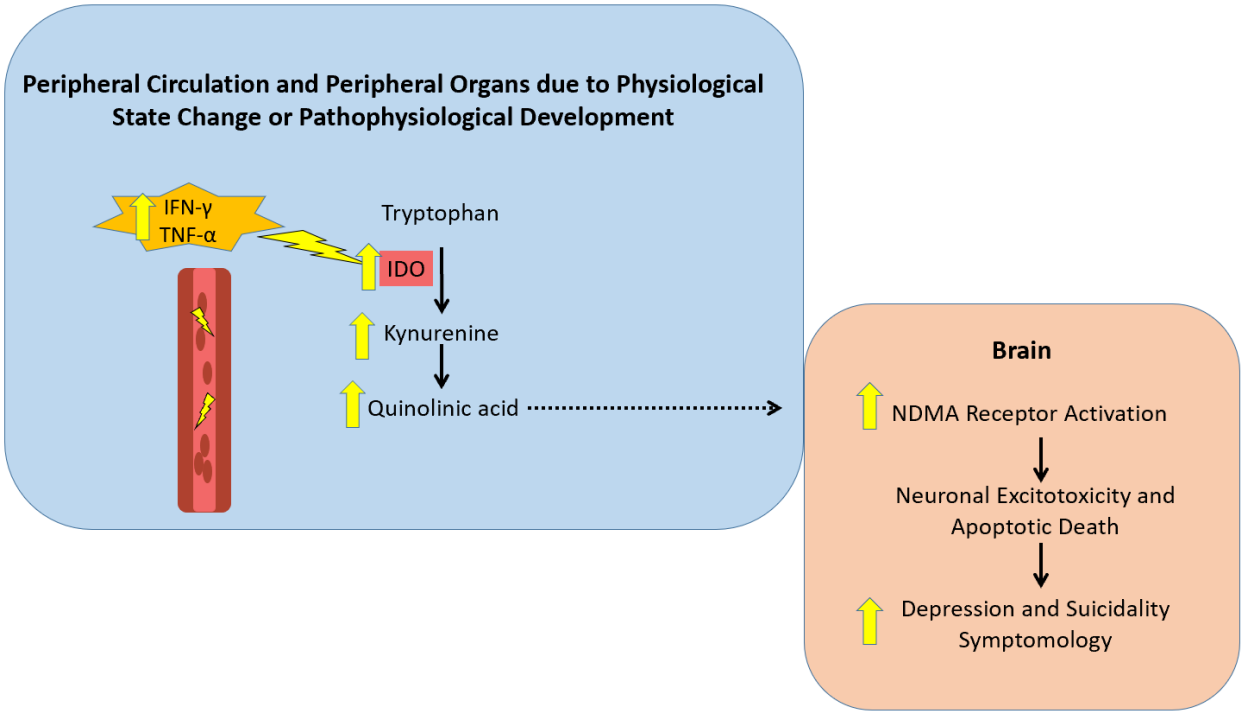

Figure 6.1 Working model of the development of depressive and suicidal symptoms
Peripheral inflammation leads to increased kynurenine pathway metabolism, generating higher levels of the neurotoxic metabolite quinolinic acid. This can reach the brain, causing increased NMDA receptor activation, increased neuronal excitotoxicity and apoptotic death, and increased depression and suicidality symptomology.

The Cause of Peripheral Inflammation

As seen in our working model (Figure 6.1) increases of inflammation and the kynurenine pathway in the periphery is hypothesized to impact depression and suicidality symptomology. In pregnancy, we hypothesis this is the placenta, however we did not establish the cause of the increased inflammatory and pathway analytes in Chapter 3.

One hypothesis is the inflammation may be caused from bacterial infection, chronic autoimmune diseases, infection or reactivation of viruses, and is an overexaggerated response from primed monocytes. This hypothesis is being analyzed in other cohorts in our laboratory. We have analyzed the IgG levels of cytomegalovirus (CMV) and herpes simplex 1 (HSV1) in our peripartum cohort and do see interesting trends of sharp reactivation during the progression of pregnancy (Figure 6.2) with a large proportion of women in our cohort testing positive for CMV (61%) and HSV1 (50%). In the future we aim to establish the contribution of viruses to the proinflammatory changes in blood and placenta and how they influence the development of depression.

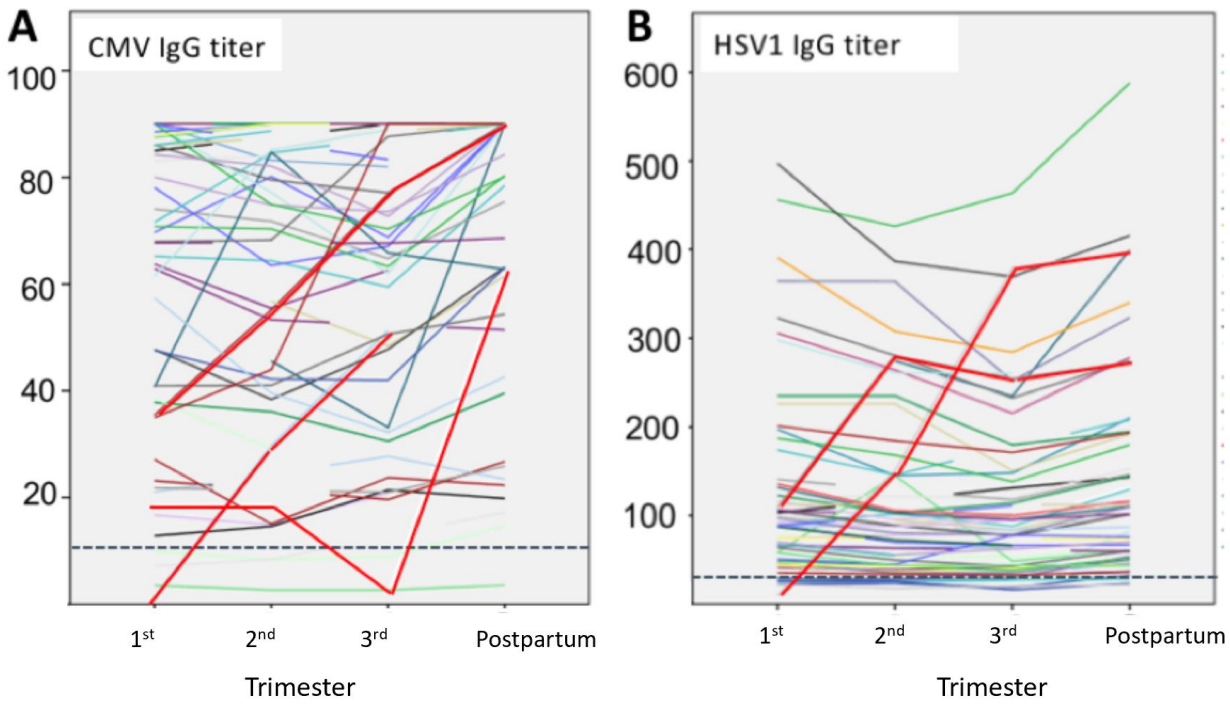

Figure 6.2 Antibody titer levels throughout pregnancy A) CMV antibody titers (U/mL B) HSV1 antibody titers (U/mL). Mean of duplicate values shown. Both fluctuated in certain patients throughout pregnancy and postpartum. The titer level of each woman is indicated with a colored line. Dotted lines indicate the cut-off for sero-positivity.

A secondary hypothesis, in pregnancy, is changes in hormones may be causing changes in psychiatric symptomology. We have analyzed our cohort in Chapter 4 and have found estradiol levels may be related to depression and suicidality in a severity step manner (Figure 6.3). More research into the kynurenine pathway, inflammation and how pregnancy relates to hormonal changes needs to be studied to get a whole model pathway.

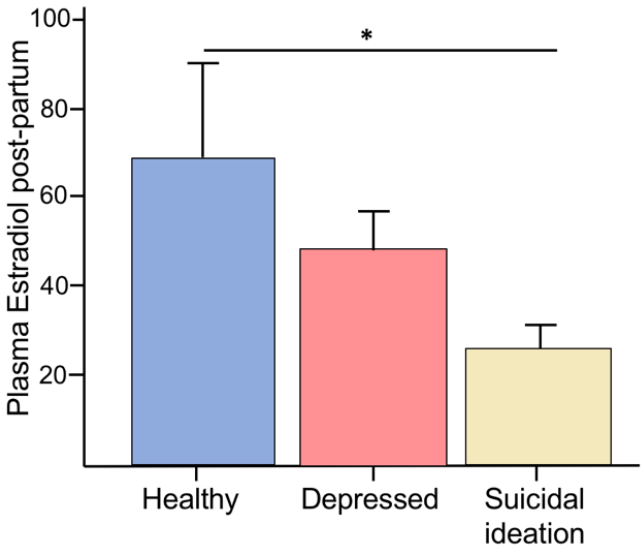

Figure 6.3 Plasma estradiol levels postpartum Plasma estradiol (pg/ml) was lowest in women with suicidal ideation in the post-partum. Unadjusted data shown, mean ±SEM. Age corrected ANOVA, $p<0.05$. An ANOVA corrected for age was significant for the three groups; women with depression and no suicidal ideation (n=61), women with depression and suicidal ideation (n=43), and healthy control women in the post-partum (n=61).

Translational Implications

Our studies suggest that monitoring both proinflammatory and kynurenine pathway metabolites in women who undergo pregnancy, the postpartum period or psychological disorders may be beneficial for the health of the woman. By monitoring closely this would allow for intervention and treatment. However, further studies must be conducted.

Future Directions

While the studies presented here show a lot of promising data, there is still a lot to be discovered to have a full understanding of the role of the kynurenine pathway and inflammation in women. We plan to look at WBCs in peripartum women, as we have found promising data that they are changed in women that are depressed or suicidal. Further, we aim to recruit for a new study, with a wider diversity of demographics, to be able to analyze the differences between different ethnicities, incomes, and support.

The data from this dissertation suggests inflammation plays a large role in PPD and suicidality. We subsequently plan to analyze whole blood, white blood cells, and monocytes that were challenged with the inflammatory inducing agent LPS to analyze the difference in response between women who were susceptible to depression and suicidality during and after pregnancy compared to healthy controls. We hypothesize the susceptible women will have a more robust response to the immune challenge. Ultimately, uncovering the biological role of the kynurenine pathway and inflammation in women will be a collaborative effort which will involve multiple stake-holders including research scientists, medical professionals, and financial support and interest. Although there is a lot to still be determined, the future looks bright for finding answers to women's illnesses by analyzing inflammation and the kynurenine pathway. There is hope to not only treat these women, but to develop panels that screen women before the symptoms ever progress into a diagnosis.

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