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Endothelial and inflammation markers in schizophrenia and bipolar disorder

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2. List of studies

Study I

NOTCH4 Gene Expression is Up-regulated in Bipolar Disorder

Ingrid Dieset, Srdjan Djurovic, Martin Tesli, Sigrun Hope, Morten Mattingsdal, Annika Michelsen, Inge Joa, Tor Ketil Larsen, Ingrid Agartz, Ingrid Melle, Jan Ivar Røssberg, Pål Aukrust, Ole A. Andreassen, and Thor Ueland

American Journal of Psychiatry, 2012 Dec 1;169 (12)

Study II

Cardiovascular risk factors during second generation antipsychotic treatment are associated with increased C-reactive protein

Ingrid Dieset, Sigrun Hope, Thor Ueland, Thomas Bjella, Ingrid Agartz, Ingrid Melle, Pål Aukrust, Jan-Ivar Røssberg and Ole A. Andreassen

Schizophr Research 2012 Sep; 140(1-3):169-74.

Study III

Elevated plasma levels of von Willebrand factor is associated with larger basal ganglia volume – relationship to schizophrenia

Ingrid Dieset, Unn Kristin Haukvik, Ingrid Melle, Jan Ivar Røssberg, Thor Ueland, Sigrun Hope, Anders M. Dale, Srdjan Djurovic, Pål Aukrust, Ingrid Agartz and Ole A. Andreassen

Submitted

3. Abbreviations

| | |
|--------|--|
| ANOVA | Analysis of Variance |
| ANCOVA | Analysis of Covariance |
| BBB | Blood brain barrier |
| BP | Bipolar disorder |
| CNS | Central Nervous System |
| CMV | Cytomegalo Virus |
| CRP | C-reactive Protein |
| DALY | Disability Adjusted Life Years |
| DNA | Deoxyribonucleic Acid |
| DSM-IV | Diagnostic and Statistical Manual of mental Disorders |
| eQTL | Expression Quantitative Trait Loci |
| GWAS | Genome Wide Association Studies |
| HSV | Herpes Simplex Virus |
| IDS | Inventory of Depression Scale |
| IL | Interleukin |
| MHC | Major Histocompatibility Complex |
| mRNA | Messenger Ribonucleic Acid |
| OPG | Osteoprotegerin |
| PANSS | Positive and Negative Syndrome Scale |
| SCID | Structured Clinical Interview for DSM-IV Axis I disorders |
| SNP | Single Nucleotide Polymorphism |
| SZ | Schizophrenia |
| TNF | Tumor Necrosis Factor |
| TOP | Tematisk område psykose (Thematically Organized Psychosis) |
| YMRS | Young Mania Rating Scale |
| vWf | von Willebrand Factor |
| WHO | World Health Organizatio |

4. Summary

Schizophrenia and bipolar disorder are complex disorders, with an etiology that involves multiple genes and a variety of environmental risk factors. Despite a high heritability rate, very little is known about the underlying biological mechanisms, but several molecular pathways are probably involved. The diagnostic criteria are descriptive based on partly overlapping symptom profiles and current treatment options only offer symptom relief and are often accompanied with adverse effects. In the recent years, promising results from genetic, molecular as well as epidemiological studies have implicated alterations involving inflammatory mechanisms to be involved in the pathophysiology of both disorders.

In this project, we investigated the role of inflammation and endothelial markers and their putative involvement in schizophrenia and bipolar disorder pathology. By using a translational approach we explored:

- i) the expression of *NOTCH4* (a gene within the major histocompatibility complex) previously found to be associated with schizophrenia in genome wide association studies in schizophrenia and bipolar disorder and the association with potential expression quantitative trait loci (eQTL) within the *NOTCH4* gene
- ii) the role of inflammation in relation to cardiovascular disease (CVD) risk factors associated with second generation antipsychotic treatment
- iii) whether inflammation and altered endothelial activity are associated with brain morphology in schizophrenia and bipolar disorder.

The expression of *NOTCH4* was significantly increased in patients with bipolar disorder and *NOTCH4* expression was associated with three loci within the *NOTCH4* gene. Overweight

and increased glucose levels were associated with elevated levels of CRP in patients treated with second generation antipsychotics. Von Willebrand factor levels were highly associated with basal ganglia volume across all groups, in particular globus pallidus volume. This association remained significant after adjustment for diagnoses and antipsychotic treatment.

Taken together, the findings support that i) mechanisms related to endothelial activity and inflammation and more specifically the NOTCH4 pathway are involved in bipolar disorder pathophysiology ii) cardiovascular disease risk factors and potentially atherosclerosis induced by second generation antipsychotics may in part be mediated through inflammatory mechanisms iii) and finally that von Willebrand factor, an endothelial marker also known to be involved in inflammation may have a role in the pathophysiological process underlying the enlarged basal ganglia volume seen in schizophrenia.

In summary, the results indicate that endothelial-related inflammation is involved in both disorders but through different molecular mechanisms.

5. Introduction

In the age group between 10-24 years no other groups of diseases are more costly in terms of disability adjusted life years (DALYs) than mental disorders (1), yet very little is known about the underlying biological mechanisms. The diagnostic criteria for the major psychiatric diseases such as bipolar disorder and schizophrenia are solely based on descriptive characterizations of symptoms.

In the last century, large efforts have been made in trying to find the cause and eventually find a cure for these diseases. Many interesting hypotheses have been proposed, some have been rejected and some are still being investigated. However, we are still only marginally closer to understanding the pathophysiology of schizophrenia and bipolar disorder. We know that intervention in the early course to some extent can prevent or lessen the devastating disability associated with chronic severe mental disorders. Identifying those at risk of developing severe mental disorders at a presymptomatic stage is extremely challenging as the prodromal signs and symptoms often are extremely subtle and could represent a variation of normality.

Another major challenge is identifying sub-groups within these heterogeneous disorders, in order to provide treatment that improves the patients' prognosis. Hence, the field of psychiatric research has long been searching for biological markers that could help identify patients at risk of developing severe mental disorders at an early stage.

The role of the human immune system has mainly been studied in somatic illness, but in the decade prior to the project the evidence implicating inflammation in the pathogenesis of schizophrenia (2) and bipolar disorder (3) started to appear. As the growing, but divergent body of literature on this topic illustrates, the exact answer as to which parts of the immune system are involved in schizophrenia and bipolar disorder and the mechanisms by which

inflammatory activity contributes to the development and course of these disorders remains unclear.

5.1 Schizophrenia and bipolar disorder

Schizophrenia is a mental disorder with severe symptoms and functional deficits, with an early age onset and a lifetime prevalence of 1% (4). The disorder has a high heritability (0.7-0.8) and a complex multifactorial cause (4). It is a devastating disease for the patient diagnosed, with high financial costs for society. According to the WHO, it is one of the world's most costly diseases with 80% of the afflicted patients being unemployed and accounting for a large percentage of medical disability at the age between 15 and 44 years.

The emergence of psychosis often marks the patient's first encounter with the health care system. Symptoms such as auditory hallucinations, bizarre delusions, and disorganized speech, typically manifest itself at the age of 18-25 years (4) and are often categorized as positive symptoms. Negative symptoms, such as anhedonia, flat affect, social withdrawal and lack of motivation are less obvious to the surroundings, but nevertheless very debilitating for the patient and often more difficult to treat.

Bipolar disorder is characterized by recurrent abnormal mood variations ranging from mania, euthymia and depression(5). The clinical course varies both in terms of number of episodes and the degree of severity. The DSM-IV differentiates between bipolar disorder I which is characterized by a combination of depressive and manic episodes and bipolar disorder II which typically manifests as a combination of depressive episodes and hypomania. In addition to elevated mood, a manic episode is typically accompanied by psychosis and often leads to severe impairment in social function and hospitalization. In comparison, hypomania is not as

severe as it per definition never leads to hospitalization, social impairment or psychotic symptoms. Similar to schizophrenia, the life time prevalence for bipolar disorder is estimated to 1% whereas the mean age at onset is approximately 23-24 years (5).

5.1.1 Diagnoses

Psychiatric diagnoses are descriptive and diagnostic conclusions are entirely based on self-reports and observations of symptoms, behavior and assessments of the patient's level of function. The Diagnostical and Statistical Manual of Mental Disorders (DSM) provides a classification system based on categorizing signs and symptoms in defining a diagnosis (6).

Critics have questioned this method claiming that several symptoms and criteria overlap and that the allocation of diagnoses is arbitrarily (7). Both schizophrenia and bipolar disorder are syndromes with partly overlapping signs and symptoms. Schizophrenia and bipolar disorder I share many clinical features such as psychotic symptoms, social and functional impairment and a chronic, relapsing course. In fact a combination of symptoms qualifies according to the DSM-IV system for the diagnosis schizoaffective disorder. Thus instead of looking upon schizophrenia and bipolar disorder as separate categorical diagnostic entities, some argue that the two disorders should be perceived along a dimensional continuous line (8). The discussion about whether schizophrenia and bipolar disorder represent two separate diagnostic entities with different etiology or clusters of symptoms along the same continuum is not a new one. In fact the ongoing discussion among contemporary psychiatrists very much mimics the debate from the mid nineteenth- and early twentieth century, when Guislain's concept of "unitary psychosis" (9) and his theory that all mental disorders shared the same anthological cause was

challenged by Emil Kraepelin's introduction of the dichotomy "dementia praecox" and "manic depression" (10).

Another objection against the DSM-system is that the criteria only embrace symptoms that have been present for some time. The identification of prodromal stages, where symptoms such as unusual thought content, cognitive deficits and minor functional deficits predominate, has led to an understanding of the disease as a neurodevelopmental disorder.

Large, longitudinal cohort studies focusing on prodromal symptoms in adolescents have reported that symptoms such as altered thought content, social isolation, change in behavior and loss of function have large predictive powers for identifying psychosis later in life (11). However, this high sensitivity is not accompanied by a high specificity as these prodromal features are difficult to distinguish from normal teen behavior and might be a variation of normality or signs of other psychopathology.

Despite its many flaws and shortcomings, the DSM-IV is one of the best available tools for diagnosing severe mental disorders today. In addition, the Structured Clinical Interviews for DSM-IV (SCID) serve as guidance in the clinical interviews (12).

The patient subjects included in this study are diagnosed according to the DSM-IV as having either a schizophrenia spectrum disorder (schizophrenia, schizophreniform or schizoaffective), a bipolar disorder spectrum disorder (bipolar disorder I, bipolar disorder II) or bipolar disorder not otherwise specified (NOS) or other psychosis (psychosis NOS or depressive psychosis).

Before embarking on this PhD project one of the questions was whether or not the inflammatory activation seen in schizophrenia and bipolar disorder was similar or different in the respective diagnostic groups. Thus, we chose to investigate and compare all three patient

groups against each other and in comparison with healthy controls. In doing so, we hoped that the results from these studies would provide some biological evidence enlightening the long, but ongoing continuum vs. dichotomy debate of psychotic disorders.

5.1.2 Treatment

There is no cure for schizophrenia and bipolar disorder, but relatively effective symptomatic treatment options exist in terms of psychosocial interventions and medication. Medication is recommended for everyone but should be supplemented with additional psychosocial interventions, such as training in coping with everyday life activity, self-help groups, family oriented education and psychotherapy. As these patients often face many years of illness, long term integrated treatment is particularly important in order to minimize the burden on the patient and their families.

In the following only medication will be discussed further. Antipsychotics are used as the primary medical intervention in schizophrenia and often as a secondary choice after mood stabilizing drugs in bipolar disorder (13). Antipsychotics are highly efficient with a number needed to treat ranging from 3 to 7 (14), which makes it unethical to do placebo controlled studies. The so-called first generation antipsychotics (FGA) were first discovered in the 1950'ies and revolutionized psychiatric treatment, enabling patients to leave the large asylums where they used to be "stored". Their mode of action is mainly antagonizing dopamine (D_2) receptors (15). The downsides of FGA are several adverse effects such as dyskinesia, akinesia and parkinsonism and these medications have not proven to be very effective in treating negative symptoms. Second generation (SGA) antipsychotics were introduced in the 1990'ies with an antagonizing effect on serotonin receptors ($5-HT_2$) in addition to dopamine receptors

(15). They proved to have less extra-pyramidal side effects, but were associated with dyslipidemia, hyperglycemia and overweight which in turn increased the incidence of metabolic syndrome and cardiovascular disease (CVD) dramatically (16). Despite massive effort in trying to identify the cause of these adverse effects, the pharmacological mechanisms underlying the induction of these metabolic side effects remain uncertain. In 2001, the largest non-commercial clinical study (the CATIE) initiated a double-blind randomized trial comparing the effects- and side effects of first- and second generation antipsychotics (16) . One of the sub-studies within this project investigated the relationship between metabolic parameters and inflammatory variables in a longitudinal cohort (17). The results from this study showed that an increase in metabolic syndrome (MetS) induced by second generation antipsychotics was accompanied by an elevation in inflammatory markers (17).

Second generation antipsychotics are also used in treating bipolar disorder particularly in the management of mania and psychotic symptoms (13), but the first drug of choice is usually a mood stabilizer (either Lithium or an anticonvulsant) (13). Lithium is effective in the treatment of mania, but is also used in treating depression and as long term stabilizing prophylactic treatment. The three most widely used anticonvulsants are Valproic acid, Lamotrigine and Carbamazepine. In addition, a selective serotonin reuptake inhibitor (SSRI) is often recommended in depressive phases (13). Some theories regarding the pharmacological mechanisms of Lithium, anticonvulsants and SSRI will be discussed in further detail later.

5.1.3 Etiology

Another major topic which has been a struggle for centuries in the field of psychiatry is the lack of known etiology in schizophrenia and bipolar disorder. Again, a walk down the historical lanes differs little from the contemporary debate we are witnessing today. In the late nineteenth and early twentieth century the debate was dominated by Kraepelin (1850-1929) who was convinced that “dementia praecox” and “manic depression” exist in nature and could best be studied as biological diseases, Wernicke (1848-1905) who introduced the concept of neuronal science, Watson (1878-1958) who introduced “behaviorism” and finally Freud (1856-1939) who argued a psychoanalytical model for explaining psychopathology.

In an effort to unite these three approaches, Engel introduced the biopsychosocial model in 1977 (18). The underlying idea was that disease should be looked upon as a result of an interaction between biology and psychology, with environmental (or social) factors as an intermediate agent. The biopsychosocial model as a theoretical framework in the clinical field of psychiatry has received much critique, but in the last decade the integrative idea behind the model has influenced psychiatry research and stimulated for studies investigating the effects of gene x environment (19), infection x genetic vulnerability (20) and gene x stress (21) on schizophrenia and bipolar disorder. As displayed in figure 1a, the interplay between genes, environment and phenotype is reciprocal in the sense that genes may be modified by environmental factors and vice versa - genes may affect environmental sensitivity (e.g. patients may be genetically prone to substance abuse which increases the risk for developing schizophrenia and bipolar disorder). Likewise, there is a mutual relationship between the environment and the phenotype. Every single component in this complex model may affect many levels ranging from gene expression, proteins, neuronal circuits and brain anatomy (figure 1b).

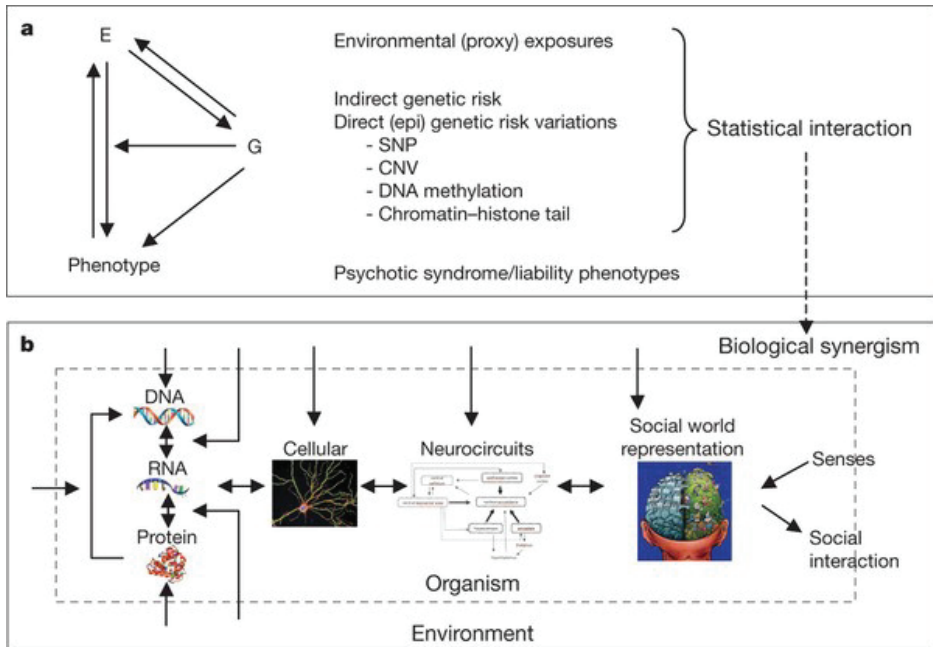


Figure 1. Model displaying the complexity of psychosis etiology

(van Os et al. Nature 2009, printed with permission)

5.1.4 Genes

The heritability rate, i.e. the proportion of the variance of the disease explained by genes is approximately 70-80% for schizophrenia and bipolar disorder and some studies have reported a genetic overlap between the two diseases (22).

Around a decade ago multiple genome wide association studies (GWAS) emerged with the intention of launching new potential candidate genes for schizophrenia and although the early GWAS focused on the genetic risk in schizophrenia, studies reporting a shared genetic risk of schizophrenia and bipolar disorder soon emerged (23). The results revealed either rare

variants of copy number variations (CNV's) with high odd scores or common single nucleotide polymorphisms (SNP's) with very low odd scores.

In 2009 when this thesis was being planned, Nature, the world's leading scientific journal, made an attempt to summarize the most important findings from the GWAS and concluded that no apparent candidate gene had appeared but that one region within the major histocompatibility complex (MHC) on chromosome 6 emerged as a promising area for further study (22,24,25). The genes within the MHC are mainly organized into three regions, the MHC I which contains the human leukocyte antigens (HLA) often linked to autoimmune diseases, the MHC II containing genes encoding the α and β chains and MHC III which contains genes encoding complement factors and cytokines such as the tumor necrosis- α . Situated on MHC III is also the *NOTCH4* gene which is one of the promising genes associated with schizophrenia (25) as well as being involved in autoimmune diseases (26,27)

Despite high heritability rates for schizophrenia and bipolar disorder and large genome wide association studies, very few genes have emerged as convincing susceptibility genes. The explanation of this "missing heritability" remains uncertain. One possibility might be that there is a considerable epigenetic inheritance involved. This theory implies that heritable changes in gene expression are not necessarily caused by alterations in the DNA strand but rather along the process towards the final phenotype. Such epigenetic effects might for example be changes in methylation or changes in the packaging of the DNA.

5.1.5 Environmental factors

Given that the heritability in schizophrenia and bipolar disorders does not explain all of the variance, the question is what about the remaining unexplained variance.

Large and well-designed epidemiological studies have identified a number of environmental risk factors for schizophrenia and bipolar disorder. Urbanization (28), famine (29), migration (30), substance abuse and pre- and post natal birth complications are all well known risk factors for developing schizophrenia. There is less evidence concerning environmental impact in bipolar disorder, but potential common environmental risk factors for both diseases are high paternal age (31), cannabis use (32,33) and childhood trauma (34,35).

Some of these environmental factors occur in pre- or early post natal stages, while others occur later in life. Many environmental factors might not necessarily explain the cause of the disease, but nevertheless contribute to the cluster of symptoms that form the clinical picture of schizophrenia and bipolar disorder. Life style factors such as poor diet and substance abuse might affect the clinical outcome to some degree (36).

An intriguing possibility is that the environmental factors affect disease development due to epigenetic mechanisms. Animal studies have shown that inducing malnutrition in the mother causes epigenetic changes in the offspring (37), thus environmental factors such as pre- and post natal diet, drugs, medication, stress and trauma may cause epigenetic changes which in turn alter gene expression patterns and ultimately result in the schizophrenia and bipolar phenotypes.

5.1.6 Phenotypes

The final product of the genetic, environmental and epigenetic input is the phenotype. Clinical phenotypes in schizophrenia are typically hallucinations, delusions, apathy, as well as disorganized thought content, speech and behavior. In bipolar disorder the clinical phenotypes are disabling variation in mood, with depression at the one end of the scale and mania with psychotic features at the other end. However, it is very difficult to identify the core mechanistic phenomenon, as behavior or even symptoms may be unspecific or secondary to the disease process. Further, the same underlying pathophysiological process could lead to a range of clinical characteristic and conversely, several mechanisms could converge on the same clinical presentation of symptoms.

The disease associated changes at the neurobiological or molecular level are at best subtle and lack the predictive validity necessary in order to be defined as a disease phenotype. The genetic complexity in schizophrenia and bipolar disorder implies several genes and the pathophysiological etiology probably involves multiple interactions between gene x gene and gene x environment. In an attempt to bridge the gap between genes and phenotype in psychiatry, the concept of endophenotype was introduced in psychiatric research (38). An endophenotype is sometimes referred to as an intermediate phenotype and must i) be associated with the illness ii) be heritable iii) be state-independent iv) co-segregate with an illness and v) be present in non-affected family members at a higher rate than in the general population (38). Examples of endophenotypes might be physiological, biochemical, neurocognitive function or neuroanatomical changes associated with the disease (38).

Several potential endophenotypes have been proposed in schizophrenia and bipolar research. Imaging studies have reported various structural brain abnormalities in the brains of

schizophrenia and bipolar disorder, such as reduced cortical volumes and thickness, enlarged ventricles and basal ganglia, and smaller hippocampus volumes (39,40) . Some studies have indicated neurocognitive deficits (41,42), while others have implicated molecular changes (43). The difference in the definition of an *endophenotype* and a *biological marker* is vague and the two terms are often used as synonyms. The term *endophenotype*, however, provides a clear causal direction to the genes whereas the term *biological marker* may be defined as an indicator of any biological or physiological process. Furthermore, a biological marker may also be used to measure effects of treatment interventions. In addition, an endophenotype assume disease state-independence which would require longitudinal studies and presence in non-affected family members (38) which is not possible to prove within the case-control design of this thesis. For these reasons the term *biological marker* will be used further in this project.

As advances in biological research have gained momentum, biological characteristics associated with the schizophrenia and bipolar disorder has emerged as potential phenotypes. The one consistent biological mechanism explaining the cause of schizophrenia and/or bipolar disorder is yet to be found.

Many of the hypotheses regarding the biophysiological mechanisms in schizophrenia and bipolar disorder are more or less derived from the observed effect of available medication and its respective pharmacological mode of action on receptor levels.

With the introduction of neuroleptics in the 1950'ies, the “dopamine hypothesis” was launched. Since then multiple attempts have been made in order to explain a putative link between schizophrenia and excess production of dopamine in the brain (44). Although treatment with dopamine antagonists reduced the load of positive symptoms such as hallucinations and to some degree delusions, they did not drastically improve negative

symptoms and cognitive deficits. As the focus on cognitive symptoms increased, theories hypothesizing that disturbances in the glutamate signaling pathway and reduced activity in GABA inhibitory neurons could play a role in schizophrenia etiology emerged (45). Serotonin has also been suggested as a potential cause for schizophrenia because of the psychosis like symptoms induced by drugs such as LSD (46).

Similar to schizophrenia, the observed effect of pharmacological treatment of bipolar disorder has generated hypotheses involving the various neurotransmitter systems. A hyper-dopaminergic state has been suggested in mania and inversely a hypo-dopaminergic state in depressive phases (47). In the same vein, studies indicating a lowering effect of lamotrigine on glutamine levels have generated theories implying the glutamatergic system (48). The exact molecular mechanisms underlying lithium's stabilizing effect on affective symptoms are not entirely known, but the inhibitory effect on the glycogen synthase kinase 3 (GSK-3) have implied this pathway to be involved both directly (49) and indirectly by blocking GSK-3's ability to promote inflammation (50). Others have suggested that both valproate acid and lithium works by inositol depletion (51).

A few other theories that go beyond the pharmacological properties of the various treatment regimens have emerged. The hypothalamic-pituitary-adrenal (HPA) axis has been proposed by many to be involved in both schizophrenia (52) and bipolar disorder (53). Lastly, several genetic studies implicating the gene *CACNA1c* in both schizophrenia and bipolar disorder (54,55) have generated hypotheses suggesting a dysfunction in the calcium signaling pathway to be involved.

5.2 The immune system and the endothelium

We are still unable to offer curable treatment options for people with schizophrenia and bipolar disorder and unfortunately the available medication are not equally effective for everyone. After years of exploring the traditional neurotransmitter systems (dopamine, glutamate, GABA and histamine), several groups have reported results suggesting that the immune system might be involved in psychosis pathology.

The human immune system is extremely complex and any attempt to present a description within the format of this thesis is bound to be superficial. Briefly summarized the immune system consists of two arms, the innate immune system and the adaptive immune system which interact on many levels.

Innate immunity is present at birth and does not need any priming before being activated against hostile agents. Pathogens such as lipopolysaccharides from the bacteria cell wall or components released as a result of tissue damage bind to toll-like receptors (TLR) which via an intra-cellular transduction cascade initiate the release of pro-inflammatory cytokines and chemokines. The adaptive immune system is activated by the innate system and is capable of “memorizing” the reaction so that it acts fast when re-confronted with the same pathogen. The activity in the adaptive immune system could be classified as humoral response or cellular response. The humoral response is initiated by a presentation of an antigen to a B-cell which in turn produces antibodies, whereas the cellular response is induced upon presentation of an antigen on a T-helper (CD4) cell MHC II receptor. T-helper cells type 1 (Th1) recruit and activate B-cell activity, whereas T-helper cells type 2 (Th2) activate cytotoxic T-cells (CD8+).

| | | |
|--|--------------------|---|
| Innate immune system: | | Adaptive immune system: |
| Macrophages | | Humoral immunity: |
| Neutrophils | Eosinophils | B-lymphocytes |
| Dendritic cells | Basophils | |
| Natural killer cells | Epithelial barrier | Cellular immunity: T-lymphocytes: cytotoxic T-cells |
| Mast cells | Plasma proteins | (CD8+) and helper T-cells (Th1 and Th2) |
| Common innate and adaptive immune system: | | |
| Natural killer T-cells and cytokines | | |

Table 1. Components in the innate and adaptive immune system

Cytokines are small protein molecules produced and secreted by cells in both the innate and adaptive immune system. Each cytokine has a corresponding receptor on a cell surface and is involved in intra- and inter cellular communication. Upon binding to a receptor, the cytokine is able to initiate a down-stream reaction activating an intra-cellular signaling cascade which can up- or down regulate genes (56).

Microglia cells are the equivalents to the macrophages in the CNS and comprise about 10% of the cells in the brain. They are the first line defense and are able to express antigens upon activation of TLR's or MHC molecules. During the development of the brain, microglia cells play a vital role in synaptic pruning. In addition dendritic cells, astrocytes mast cells and oligodendrocytes are regarded as contributors to the immune-related activity in the brain.

The endothelium is the layer of cells that constitute the inside lining of the entire vasculature and the lymphatic vessels. Endothelial cells have several functions, including serving as a physical barrier between the circulation and the rest of the organism controlling the passage of

molecules into the blood stream, as well as participating in the coagulation and inflammation process (57). In addition endothelial cells are involved in the formation of new vessels (58). Endothelial dysfunction is a pathological state in which the collaboration with the coagulation system and immune system (59) works in an inexpedient way promoting disease such as atherosclerosis. Interestingly, a state of mental stress has been found to induce endothelial dysfunction in otherwise healthy individuals (60,61).

The BBB consists of endothelial cells with high numbers of mitochondria, high numbers of tight junctions and reduced pinocytic activity compared to endothelial cells in other organs (62). Until quite recently the central nervous system was believed to be well protected from the periphery by i) the blood brain barrier (BBB) which prevented the leukocytes from entering the CNS ii) lack of lymphatic drainage from the CNS to the lymph nodes and iii) lack of MHC II molecules in the brain. Recent data have however modified the “truth” about the CNS immune privilege. Firstly, there is some passage of leukocytes and cytokines across the BBB (63). Secondly, it has been proven that there is lymphatic drainage from the CNS via the cervical lymph nodes (64). Finally, it has been shown that upon activation T-lymphocytes migrate into the CNS where they release pro-inflammatory cytokines which stimulate the expression of MHC II molecules (62).

Cytokines are also acting as signaling molecules, thus the increase in inflammatory activity may be a secondary effect of primary alterations in the development of neural synapses and neural signaling cascades. Recent studies suggest that molecules traditionally regarded as “immune molecules” also play a central role in the development of the central nervous system (65). MHC molecules have a negative effect on neural development in the sense that they inhibit differentiation, synapse formation and limit neural plasticity (66).

5.3 The immune system and psychiatric disorders

Already in 1929, Tramer reported an increased risk of developing schizophrenia in offspring born during winter season (67). Interestingly, two of his more known contemporaries Bleuler and Kraepelin, had previously speculated that infection might play a role in the development of psychosis (68). Several studies reported that infection in the mother pre-natally or in early childhood increased the risk of developing schizophrenia later in life (69,70). Multiple pathogens were proposed as possible contributors to severe mental disorder, ranging from influenza virus, CMV, toxoplasmosis, rubella, mumps, measles, polio, varicella, HSV, chlamydia, pertussis and candida (71-73). However, the lack of a single bacteria or virus as a probable cause for schizophrenia, urged a shift of focus from searching for a particular pathogen to exploring the inflammation process itself. Several studies have investigated peripheral inflammation in schizophrenia and bipolar disorder using plasma samples (2,3) and a few research groups have investigated the inflammatory activity in the cerebrospinal fluid (CSF) samples (74). Elevated levels of tumor necrosis factor alpha (TNF- α), high sensitivity c-reactive protein (hsCRP), and interleukin-2 (IL-2) are associated with both schizophrenia and bipolar disorder, whereas elevated levels of IL-1 receptor antagonist (IL-1Ra) and interleukin-6 (IL-6) are associated with schizophrenia (2,3,75-77). Inflammation stimulates the production of kynurenic acid which have been found to be elevated in the cerebrospinal fluid in schizophrenia patients (78) and interestingly an up-regulation of the kynurine pathway have been found in post mortem brain tissues in both schizophrenia and bipolar disorder (79).

In 2006, a large national registry study from Denmark reported that several autoimmune disorders were more prevalent among schizophrenia patients and their relatives. Having an auto-immune disease increased the risk of developing schizophrenia with as much as 45% (80). The results from this epidemiological study raised the question whether schizophrenia

patients could have a particular vulnerability involving the human leukocyte (HLA) system in the major histocompatibility complex (MHC).

Indeed, a few years after the Danish population study, the first genetic association studies supporting this theory appeared (22,25). The discoveries revealing associations between schizophrenia and bipolar disorder and SNP's in the major histocompatibility complex (MHC), a region dense with immune related genes, implied that these patients might be high-inflammatory responders, but in a low-grade chronic fashion (81,82). In support of this theory, microarray studies have found an up-regulation of immune related genes in oligodendrocytes (83) and endothelial cells (84) in schizophrenia. Furthermore, there is evidence of increased inflammatory activity also in the first-degree relatives of schizophrenia and schizoaffective patients (85) suggesting that abnormalities in the immune system might be an endophenotype.

5.4 Embracing the complexity-translational approach

Schizophrenia and bipolar disorders have been studied at many different levels. Methods range from descriptive studies focusing on clinical aspects, epidemiological studies investigating environmental factors, imaging studies depicting brain morphology, molecular and genetic studies. However, in many study projects the theoretical and methodological model is limited to one of these levels.

Another problem in psychiatry research is the lack of available live tissue. Despite many efforts, we have yet to see the perfect animal model for schizophrenia and bipolar disorder. Post mortem tissue banks (e.g. www.stanleygenomics.com) for microarray studies have to some extent been successful initiatives, but also these studies face major methodological

problems such as low numbers of study subjects, problems with tissue processing and fixation and limited control over confounding factors such as medication.

Initially the vision behind translational research was to bring results from the lab to the bedside. In the case of psychiatry research where disease mechanisms are still unknown, we don't have access to live tissue material or a perfect animal model and the diagnoses are still based on descriptions of symptoms, the order of direction might better be reversed in the sense that information from the patients should be brought back to the lab. In an attempt to bridge the gap between basic science and applied research in psychiatry research, the concept of translational research has been modified and introduced with the underlying idea of combining different methods in order to capture the complexity of diseases (86) . The TOP study protocol has gathered data at many different levels and provides a unique opportunity to create a study design capturing the heterogeneity of these diseases.

6. Aims of the thesis

The overall goal for this thesis was to provide new insight about underlying pathophysiological mechanisms involving inflammation and endothelial dysfunction in schizophrenia and bipolar disorder. Our ambitions were to identify biological markers which may be used to identify the diseases at an early stage and lay grounds for further research on new targets of medical intervention that would help prevent the disease and improve the quality of life for the patients.

This current project based its overall strategy on a multilevel model exploring the relationship between:

- i) immune related genes, gene expression, peripheral plasma inflammation and endothelial markers

and

- ii) clinical phenotypes (diagnoses), treatment and brain abnormalities.

Recognizing the complexity of these diseases, we aimed at controlling for as many potential confounders as possible ranging from a several environmental factors (e.g. substance abuse, smoking, medication) to symptom load (psychotic symptoms and affective symptoms).

Three studies were conducted with the following sub-aims:

Study I:

1. To determine the peripheral expression of the immune related *NOTCH4* gene in schizophrenia and bipolar disorder.

2. Identify putative expression Quantitative Trait Loci (eQTLs) in and around the *NOTCH4* gene.

Study II:

1. To determine the relationship between inflammation and cardiovascular risk factors in schizophrenia and bipolar disorder.
2. To determine whether a putative relationship between cardiovascular risk factors and elevated levels of inflammation markers is related to treatment with second generation antipsychotics.

Study III:

1. To determine whether there is an association between plasma levels of six inflammation markers and one endothelial marker and brain structure measurements.
2. To determine whether a putative association between inflammation and endothelial activation is specific for bipolar disorder or schizophrenia diagnoses.

7. Methods and Materials

The Thematically Organized Psychosis (TOP) Study is a collaborative study involving Oslo University Hospital and Stavanger University Hospital. The study is funded by the Oslo University Hospital, University of Oslo, Regional Health Authorities and the Research Council of Norway.

7.1 Referral and assessment routines of patients

Patients were recruited consecutively from out-patient and in-patient psychiatric units. Inclusions of patients referred from the acute wards were awaited until the patients were clinically stable enough to give written informed consent and participate fully in the structured interviews. Patients who did not speak Norwegian were excluded due to reliability requirements. Psychiatric assessments were performed by trained research fellows who were all clinicians (medical doctors or psychologists) and the physical examination was performed by medical doctors. After completing the protocol, the referring clinician received a full report with somatic and psychiatric assessments of the patient.

7.1.1 Diagnosis

Lifetime diagnosis were based on Structured Clinical Interview for DSM-IV Axis I disorders (SCID I module A-E) (6) and information from medical records. When necessary, co parental information was assembled with the patient's approval. Research fellows participated in the training program at UCLA (CA, USA) and participated in regular consensus meetings. A

satisfactory inter-reliability based on the UCLA training procedure was achieved with an overall agreement of 82%, $\kappa = 0.77$ (95% CI: 0.60-0.94).

7.1.2 Symptom assessments

Current psychotic symptoms were assessed using the Positive and Negative Syndrome Scale (PANSS) (87) with an intraclass correlation coefficient (ICC) of 0.73 (95% CI:0.54-0.90).

Current depressive symptoms were rated using the Inventory of Depressive Symptomatology-Clinician rating (IDS-C) (88) and current manic symptoms were assessed using the Young Mania Rating Scale (YMRS) (89).

Current level of functioning was assessed using the Global Assessments of Functioning Scale (GAF) (90). The ICC was 0.86 (95% CI: 0.77-0.92) for GAF-symptom and 0.86 (95% CI: 0.77-0.92) for GAF-function.

7.1.3 Disease course

Number of previous affective (depressive, hypomania and manic) and psychotic episodes as defined by DSM-IV, as well as number of previous hospital admissions to psychiatric acute wards was recorded.

7.1.4 Medication

Detailed records of the patient current medication was obtained through medical records and information from the patients. In cases of discrepancy between the records and the

information given by the patient, the treating clinician was consulted. Defined daily dosages (DDD) were calculated in accordance with the guidelines from the World Health Organization Collaborating Center for Drug Statistics Methodology (<http://www.whocc.no/atcdd>).

7.1.5 Illicit substances and alcohol

All patients were screened and diagnosed according to the E module in the SCID manual. Patients were asked which and how many times they had consumed illicit substances over the last 2 weeks. All participants were asked how many international units (IE) of alcohol they had consumed over the last two weeks.

7.2 Referral and assessment routines for healthy controls

An age and sex matched sample of healthy controls from the same catchment area were randomly selected from national statistical records using a computer based program. The participants were contacted by a letter of invitation. All controls were evaluated with clinical interviews and the Primary Care Evaluation of Mental Disorders (91) and excluded if they or first degree relatives had a history of severe psychiatric disorder (major depression, bipolar disorder or schizophrenia). Other exclusion criteria were substance or alcohol abuse or dependency, medical conditions involving the central nervous system and severe head injury.

7.3 Sample

A total of 986 subject were included in all three studies, 298 healthy control subjects and 688 patients. All patients and controls were enrolled in the time period from 2003-2008.

Some of the subjects were used in two or all studies (see Table 1.).

Table 1. Sample overlap

| No overlap | Overlap study 1,2 and 3 | Overlap study 1 and 2 | Overlap study 1 and 3 | Overlap study 2 and 3 | Total N |
|--------------|----------------------------|--------------------------|--------------------------|--------------------------|--------------|
| N=686 | N=105 | N=56 | N=75 | N=64 | N=986 |

In study I, whole blood for measurement of NOTCH4 mRNA was collected from a total number of 211 healthy control subjects and 479 patients. A total of 435 patients and controls with Caucasian ethnicity were genotyped.

In study II where we investigated the effect of second generation antipsychotics on immune marker levels, we used a patient group as a comparison group instead of healthy controls. The comparisons were patients not treated with second generation antipsychotics, but otherwise similar to the subject group in terms of demographic and clinical variables. This approach was deliberately chosen in order to minimize the confounding influence of environmental factors such as poor life style habits, drug and alcohol use. Briefly, 361 patients with bipolar disorder, schizophrenia and psychosis not otherwise specified were recruited. The sample was divided into two subgroups according to treatment with second generation antipsychotics (n=232) or not (n=129).

In study III, a total of 216 patients and 140 healthy control subjects with eligible plasma samples and MRI brain scans were included in the study.

7.4 Inflammation and endothelial markers

7.4.1 C-reactive protein (CRP)

C-reactive protein (CRP) is called an acute-phase protein because of its ability to mobilize instantly in the cases of inflammation or tissue damage (92). It is primarily synthesized in the liver, but also in the kidney and in vessel walls especially in atherosclerotic tissue (93). Studies have shown that elevated CRP increased the risk for CVD and some have suggested to include high sensitivity (hs) CRP measurements in cardiovascular risk protocols (94).

7.4.2 Interleukin-6 (IL-6)

Along with CRP, interleukin-6 (IL-6) is considered a mediator of acute phase response (95). In addition interleukin-6 has been shown to be inversely associated with hippocampus grey matter volume (96).

7.4.3 Tumor necrosis factor (TNF)

Tumor necrosis factor (TNF) earned its name because of its ability to induce apoptosis of cancer cells, later however it has become well known for its role in inflammatory processes (97). TNF- α acts on two different types of receptors; TNF-R1 which is expressed in most tissues and TNF-R2 which is mainly expressed in lymphoid cells (98).

7.4.4 Interleukin-1 receptor antagonist (IL-1Ra)

The interleukin-1 receptor antagonist (IL-1Ra) is a member of the IL-1 family and serves as an antagonist of the inflammatory actions of IL-1 α and IL-1 β . It is mainly synthesized in macrophages and neutrophils (99). Plasma levels of IL-1Ra reflects a recent activity in IL-1 and is thus used as an indicator of inflammatory response (100). Besides being implicated in schizophrenia and bipolar disorder (101), increased activity in the IL-1 family has also been associated with diabetes type II (99).

7.4.5 CD40 ligand (CD40L)

CD40 ligand (CD40L) is also a part of the tumor necrosis family, expressed in lymphocytes, endothelial cells and macrophages and particularly known for being involved in the formation of atherosclerotic plaque (102).

7.4.6 Osteoprotegerin (OPG)

Osteoprotegerin (OPG) is a cytokine receptor and belongs to the tumor necrosis receptor (TNF) family (103). OPG is well known for its involvement in bone metabolism and its ability to inhibit osteoclast activity, but lately it has become clear that OPG mRNA is expressed in various other tissues including the brain and several studies have implicated OPG to be involved in other disease pathogenesis, in particular vascular disease (104).

7.4.7 Von Willebrand factor (vWf)

Von Willebrand factor (vWf) is a large glycoprotein in plasma. It is synthesized and stored in endothelial cells and its main function is in hemostasis mediating platelets adhesion and binding of the blood clotting factor VIII (105). Low levels of vWf factor leads to bleeding disorders and elevated plasma levels increases the risk of thrombosis (106,107).

7.4.8 NOTCH4

NOTCH4 is one of four transmembrane proteins that constitute the NOTCH family (NOTCH1-4) (108). Together they form a signaling system which plays a crucial role in neural cell growth and T-cell mediated immune responses. The NOTCH4 protein in particular has been implicated in endothelial cell regulation and vascular inflammation (109).

7.5 Analyses of inflammatory and endothelial markers

Plasma levels of sTNF-R1, OPG, IL-1 receptor antagonist (IL-1Ra) and IL-6 were measured using enzyme immunoassays (EIA) obtained from R&D systems (Minneapolis, MN). Plasma sCD40 ligand (sCD40L) was analyzed using EIA obtained from Bender Medsystem (Vienna Austria), whereas high sensitivity CRP (hsCRP) and vWf were measured with EIA using antibodies from DakoCytomation (Oslo, Norway). vWf levels are given as plasma concentration percent (%), where the standard curve is based on samples from healthy individuals and normal range is set to 70-130 %. Intra-and interassay coefficients of variance were <10 % for all assays.

7.6 mRNA analyses

Total RNA was isolated from whole blood using the Tempus 12-Port Isolation kit (Applied Biosystems; Ambion, Austin, TX, USA) and quantified using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For further description of the procedure regarding the quantification of mRNA and isolation of cellular subpopulations for *NOTCH4* mRNA expression analysis see supplementary data published in study II.

7.7 DNA genotyping

19 SNPs in and around the *NOTCH4* gene were genotyped using Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix Inc. Santa Clara, CA.). Standard GWAS quality control procedures were followed. One additional SNP (rs3131296) which in other studies had been found associated with schizophrenia and bipolar disorder (25,110) was imputed by IMPUTE2 (111) using 1000 genomes Utah residents with ancestry from northern and western Europe as reference.

7.8 CVD risk factors

Fasting plasma levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG) and glucose were measured at Department of Clinical Chemistry, Oslo University Hospital, using an Integra 800 instrument from Roche Diagnostics, according to standard methods. Blood pressure was measured manually. Weight and height were measured with standard methods under equal conditions and BMI (kg/m²) calculated.

Cut off values for CVD risk factors were defined according to the guidelines of the American Heart Association and the American Diabetes Association (112): TC \geq 5.2 mmol/L, HDL-C <1 mmol/L (male) <1.3 mmol/L (female), fasting TG \geq 1.7 mmol/L, fasting plasma glucose \geq 5.6 mmol/L, overweight: BMI \geq 25 kg/m², systolic BP \geq 130 mmHg, diastolic BP \geq 85 mmHg.

7.9 MRI

All participants underwent MRI scanning on a 1.5 T Siemens Magnetom Sonata scanner (Siemens Medical Solutions, Erlangen, Germany) equipped with a standard head coil. After a conventional 3-plane localizer, two sagittal T1-weighted magnetization prepared rapid gradient echo (MPRAGE) volumes were acquired with the Siemens tfl3d1_ns pulse sequence (TE = 3.93 ms, TR = 2730 ms, TI = 1000 ms, flip angle = 7°; FOV = 24 cm, voxel size = 1.33 x 0.94 x 1 mm³, number of partitions = 160) and subsequently averaged together, after rigid-body registration, to increase the signal to noise ratio.

7.10 Statistics

The SPSS software package for Windows, version 16 or 18 (SPSS, Chicago, IL) was used for comparing groups and variables (Chi-Square test and Kruskal-Wallis test with Mann-Whitney U-test for post hoc), analyses of association (bivariate correlation analyses and linear regression analyses), analyses of variance (ANOVA and ANCOVA, with Tukey test for post hoc). Data normality was assessed using the Kolomogorov-Smirnov test and all skewed data was log-transformed when necessary.

In study II where we tested 20 SNP's for association and potential diagnoses interaction with NOTCH4 levels we applied the allelic test in PLINK (113).

In study II, the level of significance was set to $p < 0.05$. In study I and III the level of significance was set according to the number of tests performed using the Bonferroni method for correction.

7.11 Ethics

The study was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate, whereas the biobank is approved by the Norwegian Directorate of Health. All participants gave a written informed consent. They were thoroughly informed that they could withdraw from the project at any time without restrictions. If patients allowed, a written report summarizing the results from the interviews, the physical check-up, neurocognitive assessments and the MRI scans were presented to the participants, as well as to the clinician in charge of the patient. In cases where physical abnormalities were found either among patients or healthy controls, the TOP study group ensured that adequate measures were taken for further clinical assessments and treatment.

8. Results

Study I

The purpose of this study was to investigate the *NOTCH4* gene expression in bipolar disorder and schizophrenia compared to healthy controls and to identify putative expression quantitative trait loci in and around the *NOTCH4* gene.

We measured and compared *NOTCH4* mRNA in whole blood in 690 subjects (patients: n=479 and healthy controls: n= 211) and adjusted for a range of confounders. Furthermore, we genotyped 20 SNPs and investigated possible associations between expression quantitative trait loci and *NOTCH4* expression.

We found a strong association between *NOTCH4* expression and bipolar disorder, also after adjustments for a range of confounders and multiple testing ($p < 0.0001$). In addition, seven single nucleotide polymorphisms within the *NOTCH4* gene region were associated with enhanced *NOTCH4* mRNA levels (adjusted $p = 0.05 - 1.4 \times 10^{-6}$). Three of these expression quantitative trait loci were independent (not linkage disequilibrium).

The results from study I indicate that the association between *NOTCH4* DNA markers and bipolar disorder is related to altered function at the mRNA level. This supports that the *NOTCH4* pathway is involved in the pathophysiology of bipolar disorder.

Study II

In the second study, we investigated whether there is a relationship between CVD risk factors and inflammation in schizophrenia or bipolar disorder, and if second generation antipsychotics (SGA) interact.

We included 361 patients in a naturalistic cross-sectional study, 235 subjects on current SGA treatment and 126 subjects not treated with SGA as controls. Cardiovascular parameters were measured and current medication recorded. Fasting plasma levels of cytokines: hsCRP, sTNF-R1, OPG, sCD40L, IL-1Ra, vWf and IL-6 were measured.

In this relatively young sample of patients with a mean age of 33.3 years, the following CVD risk factors were associated with elevated inflammation markers after adjusting for confounders: BMI, triglycerides and glucose with hsCRP ($p=0.041-0.001$), HDL-cholesterol and triglycerides with sTNF-R1 ($p=0.009-0.001$) and triglycerides with vWf ($p=0.004$). In patients treated with SGA, elevated hsCRP was significantly associated with high BMI ($p=0.012$), and with high glucose levels ($p=0.003$).

Several CVD risk factors are associated with elevated levels of inflammation markers in young patients with severe mental illness. The interaction between SGA and CVD risk factors on hsCRP levels might indicate a specific inflammatory activation related to SGA induced overweight and hyperglycemia. This suggests that hsCRP could be a valuable marker for future cardiovascular events, particularly in patients treated with SGA.

Study III

Increased inflammation, endothelial cell activation, and structural brain abnormalities have been reported in both schizophrenia and bipolar disorder, but the relationships between these factors are unknown. In study III, we aimed to identify associations between markers of inflammatory and endothelial activation and structural brain variation in psychotic disorders within the schizophrenia and affective spectra.

We measured vWf as a marker of endothelial cell activation and six inflammatory markers (TNF-R1, OPG, IL-1Ra, IL-6, hsCRP, CD 40L) in plasma and 16 brain structures obtained from MRI scans of 356 individuals (schizophrenia spectrum; n=121, affective spectrum; n=95, healthy control subjects; n=140). Linear regression analyses were conducted and adjusted for multiple testing and the relationship between the inflammatory and endothelial markers and brain measurements were investigated across groups.

There was a positive association ($p=2.5 \times 10^{-4}$) between plasma levels of vWf and total volume of basal ganglia which remained significant after correction for multiple testing.

Schizophrenia spectrum diagnosis was independently associated with basal ganglia volume ($p=0.001$) and vWf ($p=0.001$). Treatment with first generation antipsychotics was associated with basal ganglia volume only ($p=0.009$). After adjusting for diagnosis and antipsychotic medication, vWf remained significantly associated with increased basal ganglia volume ($p=0.002$), in particular right globus pallidus ($p=3.7 \times 10^{-4}$). The relationship between vWf and basal ganglia volume was linear (parallel slopes) in all groups, but the intercept was significantly higher in the schizophrenia group ($df=2$, $F=8.2$, $p=3.4 \times 10^{-4}$). There were nominally significant associations between some of the inflammatory markers and ten of the brain structures measured.

The results from study III indicate a positive correlation between vWf levels and basal ganglia volume, in particular globus pallidus, independent of diagnosis. This suggests a link between endothelial cell activation and basal ganglia morphology. Any given level of vWf was associated with larger basal ganglia volume in schizophrenia suggesting a disease-specific association to high levels of vWf.

9. Discussion

9.1 Main results

The three studies in this thesis, all explore different aspect of inflammatory and endothelial activity in schizophrenia and bipolar disorder. By using a translational approach and applying a wide range of methods on a large and well described patient sample, the three studies shed light on this potential relationship from different angles.

In the following, we will discuss the novel findings derived from each of the three studies in a clinical perspective and in light of the existing literature. The potential biophysiological mechanisms underlying these findings are discussed in the three papers and will thus not be the main focus here. Furthermore, we will discuss some limitations and aspects regarding the implications related to each of the three studies in more depth and suggest directions for future research.

9.1.1 Inflammation and endothelial dysfunction at the DNA and mRNA level

Our first study suggests a role of *NOTCH4* in bipolar disorder pathophysiology and indicates that mechanisms related to neurodevelopment, endothelium and inflammation could be involved.

Association signals in immune related genes have been one of the most promising finding from the recent large whole genome studies in schizophrenia, especially the *NOTCH4* gene, which is situated within the MHC complex (81). Quite recently another study managed to

replicate a weak association with the *NOTCH4* gene in a bipolar disorder sample (114). As the association first and foremost had been reported in schizophrenia (25,115-117), we hypothesized initially that *NOTCH4* mRNA levels would be elevated in schizophrenia. Surprisingly, the *NOTCH4* expression was significantly higher in bipolar disorder compared to both healthy control subjects and schizophrenia. Quite recently, a microarray study reported that inflammation up-regulated *NOTCH4* expression in healthy controls but not in schizophrenia (118).

We did not find any interaction effect between SNPs within the *NOTCH4* gene and bipolar disorder diagnosis. There are many possible explanations for this. First, the SNPs that we genotyped may not be functional and we do not know for certain which (if any) role these particular SNPs play in the process towards the NOTCH4 protein. It is, however, encouraging that the SNPs that we identified to be associated with *NOTCH4* expression levels were in close proximity with each other. Second, there might be several environmental factors (e.g. substance abuse, toxins, drugs) affecting *NOTCH4* expression that we have not taken into account. Third, there might very well be epigenetic factors (e.g. methylation) that regulate *NOTCH4* expression levels. Fourth, the MHC region harbor many immune related genes involved in a range of diseases, in particular autoimmune diseases such as multiple sclerosis, celiac disease and diabetes type I. It is very likely that there is a high degree of epistasis in this region (119). Genes within the same region might have an inter-playing regulatory effect and there might be interactions between MHC genes and genes located at other sites that contribute to *NOTCH4* expression in particular and disease susceptibility in general. Lastly, there might be unknown non-coding microRNA affecting the *NOTCH4* expression in bipolar disorder.

The increased *NOTCH4* expression was seen in T-lymphocytes and we found no indication of increased expression in other blood cells. In line with other studies implicating increased T-cell activity in bipolar disorder(120) our results suggest a specific T-cell mediated immune response in bipolar disorder. In contrast to another study (121), we did not find any variation according to symptom levels. A possible explanation for this might be that the patients included in this study were quite stable in terms of affective symptom load. A longitudinal study investigating *NOTCH4* expression levels in different phases of the disease might have provided different results.

As previously mentioned, we had little background literature for guidance when determining which confounders to take into account in the first study. One of the confounders that correlated inversely with *NOTCH4* expression in the exploratory analyses was age, i.e. younger patients had higher *NOTCH4* mRNA levels. We know little about of how age is affecting NOTCH4 activity, but in light of studies reporting a link between an early debut and a more serious course (122) and the evidence implicating bipolar disorder to be a neurodevelopmental disease one might speculate whether NOTCH4 activity is more evident in the early course of the disease and perhaps measuring NOTCH4 could serve help identifying sub-groups of patients in prodromal or early phases. Note again, however, that these questions cannot be answered in a case-control study, but has to be elucidated further in longitudinal studies. Two other interesting aspects regarding confounders are the positive correlations between *NOTCH4* expression levels and lamotrigine and alcohol respectively. We might assume that lamotrigine which is a prescribed medication for treating affective symptoms and alcohol which is often used as self medication in adjusting mood variations (123) to some degree reflect a certain degree of disease severity. On the other hand, these

associations might merely reflect that the biochemical components in lamotrigine and alcohol modify NOTCH4 metabolism.

The NOTCH signaling pathways have been implicated in several other diseases such as cancer (124,125) and lately several studies have reported NOTCH signaling to play a central role in CVD as well (126,127). Cardiovascular health issues have been paid less attention in bipolar disorder compared to schizophrenia, but studies have shown that the prevalence of metabolic syndrome is increased (128) and cardiovascular mortality is nearly doubled in bipolar disorder (129) compared to a normal population. The inverse correlation between BMI and *NOTCH4* expression levels indirectly argue against a link between CVD and NOTCH4 activity in bipolar disorder. Nevertheless, as we were not able to provide fasting plasma samples of blood lipids and glucose in the healthy control group our study did not elucidate this aspect. Another interesting link is the strong implication of increased NOTCH4 activity in certain types of breast cancer (125). Although there are few conclusive studies evaluating cancer risk in patients with bipolar disorder and most of them report no increased cancer risk, one study reports a weak association between bipolar disorder and enhanced risk for breast cancer in women (130).

9.1.2 The relationship between CVD and inflammation in patients treated with second generation antipsychotics

In the second study we show that several inflammatory markers were associated with CVD risk factors in a relatively young sample of patients and that overweight and hyperglycemia were associated with elevated hsCRP levels in patients treated with second generation antipsychotics (131). This could either suggest that SGA modulate the relationship between

CVD risk factors and inflammation or that SGA induced CVD side effects are mediated through mechanisms involving the immune system. In the years prior to this thesis, a large amount of studies reporting serious metabolic adverse effects of second generation antipsychotics appeared (132-135). Around the same time, several studies regarding other patient groups proposed a link between inflammation and CVD (94,136). In 2009, the first report combining these two lines of new knowledge was published (17) and provided some support to our postulation that inflammatory mechanisms could be involved in the metabolic side effects associated with second generation antipsychotics. Meyer and colleagues investigated markers of inflammation (CRP, ICAM-1, VCAM-1 and E-selectin) at baseline and after three months of treatment with olanzapine, pherphenazine, risperidone and ziprasidone. The authors concluded that metabolic side effects induced by treatment were accompanied with elevated levels of CRP. In 2011, Kim et al. published results indicating that insulin resistance was the strongest predictor of CRP levels in a cohort of 64 schizophrenia patients treated with SGA (137). In line with our own results, Kim et al. found no difference between the different types of SGA with respect to CRP levels, while Meyer et al. found that olanzapine treatment accounted for the greatest increase in CRP. Together with these previous reports in the literature, our findings support the hypothesis that SGA treatment may induce cardiovascular risk factors by activating inflammatory processes.

Bearing in mind that our study is a case-control study, it is important to consider the possibility that the relationship between cardiovascular risk factors and inflammation might represent other aspects related to schizophrenia and bipolar disorder than SGA treatment. Impaired glucose metabolism and high BMI (138,139), as well as increased inflammatory activity have been reported in drug-naïve schizophrenia patients (140). Another possibility might be that stressful events activate the hypothalamic-pituitary-adrenal axis (HPA) in

inducing cortisol secretion which eventually affects glucose and fat metabolism. In 2012, one study reported an association between childhood maltreatment, higher BMI and elevated CRP levels in a sample of first-episode psychosis patients. The authors suggested that stressing events in the childhood may lead to increased inflammations which in turn contribute to higher BMI (141). One could speculate whether patients with a particular vulnerability (e.g. childhood trauma) are also the same patients who experience the most severe symptom load and are thus more likely to be treated with medication later in life.

On the other hand if stress and disease severity was in fact the main explanation for the relationship between CVD and inflammation, one would expect that symptom scores (measured with PANSS, YMRS and IDS) and/or duration of illness would affect our results. Indeed, SGA treated patients in our sample did display significantly higher PANSS scores compared to the non-treated group. In contrast and somewhat surprisingly, duration of illness was in fact longer in the non-treated group. In either case, neither symptom scores nor duration of illness affected the analyses exploring the relationship between inflammatory markers and CVD risk factors. It is, however, difficult to interpret the relationship between disease severity and treatment in a naturalistic study such as this one.

The evidence linking CVD, particularly atherosclerotic disease, and inflammation is emerging across different patient groups (136,142,143). Our study implicating SGA as an additional mediator promoting this relationship might shed light on why this patient group is so vulnerable in terms of excess cardiovascular morbidity and mortality. It is, however, important to bear in mind that not all patients who are treated with SGA develop metabolic syndrome or CVD. In fact, the prevalence of pathologically increased CVD risk factors in our sample was not different in the SGA groups compared with the non SGA group. The interactions analyses performed in the second study showed that among those with elevated

BMI and elevated glucose level, the hsCRP levels were significantly higher in the SGA group. Note, however, that the variance in hsCRP level is high (Paper II, Table 3.).

Some of this potential vulnerability might be due to genetic factors influencing cytokine production. In fact some argue that individuals can be stratified as high and low-inflammatory responders according to their genetic makeup (144). Interestingly, recent results from our own group have shown that there is considerable genetic pleiotropy implicating a link between schizophrenia and CVD risk factors (145). Several loci, including SNPs within immune related genes on chromosome 6, were associated with both schizophrenia and CVD phenotypes. This might indicate that there is a sub-group within the group treated with SGA that is particularly vulnerable in terms of developing atherosclerotic disease.

9.1.3 The relationship between inflammation and endothelial dysfunction and brain morphology

The main finding of the third study was that vWf concentration was associated increased basal ganglia volume. Some studies have suggested that loss of brain tissue in most structures and larger basal ganglia in schizophrenia are due to treatment with first generation antipsychotics (146,147). While others have reported that these anatomical abnormalities are also present in first-episode and drug-naïve patients (39,147), leaving the overall conclusions regarding the effect of antipsychotics on brain tissue inconclusive. The inconsistency in these results raises the question whether there are other mechanisms besides those involved in the pharmacological properties of antipsychotics that contribute to the brain morphological alterations seen in schizophrenia and bipolar disorder. Our results, implicating vWf as a

potential contributor in the pathophysiological mechanisms underlying increased basal ganglia volume, suggest that endothelial dysfunction and possibly inflammation might be involved.

Although our third study is to the best of our knowledge the first study to explore the relationship between vWf and brain structure measurements, there are several other studies that have demonstrated a link between inflammation and brain morphology. Taki et al. found that increased levels of CRP was associated with smaller grey matter volume in a sample of healthy elderly subjects and they proposed that a mild state of inflammation possibly related to atherosclerotic processes might lead to neuronal loss and volume reductions (148). Another group found IL-6, TNF- α and osteoprotegerin to be associated with smaller total cranial brain volume in a cohort of healthy individuals (149). Finally, members of our own group found an association between a single nucleotide polymorphism within the MHC complex and enlarged ventricles (150).

As our pre-study hypothesis was rather broad in the sense that we postulated a relationship between inflammation and endothelial dysfunction in general and brain morphological changes, we chose a rather conservative method of correction for false positive results. In light of the results from others outlined above, perhaps we should have investigated the nominally significant associations in more depth. As recent results have shown progressive brain changes related to general grey matter volume loss and increased CSF in the frontal lobe in children and adolescents with early onset schizophrenia (151), one might argue in retrospect that the association between CRP levels indicating a general state of inflammation and cortical substructure volumes should have been explored further in our study.

Although several lines of evidence suggest that peripheral inflammatory activity reflect the environment in the central nervous system (63), an important question is the validity of

plasma measurements in relation to brain imaging studies. Accepting that we don't have access to live brain tissue, we recognize that second next best approach would have been investigating CSF samples in relation to structural brain abnormalities. The procedures accompanying CSF samples are more invasive for the patient than plasma samples and require more resources which naturally limit the prospects of large sample sizes. Ideally, any results regarding relationships between processes in the central nervous system and peripheral inflammatory activity should be replicated in smaller samples of CSF.

Another issue in study III that needs further elaboration is the measurements of vWf levels in the different diagnostic groups. In contrast to another study from our group (77) on a slightly different, but yet overlapping sample, vWF were not significantly higher in bipolar disorder compared to healthy controls. In Hope et al.'s study mean vWf level was higher in the bipolar group (102 vs. 95.6) and lower in the schizophrenia group (109 vs. 113.7) compared to our results. The reason for this discrepancy might be due to the fact that the current study (study III) included patients with the diagnosis depressive psychosis (mean vWf: 82.6) in the "affective spectrum" group and psychosis not otherwise specified (mean vWf: 118.1) in the "schizophrenia spectrum" group. In line with this explanation, we have previously found in another study that vWf levels were increased in mania and decreased in depressive states (152).

9.1.4 Endothelial related inflammation - a missing link?

To summarize, the results from all our three studies indicate that endothelial dysfunction and inflammation might be involved in schizophrenia and bipolar disorder pathophysiology. Of the eight biological markers we investigated, NOTCH4 and vWf are both well known to be

directly involved in both inflammatory processes and endothelial dysfunction. The major part of circulating CRP in the plasma derives from the liver, but recently it has become increasingly clear that CRP is also produced in endothelial cells and contributes to endothelial dysfunction and the formation of atherosclerotic plaque (102,153).

As previously mentioned, there has been a shift from looking at schizophrenia and bipolar disorder as neurodegenerative to neurodevelopmental disorder. Our results do not support the one view or the other. However, if we assume that schizophrenia and bipolar disorder are neurodevelopmental disorders and allow ourselves to speculate some, there might be a dysfunctional interplay between the vascular system and the nervous system during embryogenesis mediated by the immune system.

We know that prenatal exposure to maternal infection alters cytokine expression in the placenta, amniotic fluid and fetal brain (154). In line with the neurodevelopmental hypothesis, it has been suggested that exposure to increased inflammation in critical stages of neural development enhances the risk of developing schizophrenia (155). On the other hand, all our three studies raise the possibility that increased inflammation in severe mental disorders might also be influenced by exogenous factors that often follow the course of the disease at a later stage. Although the individual genetic makeup is set, there are a range of environmental factors that could influence the individual genetic expression, the various physiological systems and ultimately the clinical outcome.

9.2 Strengths and limitations

A major advantage in this project (Study I-III) is the translational approach. By using information from gene and gene expression analyses, plasma measurements, brain imaging

data and clinical characteristics this study has explored several potential biological pathways involving inflammation and endothelial dysfunction. As schizophrenia and bipolar disorder are heterogeneous diseases where a combination of biological and environmental factors are in reciprocal interplay with each other, using multi-disciplinary methods provide new insight into the complexity of these diseases. On the other hand, this approach limits the ability to explore each aspect in depth and thus calls for further follow-up studies.

An overall limitation for the study as a whole was its cross-sectional, naturalistic design which offers no certain explanation concerning causality. As mentioned several times, we can not rule out that the associations presented are influenced by confounding factors not taken into account. On the other hand, the study sample is large and very well described, both in terms of psychometric- and somatic variables, which allowed us to control for a range of confounders. In addition, the mean age in the sample is relatively young, the gender equation was fairly equal and the mean duration of illness rather short.

Bearing in mind our previous discussion regarding the potential overlap of clinical symptoms in mental disorders, large samples in psychiatry research are often heterogeneous and it is difficult to ensure a high reliability. In the TOP study protocol, the clinicians that included the patients were trained extensively and calibrated against each other. Thus, there was a high overall agreement for diagnostic evaluations and the inter rater reliability for symptom scores was high.

In study II, the background literature on cardiovascular risk factors and inflammation was available and allowed us to develop predefined hypotheses in beforehand. All the seven inflammatory markers that were analyzed had been shown to be associated with CVD in previous studies. Nevertheless, we performed many analyses without correcting for multiple testing which no doubt could have led to type I errors (false positive results). In studies I and

III, the available background literature was scarce thus limiting our ability to choose confounders on an evidence basis. Using an exploratory approach in searching for potential confounders necessitates strict corrections for multiple testing. In study I and III, Bonferroni correction was used, which is considered to be rather conservative and might have led to type II errors (false negative results).

There are further limitations concerning each study that also need to be addressed in detail. As regards to the first study, collection of whole blood using Tempus tubes is widely used in large-scale eQTL studies. However, peripheral blood contains a variety of cell types and targets of interest could potentially be influenced by the different contributing cell types and/or proportion of these. Although this variability may confound the findings in our study, there are several points that argue against it. First, since patients with ongoing infections, autoimmune disorders and infectious diseases were excluded the increased *NOTCH4* mRNA levels were most likely not explained by differences in the relative proportions of cell types due to inflammatory responses. Further, if such cell type changes were present, they would probably induce noise, and not be systematically biased towards one diagnostic group. Second, although the quality of gene expression profiling is limited by the lack of live tissue biopsies from the affected area in question, the trend towards an association between the eQTLs near the *NOTCH4* gene and *NOTCH4* mRNA levels in bipolar disorder patients could reflect a uniform increase also in other relevant tissues such as the brain.

In the second and third study (paper II and III) where we investigated the impact of antipsychotics on the relationship between inflammation and CVD and inflammation and brain morphology respectively, the limitations of the cross sectional design warrant particular caution in interpretation of the results. A double blind randomized design is no doubt the ideal approach when investigating the effect of treatment. As antipsychotics are effective

treatments for psychotic disorders, investigating these relationships in a randomized trial comparing antipsychotics with placebo would be ethically difficult. In order to minimize the potential “noise” of life-style factors such as smoking, lack of exercise and poor diet which obviously could have affected the results, we chose to use un-medicated patients as controls instead of healthy comparison subjects in study II. In study III, we used healthy controls as comparison subjects, but investigated the potential impact and adjusted for a range of confounders.

9.3 Future directions

9.3.1 Clinical implications

As previously outlined, it is important to identify patients with increased risk of developing psychosis and intervene as early as possible in order to minimize disease morbidity. One of the means of intervention is second generation antipsychotics with an accompanied high risk of metabolic side effects, thus it is equally important to identify those who are at risk of developing metabolic syndrome at an early stage. Our evidence points to hsCRP as an important marker to be considered when monitoring second generation antipsychotic treatment and its effect on cardiovascular health and diabetes risk, especially in young patients. As CVD risk increases continuously beginning at $CRP \geq 1$ mg/L (17), our results suggest that measuring hsCRP in SGA treated patients may be beneficial in identifying those at risk of developing metabolic syndrome.

Results from several randomized trials have indicated that non-steroid anti-inflammatory drugs (NSAIDs) as adjuncts to antipsychotic treatment might have a positive effect on positive and negative symptom load in schizophrenia (156-159). It is tempting to hypothesize

that such an augmentation might also have an additional effect in terms of reducing the risk for CVD. It is, however, important to note that the maximum follow-up time in these studies is three months (158) which is far from enough time to evaluate the long term effect of NSAIDs both in terms of potential beneficial effects and side effects. Furthermore, these results support that combining antipsychotics with anti-inflammatory treatment could decrease the incidence of metabolic syndrome (160).

The results from study II could prove to have significant clinical impact as the development of NOTCH4 targeted inhibition therapies has received much attention in other patient groups particularly in cancer treatment and atherosclerosis (127,161). Our results implicate that patients with bipolar disorder display enhanced NOTCH4 activation which open up for studies of new treatment options also in this patient group.

Taken together with recent results reporting morphological changes in drug-naïve children with early onset schizophrenia, our results from study III indicating a relationship between endothelial dysfunction and brain morphology is of clinical concern. Elevated levels of vWf increases the risk of thrombosis and ischemic stroke (162), thus monitoring vWf levels in patients with schizophrenia at an early stage might serve as a potential marker for identifying those at risk of developing thrombotic vascular disease.

9.3.2 Scientific implications

These three studies have strengthened the body of evidence implicating inflammatory mechanisms and endothelial dysfunction to be involved in the underlying pathophysiological mechanisms in bipolar disorder and schizophrenia. Major implications are that NOTCH4 could be potential biomarker for bipolar disorder, CRP could be a potential predictive marker

of CVD in schizophrenia and bipolar disorder and finally vWf could be a potential contributor to the pathologically increased basal ganglia volume in schizophrenia. At this point, it is important to recognize that further research is needed in order to confirm these findings and most importantly transform this new knowledge into clinically useful tools and new potential targets for medical intervention.

The recent evidence implicating NOTCH4 in atherosclerotic disease (126), calls for investigations exploring the relationship between CVD and NOTCH4 in bipolar disorder. In the extension of this new knowledge, another interesting future project would be investigating the effect of NOTCH4 inhibitors in a bipolar disorder sample using a randomized double blind design with psychiatric and cardiovascular outcomes. Finally, the putative relationship between *NOTCH4* (both on DNA and RNA level) and brain abnormalities should be explored.

The results from study II which implicate inflammatory mechanisms in SGA induced cardiovascular risk, call for longitudinal studies exploring CRP as potential marker for identifying those at risk for developing metabolic syndrome and CVD. As mentioned, a few studies have investigated the effect of COX-2-inhibitors and NSAIDs on clinical psychiatric symptom, but with short follow-up time. Future studies should have considerably longer follow-up time and explore the effect of mild anti-inflammatory medication as an adjunct to antipsychotic treatment with both psychometric and cardiovascular risk factors as outcome variables.

The results from study III, implicate endothelial and inflammatory mechanisms underlying morphological changes in schizophrenia and bipolar disorder, but an extensive amount of research is needed in order to confirm these results. Exploring the relationship with CSF

samples and targeted microarray studies exploring vWf expression in the basal ganglia would shed further light on this issue.

9.4 Conclusion

Schizophrenia and bipolar disorder are complex and heterogeneous disorders and there are probably many pathophysiological disease mechanisms involved. The high heritability rate implies genetic mechanisms, but the body of evidence gathered from several GWAS in the recent years indicates that several genes are involved each with a rather small odds ratio. We and others have implicated several different peripheral biological mechanisms, clinical studies have proved that both disorders present with a variety of clinical symptoms and finally epidemiological studies have pointed towards a wide range of environmental factors.

Another complicating matter is the question as to whether schizophrenia and bipolar disorder are two different diagnostic entities or represent clusters of symptoms along the same dimensional line.

This project has provided novel findings implicating inflammation and endothelial dysfunction to play a part in the pathophysiological mechanisms underlying schizophrenia and bipolar disorder. In particular we have shown:

1. A strong association between *NOTCH4* expression and bipolar disorder, also after adjustments for a range of confounders and multiple testing. This suggests the NOTCH4 pathway to be involved in bipolar disorder pathophysiology
2. Several CVD risk factors are associated with elevated levels of inflammation markers in young patients with severe mental illness. Furthermore, the interaction between SGA and

CVD risk factors on hsCRP levels might indicate a specific inflammatory activation related to SGA induced overweight and hyperglycemia. This suggests that hsCRP could be a valuable marker for future cardiovascular events, particularly in patients treated with SGA.

3. A strong positive correlation between vWf levels and basal ganglia volume, in particular globus pallidus, which was independent of diagnosis. This suggests a link between endothelial dysfunction and basal ganglia morphology. Furthermore we found that any given level of vWf was associated with larger basal ganglia volume in schizophrenia compared to bipolar disorder and healthy controls. Taken together with previous findings of increased vWf levels in schizophrenia, our results suggest that high levels of vWf might account for the enlarged basal ganglia volume specific for this disease.

4. Finally, the results presented in study I and III in this project indicate that there might be different molecular mechanisms related to inflammation and endothelial dysfunction involved which are disease specific for bipolar disorder and schizophrenia respectively.

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