# AUTOIMMUNITY IN SCHIZOPHRENIA: CULPRIT OR BYSTANDER?

HANS C. VAN MIERLO

UMC Utrecht Brain Center

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**Hans Christian van Mierlo**

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# Autoimmunity in schizophrenia: culprit or bystander?

Auto-immuniteit in schizofrenie:
dader of omstander?
(met een samenvatting in het Nederlands)

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**Hans Christian van Mierlo**

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te Sittard

**Promotor:**
Prof. dr. R.S. Kahn

**Copromotor:**
Dr. L.D. de Witte

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# Chapter 1

## General introduction

In this introduction a brief overview of the characteristics of schizophrenia and the functioning of the immune system in health and disease is given. Followed by a summary of the current literature on the link between the immune system and schizophrenia. Finally, the aim and outline of this thesis is discussed.

## 1.1 Schizophrenia

Schizophrenia is a chronic psychiatric disorder characterized by recurrent or persisting psychotic episodes, alongside motivational and cognitive symptoms. It is a heterogeneous disorder, as there are large variations between patients in severity of symptomatology and outcome. It is estimated that the lifelong prevalence of schizophrenia is around 1%, although there are geographical differences in prevalence rates.[1,2]

The onset of the disorder is thought to occur during the so-called prodromal phase in early adolescence, or possibly earlier, marked by social withdrawal and cognitive decline. More pronounced symptoms, such as a first psychotic episode, often debut during late adolescence or early adulthood.[1,2] Effective treatment is available for psychotic symptoms in the form of antipsychotic medication, which partially block dopaminergic neurotransmission. However, these medications have various potential side effects, such as weight gain and extrapyramidal symptoms and are not effective for all patients with psychotic symptoms. Unfortunately, interventions for the motivational and cognitive dysfunctions that occur in patients with schizophrenia, are still very limited.[3,4]

Despite large efforts from various research groups, the exact pathogenesis of schizophrenia remains unknown. Presumably, the disease is caused by interplay between genetic, developmental and environmental factors. Twin studies have found that the estimated heritability of schizophrenia is approximately 80-85%.[5] Recent genome wide association studies have identified over a hundred single nucleotide polymorphisms that each result in a small increase in the risk of developing schizophrenia.[6] In addition to this, several environmental factors have been identified that also result in a small

increase the risk of developing schizophrenia. These, amongst others include: obstetric and perinatal complication, migration, living in an urban region, being born in winter or using cannabis.[7]

## 1.2 A search for "the schizophrenia virus"

It has long been suggested that the immune system is involved in the pathogenesis of schizophrenia. The German psychiatrist Kreaplin already wrote about the link between fever and psychiatric symptoms in 1881[8] and in 1926 the American psychiatrist Menninger described a cohort of patients with psychotic symptoms and suggested that these symptoms might be related to a recent influenza-epidemic.[9] In the following years hundreds of studies have been performed in search of a pathogen that might be the cause of schizophrenia.[10–12] Although this pathogen has not been found to date, these studies did give rise to several new hypotheses on the potential link between the immune system and schizophrenia.

## 1.3 Immune system in health

The aim of the immune system is to protect the body against infectious pathogens such as parasites, fungi, viruses and bacteria. In addition to this, it is also involved in recognizing and clearing damaged or altered cells and tissue.

A further subdivision of the immune system is made into the innate and adaptive (or acquired) immune system. The innate immune system is capable of setting up a quick and first response after contact with a potential pathogen. In response to contact with this potential pathogen the innate immune system will also activate the adaptive immune system. The adaptive immune system will then set up a specific immune response tailored for the potential pathogen. Due to its immunological memory, the adaptive immune system is capable of setting up a quick and specific immune response when contact with the same pathogen occurs at a later time-point.

These two systems collaborate intensively in order to provide an adequate immune response. Communication within or between these systems occurs via cell-cell contact or production of signaling proteins, so called cytokines.[13,14]

## 1.4 Neuro-immunology

Cells of the immune system are also found within the brain. Microglia reside within the brain parenchyma and macrophages can be found around blood vessels. Apart from these cells, monocytes and lymphocytes will also migrate from the blood towards the parenchyma during an inflammatory response. Once present within the parenchyma, monocytes will differentiate into macrophages.

Microglia share several similarities with macrophages, but already migrate towards the brain during prenatal development. These cells are involved in recognizing potential pathogens and initiating an immune response within the brain. During the last decade it has become clear that they also play a key-role in brain maintenance, for example by removing non-functional synapses.[15]

## 1.5 Immune system in disease

Recognizing potential pathogens or transformed cells and initiating an adequate immune response are strictly regulated complex processes. During various steps within these processes problems can occur. In patients with an immunodeficiency, which can be innate or acquired, the immune system is not capable of initiating an adequate immune response. There are also disorders in which the immune system is erroneously activated or initiates an excessive immune response. These include allergies in which the immune system falsely recognizes harmless environmental factors as a potential pathogen. But also autoimmune diseases in which the adaptive immune system, after being activated by the innate immune system, initiates a chronic immune response directed against auto-antigens, this immune response results in tissue inflammation and cell damage.[13,14,16] In order for a disease to be classified

as an auto-immune disease the German-American immunologist Witebsky suggested a number of criteria in 1957,[17] which have been modified in 1993[18] and now state as following:

- Presence of disease-specific antibodies or autoreactive T-lymphocytes in affected tissue
- Ability to reproduce the disease in an animal model by transferring antibodies or autoreactive T-lymphocytes
- Circumstantial clinical evidence such as a favorable response to immunosuppression
- Family or personal history of autoimmune disorders or association with certain MHC variants

## 1.6 The immune system and schizophrenia

*1.6.1 Clinical clues*

One of the clinical characteristics of autoimmune diseases is their relapsing and remitting course. Such a fluctuating course, especially of psychotic symptomatology, is also present in some patients with schizophrenia.[19] In addition to this, it has been suggested that antipsychotic drugs might have some immunosuppressive effects, especially clozapine. The exact mechanism behind the immune alterations found in patients treated with clozapine is unfortunately still unknown.[20]

*1.6.2 Epidemiology*

Epidemiological studies have found evidence in support of a link between schizophrenia and the immune system.[21] Multiple studies have found an association between prenatal maternal infections with different viruses (including various herpes viruses), bacteria and the parasite Toxoplasma gondii and a two to five fold increase in the risk of developing schizophrenia. In addition to this, a maternal pro-inflammatory cytokine profile has also been associated with an increased risk of developing schizophrenia.[22] A slight increase in the prevalence of schizophrenia is found in children born in winter or spring; this could also be related to the higher occurrence of infections during the winter season.[23,24]

Population-based studies performed in Denmark and Taiwan found that patients with schizophrenia have an elevated risk of autoimmune and atopic disorders.[25–27] Interestingly, several studies have found a negative association between rheumatoid arthritis and schizophrenia.[28]

Lastly, it has been shown that patients with schizophrenia more often have a history of hospitalization due to an infectious disorder[29] and infections during childhood are associated with a nearly two-fold increased risk of developing a non-affective psychotic disorder.[30]

### *1.6.3 Genetic findings*

As mentioned, the most recent genome wide association studies on schizophrenia, which included more than 35.000 patients, have found over a hundred genome-wide significant loci. These findings suggest that variations in numerous genes alter the risk of developing schizophrenia. Several of the identified loci are located near genes that play an important in the immune system. The most significant loci are located within the major histocompatibility complex (MHC), a region on chromosome 6 that contains the genetic code for dozens of immune-related genes.[6,31] It has recently been discovered that the strongest genetic association within this region partly arises from variations within the C4A gene. C4 is a part of the complement system, which plays an important role in coordinating the immune response of the adaptive immune system. However, this protein also seems to be involved in brain development, by adjusting the neuronal network through synaptic pruning.[32]

Another genetic finding suggestive of a link between the immune system and schizophrenia includes the high rate of immune system abnormalities such as thymic hypoplasia and impaired t-cell development in patients with 22q11.2 Deletion Syndrome. This syndrome has been associated with a 20-25 times increased relative risk of developing schizophrenia.[33,34]

### *1.6.4 Leukocytes*

As infection or inflammation results in alterations in leukocytes, various studies have compared these cells in blood samples of patients with schizophrenia and healthy controls. A meta-analysis on lymphocytes in blood of patients

with schizophrenia found changes in the percentage and absolute number of lymphocytes in schizophrenia. Findings included an increased absolute number of T-lymphocytes in medication-naïve patients with a first episode psychosis, with an increased ratio between T-helper cells (CD4) and cytotoxic T-cells (CD8). This ratio decreases after initiating treatment with antipsychotic medication. Furthermore, an increase in the percentage of CD4 and CD56 positive cells was found in patients with an acute relapse.[35] In addition to this, several small studies suggest that monocytes might have a more pro-inflammatory phenotype in schizophrenia, due to increased production and excretion of cytokines.[36]

There is unfortunately a lack of large studies systematically assessing the different types of leukocytes in cerebrospinal fluid (CSF) of patients with schizophrenia. Two small studies found a slight increase in monocytes, macrophages and lymphocytes in CSF of patients with schizophrenia as compared to healthy controls. The number of monocytes normalized after initiating treatment with antipsychotics.[37,38]

*1.6.5 Cytokines*

As cytokines are key cell signaling proteins that coordinate the immune response, their presence has been extensively studied in blood, CSF and brain tissue of patients with schizophrenia. Cytokine levels can show large variations based on multiple environmental factors, which makes these studies vulnerable to confounding.[39]

A meta-analysis on cytokines in blood found increased levels of IL-1β, IL-6 and TGF-β in patients with a first episode psychosis and acute relapse. These cytokine levels normalized after treatment with antipsychotic medication was initiated. Levels of IL-12, IFNγ, TNFα and soluble IL-2 receptor were increased in patients with a relapse and during treatment with antipsychotic medication.[40]

A similar meta-analysis was conducted on studies measuring cytokine levels in CSF. Results showed an increase in levels of IL-1β, IL-6 and Il-8 and a decrease of soluble IL-2 receptor.[41]

Studies on cytokine levels in post-mortem brain tissue show heterogeneous results, which also differ per brain region.[42] One meta-analysis found an increased level of IL-1β to be the most consistent finding in these studies.[43]

*1.6.6 C-reactive protein*

C-reactive protein (CRP) is an acute phase protein produced by the liver. It is globally used as a general marker of inflammation. Several studies have found a slight increase in the levels of this protein in blood of patients with schizophrenia. CRP levels remain elevated after treatment with antipsychotic drugs and show an association with the severity of positive symptoms, age and BMI.[44]

*1.6.7 Antibodies*

Increased levels of various non-specific autoantibodies have been found in the blood of patients with schizophrenia. However, the clinical relevance of most of these autoantibodies remains uncertain.[45]

An exception to this, are antibodies directed against neuronal cell-surface proteins. These antibodies are capable of having a direct pathogenic effect by disrupting neurotransmission and causing a form of antibody mediated encephalitis.[46] The most well-known example of such a disorder is anti-NMDA receptor encephalitis, in which the patients' immune system produces IgG antibodies against the GluN1 subunit of the NMDA-receptor, leading to disruption of glutamatergic neurotransmission.[47] This results in a disorder characterized by both severe psychiatric and neurological symptoms.[48]

Anti-NMDA receptor encephalitis was described for the first time in 2007 as a paraneoplastic phenomenon in a cohort of woman with an ovarian teratoma,[49] but since then several cases have been reported in both males and females without an associated tumor.[50] The discovery of this disorder has sparked a growing interest in the role of these and other potentially clinically relevant neuronal antibodies in psychiatric disorders.[51] It has also been suggested that patients with anti-NDMA receptor encephalitis might be misdiagnosed with disorders such a schizophrenia and bipolar disorder.[52] Various studies have therefore screened large cohorts of patients with psychotic disorders for the presence of GluN1 and other antibodies against neuronal surface-proteins, with divergent results thus far.[53,54]

Serum, CSF and brain tissue of patients has also been screened for the presence of antibodies against various viruses, bacteria and parasites. Increased prevalence of such antibodies might be of relevance as various pathogens are capable of residing in the nervous system in a latent state and could become reactivated during a later time-point. Multiple studies have found an increased seroprevalence of Herpes simplex virus 2 and the parasite Toxoplasma gondii. However, several factors could have confounded these results.[10]

### *1.6.8 Microglia*

As microglia are the resident immune cells of the brain and seem to play a key role in shaping the neuronal network, there has been a growing interest in examining their functioning in schizophrenia. Thus far, this has mostly been done by conducting positron emission tomography (PET) studies in vivo and by examining post-mortem brain tissue. For PET-studies a tracer binding to the 18kD translocator protein (TSPO) is used. Current evidence suggests that activation of microglia results in an increased expression of this protein, although this assumption is not undisputed. Studies using this technique in schizophrenia show some elevated binding in grey matter, but methodological differences between studies also seem to have a large influence on these findings.[55]

A meta-analysis on post-mortem brain studies found a significantly increased density of microglia, although one important included study has been retracted.[43] Findings on microglia related markers in post-mortem tissue show heterogeneous results, which could be due to differences in analytical techniques used, lack of correction for use of medication, inclusion of various brain regions or of patients in different stages of the disorder.[42]

### *1.6.9 Clinical trials*

Several randomized clinical trials have examined whether drugs with anti-inflammatory properties, including non-steroidal anti-inflammatory drugs (NSAID), acetylsalicylic acid and the antibiotic minocycline have any efficacy in patients with schizophrenia. These trials have found some positive effects for the addition to antipsychotic treatment of N-acetyl cysteine, acetylsalicylic acid and estrogens in schizophrenia. N-acetyl cysteine and estrogens are not primary immunosuppressive drugs, but it has been suggested that they possess at least some anti-inflammatory properties.[56–58] In addition to this, a

small open-label trial including 6 patients using an IL-6 receptor monoclonal antibody (tocilizumab), showed some beneficial effects on cognition.[59] While a larger trial on the same drug, including 36 patients, found no positive effect on any behavior outcome measure.[60]

## 1.7 Why is autoimmunity relevant in schizophrenia?

In the 1930s the German neuropathologist Facius suggested that dementia praecox might be caused by antibodies directed against a part of the brain.[8] This new hypotheses triggered several follow-up studies examining the presence of autoantibodies in what was later called, schizophrenia.[61,62]

Autoimmunity is characterized by defects in the elimination or control over self-reactive lymphocytes.[63] In schizophrenia autoantibodies could be responsible for directly or indirectly altering brain functioning. As mentioned, autoantibodies directed against neuronal cell-surface proteins are capable of directly disturbing neurotransmission which could result in, among other, psychotic symptoms.[64] Activation of B-cells and production of non-specific autoantibodies could result in a more general form of immune system activation, which could also disturb the functioning of the central nervous system as has been suggested in various autoimmune disorders.[65–67]

Indirect evidence in support of this hypothesis include the increased prevalence of autoimmune disorders in schizophrenia,[28] increased levels of various non-specific autoantibodies found in the blood of patients with schizophrenia[45] and the increased expression of genes associated with schizophrenia in B-cells.[6]

## 1.8 Conclusion

In summary several lines of evidence suggest that the immune system shows, at least some, abnormalities in patients with schizophrenia. However, many uncertainties surround these findings, as there are various factors that are capable of influencing the immune system and multiple components of the immune system do not limit themselves to a single function.

The aim of this thesis is to further examine the role of autoimmunity in schizophrenia. This is done by examining: 1) Non-specific peripheral autoantibodies, which could be a marker for autoimmunity 2) Antibodies directed against various neurotropic pathogens with could trigger autoimmunity 3) The functioning of B-cells in schizophrenia 4) Specific antibodies directed against neuronal cell-surface proteins which are capable of having a direct effect on the central nervous system.

## 1.9 Outline of thesis

Antinuclear antibodies are found in various autoimmune disorders. These antibodies have also been associated with schizophrenia. In **chapter 2** the prevalence of antinuclear antibodies is examined in a large cohort of patients with schizophrenia and healthy controls.

In **chapter 3** the additional value of performing routine extensive laboratory research in patients with a first episode psychosis is examined. This routine examination included measuring anti-nuclear and anti-thyroid peroxidase antibodies, C-reactive protein and erythrocyte sedimentation rate (ESR).

As contact with or reactivation of a neurotropic pathogen could trigger an immune response within the central nervous system, exposure to six of such pathogens is examined in in a cohort of patients with schizophrenia and controls in **chapter 4**.

Subsequently, an overview and summary of the current literature on the potential link between B-cells and schizophrenia is given in **chapter 5**. Based on these data we critically appraise whether various B-cells mediated pathological mechanisms identified in autoimmune disorders are likely to play a role in schizophrenia.

In **chapter 6** a description of 3 patients with anti-NMDA receptor encephalitis with an onset with severe psychiatric symptoms is given. In addition to this, the results of routinely screening a naturalistic cohort of patients admitted with a first episode psychosis for anti-NMDA receptor antibodies are discussed.

To provide further insight in the prevalence of anti-NMDA receptor antibodies in patients with schizophrenia, we describe the results of screening 3 research cohorts for these antibodies in **chapter 7**. These cohorts consisted of patients with a first episode psychosis and patients with schizophrenia with a long duration of illness.

In **chapter 8** various molecular techniques are used to screen for novel autoantibodies against multiple candidate antigens in serum of patients with schizophrenia.

In **chapter 9** a summary of the findings described in this thesis is given, followed by a general discussion and recommendations for future studies.

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# Chapter 2

## The prevalence of antinuclear antibodies in patients with schizophrenia spectrum disorders: results from a large cohort study

Hans C. van Mierlo[a], Lot de Witte[a], Ronald H.W.M. Derksen[b], Henny G. Otten[c] and the GROUP investigators

[a] Department of Psychiatry, UMC Utrecht Brain Center, University Medical Center Utrecht, Utrecht, The Netherlands

[b] Department of Rheumatology and Clinical Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

[c] Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

Genetic Risk and Outcome of Psychosis (GROUP) Investigators

Richard Bruggeman[1], Wiepke Cahn[2], Lieuwe de Haan[3], René S. Kahn[2], Carin J. Meijer[3], Inez Myin-Germeys[4], Jim van Os[4], Durk Wiersma[1]

[1] University Medical Center Groningen, Department of Psychiatry, University of Groningen, The Netherlands

[2] University Medical Center Utrecht, Department of Psychiatry, Brain Center Rudolf Magnus, The Netherlands

[3] Academic Medical Center University of Amsterdam, Department of Psychiatry, Amsterdam, The Netherlands

[4] Maastricht University Medical Center, South Limburg Mental Health Research and Teaching Network, EURON, Maastricht, The Netherlands

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## Abstract

An increased prevalence of autoantibodies has been found in patients with schizophrenia, suggesting a role for autoimmunity in schizophrenia pathogenesis. We examined the presence of antinuclear antibodies (ANAs), with further determination of specific antibodies, in 368 patients with a schizophrenia spectrum disorder and 283 healthy controls. No significant difference in prevalence of ANAs between patients (8%) and controls (11%) was found. We did not find an association between ANAs and schizophrenia spectrum disorders. We discuss potential reasons for the discrepancy with some previous studies, such as inclusion of patients using chlorpromazine, which can induce ANAs.

## Introduction

Several lines of evidence suggest that the immune system is involved in the etiopathology of schizophrenia. This theory is supported by the findings of an increased prevalence of autoimmune diseases among patients with schizophrenia[1] and a higher frequency of several autoantibodies in the blood of patients with schizophrenia, as shown by a recent systematic review,[2] including antinuclear antibodies (ANAs). The presence of ANAs is used to support the clinical diagnosis of various autoimmune disorders and can be seen as a marker of autoimmunity.[3]

Ezeoke et al.[2] concluded that ANAs are significantly more prevalent in patients with schizophrenia as compared with controls (22.2% versus 6.7%). The authors, however, mention that there is a marked heterogeneity and inconsistency among included studies and that additional studies are needed that control for potential confounding factors, such as clinical status, age, genetic background and medication.

ANAs are autoantibodies that react with intracellular components[4] and can be further divided into antibodies directed against double-stranded DNA (dsDNA) or specific proteins such as SSA.[5,6] Examples of autoimmune diseases with a high prevalence of ANAs (up to 93%) are systemic lupus erythematosus (SLE), systemic sclerosis, Sjögren's syndrome, mixed connective tissue disease, inflammatory myopathies -and some autoimmune hepatic disorders.[7–9] However, ANAs are also frequently found in the general population with an estimated prevalence ranging from 1 to 20%. The wide range in reported prevalence is caused, among others, by differences in assays used, cutoff values used for a positive result and characteristics of the studied population. The prevalence of ANAs is higher in females than in males and increases with age.[10]

The aim of this study was to examine the prevalence of ANAs among patients with a schizophrenia spectrum disorder as compared with healthy controls and to subsequently analyze the presence of specific antibodies in participants testing positive for ANAs. Previous studies in patients with schizophrenia spectrum disorders often consisted of small cohorts; the present study is one

of the largest case-control studies to date. It includes the most important confounding factors and does not include patients on chlorpromazine, medication that can induce ANAs.

## Methods

*Data collection*

The data from this study were collected as part of the ongoing multicenter longitudinal Genetic Risk And Outcome of Psychosis (GROUP) study, started in the Netherlands in 2006.[11] This study was approved by the human ethics committees of the University Medical Centers of Utrecht, Amsterdam, Maastricht and Groningen. All included patients and healthy volunteers provided written informed consent before participating.

This study consists of 368 patients and 283 healthy controls whose plasma samples were available for analysis. Plasma samples were collected during a follow-up visit in 2009.

*Population*

Patients were recruited from mental health centers throughout the Netherlands in 2006. Healthy controls were recruited via mailings to random addresses in the catchment region. Inclusion criteria for patients were: fluent in Dutch and diagnosis of a schizophrenia spectrum disorder at follow-up according to the Comprehensive Assessment of Symptoms and History (CASH)[12] or Schedules for Clinical Assessment in Neuropsychiatry (SCAN)[13] interview. Eligible healthy controls had to meet the following criteria: fluent in Dutch, no history of a lifetime psychotic disorder or lithium use and no first- or second-degree family member with a lifetime psychotic disorder. All subjects with a psychosis due to a general medical condition were excluded from this study. Data on current or previous comorbid physical disorders were collected through questionnaires.

*Plasma analysis*

Blood was drawn by venous puncture and after centrifugation plasma samples were stored in -80$^0$C until further analysis.

Plasma samples from all participants were tested for ANAs by indirect immunofluorescence using a commercially available assay, according to the manufacturers protocol (Euroimmun, Lübeck, Germany). In short, plasma samples were diluted 1:100 and incubated with HEp-20-10 cells and primate liver substrates. After washing, attached antibodies were stained using a fluorescein-labeled antibody against human IgG. Nuclear staining was evaluated by two independent raters unaware of subject status and rated negative (absent or weak staining) or positive (moderate or strong staining). Subsequently, ANA positive samples were examined for specific antibodies using a commercially available Lineblot assay according to the manufacturers protocol (Profile 3 Lineblot, Euroimmun, Lübeck, Germany). In brief, plasma samples were diluted 1:101 and incubated on a membrane strip containing 14 antigen extracts (histones, nucleosomes, dsDNA, PCNA, centromere protein B, PM/Scl, Scl-70, SS-B, SS-A, Sm, nRNP, mitochondrial M2, ribosomal-P and Jo-1). Subsequently, the strips were stained using an alkaline phosphatase-labeled anti-human IgG antibody, which was visualized by an NBT/BCIP substrate solution. Assays were interpreted using scanning software provided by the manufacturer.

*Statistical analysis*

Differences in baseline characteristics between the two diagnostic groups were examined using chi-square and Mann Whitney U tests when appropriate. To assess whether the prevalence of ANAs was significantly different between the group of patients and the control subjects chi-square tests were used. A multiple logistic regression model was used to examine the influence of diagnostic group, gender, age and ethnicity on ANA status.

## Results

Table 1 shows an overview of the characteristics of both patient and control subjects. In total 29 patients (8%) and 32 controls (11%) tested positive for ANAs. This difference was not significant: $X^2 = 2.212$ $p = 0.137$. The ANA assay was strongly positive in 6/29 (21%) patients and 7/32 (22%) control subjects.

Age, ethnicity and gender have previously been shown to be associated with ANAs. In our study significantly more patients compared to control subjects were male (76% of the patients versus 42% of the control subjects). When we analyzed both gender groups separately, 21 out of 281 male patients (7%) and 11 out of 118 male control subjects (9%) tested positive for ANAs ($X^2$= 0.385 $p$= 0.535), whereas in the group of females 8 out of 87 patients (9%) and 21 out of 165 controls (13%) tested positive for ANAs ($X^2$= 0.698 $p$= 0.404).

Using multiple logistic regression analysis we did not find a significant influence of age, gender or ethnicity on ANA status (data not shown).

To assess the potential influence of antipsychotics on the ANA status, we examined the prevalence of ANAs in the group of patients currently using antipsychotics (N= 257) as compared to the group of patients currently not using antipsychotics (N= 37). The prevalence of ANAs was 6% and 8%, respectively (no significant difference: $X^2$= 0.190 $p$= 0.663). Furthermore, we found no significant difference in the prevalence of ANAs between patients currently using first (N=24) and second generation (N=183) antipsychotic agents ($X^2$= 0.206 $p$= 0.650).

**Table 1.** Characteristics of patients and controls.

| | Patients (N= 368)[a] | Controls (N=283) | Group comparison test |
|---|---|---|---|
| **Gender M/F (%males)** | 281/87 (76%) | 118/165 (42%) | $p$< 0.01 |
| **Mean age (SD) in years Range** | 30.5 (±7.0) 18-55 | 34.5 (±10.5) 18-55 | $p$< 0.01 |
| **Ethnicity Caucasian yes/ no/NA** | 310/50/8 | 263/16/4 | $p$< 0.01 |
| **Reported comorbid auto-immune disorder yes/no/NA** | 3/187/178 | 9/177/97 | $p$= 0.072 |
| **ANAs positive/negative** | 29 (8%) / 339 (92%) | 32 (11%) / 251 (89%) | $p$= 0.137 |
| **Specific ANAs** | | | |
| **Anti-histones** | 2 | 1 | |
| **Anti-PCNA** | 1 | 1 | |
| **Anti-SSA** | 1 | 1 | |
| **Anti-mitochondrial M2** | 0 | 1 | |

[a]Diagnosis: 295 schizophrenia, 58 schizoaffective disorder, 15 schizophreniform disorder. Mean duration of illness 7.3 years (±4.3), range 2-43 years. Using antipsychotics y/n/NA: 257/37/74. Clozapine: 58, olanzapine: 54, risperidone: 29, aripiprazol: 27, quetiapine: 15, haloperidol: 12, flupentixol: 4, zuclopenthixol: 4, bromperidol: 2, pimozide: 2, multiple antipsychotics: 41 and not specified antipsychotics: 9.

All 61 samples that tested positive for ANAs (29 originating from patients and 32 from control subjects), were tested for specificity in a line blot assay. In total 8 samples had a positive line blot result against the following antigens: histone, pCNA, SSA and a mitochondrial antigen. Positive results were equally distributed among patients and control subjects (Table 1).



## Discussion

Previous studies have not shown an unambiguous relation between ANAs and schizophrenia spectrum disorders (Table 2). Recognized reasons for contradictory results are differences in the techniques used to detect ANAs, differences in definition of positive results, variation and shortcomings in studied populations and whether or not control subjects were included.[2]

We found a similar prevalence of ANAs in patients with a schizophrenia spectrum disorder and control subjects, using indirect immunofluorescence on HEp-2 cells to detect ANAs, which is the gold standard for their detection.[3] Many published studies consisted of small cohorts and did not include a control group. An overview of previous case-control studies is provided in table 2. Furthermore, most studies did not correct for gender and age.[2] The present study does not suffer from these shortcomings.

Another important reason for the discrepant results in studies on ANA frequencies can be the use of medication. Various earlier studies have included a large proportion of patients using chlorpromazine[14–18] or did not describe medication use. Chlorpromazine is known to induce ANAs.[19,20] None of the patients included in our study was on chlorpromazine as this drug is not available in the Netherlands since 2008,[21] unfortunately we cannot exclude that some of the included patients might have used chlorpromazine before 2008.

ANA positivity can be caused by antibodies with a wide variety of fine specificities. Only a part of these antibodies are well described and have clinically relevant associations with specific autoimmune disorders.[3] A common way to detect fine specificity of ANAs is by line blot techniques. The

method we used can detect reactivity against 14 antigens. The frequency of a positive result with line blot in the ANA positive samples was low and no clinically relevant results were found. Most importantly, frequencies of positive results were similar for patients and control subjects.

In conclusion our study shows that the prevalence of ANAs, with determination of specific antibodies, is similar in patients with a schizophrenia spectrum disorder and healthy controls. Our study suggests that previous findings on autoantibodies in schizophrenia should be interpreted with care, paying attention to potential confounders, and that further validation in large cohorts is needed before conclusions can be drawn.

**Table 2.** Overview of case-control studies on the prevalence of ANAs in patients with schizophrenia spectrum disorders.

| Reference | Patients | ANA positive/% | Controls | ANA positive/% | ANA test |
|---|---|---|---|---|---|
| Gottfries and Gottfries, 1974[22] | 250 patients with schizophrenia | 56/22.4% | 77 | 7/9.1% | IF |
| Zarrabi et al., 1979[18] | 74 patients with schizophrenia | 29/39.2% | 15 | 0/0% | IF |
| Villemain et al., 1989[23] | 16 patients with schizophrenia | 5/31.3% | 10 | 2/20% | IF on mouse liver |
| Canoso et al., 1990[15] | 184 males with chronic psychosis | 45/24.5% | 35 | 0/0% | IF on HEp-2 cells |
| Yannitsi et al., 1990[17] | 179 patients with schizophrenia | 86/48.0% | 150 | 10/6.7% | IF on HEp-2 cells |
| Ganguli et al., 1992[24] | 225 patients with schizophrenia or schizoaffective disorder | 30/13.3% | 327 | 20/6.1% | IF on HEp-2 cells |
| Sirota et al., 1993[25] | 108 patients with schizophrenia | 42/38.9% | 210 | 8/3.8% | ELISA |
| Spivak et al., 1995[26] | 85 patients with schizophrenia | 18/21.2% | 37 | 2/5.4% | IF on HEp-2 cells |
| Zorrilla et al., 1996[27] | 56 patients with schizophrenia | 7/12.5% | 84 | 18/21.4% | IF on HEp-2 cells |
| Laske et al., 2008[28] | 34 patients with schizophrenia | 2/5.9% | 50 | 0/0% | IF on rat liver |
| Sidhom et al., 2012[29] | 60 patients with schizophrenia or schizoaffective disorder | 7/11.6% | 41 | 5/12.2% | IF on rat tissue |

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# Chapter 3

## Clinical consequences of extensive routine laboratory investigations in patients with a recent onset psychotic disorder

Hans C. van Mierlo[a*], Dominique V.C. de Jel[a*], Saskia J.M.C Palmen[a], Nicoletta M. van Veelen[a], Christiaan H. Vinkers[a] and Lot D. de Witte[a]

[a] Department of Psychiatry, UMC Utrecht Brain Center, University Medical Center Utrecht, Utrecht, The Netherlands

[*] Contributed equally to this work

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## Introduction

A large variety of non-psychiatric illnesses, including infectious, autoimmune, neurodegenerative and metabolic disorders is able to cause psychotic symptoms during their course.[1,2] Such cases are labeled as secondary psychosis or psychosis due to a general medical condition and routine laboratory investigations in patients with a first episode psychosis could aid in their detection. In addition to this, laboratory investigations could be of assistance in detecting relevant comorbid somatic disorders.[3] Previous studies examining the relevance of routine laboratory investigations in patients with various psychiatric disorders found a low yield of clinically relevant results,[4] but studies specifically investigating patients with a first episode psychosis are lacking.

This retrospective chart review study aims to describe the results and relevance of routine laboratory investigations in patients with a psychotic disorder referred to the tertiary psychosis unit of the University Medical Center Utrecht (the Netherlands) between February 2012 and October 2014. These patients underwent a blood examination consisting of 23 tests, as part of their diagnostic work-up, if possible within 24 hour after admission. All laboratory investigations were performed in the accredited diagnostic laboratory of the hospital.

## Methods

In total, we collected data of 37 outpatients and 178 inpatients, with an average age of 23.6 years (±5.9), 74% was male, 63% had a first episode psychosis and the median duration of psychotic symptoms was 2 months (IQR: 9.0). Of these patients 58% was using antipsychotic medication during referral and 85%during discharge. Diagnoses were as follows: 110 psychosis N.O.S., 42 schizophrenia, 35 bipolar disorder, 11 schizophreniform disorder, 5 substance induced psychotic disorder, 5 schizoaffective disorder, 4 brief psychotic disorder and 3 psychotic depression.

## Results

An overview of the results of the laboratory investigations is shown in Table 1. For 15 out of the 23 tests ≥90% of the patients had a result within the reference values of our laboratory. For the other eight tests, deviations ranged between 13% and 74%, the highest deviations were found for calcifediol (74%), CK in males (33%) and vitamin B6 (25%). Of the 4415 laboratory tests that were conducted, 290 (7%) test results gave rise to an alteration in clinical care. These consisted of 144 (3%) test results that led to additional follow-up blood tests, 133 (3%) to starting vitamin supplementation, 7 (0.2%) to stopping vitamin supplementation, 3 (0.1%) to a possibly newly diagnosed comorbid disorder and 3 (0.1%) to a change in antipsychotic medication.



Levels of blood lipid spectrum, glucose, HbA1C and prolactin can increase during treatment with antipsychotic medication, as was also found in this cohort. Most guidelines therefore advise to frequently measure these laboratory parameters; our results seem to underline these recommendations. Increased levels of ALP, GGT, AST and ALT are known side effects of antipsychotic medication. However, which cutoff values should lead to a switch in antipsychotic medication is still uncertain. Increased levels of these parameters ranged from 2% to 24% in this cohort. In most cases the increase in these enzyme levels was mild, asymptomatic and transient, as also described in other cohorts,[5,6] and in only one case it was decided to switch medication.

Despite broad laboratory screening no patients were diagnosed with psychosis due to a general medical condition, including neuropsychiatric systemic lupus erythematosus and Hashimoto encephalopathy.[7,8] Anti- nuclear and anti-TPO antibodies were detected in several patients, however without further clinical signs of these disorders. Based on our results, these antibodies should only be tested in patients with a high clinical suspicion of these disorders to prevent false positive and nonspecific results.

A large proportion of the included patients had a vitamin D deficiency, which has been associated with psychosis previously, but the relevance of supplementation is still uncertain.[9] Despite this uncertainty vitamin D was frequently supplemented in this cohort. Several patients had increased levels

**Table 1.** Overview of laboratory investigations.

| | Reference value | Mean with range | Normal | Increased |
|---|---|---|---|---|
| **Sodium** | 136-146 mmol/L | 139.6 (±1.9, 133-145) | 203 (98%) | 0 (0.0%) |
| **HbA1c** | 20-42 mmol/mol | 34.3 (±9.2, 25-72) | 184 (97%) | 5 (3%) |
| **ALP** | 0-120 U/L | 83.5 (±36.5, 25-377) | 186 (91%) | 19 (9%)* |
| **GGT** | 0-55 U/L (M)<br>0-45 U/L (F) | 23.9 (±15,1, 7-86)<br>18.8 (±16.9, 8-129) | 146 (95%)<br>52 (98%) | 7 (5%)<br>1 (2%) |
| **AST** | 0-35 U/L (M)<br>0-30 U/L (F) | 28.7 (±18.8, 10-134)<br>23.6 (±8.7, 10-44) | 125 (81%)<br>41 (76%) | 28 (19%)<br>13 (24%) |
| **ALT** | 0-45 U/L (M)<br>0-35 U/L (F) | 32.8 (±36.5, 3-289)<br>22.8 (±13.5, 7-63) | 126 (82%)<br>47 (87%) | 27(18%)<br>7 (13%) |
| **CK** | 0-170 U/L (M)<br>0-145 U/L (F) | 266.8 (±606.0, 5.3-6412)<br>112.7 (±95.4, 16-469) | 88 (67%)<br>39 (78%) | 43 (33%)<br>11 (22%) |
| **CRP** | 0-10 mg/L | 3.2 (±6.3, 0.5-65) | 180 (95%) | 12 (5%) |
| **Cholesterol** | 3.5-6.5 mmol/L | 4.2 (±0.9, 2.1-6.7) | 166 (82%) | 1 (%) |
| **Triglycerides** | 0.0-2.0 mmol/L | 1.1 (±0.6, 0.2-3.5) | 181 (90%) | 21 (10%) |
| **LDL** | 0.0-3.5 mmol/L | 2.4 (±0.7, 0.9-4.9) | 182 (92%) | 16 (8%) |
| **HDL** | > 0.9 mmol/L (M)<br>> 1.1 mmol/L (F) | 1.2 (±0.3, 0.5-2.0)<br>1.4 (±0.4, 0.9-3.1) | 133 (90%)<br>42 (79%) | 0 (0%)<br>0 (0%) |
| **Vitamin B1** | 65-200 nmol/L | 134.7 (±27.0, 50-253) | 182 (97%) | 4 (2%) |
| **Vitamin B6** | 35-120 nmol/L | 118.1 (±174.8, 38-2351) | 139 (75%) | 46 (25%) |
| **Vitamin B12** | 130-700 nmol/L | 284.7 (±131.9, 81-1100) | 180 (96%) | 1 (1%) |
| **Glucose** | 3.6-5.6 mmol/L | 5.1 (±1.1, 3.6-14.8) | 177 (94%) | 11 (6%) |
| **Hemoglobin** | 8.6-10.7 mmol/L (M)<br>7.4-9.6 mmol/L (F) | 9.5 (±0.6, 7.6-11.4)<br>8.4 (±0.6, 6.8-9.5) | 141 (93%)<br>52 (96%) | 3 (2%)<br>0 (0%) |
| **ESR** | 1-11 mm/1H (M)<br>2-24 mm/1H (F) | 4.3 (±5.6, 1.0-51)<br>10.6 (±12.7, 2.0-66) | 123 (96%)<br>47 (92%) | 5 (4%)<br>4 (8%) |
| **Anti-TPO antibodies** | 0-60 U/mL | 43.4 (±236.7, 0-3000) | 175 (97%) | 6 (3%) |
| **TSH** | 0.35-5.0 mIU/L | 1.7 (±0.8, 0.05-4.4) | 204 (99%) | 0 (0%) |
| **25(OH)D** | 50-100 nmol/L | 43.2 (±26.6, 20-152) | 49 (26%) | 7 (4%) |
| **Prolactin** | 0.1-0.65 IU/L (M)<br>0.1-1.2 IU/L (V) | 0.46 (±0.3, 0.5-1.6)<br>0.66 (±0.5, 0.1-2.26) | 104 (78%)<br>44 (86%) | 29 (22%)<br>7 (14%) |
| | | | **Absent** | **Present** |
| **Anti-nuclear antibodies** | | | 126 (96%) | 5 (4%) |

*14 cases had normal levels for their age (<19 year) # 1 case had normal levels for his age (<18 year)

| Decreased | Missing | Alteration in clinical care | Additional follow-up | Switching medication | Vitamin start/stop | New diagnosis |
|---|---|---|---|---|---|---|
| 4 (2%) | 8 | 2 (1%) | 2 | 0 | 0 | 0 |
| 0 (0%) | 26 | 2 (1%) | 0 | 0 | 0 | 2 |
| 0 (0%) | 10 | 5 (2%) | 5 | 0 | 0 | 0 |
| 0 (0%)<br>0 (0%) | 7<br>2 | 8 (4%) | 8 | 0 | 0 | 0 |
| 0 (0%)<br>0 (0%) | 7<br>1 | 24 (12%) | 24 | 0 | 0 | 0 |
| 0 (0%)<br>0 (0%) | 7<br>1 | 17 (8%) | 16 | 1 | 0 | 0 |
| 0 (0%)<br>0 (0%) | 29<br>5 | 9 (5%) | 8 | 1 | 0 | 0 |
| 0 (0%) | 23 | 3 (2%) | 3 | 0 | 0 | 0 |
| 35 (17%) | 13 | 1 (0%) | 1 | 0 | 0 | 0 |
| 0 (0%) | 13 | 21 (10%) | 21 | 0 | 0 | 0 |
| 0 (0%) | 17 | 16 (8%) | 16 | 0 | 0 | 0 |
| 14 (10%)<br>11 (21%) | 13<br>2 | 0 (0%) | 0 | 0 | 0 | 0 |
| 1 (1%) | 28 | 3 (2%) | 0 | 0 | 3 | 0 |
| 0 (0%) | 30 | 3 (2%) | 1 | 0 | 2 | 0 |
| 7 (4%) | 27 | 5 (3%) | 1 | 0 | 4 | 0 |
| 0 (0%) | 27 | 8 (4%) | 8 | 0 | 0 | 0 |
| 8 (5%)[#]<br>2 (4%) | 8<br>1 | 5 (2%) | 4 | 0 | 0 | 1 |
| 0 (0%)<br>0 (0%) | 32<br>4 | 2 (1%) | 2 | 0 | 0 | 0 |
| 0 (0%) | 34 | 1 (1%) | 1 | 0 | 0 | 0 |
| 1 (0%) | 10 | 1 (0%) | 1 | 0 | 0 | 0 |
| 129 (70%) | 30 | 131 (71%) | 0 | 0 | 131 | 0 |
| 0 (0%)<br>0 (0%) | 27<br>4 | 23 (13%) | 22 | 1 | 0 | 0 |
|  | 84 | 0 (0%) | 0 | 0 | 0 | 0 |

of vitamin B1, B6 and B12, which could be explained by the excessive use of over the counter supplementations.

Previous studies have also described increased levels of CK in patients with psychosis, which could be due to psychomotor agitation, medication, drugs, intramuscular injections and rhabdomyolysis.[10]

## Discussion

The results presented in this study are limited by referral bias, its retrospective nature and the lack of long-term follow-up. Strengths include the extensive clinical characterization, short-term follow-up and the naturalistic nature of this cohort.

In conclusion, extensive laboratory investigations were of no additional value in detecting psychosis due to a general medical condition in this cohort. Although some laboratory outcomes resulted in changes in clinical care, the majority of these changes concerned vitamin supplementation or additional follow-up. As such, the additional value of routing laboratory investigations in the early phase of a psychotic disorder seems limited.

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# Chapter 4

## The association between antibodies to neurotropic pathogens and schizophrenia: a case-control study

Lot D. de Witte[a*], Hans C. van Mierlo[a*], Manja Litjens[a], Hans C. Klein[b], Sabine Bahn[c], Ab D. Osterhaus[d], and the GROUP investigators

[a] Department of Psychiatry, UMC Utrecht Brain Center, University Medical Center Utrecht, Utrecht, The Netherlands

[b] Department of Psychiatry, University of Groningen, Groningen, The Netherlands

[c] Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, UK & Department of Neuroscience, Erasmus MC, Rotterdam, The Netherlands

[d] Department of Viroscience, Erasmus University Medical Center, Rotterdam, the Netherlands

[*] Contributed equally to this work

Genetic Risk and Outcome of Psychosis (GROUP) Investigators

Richard Bruggeman[1], Wiepke Cahn[2], Lieuwe de Haan[3], René S. Kahn[2], Carin J. Meijer[3], Inez Myin-Germeys[4], Jim van Os[4], Durk Wiersma[1]

[1] University Medical Center Groningen, Department of Psychiatry, University of Groningen, The Netherlands

[2] University Medical Center Utrecht, Department of Psychiatry, Brain Center Rudolf Magnus, The Netherlands

[3] Academic Medical Center University of Amsterdam, Department of Psychiatry, Amsterdam, The Netherlands

[4] Maastricht University Medical Center, South Limburg Mental Health Research and Teaching Network, EURON, Maastricht, The Netherlands

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## Abstract

Exposure to neurotropic pathogens has been proposed as an environmental risk factor for schizophrenia and can be evaluated by measuring pathogen-specific immunoglobulin G (IgG). Seroprevalence of pathogen-specific IgG reflects prior exposure, whereas IgG levels are associated with re-activity or re-infection. Several studies have examined these parameters in schizophrenia. However, results still remain inconclusive, as several previous studies did not correct for important confounding factors. We examined the seroprevalence and titer of IgG antibodies against herpes simplex virus-1 and -2 (HSV-1/HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV) and *Toxoplasma gondii* (TG) in plasma of 368 adult patients with a schizophrenia spectrum disorder and 282 controls using ELISA. We did not find evidence for an increased exposure to HSV-1, HSV-2, EBV and TG in patients. There was a significantly higher seroprevalence of VZV (98.9% vs. 95.6%, $p< 0.05$) and CMV (40.4% vs. 27.7%, $p< 0.001$) in controls as compared to patients, which did not remain statistically significant after adjustment for various potential confounders. We did not find significant differences in antibody titers of seropositive patients and controls for any of the six pathogens. Our results do not support the hypothesis that increased exposure to neurotropic pathogens after birth is associated with schizophrenia.

## Introduction

While the pathogenesis of schizophrenia is still largely unclear, it is generally accepted that this disease is caused by the interplay between genetic and environmental factors. Micro-organisms that can infect the central nervous system (CNS), the so-called neurotropic pathogens, have been proposed as candidate environmental factors for a long time.[1,2] This hypothesis is supported by the fact that genes associated with schizophrenia are involved in immune processes, including host-pathogen interactions.[3–5] Other support includes the increased risk for schizophrenia after prenatal[6] or childhood infections[7] and the increased prevalence of schizophrenia amongst people born in winter season[8] and in urban areas.[9]



Specific neurotropic pathogens have been proposed as candidate environmental risk factors for schizophrenia.[1] These include pathogens that infect a substantial part of the population, such as several types of herpes viruses (herpes simplex virus-1 and -2, Epstein-Barr virus and cytomegalovirus) and the parasite *Toxoplasma gondii*. Another common characteristic of all these pathogens is their ability to cause a latent infection by slowing down their replication and remaining undetected from the immune system by pathogen-specific mechanisms.[10] These pathogens may therefore affect crucial CNS functions and neurodevelopmental processes during primary infection, but also afterwards.

Prior exposure to a specific pathogen can be determined by analysing immunoglobulin G (IgG) class antibodies. Early during a primary infection immunoglobulin M (IgM) is produced. However, following maturation of B cells, immunoglobulin class switching takes place and IgG is synthesized.[11] This production generally lasts for the entire lifespan to protect the infected person against re-infection. Measuring pathogen-specific IgG is therefore indicative of exposure to a certain pathogen. An exception is the first six months after birth; in this period the new-born still has maternal IgG. Measuring IgG in new-borns therefore reflects maternal exposure to pathogens. In cases of a chronic infection, re-infection or re-activation the production of IgG increases. Therefore, measuring the level of IgG can provide information about an on-going or resumed replication of the pathogen.

The association between schizophrenia and exposure to neurotropic pathogens, including herpes viruses and *Toxoplasma gondii*, has been investigated in several types of cohorts using different kinds of material (blood, cerebrospinal fluid (CSF) and post-mortem tissue) and techniques (PCR and pathogen-specific antibodies). These previous findings have been thoroughly reviewed elsewhere.[6,7,12–14] Various studies measured the seroprevalence and levels of pathogen-specific IgG in serum and CSF in cross sectional cohorts to assess exposure and re-activity or re-infection of these pathogens during life. Some studies found spectacular increases in seroprevalence or IgG titers in patients with schizophrenia as compared to controls. However, other studies could not replicate these findings. Important confounders such as age, gender, ethnicity and urbanicity were not always included in these studies, which could have contributed to inconsistent results.

This study aims to investigate whether prior exposure to a broad range of neurotropic pathogens, including herpes simplex virus-1 and -2 (HSV-1/HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV) and *Toxoplasma gondii* (TG), is associated with schizophrenia and whether signs of increased rates of replication can be found in patients as compared to healthy controls. This study is one of the larger studies to date and it accounts for the most important confounders including age, gender, ethnicity, level of education, urbanicity at birth and size of household.

## Methods

*Study population*

The Genetic Risk And Outcome of Psychosis (GROUP) study, started in 2006, is a large multicenter study in the Netherlands. Details of the study design have been described elsewhere.[15] In short, patients with a schizophrenia spectrum disorder were recruited from mental health centers throughout the Netherlands. Healthy controls were recruited through mailings to random addresses in the catchment region. Inclusion criteria for patients were: fluent in Dutch and diagnosis of a schizophrenia spectrum disorder according to the Comprehensive Assessment of Symptoms and History (CASH)[16] or Schedules for Clinical Assessment in Neuropsychiatry (SCAN)[17] interview using the DSM-

IV criteria. Eligible healthy controls had to meet the following criteria: fluent in Dutch, no history of a lifetime psychotic disorder or lithium use and no first- or second-degree family member with a lifetime psychotic disorder. At the second follow-up visit in 2009 plasma samples were collected from a subpopulation of the study and stored at -80°C until further analysis. We included all available plasma samples from patients with a diagnosis of schizophrenia, schizophreniform disorder or a schizo-affective disorder at follow-up. The study was approved by the human ethics committees of the University Medical Centers of Utrecht, Amsterdam, Maastricht and Groningen. All included patients and healthy volunteers provided written informed consent before participating. Using questionnaires the highest level of completed education and size of the current household were assessed. Urbanicity of place of birth was assessed as previously described.[18] This variable was dichotomized into being born in a low (0) or high urbanicity region (1). Data on current or previous comorbid physical disorders were collected through questionnaires.



*Measurement of IgG*

IgG class antibodies against HSV-1, HSV-2, VZV, EBV, CMV and TG were determined by commercial enzyme-linked immunosorbent assay (ELISA) tests (IBL Laboratories, Hamburg, Germany) according to the manufacturer's protocols. Sensitivities and specificities of these tests are all >95%. In short, plasma samples were diluted and applied to 96-well microtiter plates that were pre-coated with pathogen specific antigens. Patients and controls were equally distributed on the plates. After 1-hour incubation and extensive washing, an enzyme-labeled anti-human IgG antibody was added for 30 minutes. Bound IgG was visualized by adding tetramethylbenzidine substrate, followed by adding $H_2SO_4$. The absorbance was measured at 450 nm using an ELISA microwell plate reader. A cut-off control sample was provided with the test for the qualitative interpretation of the results. Samples above the cut-off samples were scored positive, below the cut-off negative. IgG levels for CMV, HSV-1 and HSV-2 were quantified by calculating (patient absorbance value x 10)/(absorbance value of the cut-off) from all seropositive subjects and expressed as units/mL. IgG levels for EBV, VZV and TG were determined by using the calibration curve provided with the test and expressed as units/mL.

*Statistical analysis*

Differences in baseline characteristics between the two diagnostic groups were examined using chi-square and Mann Whitney U tests when appropriate. To assess whether the prevalence of IgG antibodies against the six pathogens differed significantly between the group of patients and the control subjects chi-square tests were used. Titers of positive cases were compared between patients and controls using Mann Whitney U tests. A multiple logistic regression model was used to calculate odds ratios (OR) for schizophrenia spectrum disorders in the groups testing positive for exposure to the various pathogens as compared to the groups testing negative. This model was adjusted for age, gender, ethnicity, level of education, urbanicity of place of birth and size of household.

## Results

Plasma samples of 368 patients and 282 healthy controls were examined. An overview of the demographics and clinical characteristics of patients and controls is depicted in Table 1.

As shown in table 2 no significant differences between patients and controls were found for HSV1 ($X^2$= 2.761 $p$= 0.097), HSV2 ($X^2$= 0.406, $p$= 0.524), EBV ($X^2$= 0.328, $p$= 0.567) and TG ($X^2$= 0.060, $p$= 0.806) seropositivity. A significantly higher seroprevalence of VZV (98.9% versus 95.6%, $X^2$= 6.068, $p$< 0.05) and CMV (40.4% versus 27.7%, $X^2$= 11.621, $p$< 0.001) was found in controls as compared to patients, without adjustment for possible confounders. The IgG titers of the positive cases did not differ significantly between patients and controls for any of the six pathogens.

The effects of adjusting for possible confounding factors are shown in table 3. When applying the possible confounders separately the negative association with VZV and CMV remained significant. When applying the fully adjusted regression model no statistically significant association was found between seropositivity for any of the 6 pathogens and schizophrenia spectrum disorders.

**Table 1.** Demographics and clinical characteristics of patients and controls.

| | Patients (N=368) | Controls (N=282) | Group comparison |
|---|---|---|---|
| **Mean age (SD) in years Range** | 30.5 (±7.0) 18-55 | 34.5 (±10.5) 18-55 | p< 0.01 |
| **Gender M/F (%males)** | 281/87 (76%) | 118/164 (42%) | p< 0.01 |
| **Diagnosis** | Schizophrenia: 295 Schizoaffective disorder: 58 Schizophreniform disorder: 15 | | |
| **Mean duration of illness in years Range** | 7.4 (±4.3) 2-43 | | |
| **Currently using antipsychotics yes/no/NA** | 257/37/74 | | |
| **Ethnicity Caucasian yes/no/NA** | 310/50/8 | 262/16/4 | p< 0.01 |
| **Completed selective secondary education or higher yes/no/NA** | 241/126/1 | 274/8/0 | p< 0.01 |
| **High urbanicity yes/no/NA** | 109/227/32 | 87/181/14 | p= 0.995 |
| **Single household yes/no/NA** | 148/167/53 | 57/202/23 | p< 0.01 |
| **Reported current or previous atopic, inflammatory or autoimmune disorder yes/no/NA** | 94/96/178 | 96/89/97 | p= 0.640 |

NA = not available

**Table 2.** Prevalence and titer of IgG antibodies against pathogens in patients and controls.

| | Patients (N=368) positive/ negative (%positive) | Controls (N=282) positive/ negative (%positive) | Group comparison | Patients IgG level (median, IQR) | Controls IgG level (median, IQR) | Group comparison |
|---|---|---|---|---|---|---|
| **HSV-1** | 133/235 (36.1%) | 120/162 (42.6 %) | p= 0.097 | 31.0, 19.2 | 33.3, 20.6 | p= 0.145 |
| **HSV-2** | 11/357 (3.0%) | 11/271 (3.9%) | p= 0.524 | 19.4, 4.9 | 30.9, 32.8 | p= 0.178 |
| **VZV** | 352/16 (95.6%) | 279/3 (98.9%) | p< 0.05 | 77.2, 142.6 | 83.2, 146.6 | p= 0.451 |
| **EBV** | 264/104 (71.7%) | 208/74 (73.8%) | p= 0.567 | 19.8, 6.3 | 19.9, 7.5 | p= 0.906 |
| **CMV** | 102/266 (27.7%) | 114/168 (40.4%) | p< 0.001 | 33.7, 16.0 | 34.5, 18.5 | p= 0.856 |
| **TG** | 68/300 (18.5%) | 50/232 (17.7%) | p= 0.806 | 227.8,107.7 | 197.6,102.9 | p= 0.232 |

**Table 3.** Exposure to pathogens and risk of schizophrenia spectrum disorder.

| Adjustment for confounding | Sample size | HSV1 | HSV2 | VZV | EBV | CMV | TG |
|---|---|---|---|---|---|---|---|
| | | Odds Ratio (95% confidence interval) | | | | | |
| **Unadjusted** | 650 | 0.764 (0.556-1.050) | 0.759 (0.324-1.777) | 0.237 (0.068-0.820)* | 0.903 (0.637-1.280) | 0.565 (0.406-0.786)** | 1.052 (0.703-1.574) |
| **Age** | 650 | 0.885 (0.636-1.231) | 1.136 (0.469-2.754) | 0.229 (0.065-0.809)* | 1.011 (0.706-1.447) | 0.598 (0.426-0.838)** | 1.318 (0.864-2.010) |
| **Gender** | 650 | 0.839 (0.597-1.178) | 1.403 (0.569-3.459) | 0.205 (0.056-0.750)* | 1.023 (0.704-1.485) | 0.651 (0.458-0.925)* | 0.979 (0.637-1.506) |
| **Ethnicity** | 638 | 0.728 (0.526-1.009) | 0.805 (0.342-1.892) | 0.248 (0.071-0.870)* | 0.877 (0.615-1.250) | 0.509 (0.362-0.717)** | 1.125 (0.744-1.702) |
| **Educational level** | 649 | 0.701 (0.495-0.993)* | 0.952 (0.392-2.311) | 0.224 (0.062-0.807)* | 0.968 (0.662-1.416) | 0.618 (0.432-0.882)** | 0.883 (0.564-1.382) |
| **Urbanicity** | 604 | 0.736 (0.528-1.025) | 0.883 (0.353-2.206) | 0.260 (0.074-0.915)* | 0.887 (0.618-1.273) | 0.500 (0.354-0.706)** | 0.967 (0.639-1.464) |
| **Single household** | 574 | 0.822 (0.579-1.166) | 0.705 (0.273-1.826) | 0.265 (0.073-0.957)* | 0.885 (0.605-1.296) | 0.585 (0.406-0.845)** | 0.862 (0.548-1.355) |
| **Fully adjusted** | 524 | 0.773 (0.490-1.217) | 2.160 (0.639-7.298) | 0.265 (0.057-1.227) | 1.128 (0.700-1.815) | 0.663 (0.416-1.057) | 0.992 (0.548-1.795) |

*= $p<0.05$ **= $p< 0.01$

## Discussion

In the present study we did not find evidence for an increased exposure to HSV-1, HSV-2, EBV and TG in plasma of patients with schizophrenia spectrum disorders as compared to healthy controls. We found a significantly higher seroprevalence of VZV and CMV IgG in controls as compared to patients. However, these differences did not remain statistically significant after correcting for multiple possible confounders. No differences in IgG titers were found between patients and controls for the seropositive cases.

A meta-analysis of previous findings on various infectious agents in schizophrenia did not find evidence for an increased exposure to HSV-1, VZV, EBV and CMV in patients.[13] Our results are in accordance with this meta-analysis. However, this meta-analysis did find a higher exposure to HSV-2 and TG in patients. We were unable to replicate these findings in our study. The authors of the meta-analysis mention that results on HSV-2 are strongly dependent on one large study that used blood samples obtained at birth and therefore assessed maternal IgG.[19] This study design is incomparable to ours, which could explain the conflicting results.

In contrast to our results, numerous studies have found an association between schizophrenia and IgG type TG antibodies, described in several meta-analyses.[12–14,20] Publication bias and the influence of confounders are most likely involved in this discrepancy. In 2012, Arias et al. described that studies that did not find an association between TG and schizophrenia seemed to have a more thorough design than those that do find this association.[13] Since then, several larger studies were also unable to detect a difference between patients and controls in TG antibody levels[21–24] or seropositivity.[24,25] Moreover, Sutterland et al. found signs of publication bias in his recent meta-analysis, although a significant positive association between TG and schizophrenia remained after correcting for this bias.[20] Importantly, age, ethnicity, urbanicity and social contact are associated with exposure to TG. In most studies age and gender were included as confounders, but the inclusion of the others was highly variable. In the present study we were able to control for age, gender, ethnicity, level of education, urbanicity at birth and size of household. In addition to this, contact with felines and consumption of raw meat are major risk factors for

TG infection. A previous study that included these confounders found that they had a significant effect on the association between schizophrenia and TG.[26] Unfortunately data on these confounders were not available in the present study, as well as most of the previous studies, and could therefore be involved in the inconsistent results.

Interestingly, we found a significant higher exposure to CMV in controls in our unadjusted model, which remained significant after adjusting for multiple possible confounders separately, but not after applying the fully adjusted model. This could be due to lack of statistical power. Two other recent studies also found a significantly higher exposure[27] and titer[23] of CMV in controls as compared to patients. These results seem contra-intuitive but could be explained by a decreased exposure to CMV during life due to the more isolated lifestyle of patients, a protective effect of CMV infection on the risk of developing schizophrenia or as sign of an altered immune function in schizophrenia.[23] Some support for the latter hypothesis comes from a metagenomic analysis performed with the virus discovery method VIDISCA-454 that revealed a significantly lower viral prevalence in a group of pregnant mothers of offspring with schizophrenia. Consistent with the existing inverse correlation between the level of these viruses and the immunocompetence of an individual, Canuti et al. hypothesized the presence of a higher immune activity during pregnancy in mothers whose offspring later develop a psychotic disorder as compared to controls.[28] In addition to this, CMV is known for modulating MHC class I antigen presentation pathways[29] and variations in the MHC region on chromosome 6p21.3-22.1 are highly associated schizophrenia.[3]

We were unable to retrieve other studies that also found an increased exposure rate to VZV in controls as compared to patients. However, only very little patients and controls tested negative for VZV, which makes it unlikely that the difference between patients and controls is relevant for our understanding of schizophrenia.

Strong aspects of our study include the large sample size and the correction for multiple relevant confounders. Our study also has several limitations. As described earlier we did not have data on all relevant confounders. Furthermore, this study is limited by its cross sectional design. Patients could

indeed have had an increased exposure to the examined pathogens during prenatal[6] or childhood[7] periods, but these differences might not be detectable anymore in adulthood as exposure to these pathogens in later stages of life might be similar or higher in controls. Signs of increased replication of neurotropic pathogens could be state-specific and only detectable in acutely ill patients or patients with a first episode psychosis which are lacking in this study. Lastly, plasma levels of IgG do not necessarily reflect intrathecal production of antibodies and are therefore apt to underestimate brain immune responses against these pathogens.

In conclusion, we found no evidence to support the hypothesis that infection with HSV-1, HSV-2, VZV, EBV, CMV and TG after birth plays a role in the pathogenesis of schizophrenia. However, we emphasize that findings from prenatal cohorts, childhood cohorts before disease onset and adult patients should be clearly distinguished to further unravel the link between infectious agents and schizophrenia.

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# Chapter 5

## B-cells and schizophrenia: a promising link or a finding lost in translation?

Hans C. van Mierlo[a], Jasper C.A. Broen[b,c], René S. Kahn[d,e], Lot D. de Witte[d,e]

[a] Department of Psychiatry, UMC Utrecht Brain Center, University Medical Center Utrecht, Utrecht, The Netherlands

[b] Laboratory of Translational Immunology, Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

[c] Department of Rheumatology & Clinical Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

[d] Department of Psychiatry, Icahn School of Medicine, New York, United States of America

[e] Mental Illness Research, Education and Clinical Center (MIRECC), James J Peters VA Medical Center, Bronx, New York, United States of America

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## Abstract

Recent genetic studies have suggested a potential role for B-cells in the pathogenesis of schizophrenia. Greater insight in the functioning of B-cells in patients with schizophrenia is therefore of importance. In this narrative review we aim to give an overview of the current literature on B-cells and schizophrenia. We found no evidence for altered numbers of these cells in blood. We did find support for increased levels of B-cell related cytokines and certain autoantibodies. Studies on B-cell development and function, or their numbers in cerebrospinal fluid or brain tissue are very limited. Based on the available data we appraise whether various B-cell mediated pathological mechanisms are likely to play a role in schizophrenia and provide directions for future research.

## Introduction

Over the last decade the high heritability of schizophrenia has fuelled a massive effort to elucidate genetic factors involved in the pathogenesis of this disorder. In 2014 this resulted in a landmark genome wide association study (GWAS) on schizophrenia. This study included 36,989 patients and 113,075 controls and identified 108 genome wide significant loci. In order to translate these genetic findings to potential disease mechanisms, associated loci were mapped onto sites of active enhancers in different tissues and cell lines. Interestingly, schizophrenia associations were most significantly enriched at enhancers active in $CD19^+$ and $CD20^+$ B-cells.[1] An association with B-cells was also suggested by a cross-disorder genetic pathway analysis of depression, bipolar disorder and schizophrenia.[2] Although these explorative analyses are accompanied by several uncertainties and limitations,[3] they generate novel hypothesis for follow-up research. A clear overview of other evidence supportive of a link between B-cells and schizophrenia pathogenesis is therefore of importance.



Based on the specific properties of B-cells, one can speculate about the possible role of B-cells in the pathogenesis of schizophrenia. B-cells have various functions within the adaptive immune system. They are best known for the production of immunoglobulins, also named antibodies. In addition, they are capable of presenting antigens to T-cells through their MHC-II complex. Furthermore, they can secrete cytokines and chemokines involved in regulation of immune responses and tissue repair.[4] A dysfunction of B-cells could therefore hypothetically lead to autoimmunity, hyperinflammation or immune deficiencies.[5,6] In addition, it has recently been shown that a specific type of B-cells is involved in early neurodevelopmental processes, resulting in a new hypothesis on how B-cells could contribute to schizophrenia.[7,8]

When scrutinizing the literature about immunology in schizophrenia, various studies have found phase dependent immune system abnormalities in patients, including altered levels of cytokines and altered numbers or activation states of immune cells in blood, cerebrospinal fluid (CSF) and brain tissue.[9] Epidemiological studies have also shown a significant association between schizophrenia and the prevalence of autoimmune disorders, as

well as prenatal and childhood infections.[10–12] Together, these data have resulted in various hypotheses regarding the involvement of the immune system in the pathogenesis of schizophrenia, including a form of low grade inflammation in the brain resulting in cell damage or cell death,[13] activation of the immune system due to environmental factors such as early life stress or infections with certain pathogens,[12] increased or altered microglial activation resulting in excessive synaptic pruning[14] and a form of autoimmunity with increased presence of specific pathogenic autoantibodies.[15] Although these studies do not directly implicate alterations in B-cells, various B-cell features are involved in these processes, including the production of cytokines and antibodies.

In light of the aforementioned genetic studies that suggest a role for B-cells in the pathogenesis of schizophrenia and the more circumstantial evidence gathered from epidemiological and translational studies, we are convinced that greater insight in the functioning of B-cells in schizophrenia could aid in deriving more insight in the immunologic aspects of this disorder. In this narrative review we therefore aim to give an overview of the current literature on B-cells and schizophrenia. Results of these studies are discussed within the scope of physiological B-cell functioning and pathological mechanisms identified in this process in various autoimmune disorders.

## Methods

Relevant literature was collected through a literature search conducted on PubMed and Embase on April 17 2019 (see Figure 1). Only English studies were included, no year restrictions were applied. The following keywords were used for this search: (schizophrenia OR psychosis OR psychotic) AND (B-cell OR B cell OR B-cells OR B cells OR B-lymphocyte OR B lymphocyte OR B-cells OR B lymphocytes OR CD19 OR CD20). Candidate studies had to meet the following inclusion criteria: Human or animal study providing information on B-cells or related cytokines/chemokines in schizophrenia or schizophrenia animal model, published as a peer reviewed article. Conference abstracts were excluded.

This search resulted in 425 unique articles, 19 studies met our inclusion criteria. The reference lists of all relevant articles were screened, which resulted in 10 additional articles. Additional background information or data from other studies on this topic were collected using recent review articles on immunology, B-cells, schizophrenia and various autoimmune disorders.

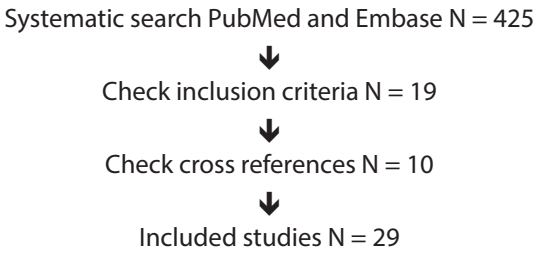


**Figure 1.** Flowchart.



## Results

We included 29 studies in this review. Table 1 shows the studies focused on measuring B-cell numbers in blood of patients with schizophrenia as compared with controls, or alterations in these numbers during treatment with medication. An overview of the other studies retrieved through our systematic search is given in Table 2. A visual overview of normal B-cell development and activation is given in Figure 2. An overview of B-cell related findings in schizophrenia is given in Figure 3.

*Origin of B-cells*

B-cells are part of the adaptive immune system and mediate the humoral immune response. They originate from hematopoietic stem cells in the bone marrow. Differentiation of these stem cells into immature B-cells is a multi-step process revolving around rearrangement of immunoglobulin (Ig) heavy-chain and light-chain gene segments. As a result of this rearrangement each B-cell expresses a unique B-cell receptor, which is a membrane-bound form of the immunoglobulin that they will produce once activated. B-cell activating factor (BAFF) is a cytokine that provides important survival signals

during B-cell differentiation. Increased levels of this cytokine have been found in various autoimmune disorders.[16] One study examined levels of this cytokine in patients with schizophrenia and found a significantly decreased level in serum samples of patients (N=60) as compared with controls (N=28).[17]

*Peripheral B-cells*

After the first differentiation steps in the bone marrow, B-cells enter the bloodstream and migrate towards the spleen to complete their maturation to naïve B-cells. A part of these naïve B-cells remains within the spleen. The rest migrates through the blood and lymphatic system towards other secondary lymphoid organs, such as the lymph nodes.[6] CXCL13 is a strong B-cell attracting molecule, highly expressed in secondary lymphoid organs. A negative association has been described between CXCL13 serum levels and scores on the Positive and Negative Syndrome Scale (PANSS) in female patients (N=54) with schizophrenia. However, no alterations in levels of this cytokine were found in the same study in the group of female patients or in the whole group of included patients (N=133) as compared with controls (N=133).[18]

As mentioned before, results of the most recent schizophrenia GWAS showed an enrichment of enhancers active in $CD19^+$ and $CD20^+$ B-cells in the 108 identified significant loci.[1] CD19 and CD20 are both trans membrane proteins expressed on mature B-cells, but not on plasma cells. These proteins are commonly used as marker to identify B-cells. CD19 is expressed from earlier developmental timepoints as compared to CD20. Increased numbers of B-cells expressing these markers have been found in blood of patients with various autoimmune disorders, including systemic lupus erythematosus (SLE), rheumatoid arthritis and multiple sclerosis.[19–21]

Several studies have examined the percentage and absolute numbers of $CD19^+$ or $CD20^+$ B-cells in blood of patients with schizophrenia as compared with controls or after antipsychotic treatment (see Table 1 for an overview). Seven of these studies have been previously included in a meta-analysis.[22] This meta-analysis did not find a significant change in absolute or relative numbers of B-cells in patients with an acute relapse, after antipsychotic treatment or during a first episode psychosis. Our updated and extended overview of studies examining B-cell numbers in blood of patients with schizophrenia also

shows little indication for alterations in B-cell numbers in patients as compared with controls.

B-cells in blood can be subdivided in naïve, memory and plasma cells. Markers such as CD27, CD20 and IgD characterize these populations. Thus far, only one study examined these specific subsets of circulating B-cells in schizophrenia. This study described an increase in the percentage of naïve B-cells ($CD19^+$ $IgD^+$ $CD27^-$) in 18 patients with chronic schizophrenia, as compared with 18 controls.[23]

In relation to treatment response, one study found a trend level decrease in the number of B-cells in 12 patients that started with clozapine.[24] This is of interest, as it has been suggested that clozapine has immunomodulatory effects.[25] Another study investigated B-cells in blood of 50 patients that participated in a randomized double blind, placebo-controlled trial of risperidone and the COX-2 inhibitor celecoxib versus risperidone and placebo. In both groups there was a small but significant decrease in the percentage of $CD19^+$ B-cells during treatment. In the celecoxib group, but not in the placebo group, the authors observed a significant relationship between the decrease of $CD19^+$ B-cells and the decrease of the PANSS negative-scale during treatment.[26]

In summary, no clear differences in peripheral B-cell numbers were found in schizophrenia. Very limited research has been done on the presence of specific B-cell subsets and their response to treatment. Two studies suggest there may be a relation between B-cell numbers and the use of medication.

**Table 1.** Overview of studies examining B-cell percentage or absolute number in blood of patients with schizophrenia as compared with controls or during treatment.

| Year | Author | Patients | Controls | Method | Marker | A | % | T | Significant outcome |
|---|---|---|---|---|---|---|---|---|---|
| 1958 | Schleifer[27] | 16 | 16 | Rosette formation | | Y | Y | | - |
| 1979 | Zarrabi[28] | 75 | ND | Rosette formation | | | Y | | - |
| 1980 | Nyland[29] | 37 | 30 | Rosette formation | | Y | Y | | - |
| 1988 | Kronfol[30] | 22 | 37 | Microscopy | | | Y | | - |
| 1989 | McAllister[31] | 34 | 33 | Flow Cytometry | CD19 | | Y | | - |
| 1989 | McAllister[24] | 12 | None | Flow Cytometry | ND | | Y | Y | Trend level decrease in percentage of B-cells (14.1% to 11.8%) during treatment with clozapine |
| 1990 | Masserini[32] | 42 | 37 | Microscopy | | Y | Y | | Increase in absolute number of B-cells in subgroup of drug-free (N=9) patients (503 cells/µl) as compared with controls (208 cells/µl) |
| 1993 | Ganguli[33] | 116 | 166 | Flow Cytometry | CD19 | Y | | | - |
| 1994 | Sasaki[34] | 26 | 32 | Flow Cytometry | CD20 | | Y | | - |
| 1996 | Cosentino[35] | 9 | 18 | Flow Cytometry | ND | Y | Y | | - |
| 1997 | Arolt[36] | 27 | 27 | Flow Cytometry | ND | | Y | | - |
| 1998 | Cazzullo[37] | 29 | 20 | Flow Cytometry | CD19 | | Y | | - |
| 1998 | Rothermundt[38] | 44 | None | Flow Cytometry | CD19 | Y | Y | | Increase in percentage of B-cells in patients (14.5%) as compared with reference range (3.0 – 12.0%) |
| 1999 | Printz[39] | 29 | 30 | Flow Cytometry | CD20 | Y | Y | | - |
| 2003 | Bilici[40] | 20 | None | Flow Cytometry | CD19 | | Y | Y | - |
| 2004 | Mazzarello[41] | 24 | 5 | Flow Cytometry | ND | Y | Y | | - |

**Table 1.** Continued

| Year | Author | Patients | Controls | Method | Marker | A | % | T | Significant outcome |
|---|---|---|---|---|---|---|---|---|---|
| 2004 | Müller | 50 | None | Flow Cytometry | CD19 | | Y | Y | Decrease in percentage of B-cells during treatment with risperidon with (N=25, 16.3% to 13.4%) and without (N=25, 15.9 to 14.4%) celecoxib addition |
| 2004 | Rudolf[42] | 31 | 31 | Flow Cytometry | CD19 | | Y | | - |
| 2007 | Maino[43] | 40 | 20 | Flow Cytometry | CD19 | | Y | Y | - |
| 2009 | Torres[44] | 8 | 7 | Flow Cytometry | CD19 | | Y | | - |
| 2010 | Steiner[45] | 26 | 32 | Flow Cytometry | CD19 | Y | | | Increase in absolute number of B-cells in patients (300 cells/µl) as compared with controls (195 cells/µl) |
| 2016 | Fernandez-Egea[23] | 18 | 18 | Flow Cytometry | Various subsets | | Y | | Increase (numbers ND) in relative numbers of naïve B-cells (CD3- CD19+ IgD+ CD27-) as compared with controls |

ND = not described, A = absolute number of B-cells described, % = percentage of B-cells described, T = Treatment study describing changes in B-cells, Y = yes.



*B-cell activation*

Naïve B-cells are activated upon encountering a specific antigen that is recognized by their B-cell receptor. Some B-cells need additional signals from T-helper cells for their activation. After activation, the B-cell will rapidly proliferate and differentiate into plasmablasts, plasma cells and memory B-cells. Plasmablasts are short-lived, generated in the early stage of infection and produce low-affinity antibodies. Immunoglobulin class switching and somatic hypermutation in plasma cells and memory B-cells subsequently result in the production of high-affinity antibodies.[46,47]

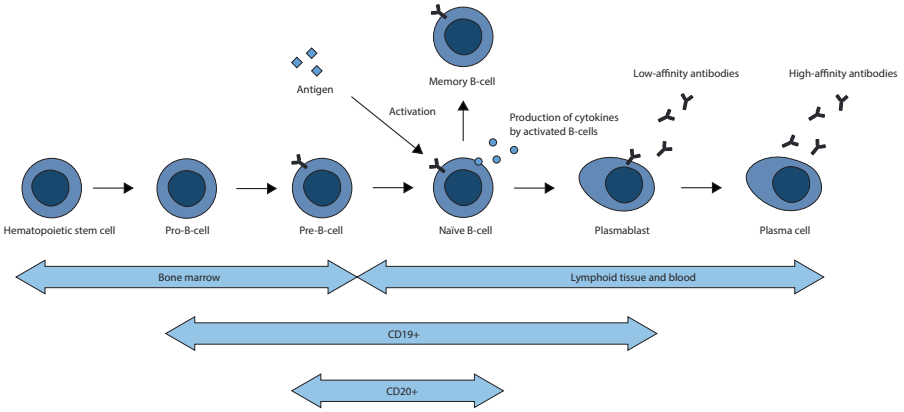


**Figure 2**. Overview of B-cell development. B-cells originate from hematopoietic stem cells in the bone marrow. During various steps these cells proliferate into naïve B-cells expressing B-cell receptors. Naïve B-cells become activated after encountering an antigen. Activated B-cells proliferate into memory B-cells or antibody secreting plasma cells.

The complex biological pathway of B-cells activation has been linked to genetic variants associated with schizophrenia, depression and bipolar disorder.[2] Interestingly, a study examining DNA methylation profiles in whole blood of 98 schizophrenia patients and 108 healthy controls identified *LAX1*, a gene with a role in B-cell activation, as hypermethylated.[48] LAX1 is a negative regulator of cell survival and proliferation and LAX deficient mice exhibit hyperresponsive T- and B-cells.[49] In addition to this, a study examining the ex-vivo signaling network of lymphocytes from patients with various psychiatric disorder, suggests that AKT1 is functionally dysregulated in B-cells of patients with schizophrenia (N=25) as compared with controls (N=100).[50] Previous studies have shown that suppression of AKT1 has a mixed and complex effect on both B-cell survival and proliferation.[51]

*B-cell effector functions: Antibody production*

Plasmablasts and plasma cells are capable of producing large amount of antibodies. Five different classes of antibodies are distinguished (IgM, IgD, IgG, IgA and IgE). IgM and IgD are present on the surface of B-cells. The amount of IgM secretion rises early after contact with a pathogen. The production of IgG, IgA and IgE occurs after isotype switching has taken place.[4,52] Antibodies can neutralize an antigen by blocking its binding to target cells, opsonize an antigen for uptake by phagocytes and activate the innate immune system.

To protect the body from autoimmunity, it is important to prevent the production of antibodies that recognize an antigen present in the human body itself, a so-called self-antigen.[6] Several positive and negative selection steps mediate this process during B-cell development. However, flaws in this process can result in the production of antibodies that are self-reactive, called autoantibodies.[4] Autoantibodies can have direct pathogenic effect by interacting with the self-antigen or result in unwanted immune system activation through the complement system or Fc-receptors. Antibodies showing self-reactivity, usually of the IgG isotype, are found in many autoimmune disorders. The production of these autoantibodies can be triggered by the presentation of intracellular self-antigens to B-cells by insufficient clearing of cell debris after apoptosis or inflammation.[5] In addition to this, self-reactivity can arise through cross-reactivity of antibodies that recognize foreign antigens that largely resemble autoantigens[53]. This process is referred to as molecular mimicry.



*Neuronal autoantibodies*

During recent years there is a growing interest in autoantibodies directed against neuronal membrane proteins, such as those found in patients with anti-NMDA receptor encephalitis.[54] The presence of these antibodies can result in direct neuronal dysfunction and disruption of neurotransmission.[55] Studies on the presence of NMDA receptor antibodies in schizophrenia show that these antibodies are rare but might play a role in a small percentage of patients diagnosed with schizophrenia.[56,57] New studies on these and other neuronal autoantibodies in schizophrenia are still appearing both with positive[58,59] and negative findings.[60–62] There is an ongoing debate about the optimal technique to test for these antibodies. Most studies use a cell-based assay expressing the protein of interest. This can be done using either live or fixed cells, which seems to have an impact on the results of the test. In addition to this, the group that discovered anti-NMDA receptor antibodies emphasizes the use of immunohistochemistry or immunocytochemistry on cultured to neurons to verify results from cell-based assays in order to prevent false-positive or false-negative results.[63] Lastly, differences in the patients included in these studies, ranging from patients with chronic schizophrenia to patients with a first episode psychosis with (mild) neurological symptoms may also explain some of the heterogeneity in outcomes.

One recent study suggests that the anti-NMDA receptor antibodies found in patients with a psychotic disorder differ from those found in anti-NMDA receptor encephalitis, presumably because they target a different epitope. The authors have used molecular imaging techniques to demonstrate the pathogenicity of these antibodies and argue that such assays should be further developed to overcome the shortcomings of other testing methods such as the cell-based assay.[59,64] Cloning of antibodies from these patients to further study their in vitro and in vivo effects, is likely to provide valuable additional insights.[65,66]

Apart from the difficulties concerning the antibody tests, there is also still a lack of large studies examining the presence of these antibodies in CSF.[67] Two studies have thus far screened CSF of patients with a psychotic disorder for these antibodies. One included 124 patients and found zero positive cases.[68] While the other included 125 patients and identified one patient with anti-NMDA receptor antibodies.[69]

Altogether, the current evidence suggests that anti neuronal antibodies may play a role in the disease pathogenesis of a small amount of patients with schizophrenia.

*Non-neuronal antibodies*

Numerous studies have also examined the presence of non-neuronal autoantibodies in blood of patients with schizophrenia, such as antinuclear antibodies, anti-gliadin and anti-TPO. A comprehensive overview of their outcomes is given in a recent meta-analysis.[70] This meta-analysis shows that multiple autoantibodies were significantly more prevalent in schizophrenia. It also shows that not all studies correct for important confounding factors and there is a large between studies heterogeneity.

One recent study, not included in this meta-analysis, specifically examined the prevalence of IgG antibodies against peptides from 15 genes genetically associated with schizophrenia. Patients (N=169) showed increased levels of IgG against 6 of these peptides derived from DPYD, MAD1L1, ZNF804A, DRD2, TRANK1 and MMP16 as compared with controls (N=187).[71]

Other studies have not looked at specific antibodies but measured general levels of antibodies. In disorders with intrathecal production of antibodies, such as MS, this production can often be detected in CSF as oligocolonal bands (OCB). Multiple studies have examined the presence of these OCB in CSF of patients with schizophrenia, with both positive and negative results.[72] Two recent studies found OCB restricted to CSF in 7-15% of the patients with schizophrenia or a psychotic disorder (N=124 and 180).[68,69] However, these results were not compared to a control group and these bands are known to also occur in controls. Peripherally produced antibodies are capable of entering the CSF after disruption of the blood brain barrier, which could be caused by various factors such as stress, trauma, inflammation and infection.[53] This process can be studied by measuring albumin and IgG levels in paired serum and CSF samples. Thus far, contradicting results have been reported regarding IgG levels in the CSF of patients with schizophrenia.[72,73]



Lastly, one study examined immunoglobulin G genotypes in 398 patients and 400 controls. Results showed a highly significant association between the susceptibility to schizophrenia and variants in the genes encoding for the heavy chain of IgG, required for antibody production.[74]

In conclusion there is evidence in support of an increased production of non-neuronal autoantibodies in blood and CSF of patients of patients with schizophrenia. Whether this is a sign of susceptibility to autoimmunity, or whether these autoantibodies have a direct role in the pathogenesis of schizophrenia remains uncertain.

*Other B-cell effector functions*

Apart from antibody production, B-cells play an important role in the regulation of the immune system by producing cytokines. Cytokines produced by activated B-cells, such as IL-2, IL-4, TNF-α, IL-6 and IL-12 are involved in the proliferation of $CD4^+$ T-cells, activation of the innate immune response and influence tissue renewal. A small subpopulation of B-cells, the B-regulatory cells, plays a role in suppressing the immune system by producing IL-10, TGF-β and IL-35.[75]

Levels of different cytokines have been extensively examined in blood of patients with schizophrenia. However, interpreting these studies is troublesome as results are often heterogeneous, state-dependent and lack correction for relevant confounders. Various studies have found increased levels of IL-6 in patients with schizophrenia, but this interleukin is also produced by various other immune cells.[76,77] In addition tot this, increased levels of TNF-α, IL-12 and TGF-β have also been found in patients with schizophrenia during various diseases phases while a decrease in IL-10 was found in patients with an acute relapse.[76]

*B-cells in the central nervous system*

In healthy individuals B-cells are virtually not detected in CSF, while in neuro-inflammatory disorders like MS the percentage of B-cells in CSF is increased.[78] To our knowledge no studies have specifically examined the amount or percentage of $CD19^+$ or $CD20^+$ cells in CSF of patients with schizophrenia. One study did assess the cell composition of CSF in patients with schizophrenia (N=30) and reported an increased frequency of activated lymphocytes as compared with controls (N=46) based on their morphology, but did not differentiate between B- and T-cells.[79] Various other studies performed a cell count in CSF of patients with schizophrenia, but did not differentiate between cell-subsets. These studies often found an increased amount of cells in a small proportion of the included patients.[72]

A study examining post-mortem brain tissue of healthy individuals has shown that B-cells are present in very low numbers in brain parenchyma.[80] In brain diseases, such as limbic encephalitis, infiltrates of B-cells are commonly found.[81] Infiltrates found in the parenchyma of MS patients usually consist primarily of T-cells and myeloid cells, although cellular aggregates rich in B-cells have been found in the meninges of some patients.[82]

Neuropathological studies of schizophrenia have not found evidence in support of the presence of large lymphocyte infiltrates in post-mortem brain tissue.[83] However, there is a severe lack of large studies specifically focusing on these cells in post-mortem brain tissue. We retrieved only two studies that systematically quantified the presence of B-cells in post-mortem brain tissue of patients with schizophrenia. In one study $CD20^+$ cells were visualized using

immunohistochemistry in posterior hippocampal tissue of 17 patients with schizophrenia and 11 matched controls. An analyses comparing all patients with controls was not presented.[84] The authors only presented the results separately for patients with residual type schizophrenia (N=7) and paranoid type schizophrenia (N=10). Patients with residual type schizophrenia showed a significant increase in the number of $CD20^+$ cells as compared with controls and patients with paranoid type schizophrenia. Another study examined the presence of $CD20^+$ cells, also using immunohistochemistry, in tissue of the hippocampus/parahippocampus, thalamus/hypothalamus, neocortex (temporal and frontal), cingulate gyrus, subcortical white matter and the choroid plexus of 20 patients and 20 controls. Presence of B-cells was scored in a semi-quantitative fashion as no cells, few cells or many cells. The only significant difference was found in hippocampus/parahippocampus tissue. In two out of twenty patients many $CD20^+$ cells were found in this brain region, while no cells were found in all of the controls and other patients.[85]



Multiple transcriptome studies have assessed the expression levels of genes in post-mortem brain tissue of patients with schizophrenia. A large RNA sequencing study, specifically examining the differential expression of 561 immune genes and 20 immune pathways, found no altered expression levels in genes or pathways related to B-cells.[86] In addition to this, a hypothesis-free transcriptome study of post-mortem cortical brain tissue from 159 patients and 293 controls found no significant alterations in CD19 or CD20.[87]

Taken together, B-cells in CSF and brain tissue of patients with schizophrenia have been poorly studied, but might be of interest in the hippocampus region. In addition to this, studies examining gene expression levels in brain tissue found no evidence in support of B-cell alterations.

*Function of B-cells during brain development*

Although various lines of evidence have suggested that the immune system plays an important role in brain development, the specific function of lymphocytes during this process remains largely unknown.[8] It was recently discovered that B-1A cells, a specific subtype of B-cells appearing during fetal development and best characterized in mice, are highly present in neonatal mouse brains. These cells secrete natural IgM, which amongst other functions,

**Table 2.** Overview of other studies retrieved through systematic search on B-cells and schizophrenia.

| Year | Author | Patients | Controls | Material | Assessment | Significant B-cell related finding |
|---|---|---|---|---|---|---|
| **Studies on brain tissue** | | | | | | |
| 2012 | Busse[84] | 11 | 17 | Posterior hippocampus tissue | CD3, CD20 and HLA-DR staining | Increase in B-cells in patients with residual type schizophrenia (N=7) as compared with controls and patients with paranoid type schizphrenia |
| 2017 | Bogerts[85] | 20 | 20 | Tissue from various brain regions | CD3 and CD20 staining | 2/20 patients showed increased CD20+ cells in hippocampus tissue |
| **Studies on cytokines or other proteins in blood** | | | | | | |
| 2013 | Ramsey[18] | 133 | 133 | Serum | Multiplex immunoassay (190 different proteins measured) | Negative association between CXCL13 serum levels and PANSS scores in 54 female patients |
| 2015 | El Kissi[17] | 60 | 28 | Serum | ELISA (5 different cytokines measured) | Decrease in level of B-cell activating factor in 60 neuroleptic free patients |
| 2018 | Lago[50] | 25 (75 with other psychiatric disorder) | 100 | Peripheral blood mononuclear cells | High-throughput flow cytometry (1764 cell subtype-epitope-ligand combinations measured) | Functional dysregulation of AKT1 in B-cells of 25 patients |
| 2018 | Whelan[71] | 169 | 187 | Plasma | ELISA (15 different antibodies measured) | Increased levels of IgG against peptides derived from 6 genes genetically associated with schizophrenia as compared with controls |
| **Studies on DNA** | | | | | | |
| 2014 | Liu[48] | 98 | 108 | DNA from blood | Methylation assay (7562 CpG sites analyzed) | Hypermethylation of *LAX1* in patients as compared with controls |

seems to promote the proliferation of oligodendrocyte precursor cells. Blocking of the IgM Fc receptor on oligodendrocyte precursor cells resulted in less myelinated axons in neonatal mouse brains. The authors of this study note that these findings may be relevant for various developmental disorders in which white matter abnormalities have been found, including schizophrenia.[7]

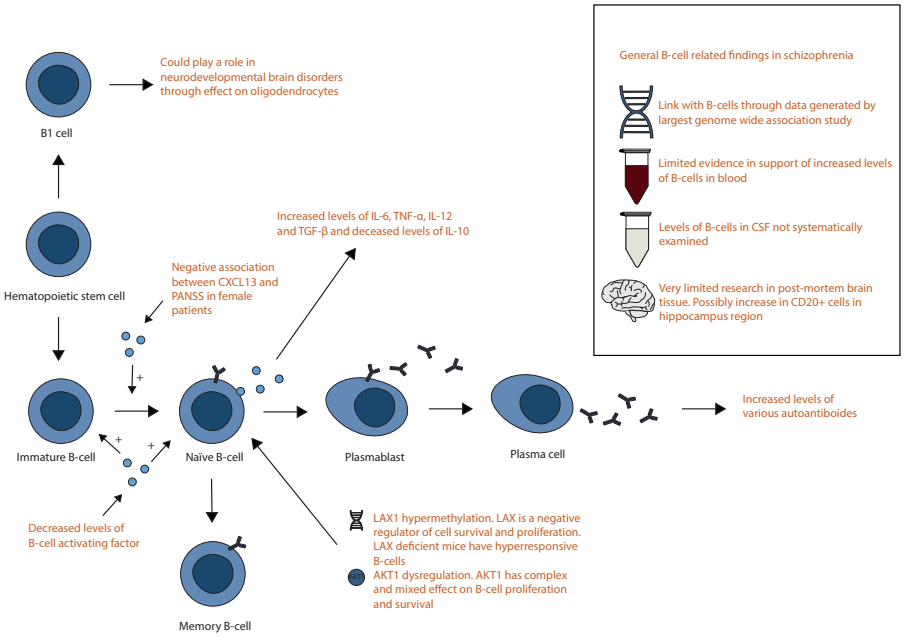


**Figure 3.** Overview of B-cell related findings in schizophrenia. Results from genetic studies suggest that B-cells might be involved in the etiology of schizophrenia. Increased levels of B-cells are not consistently found in blood of patients. Studies assessing B-cells in CSF and brain tissue have not been systematically conducted. There is some evidence in support of increased levels of CD20+ cells in the hippocampus region. Alterations in cytokines (B-cell activating factor and CXCL13), genes (hypermethylation of LAX1) and proteins (dysregulation of AKT1) involved in B-cell proliferation have been found. Increased levels of multiple cytokines, produced by various immune cells including B-cells, have been detected. Lastly, increased levels of various autoantibodies produced by plasmablast or plasma cells have been found.

## Discussion

In this narrative review we evaluated whether, apart from translational genetic findings, there is more evidence in support of a role for B-cells in the pathogenesis of schizophrenia. Few studies have directly assessed the phenotype or function of B-cells in patients with schizophrenia. Most studies

focused on measuring cell counts in blood and results of these studies do not seem to point towards an altered number of peripheral B-cells in schizophrenia. Studies on B-cells in CSF and brain tissue are limited and there is little evidence indicating parenchymal infiltrates of these cells. Increased levels of various cytokines and autoantibodies have been detected in patients with schizophrenia. Neuronal autoantibodies are known to lead to psychotic symptoms in some encephalitis patients and their role in schizophrenia is currently being investigated.

The studies included in this review have multiple potential drawbacks. Several studies consist of a rather small sample size and use heterogeneous methods and outcome measures. In addition to this, very few results have been replicated in multiple studies. Finally, none of the retrieved studies were primarily set-up to examine the role of B-cells in the pathogenesis of schizophrenia. Included studies therefore often only address one specific aspect of B-cell physiology in schizophrenia.

*Possible pathogenic mechanisms and future research*

Although recent genetic studies suggest that B-cells are associated with the pathogenesis of schizophrenia, there is a lack of a mechanistic framework explaining this association. Based on the current literature several potential mechanisms are of interest and warrant further research.

*Brain development*

Although the role of B-cells during early brain development is still largely unknown, a study in mice suggests that B-1A cells play an important role in axon myelination by stimulating oligodendrogenesis.[7] Schizophrenia is seen as a neurodevelopmental disorder and expression of related genes is strongly regulated during early neurodevelopmental phases.[88,89] In addition, several known environmental risk factors for schizophrenia occur during the pre-[90] and perinatal[91] period and white matter deficits, possible associated with neuro-inflammation, are found in affected patients.[92] Therefore, further studies on the functioning of B-cells during early development could be of interest in schizophrenia. A first step will be to replicate the presence of B-1A cells in human fetal brains and subsequently examine their gene expression profile for genes associated with schizophrenia. Furthermore, the development and

functioning of these cells could be studied in mouse models of schizophrenia risk genes, such as the 22q11.2 deletion model.

Another emerging topic of interest is the potential role of maternal autoantibodies in the occurrence of neurodevelopmental disorders. Although the current evidence does not directly support a link with schizophrenia, findings in autism[93] and mental retardation[94] are of interest given the aforementioned link between schizophrenia and pre- and perinatal complications.

*Immune activation*

As described, B-cell activation has been implicated in schizophrenia based on pathway analysis of genetic findings.[2] Genetic deficits combined with environmental triggers could cause over or under activation of this pathway resulting in increased auto reactivity, production of chemokines or activation of other immune cells. Interestingly, two studies using a hypothesis free approach found alterations in genes related to this pathway.[48,50] In addition to this, increased levels of IL-6 have been frequently associated with schizophrenia and this cytokine plays a role in B-cell related immune activation, although it is also expressed by various other immune cells.[75] Further functional studies using isolated B-cells or B-cell like cells derived from induced pluripotent stem cells from patients with a known genetic background should be performed to shed further light on possible alterations of the B-cell activation pathway in schizophrenia.



*Autoimmunity*

Autoreactive B-cells that have escaped tolerance mechanisms are capable of producing autoantibodies. As discussed, production of such autoantibodies can be triggered by environmental insults such as infections with bacteria or viruses, which has been recognized as a potential environmental risk factor for schizophrenia.[95] Evidence to date suggests that pathogenic neuronal autoantibodies only play a role in a small percentage of patients with psychosis and schizophrenia.[96] Other autoantibodies that do not directly disrupt neurotransmission could also trigger an immune response as described in neuropsychiatric SLE and result in cell-damage or –death.[97] Future studies on this topic should therefore focus on examining the

presence of such potentially pathogenic antibodies in CSF and brain tissue of patients with schizophrenia.

*Concluding remarks*

To draw a definite conclusion on the potential link between schizophrenia and B-cells further studies are needed. The current genetic findings justify a series of carefully conducted follow-up experiments for which suggestions have been done in this review. In addition to this, B-cells could be a potentially interesting target for treatment in schizophrenia, if future evidence supports the current genetic findings. Results of currently ongoing trials using immunosuppressive therapy in schizophrenia are therefore of great interest for this topic.

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# Chapter 6

## Early recognition of anti-N-methyl-D-aspartate receptor encephalitis in psychiatric patients

Hans C. van Mierlo[a], Maarten J. Titulaer[b], Marjan Kromkamp[a], Rianne van de Kraats[a], Nicoletta M. van Veelen[a], Saskia J. Palmen[a], René S. Kahn[a] and Lot D. de Witte[a]

[a] Department of Psychiatry, UMC Utrecht Brain Center, University Medical Center Utrecht, Utrecht, The Netherlands

[b] Department of Neurology, Erasmus Medical Center, Rotterdam, The Netherlands

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## Introduction and methods

Anti-NMDA receptor encephalitis (ANMDARE) is an important differential diagnosis in psychiatric patients,[1] which is emphasized by the recent paper of Jørgensen et al.[2] ANMDARE is caused by GluN1 IgG autoantibodies that lead to a characteristic multi-stage syndrome with both psychiatric and neurologic symptoms. For a more extensive description of the symptomatology we refer to Titulaer et al.[3] In the first phase of the disease psychiatric symptoms are most apparent and therefore ~75% of the patients are first seen by a psychiatrist.[1] Importantly, prompt initiation of immunosuppressive treatment improves outcome and therefore early recognition of ANMDARE is of great importance. By describing three cases and the result of screening a clinical cohort of patients with a (first) psychotic disorder for GluN1 antibodies, we highlight some important issues concerning the early recognition of ANMDARE in psychiatric patients.

## Results

Since the first description of ANMDARE in 2007 two female patients (23 and 38 years) and one male patient (18 years) were diagnosed with this disease at our hospital. The three patients were all initially admitted to a psychiatric ward with severe (manic) psychotic symptoms. Eventually they developed most of the symptoms typical for ANMDARE.[3] It took, however, considerable time until the disease was recognized. This was partly due to the unfamiliarity with this disorder, but also because some of the more subtle signs can be overlooked.

In retrospect, the patients already showed signs of ANMDARE early in the disease. They all had movement disorders, including typical orofacial dyskinesias, in two referred to as extra-pyramidal side effects. Mild or more severe signs of autonomic dysfunction were documented, but interpreted with a focus on the single parameter (e.g. 'tachycardia'), or as a sign of malignant neuroleptic syndrome. Catatonia was present in all patients and seen as part of a primary psychiatric disorder. Fluctuating consciousness was attributed to medication. Before the male patient developed evident seizures, twitching and uncontrolled movements were evaluated as of behavioral origin. Finally, more

sensitive than specific to ANMDARE, the onset of the psychiatric symptoms was relatively sudden (days-weeks), two patients had flu-like prodromal symptoms, the symptoms were heavily fluctuating and psychotic symptoms were resistant to antipsychotics.

Two patients were eventually admitted to the ICU because of status epilepticus or respiratory insufficiency. At this point, a non-psychiatric cause was highly suspected and extensive additional examinations were performed. In one female patient an ovarian teratoma seen on CT-scan was the clue for ANMDARE and serum GluN1 antibodies confirmed the diagnosis. In the male patient GluN1 antibodies were analyzed and positive in CSF, but not in serum. In the third patient the diagnosis was made four years after the onset of the disease, when a psychiatrist (MK) critically reviewed her medical history and Glun1 antibodies were still detectable, but only in CSF.

To improve the early recognition of ANMDARE, we decided to start screening for anti-NMDA-receptor autoantibodies in patients referred to our psychosis unit in January 2012. GluN1 IgG antibodies were analyzed in serum using a commercially available cell-based assay (CBA). Inconclusive or positive results were tested by immunohistochemistry as reported before.[4] In October 2014 we evaluated the additive value of this analysis. 127 patients with a psychotic disorder were screened, but none tested seropositive to GluN1 IgG. Of these 127 patients (93 males, 34 females) 82% were hospitalized and 64% had a first episode psychosis. Patients were characterized retrospectively for signs associated with ANMDARE [3]. These include: flu-like prodromal symptoms, seizures, movement disorders, signs of severe autonomic dysfunction, altered consciousness, pronounced symptom fluctuation, admission to an ICU, catatonia, acute onset severe psychosis, severe behavioral problems and a history of an ovarian teratoma. The majority (70%) of the patients in the cohort had none of the signs associated with ANMDARE. Seven patients showed between three and five of the associated signs, but none had more than five. The three described cases all had at least 7 of these signs.

## Discussion

The case reports underline that ANMDARE is present in psychiatric practice. Results from our routine screening do not indicate that serum GluN1 antibodies should be added to the diagnostic work-up of every patient with a psychotic disorder, but physicians should actively look for red flags to consider it. We cannot exclude that we missed cases by screening for serum GluN1, since only 85% of the ANMDARE patients has antibodies in serum[4] and this might be even lower for patients with isolated psychiatric symptoms (personal communication MJT). Therefore, larger CSF studies that examine the prevalence of ANMDARE in psychotic patients are needed.

In conclusion, psychiatrists should be aware of the symptoms and signs of ANMDARE and in case of a clinical suspicion test serum and CSF for GluN1. Importantly, CSF confirmation or different testing methods, including immunohistochemistry and live-neuron staining, should be applied to validate both positive and negative results.[5]

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# Chapter 7

## Absence of N-methyl-D-aspartate-receptor IgG autoantibodies in schizophrenia: the importance of cross-validation studies

Lot D. de Witte[a], Carolin Hoffmann[b*], Hans C. van Mierlo[a*], Maarten J. Titulaer[c], René S. Kahn[a] and Pilar Martinez-Martinez[b] for the European Consortium of Autoimmune Mental Disorders (CAIMED)

[a] Department of Psychiatry, UMC Utrecht Brain Center, University Medical Center Utrecht, Utrecht, The Netherlands

[b] Division Neuroscience, School for Mental Health and Neuroscience, Maastricht, the Netherlands

[c] Department of Neurology, Erasmus Medical Center, Rotterdam, the Netherlands

* Contributed equally to this work

The European Consortium of Autoimmune Mental Disorders members include Mario Losen (Division Neuroscience, School for Mental Health and Neuroscience, Maastricht, the Netherlands); Peter Molenaar (Division Neuroscience, School for Mental Health and Neuroscience, Maastricht, the Netherlands); Marc De Hert (University Psychiatric Centre Catholic University Leuven, Campus Kortenberg, Kortenberg, Belgium); Christian H. Roeder (Department of Psychiatry, Erasmus University Medical Center, Rotterdam, the Netherlands); Nico van Beveren (Department of Psychiatry, Erasmus University Medical Center, Rotterdam, the Netherlands); Bart P. F. Rutten (Division Neuroscience, School for Mental Health and Neuroscience, Maastricht, the Netherlands); Jim van Os (Division Neuroscience, School for Mental Health and Neuroscience, Maastricht, the Netherlands); and Pilar Martinez-Martinez (Division Neuroscience, School for Mental Health and Neuroscience, Maastricht, the Netherlands)

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## Introduction

Patients with anti–N-methyl-D-aspartate receptor (NMDAR) encephalitis usually develop characteristic neurologic and psychiatric symptoms. Some patients have isolated psychiatric symptoms, mimicking a schizophrenia-like psychotic episode.[1,2] Therefore, it has been hypothesized that a subgroup of patients diagnosed as having schizophrenia actually has anti-NMDAR encephalitis,[2] which would have important clinical implications. Anti-NMDAR encephalitis is caused by IgG-type autoantibodies to the GluN1 subunit of the NMDAR. Conflicting reports have been published regarding the seroprevalence of anti-NMDAR autoantibodies in schizophrenia, ranging from 0% to 8%.[2,3] Some studies that reported high seroprevalences included patients with schizophrenia with atypical features, such as seizures and movement disorders. We hypothesized that the use of a single antibody detection assay, without any further validation of the findings, may have contributed to the inconsistent findings.[3] Here, we investigated the presence of anti-GluN1 IgG autoantibodies in 475 patients with a schizophrenia spectrum disorder and included the cross-validation of positive samples.

## Methods

The current study included 3 cohorts. Cohort 1 involved 415 patients with schizophrenia who were screened over 2 years with a full laboratory screening for studying metabolic changes at the University Psychiatric Center, Katholieke Universiteit Leuven, Kortenberg, Belgium. Samples from this project have been used in the study by van Winkel et al.[4] To account for the pleiomorphic clinical presentation thought to arise from mental disorders associated with autoimmune encephalitis, we included cohort 2, which consisted of consecutively admitted patients who initially presented with psychosis and were finally diagnosed as having schizophrenia. These samples were collected at the Erasmus University Medical Center, Rotterdam, clinic for patients with suspected psychotic disorders. Samples from this project have been used in the study by Schwarz et al.[5] Cohort 3 was from the Genetic Risk And Outcome of Psychosis (GROUP) Study, a multicenter longitudinal study started in the Netherlands in 2006. Samples from this project have been used in the study by

Korver et al.[6] The cohorts described in this article were included in 2 different laboratories working simultaneously on the same research question.

The serum samples of cohorts 1[4] and 2[5] were analyzed using an in-house cell-based assay (CBA) in human embryonic kidney cells expressing human GluN1; cells were fixed with 4% paraformaldehyde. Serum samples were used in a dilution of 1:40. The CBA was validated using NMDAR-positive serum samples by interassay and interlaboratory assessment. The plasma samples of cohort 3[6] were analyzed by the CBA from EUROIMMUN (anti-glutamate receptor IIFT test), according to the manufacturer's protocol with a starting dilution of 1:10. Potentially positive samples were cross-validated by testing them in 2 other participating laboratories by EUROIMMUN CBA (1:10), by the in-house CBA performed as just described at 2 dilutions (1:40 and 1:10), and by immunohistochemistry on sagittal sections from rat brain fixed for 1 hour in 4% paraformaldehyde (1:200).[1]

Approval for this study was obtained from the institutional review boards of Erasmus Medical Center, University Medical Center Utrecht, and EPC KU Leuven; written patient consent was obtained.



## Results

The table shows the demographics of the cohorts. In cohorts 1 and 2, no patient sample was positive by CBA (Figure, A and E), whereas the results of 2 of 319 (0.6%) in cohort 3 tested positive in the EUROIMMUN CBA (Figure, B and F). The 2 samples were retested positive when using the same batch of the test and applying gradual dilutions (1:40 and 1:80) (data not shown). Further analyses of the samples in blinded studies by 2 independent laboratories using CBA (Figure, C and G) and immunohistochemistry (Figure, D and H) did not confirm the presence of the autoantibodies. Moreover, retesting the samples with a different batch of the EUROIMMUN CBA in another laboratory was negative (data not shown).

**Table 1.** Demographics, diagnostic assay and test results of patients in cohort 1, 2 and 3.

| Characteristic | Cohort 1 | Cohort 2 | Cohort 3 |
|---|---|---|---|
| Study | Van Winkel et al,[4] 2006 | Schwarz et al,[5] 2012 | Korver et al,[6] 2012 |
| Blood sample | Serum | Serum | Plasma |
| Patients | 110 | 46 | 319 |
| Gender M/F | 94/16 | 37/9 | 247/72 |
| Mean age (SD) in years | 26.2 (±7.5) | 27.4 (±8.7) | 30.6 (±7.0) |
| DSM-IV-TR diagnosis | | | |
| Schizophrenia | 45 | 40 | 250 |
| Schizophreniform disorder | 0 | 4 | 27 |
| Schizoaffective disorder | 10 | 2 | 42 |
| First episode psychosis | 55 | 0 | 0 |
| Mean duration of illness (SD) | 3.0 years (±4.1) | 2.63 years (±2.6) | 7.2 years (±3.7) |
| Laboratory | Maastricht | Maastricht | Utrecht |
| Method | In-house CBA | In-house CBA | EUROIMMUN CBA |
| Positive samples | 0 | 0 | 2 (but negative in cross-validation assays) |

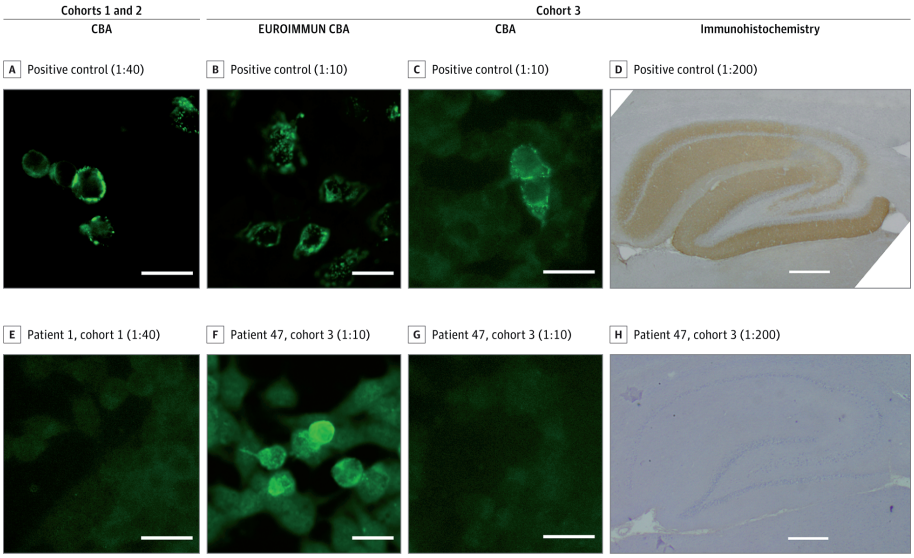


**Figure 1.** Representative Pictures of the Antibody Detection Assays. Cohorts 1 and 2 were analyzed by our in-house cell-based assay (CBA) by transfecting human embryonic kidney cells with GluN1 expression plasmid (cDNA from pENTR223.1-GRIN 1 subcloned in pcDNA3.1+; Invitrogen; V790-20 Life Technologies). Cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton-X100. Cells were incubated with patient serum followed by Alexa Fluor 488–labeled goat anti-human IgG antibody (1:1000, Molecular Probes). A patient with anti–*N*-methyl-$_D$-aspartate encephalitis was used

as a positive control (A) and schizophrenia serum samples from cohorts 1 and 2 were tested. None of the schizophrenia serum samples were found positive (eg, patient 1) (E). Cohort 3 was analyzed by the commercially available CBA from EUROIMMUN following the manufacturer's recommendations. The positive control from the kit (B) and 2 samples were positive in dilutions 1:10 up to 1:80, a representative picture of the 1:10 dilution from patient 47 is depicted in F. Retesting of the potential positive plasma samples by CBA was performed with the CBA described for cohorts 1 and 2 using a patient with anti-NMDAR encephalitis as a positive control (C) and 1:10 dilution from patient 47, which is negative (G). Scale bars in A-C and E-G = 25 µm. Immunohistochemistry on rat brain was performed with patient serum followed by secondary goat anti-human biotinylated IgG (Vector) dilution 1:2000. In D, the staining was performed using a patient with anti-NMDAR encephalitis (positive control, 1:200) and in H, patient 47 was used (1:200), the results for whom were negative; scale bars in D and H = 500 µm.

## Discussion

We showed that the prevalence of classic GluN1 IgG autoantibodies in the blood of patients with schizophrenia is very rare. Our results do not support the hypothesis that a significant subpopulation of those diagnosed as having schizophrenia are patients with misdiagnosed anti-NMDAR encephalitis. However, most patients with anti-NMDAR encephalitis are initially seen by a psychiatrist and within a month develop characteristic symptoms of the disease, leading to the diagnosis.[1,2] When nonfixed cells were used in the CBA, 23% of cases showed antibody-positive results without anti-NMDAR encephalitis (false-positive for this disease)[7]; in contrast, when fixed CBA and validation studies were used, no false-positive results were found.[1] Furthermore, it is known that antibody titers are higher during the acute phase of the disease and the results of 15% of patients test positive only in cerebrospinal fluid.[1] The main limitations of the current study were that cerebrospinal fluid was not available in these patient cohorts, most patients had chronic illness, and assessments of neurological symptoms were not available. Therefore, studies investigating cerebrospinal fluid and serum of a large number of acutely ill patients with first-episode psychosis are needed to draw the final conclusions on this topic. This study also demonstrated that the use of a single screening method may yield clinically irrelevant, false-positive results, especially in high-throughput screening with a low prior probability. Positive serum antibody results in patients with psychosis should be confirmed by alternative test methods and/or assays using cerebrospinal fluid samples.

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# Chapter 8

## No evidence for the presence of neuronal surface autoantibodies in plasma of patients with schizophrenia

Hans C. van Mierlo[a*], Marleen H. van Coevorden-Hameete[b*], Leon P. Munting[b], Esther de Graaff[b], Lot de Witte[a] and the GROUP investigators

[a] Department of Psychiatry, UMC Utrecht Brain Center, University Medical Center Utrecht, Utrecht, The Netherlands

[b] Department of Biology, Division of Cell Biology, Utrecht University

[*] Contributed equally to this work

Genetic Risk and Outcome of Psychosis (GROUP) Investigators

Richard Bruggeman[1], Wiepke Cahn[2], Lieuwe de Haan[3], René S. Kahn[2], Carin J. Meijer[3], Inez Myin-Germeys[4], Jim van Os[4], Durk Wiersma[1]

[1] University Medical Center Groningen, Department of Psychiatry, University of Groningen, The Netherlands

[2] University Medical Center Utrecht, Department of Psychiatry, Brain Center Rudolf Magnus, The Netherlands

[3] Academic Medical Center University of Amsterdam, Department of Psychiatry, Amsterdam, The Netherlands

[4] Maastricht University Medical Center, South Limburg Mental Health Research and Teaching Network, EURON, Maastricht, The Netherlands

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## Abstract

The immune system has been implicated in the etiology of schizophrenia. Autoimmunity by antibodies against neuronal cell surface antigens has been proposed as one of the pathological mechanisms. We examined plasma samples of 104 patients diagnosed with schizophrenia for the presence of autoantibodies against neuronal cell surface antigens using cultured hippocampal neurons and transfected HeLa cells. None of the samples tested positive for the presence of these autoantibodies. Based on our results it seems unlikely that autoantibodies against neuronal cell surface antigens play a role in the pathogenesis of schizophrenia, although further studies using cerebrospinal fluid are needed.

## Introduction

Different lines of evidence from the genetic,[1] epidemiological[2] and the immunological field[3] suggest that the immune system is involved in the pathogenesis of schizophrenia. However, the exact pathological mechanism explaining how the immune system contributes to schizophrenia is still unknown. In the 1960s autoimmunity was already proposed as a potential pathological mechanism.[4] More recent support for this theory is provided by studies showing a higher prevalence of autoimmune disorders in patients with schizophrenia[2] and the association of schizophrenia with genetic loci that are involved in adaptive immune responses and autoimmunity, including the MHC region.[1,5]

One of the key mechanisms involved in autoimmune disorders is the production of self-reactive antibodies, the so-called autoantibodies. Some neurological autoimmune disorders, such as anti-NMDA receptor encephalitis, are caused by autoantibodies targeting neuronal cell surface antigens. These pathogenic autoantibodies can impair the function of their target protein in various ways, for example by clustering and internalization of the receptor leading to diminished cell surface expression (for review see[6]). This leads to changes in synaptic transmission and neuronal excitability, resulting in neurologic and/or psychiatric symptoms. Isolated episodes with psychotic symptoms can occur in patients with anti-NMDA receptor encephalitis.[7] It has therefore been postulated that neuronal surface autoantibodies could cause a clinical syndrome similar to schizophrenia.[8]



Several groups have investigated the presence of neuronal autoantibodies in patients with schizophrenia. The presence of antibodies against brain antigens in general has been examined by incubating rat brain tissue slices with patients' sera. Anti-brain antibodies were found to be increased in some studies but not in others, thoroughly reviewed elsewhere.[9] The last decade, multiple studies have focused on the seroprevalence of autoantibodies targeting specific neuronal surface antibodies, such as NMDA,[10] dopamine, AMPA and GABA receptor antibodies,[11,12] with inconclusive evidence.

We hypothesized that known and/or yet unknown autoantibodies against neuronal cell surface antigens are involved in the pathogenesis of schizophrenia in a subgroup of patients. In this study we therefore set out to examine the prevalence of neuronal autoantibodies by screening plasma of 104 patients with schizophrenia using live rat hippocampal neurons. In addition, cell-based assays (CBA) were used to test these samples for autoantibodies against a selection of 24 neuronal cell surface antigens that have been associated with schizophrenia in GWAS studies.[13–16]

## Methods

*Participants*

Plasma samples from a subgroup of 104 patients diagnosed with schizophrenia and schizophreniform disorder that participated in the Genetic Risk and Outcome of Psychosis (GROUP) study in the Netherlands were used for this study. Further details of this study are described elsewhere [17]. In brief, inclusion criteria for patients participating in the original study were: (1) Age range of 16 to 50 years, (2) a diagnosis of non-affective psychotic disorder according to DSM-IV criteria, (3) good command of the Dutch language, (4) and able and willing to give written informed consent. A plasma sample of a patient with autoimmune encephalitis caused by anti-GluR1 antibodies was included as positive control.

*Commercial antibodies*

The following antibodies were used in this study: mouse anti-myc (Santa Cruz Biotechnology, 9E10), mouse anti-v5 (Life Technologies, R960-25) and Alexa 488- and Alexa 568- conjugated anti-mouse and -human IgG secondary antibodies (Life Technologies).

*DNA constructs*

DNA constructs for the 24 candidate antigens, including a tag to identify transfected cells, were either present in our lab, or a gift from other laboratories. Table 1 depicts the specifications of all DNA constructs used in this study.

**Table 1.** DNA constructs used in the cell-based assay.

| Protein | Gene | Species | Vector | Tag | Source |
|---|---|---|---|---|---|
| Glutamate receptor 1 | Gria1 | rat | pGW2 | GFP | Casper Hoogenraad, Universiteit Utrecht, Netherlands |
| Glutamate receptor 2 | Gria2 | rat | pGW2 | GFP | Casper Hoogenraad, Universiteit Utrecht, Netherlands |
| Metabotropic Glutamate receptor 1 | GRM1 | human | pGW2 | GFP | Casper Hoogenraad, Universiteit Utrecht, Netherlands |
| Metabotropic Glutamate receptor 3 | GRM3 | human | pGW2 | GFP | Casper Hoogenraad, Universiteit Utrecht, Netherlands |
| Metabotropic Glutamate receptor 5 | Grm5 | mouse | pGW2 | GFP | Casper Hoogenraad, Universiteit Utrecht, Netherlands |
| Glutamate Receptor Ionotropic, Kainate 3 | Grik3 | rat | pcDNA3.1 | Myc | Christophe Mulle, Université Bordeaux, France |
| Glutamate Receptor Ionotropic, NMDA 1 | Grin1 | rat | pEGFP | YFP | Casper Hoogenraad, Universiteit Utrecht, Netherlands |
| Glutamate Receptor Ionotropic, NMDA 2a | Grin2a | rat | pGW2 | GFP | Casper Hoogenraad, Universiteit Utrecht, Netherlands |
| Glutamate Receptor Ionotropic, NMDA 2b | Grin2b | rat | pGW2 | GFP | Casper Hoogenraad, Universiteit Utrecht, Netherlands |
| Glutamate Receptor Ionotropic, NMDA 3 | Grin3a | rat | pEGFP | GFP | Casper Hoogenraad, Universiteit Utrecht, Netherlands |
| Neuronal Membrane Protein M6a | Gpm6a | rat | pEGFP | GFP | Camila Scorticati, Universidad nacional de San Martín, Argentina |
| Glutamate Receptor Ionotropic, Kainate 4 | GRIK4 | human | pGW2 | GFP | Casper Hoogenraad, Universiteit Utrecht, Netherlands |
| Brain-derived neurotrophic factor | Bdnf | rat | pEGFP | GFP | Casper Hoogenraad, Universiteit Utrecht, Netherlands |
| Receptor tyrosine-protein kinase erbB4 | ERBB4 | human | pEGFP | GFP | Frank Jones, Tulane University Health Sciences Center, New Orleans, USA |
| Kv channel-interacting protein 1 | KCNIP1 | human | pcDNA3.1 | v5 | Priit Pruunsild, University of Heidelberg, Germany |
| Kv channel-interacting protein 2 | KCNIP2 | human | pcDNA3.1 | v5 | Priit Pruunsild, University of Heidelberg, Germany |
| Kv channel-interacting protein 3 | KCNIP3 | human | pcDNA3.1 | v5 | Priit Pruunsild, University of Heidelberg, Germany |
| Kv channel-interacting protein 4 | KCNIP4 | human | pcDNA3.1 | v5 | Priit Pruunsild, University of Heidelberg, Germany |
| 5-Hydroxytryptamine Receptor 4 (isoform d) | HTR4 | human | pcDNA3.1 | YFP | Frank Lezoualc'h, Inserm, France |
| 5-Hydroxytryptamine Receptor 4 (isoform e) | HTR4 | human | pcDNA3.1 | YFP | Frank Lezoualc'h, Inserm, France |
| Neurexin-1 | Nrxn1 | rat | unknown | GFP | Edwin Chapman, Howard Hughes Medical Institute, USA |
| D (1A) Dopamine Receptor | DRD1 | human | pIRES2-GFP | GFP | Fabienne Brilot, University of Sydney, Australia |
| D (2) Dopamine Receptor | DRD2 | human | pIRES2-GFP | GFP | Fabienne Brilot, University of Sydney, Australia |
| D (1B) Dopamine Receptor | DRD5 | human | pIRES2-GFP | GFP | Fabienne Brilot, University of Sydney, Australia |

*Immunocytochemistry of live primary hippocampal neurons*

Cultures of primary hippocampal neurons were prepared from embryonic day 18 rat brains. Neurons were grown in Neurobasal medium (Life Technologies) supplemented with B27, 0.5 μM glutamine, 12.5 μM glutamate and penicillin/streptomycin. Neurons were plated on coverslips coated with poly-L-lysine (30μg/ml) and laminin (2μg/ml) at a density of 75,000/well. Neurons were then incubated with plasma (1:50) in unconditioned medium for 1 hour at 37°C, washed in medium and fixed for 10 minutes with 4% paraformaldehyde (PFA)/4% sucrose at room temperature. Cells were incubated with the secondary-antibody in GDB buffer (0.1% bovine serum albumin (BSA), 0.4M NaCl, 15mM phosphate buffer, pH 7.4) for 1 hour at room temperature. Neurons were then washed in PBS and mounted on slides in Vectashield mounting medium containing DAPI (Vector Laboratories).

*Cell-based assay*

HeLa cells were cultured in Ham's F10/DMEM (50%/50%) containing 10% fetal calf serum and 1% penicillin/streptomycin. HeLa cells were detached using trypsin/EDTA and were plated on 16 well Tissuetek chamber slides (Thermoscientific) and were transfected using Polyethylenimine (1 mg/ml, PEI max, Polysciences, 24765-2). Cells were fixed for 10 minutes using 4% PFA and incubated overnight at 4°C with patient plasma (1:400) in PBS+ (PBS with 1% BSA and 0,1% triton). Cells transfected with Grik3 or KCNIP constructs were also incubated with mouse anti-myc tag or mouse anti-v5 tag primary antibodies. After washing, cells were incubated with secondary antibodies in PBS+ for 1 hour at room temperature. Cells were washed and mounted in Vectashield mounting medium containing DAPI (Vector Laboratories).

*Imaging*

Both the HeLa cells and the neurons were scored using a Nikon eclipse 80i. Confocal images were acquired with the Zeiss LSM 700 using the 40x (oil) objective.

## Results

We included plasma samples of 104 patients, 102 patients were diagnosed with schizophrenia and 2 patients were diagnosed with schizophreniform disorder. The cohort consisted of 81 males and 23 females. The average age of the included patients was 30.7 years (SD ±7.6, range 20-55). The average duration of illness was 7.1 years (SD ±3.9, range 2.1-23.4).

Plasma samples were examined for surface staining by live incubation on primary hippocampal neurons. The positive control showed the typical surface staining seen in patients with neuronal autoantibodies (Figure 1A). All samples in the cohort showed an aspecific background staining of which one example is shown in Figure 1B. None of the 104 plasma samples were positive using this approach. The plasma samples were next tested for autoantibodies against 24 neuronal antigens using CBA. The different tags identified the transfected cells. The positive control showed an increased staining of HeLa cells cotransfected with GluR1 and GluR2 as shown in Figure 2A. None of the plasma samples were positive for autoantibodies against any of the 24 proteins, of which one example is shown in Figure 2B.

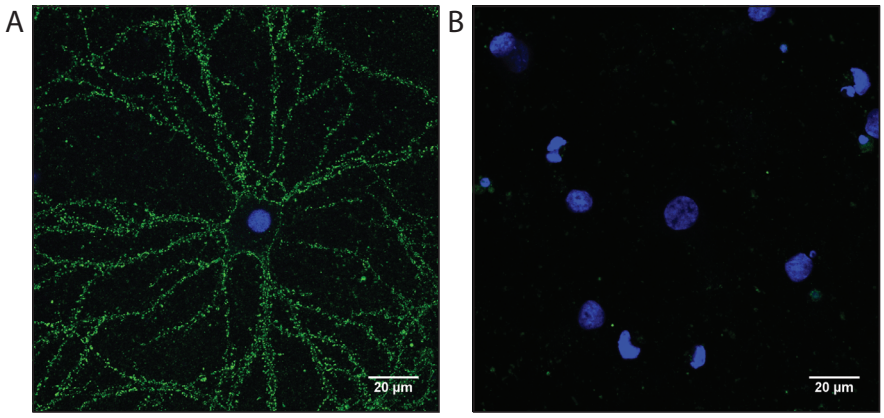


**Figure 1. A.** Representative picture of surface labeling of rat hippocampal neurons by plasma from the positive control. **B.** Typical example of background staining by a plasma sample from schizophrenia cohort. Human antibodies labeled green, nuclei blue.

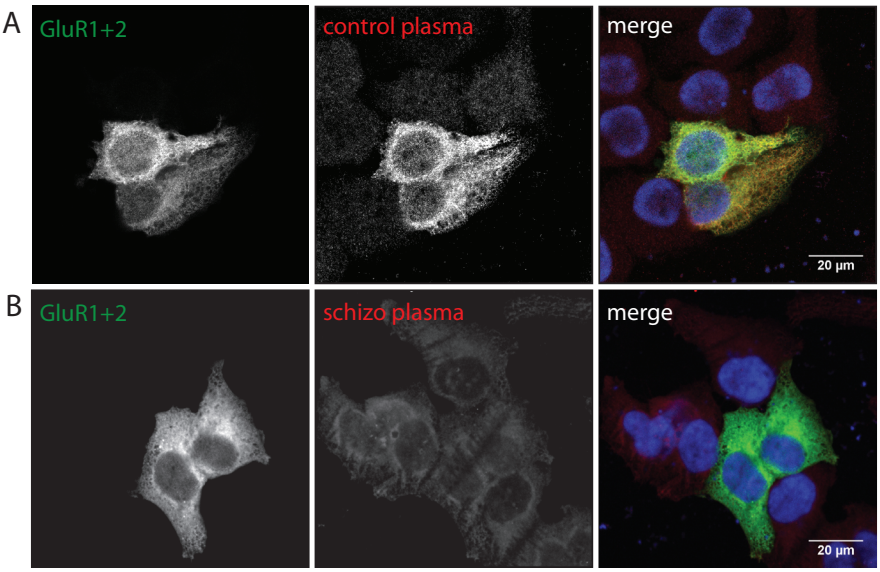


**Figure 2. A.** Representative picture of increased staining of HeLa cells cotransfected with AMPA receptor subunits GluR1 and GluR2 by plasma from the positive control with anti-GluR1 antibodies. **B.** Typical example of background staining by a plasma sample from the schizophrenia cohort. Transfected cells labeled green, human antibodies red, nuclei blue.

## Discussion

In this study we examined the prevalence of autoantibodies against neuronal cell surface antigens in patients diagnosed with schizophrenia or schizophreniform disorder. Plasma samples of 104 patients were screened using cultured hippocampal neurons and a CBA using 24 candidate neuronal membrane proteins. None of the samples were positive in either of these assays.

One other study used a similar approach by screening sera of patients with schizophrenia for neuronal autoantibodies using cultured hippocampal neurons and rat brain slices. In this study 4/80 patients and 4/40 controls showed reactivity with neuronal surface antigens, unfortunately the targets of these autoantibodies have not been identified and therefore their clinical relevance remains unknown.[18] However, the fact that these antibodies occurred more frequent in controls does not support a role for antibodies in the pathogenesis of schizophrenia.

Using the CBA we examined the presence of several known and unknown autoantibodies. In line with some prior studies we did not find autoantibodies against subunits of the NMDA-receptor[18,19] or subunits of the dopamine, AMPA and GABA receptor.[11] However, there is still an ongoing debate about the presence of anti-NMDA receptor antibodies in patients with schizophrenia as some studies reported a seroprevalence of up to 10%.[20,21] It is important to stress that the majority of the seropositive patients had antibodies of the IgA and IgM subclass, whereas only antibodies of the IgG class seem to be clinically relevant.[22–24] Therefore, in our study only antibodies of the IgG subclass were assessed.

Strengths of our study include the use of two different screening methods, offering the opportunity to explore the presence of yet unknown autoantibodies and to further validate findings on known autoantibodies. In addition, positive findings on surface autoantibodies found using a CBA can be validated using hippocampal neurons. A possible shortcoming of our study is the use of plasma samples instead of cerebrospinal fluid (CSF). For anti-NMDA receptor antibodies it has been shown that testing CSF samples has a higher sensitivity[23] and this may also apply for other neuronal autoantibody mediated disorders. Moreover, most included patients were diagnosed years ago. It seems worthwhile to perform a similar screening in acutely ill patients with a first episode psychosis, preferably using both blood and CSF samples, as some autoantibodies might only be detectable in CSF in an early phase of the disease. In addition, some types of neuronal autoantibodies have a higher prevalence in specific subgroups, for example anti-NMDA receptor antibodies in females with a teratoma.[25] Future studies could therefore focus on specific subgroups of patients with schizophrenia or psychosis, such as patients with a post-partum psychosis,[26] with neurologic or other characteristic signs of autoimmune encephalitis[27] or with a history of a malignancy.[25] Lastly, performing a similar screening in patients with bipolar disorder could prove to be of value.[28]

In conclusion this study does not support a role for autoantibodies against neuronal cell surface antigens in the pathogenesis of schizophrenia. However, studies using cerebrospinal fluid from a large group of first episode patients are needed to further evaluate the potential role of autoantibodies in schizophrenia.

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# Chapter 9

## Summary and general discussion

In this chapter a summary of the main findings described in this thesis is given. This summary is followed by a general discussion on autoimmunity in schizophrenia based on the relevant literature. Lastly, several recommendations for future studies are given.

## 9.1 Summary

In **chapter 1** the topic of this thesis is introduced and an overview of the literature that links schizophrenia and the immune system is given. Several types of studies, including genetic, epidemiologic, translational and imaging studies provide evidence in support of this hypothesis, although their results are not unambiguous and several confounding factors could play a role in their findings. In addition to this, it remains unclear whether immune system aberrancies found in schizophrenia are a causative factor or merely a circumstantial consequence of the disorder or other environmental factors.

Previous studies have reported an increased prevalence of antinuclear antibodies in patients with schizophrenia, although these studies often consisted of a small sample size. Various specific autoantibodies underlie these antinuclear antibodies, and not of all of them have been identified thus far. The presence of antinuclear antibodies is used as a supportive diagnostic marker in various autoimmune disorders. In **chapter 2** the prevalence of antinuclear antibodies was measured using indirect immunofluorescence in 368 patients with a schizophrenia spectrum disorder and 283 healthy controls. Positive cases were subsequently tested for the presence of 14 specific antibodies. In conclusion, no association was found between the presence of these antibodies and schizophrenia. The potential influence of age, gender and use of medication was also examined, but did not alter these findings.

Routine laboratory investigations in patients with a psychotic disorder could be of aid in detecting relevant comorbid disorders or cases of psychosis due to a general medical condition, such as an infection or autoimmune disorder. The retrospective study in **chapter 3** describes the results of extensive routine laboratory investigations, including 23 tests, conducted in a cohort

of 215 patients with a psychotic disorder. Most of these patients had a first episode psychosis. These routine laboratory investigations did not result in the identification of cases of psychosis due to a general medical condition. Measuring anti-thyroid peroxidase (anti-TPO) and anti-nuclear antibodies or general markers of inflammation such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) in this cohort did not result in any relevant alterations in clinical care.

Pathogens that are capable of infecting the central nervous system have long been suggested as a potential environmental risk factor for schizophrenia. These pathogens could have a direct pathogenic effect on the brain or exposure to these pathogens could result in an aberrant or persisting immune response causing secondary damage to brain. One method of examining prior exposure to such pathogens is by measuring the levels of immunoglobulin G in serum directed against these pathogens. However, previous studies on this topic often did not account for important potential confounding factors. **Chapter 4** describes the results of examining the seroprevalence and titer of IgG antibodies against herpes simplex virus-1 and -2 (HSV-1/HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV) and *Toxoplasma gondii* (TG) in plasma of 368 adult patients with a schizophrenia spectrum disorder and 282 controls using enzyme-linked immunosorbent assay (ELISA). Results show a slightly higher seroprevalence of VZV and CMV in controls as compared to patients. No significant differences were found in the seroprevalence of the other pathogens, these results remained unaltered after adjustments for various potential confounders.

In **chapter 5** an overview of the studies on the role and functioning of B-cells in schizophrenia is given, as results from the largest genome wide association study on schizophrenia suggest that these cells might be involved in the pathogenesis of schizophrenia. However, the current evidence on this topic is limited. Most studies on this topic measure B-cell counts in blood of patients with schizophrenia and studies using CSF or brain tissue are small and lack reproduction of their findings. The functioning of B-cells in CSF, brain tissue or in-vitro models of patients with schizophrenia has not been systemically assessed. Based on findings from various autoimmune disorders several mechanisms are discussed through which B-cells could be involved in the

pathogenesis of schizophrenia, these include: the production of antibodies, activation of other immune-cells or by modifying brain development.

Anti-NMDA receptor encephalitis is the most prevalent type of antibody-mediated autoimmune encephalitis and its discovery has had a major impact on both neurological and psychiatric practice. As this disorder can present with severe psychiatric symptoms, it is sometimes misdiagnosed as psychosis or mania. However, timely recognition has major implications for treatment and prognosis. To further examine how often this diagnosis remains unrecognized in psychiatric clinics, the results of routinely screening for antibodies against the GluN1 subunit of the NMDA-receptor in patients admitted to a first-episode psychosis clinic are described in **chapter 6.** In total 127 patients were screened for these antibodies using a cell-based assay, and immunohistochemistry when results were inconclusive. In this cohort 0 patients tested positive for these antibodies. In addition to this, three cases of patients with anti-NMDA receptor encephalitis with an onset with severe psychiatric symptoms are described. Their symptoms are compared to those of the patients in the cohort. Based on these findings several red flags, that warrant testing for anti-NMDA receptor encephalitis in patients with a first episode psychosis, are discussed.

Findings on the prevalence of anti-NDMA receptor encephalitis in research cohorts of patients with psychotic symptoms remain inconsistent, which might be due to a lack of validation of positive results. **Chapter 7** describes the results of screening serum or plasma for GluN1 antibodies in 3 research cohorts of patients with psychotic symptoms. In total, 475 patients were included and the cohorts consisted of patients with a first episode psychosis and patients with schizophrenia with a long duration of illness. Results showed 2 cases that tested positive for GluN1 antibodies, using a commercially available cell-based assay, but these findings could not be validated using immunohistochemistry. These results do not suggest that a significant subpopulation of patients with schizophrenia actually have an unrecognized form of anti-NMDA receptor encephalitis. In addition to this, the results of this study stress the need to perform cross-validation of positive results and to set up studies using CSF.

In **chapter 8** an effort is made to examine the relevance of other anti-neuronal membrane antibodies, including antibodies against subunits of the dopamine, AMPA and GABA receptor, in patients with schizophrenia or a schizophreniform disorder. Plasma samples of 104 patients were screened for such antibodies, using cultured hippocampal neurons and a cell-based assay using 24 candidate proteins. None of the included samples showed positive results. These results suggest that anti-neuronal membrane antibodies do not play a major role in the pathogenesis of schizophrenia.

## 9.2 Peripheral non-neuronal autoantibodies in schizophrenia

*9.2.1 Limitations of studies on this topic*

Several studies have found an increased prevalence of various non-neuronal autoantibodies in blood of patients with schizophrenia. As described in chapter 2 we were unable to replicate these findings for anti-nuclear antibodies. Small number of subjects, lack of a relevant control group, lack of reproduction studies, and differences in techniques or cutoff values used often hamper studies on autoantibodies in blood of patients with schizophrenia. It therefore remains uncertain whether increased levels of various non-neuronal autoantibodies that have been identified in blood of patients with schizophrenia have any clinical importance.[1]

*9.2.2 Interpretation of alterations in peripheral non-neuronal autoantibodies*

How findings on these autoantibodies in schizophrenia should be interpreted also remains a subject of debate. There are several reasons that could explain their presence in schizophrenia, these include: 1) Autoantibodies could be a marker of autoimmunity or increased activity of the immune system, as is the case in some autoimmune disorders. Such autoantibodies could arise from auto-reactive B-cells escaping central tolerance mechanisms. However, antibody production could also be triggered by processes such as cell death resulting in extracellular exposure of self-antigens, alterations in self-antigens through inflammation or by cross reaction after exposure to foreign antigens.[2] 2) Peripheral non-neuronal autoantibodies could be directly involved in the pathogenesis of the disease, through a still unknown mechanism

3) Their presence is an effect of the progressing disease 4) Their presence is a side-effect of certain environmental factors associated with the disease.[3,4]

### *9.2.3 Environmental factors that influence peripheral non-neuronal autoantibodies*

One potential environmental factor that seems to be of relevance in findings concerning peripheral autoantibodies is the use of psychotropic medication. A clear example of this is the association between antinuclear antibodies and the use of chlorpromazine, which is further discussed in chapter 3. Whether these autoantibodies only occur during treatment with chlorpromazine, or also persist during a certain timeframe after treatment, remains uncertain. Interestingly, other studies have shown that the use of other antipsychotics, including risperidon and clozapine, has been associated with the occurrence of antiphospholipid antibodies.[5–7]

On a more general note it has been suggested that antipsychotic medication has at least some immunomodulatory properties, as its usage has been associated with suppressing certain cytokines such as IL-1β, IL-6 and TGF-β.[8,9] The immune modulatory properties of clozapine, which have been found both in vivo and in vitro, have gained some specific interest in the literature, but the mechanisms that cause these effects still remain unclear.[10] Antipsychotics however, also increase the risk of weight gain and diabetes, disorders associated with increased inflammation.[11]

Other factors that could be of relevance in the occurrence of autoantibodies and that should be addressed in future studies on this topic include age, ethnicity, gender, smoking and BMI of included subjects.[1] One large cross-sectional study, examining the presence of antinuclear antibodies in the general population, found a higher prevalence of antinuclear antibodies among females, elderly, African Americans and people without obesity.[12] Smoking has been associated with the increased presence of autoantibodies in the autoimmune disorders SLE and rheumatoid arthritis.[13,14]

### *9.2.4 Clinical relevance of measuring peripheral non-neuronal autoantibodies*

The clinical relevance of non-neuronal autoantibodies in schizophrenia might increase if they could be used as a supportive diagnostic or treatment marker,

perhaps in combination with other immune, imaging or genetic markers.[3,15] However, differences in the prevalence rates of these autoantibodies between patients and controls are often small. Therefore autoantibodies with a higher prevalence in schizophrenia identified thus far are not usable for such a purpose.[1] High-throughput screening techniques applied for discovering novel peripheral autoantibodies or testing for autoantibodies against specific proteins associated with schizophrenia has also not resulted in identifying autoantibodies suitable for this purpose yet.[16,17]

Another function of testing for these autoantibodies could be detecting psychosis due to a general medical condition.[18] The results of one previous study suggested that screening patients with a first episode psychosis for antinuclear antibodies could be of value in timely recognizing the neuropsychiatric variant of the autoimmune disorder systemic lupus erythematous. The authors identified 2 cases of this disorder in a cohort of 85 patients with a first episode psychosis by screening for these antibodies.[19] In the study described in chapter 3, these findings could not be reproduced, which could be due to differences in cohort characteristics or referral bias.

*9.2.5 Future directions for studies on peripheral non-neuronal autoantibodies*

At present it is unclear whether the frequencies and titers of peripheral non-neuronal autoantibodies are altered in schizophrenia. In addition to this, uncertainty remains about how alterations in these autoantibodies should be interpreted and what their clinical significance is.

Additional studies, without the aforementioned shortcomings, are needed. It would be of relevance to perform large-scale longitudinal studies with multiple measurements to further examine the potential influence of disease-phase and environmental factors on the presence of these autoantibodies in schizophrenia. Such studies may also be more successful in identifying autoantibodies that are suitable as a biomarker. However, one could also argue that research on autoantibodies in the brain and CSF should be given priority, as such autoantibodies are more likely to play a direct role in the disease pathology.

## 9.3 Antibodies against neurotropic pathogens in schizophrenia

*9.3.1 Measuring exposure to neurotropic pathogens*

The results of the study described in chapter 4 do not support the notion that patients with schizophrenia spectrum disorders are more frequently exposed to several neurotropic pathogens, including HSV-1, HSV-2), VZV, EBV, CMV and TG. Previous studies have described findings both in agreement and opposing these results.[20–23] Especially for TG there are many studies in support of an association with schizophrenia, as shown in a meta-analysis on this topic.[20] It should however be noted that there a large methodological differences between the studies conducted on this topic, as immunoglobulin levels against different pathogens have been measured in maternal, newborn and adult blood samples using various techniques.[22]

Although the study described in this thesis included a considerable amount of subjects and accounted for several import potential confounding factors such as size of household, educational level and living area, additional longitudinal studies are needed to draw a definitive conclusion on this topic. Such research should be prioritized before conducting further, more functional, follow-up studies on the role of these pathogens in schizophrenia.

Of further interest is the fact that there are regional differences in the prevalence of certain strains of TG. One meta-analysis found a significant association between TG and schizophrenia after controlling for potential publication bias and concluded that additional studies examining the prevalence of specific TG strains could be of use.[20]

*3.2 Clinical relevance of neurotropic pathogens*

If neurotropic pathogens are involved in the pathogenesis of schizophrenia, their detection could aid in diagnosing specific subgroups of patients. In addition to this, these pathogens could provide novel targets for prevention and treatment. Some studies have therefore already assessed the additional value of treating patients with schizophrenia with antimicrobial agents.[24] It is, however, important to note that studies that did find positive results regarding the prevalence of neurotropic pathogens in schizophrenia often only detected

marginal or small differences between patients and controls.[22] This would imply that neurotropic pathogens that form a larger risk factor have not been identified yet or that exposure to neurotropic pathogens is one of the many risk factors for schizophrenia with quite a small effect.

*9.3.3 Future directions for studies on neurotropic pathogens*

An important limitation of the studies currently conducted on this topic is their cross-sectional design. As neurotropic pathogens are hypothesized to contribute to the pathogenesis of schizophrenia, additional data on the moment of exposure to these pathogens is needed. One way to address this would be to set up a large prospective longitudinal multi-site cohort following a random selection of newborns until (late) adulthood. During follow-up regular blood samples should be acquired to monitor when infections with neurotropic pathogens occur and to measure antibody class and levels. These data should then be compared between subject that do and do not develop schizophrenia. In addition to this, data on relevant confounders should be collected.

## 9.4 Autoantibodies against neuronal surface-proteins in schizophrenia

*9.4.1 Prevalence of neuronal autoantibodies*

The discovery of anti-NMDA receptor encephalitis has led to great enthusiasm for the idea that a part of the patients with schizophrenia might have a forme fruste of this treatable autoimmune disorder.[25] This has resulted in several studies examining the prevalence of autoantibodies directed against the GluN1 subunit of the NMDA-receptor, and other neuronal proteins, in patients with psychotic disorders and schizophrenia. These studies have shown divergent results, as depicted in table 1. There are several reasons that could explain these discrepancies and these will be discussed in the following paragraphs.

**Table 1.** Overview of studies examining the prevalence of IgG antibodies against the NMDA-receptor in patients with a schizophrenia spectrum disorder or a first episode psychosis.

| Year | Author | Cohort | Sample | Testing method | Patients | Positive | Controls | Positive | Remarks regarding positive cases |
|---|---|---|---|---|---|---|---|---|---|
| 2011 | Zandi[26] | FEP | Serum | CBA (live) | 46 | 3 | NA | NA | No titers described. No validation in CSF. No cases with tumor or seizures. |
| 2011 | Rhoads[27] | SCHZ | Serum | CBA (fixed) | 7 | 0 | 3 | 0 | |
| 2012 | Haussleiter[28] | PD | Serum | CBA (fixed) | 50 | 0 | NA | NA | |
| 2012 | Masdeu[29] | SCHZ | Serum | CBA (fixed), IHC, ICC | 80 | 0 | 40 | 0 | |
| 2012 | Tsutsui[30] | SSD | Serum | CBA (fixed) | 51 | 4 | NA | NA | No titers described. No validation in CSF. 2 cases with ovarian tumor and 2 cases with seizures. |
| 2013 | Steiner[31] | SCHZ | Serum | CBA (fixed) | 121 | 4 | 230 | 0 | Titer range: 1:100 – 1:3200. 2/4 positive in CSF. No cases with tumor or seizures. |
| 2014 | Dahm[32] | SCHZ | Serum | CBA (fixed) | 1378 | 8 | 1703 | 20 | Titer range: 1:10 – 1:320. No validation in CSF. Tumor or seizures not described. |
| 2014 | Hammer[33] | SCHZ | Serum | CBA (fixed) | 1081 | 7 | 1325 | 5 | Titer range: 1:10 – 1:320. No validation in CSF. Tumor or seizures not described. |
| 2015 | Masopust[34] | FEP | Serum | CBA (fixed) | 50 | 0 | 50 | 0 | |
| 2015 | van Mierlo[35] | PD | Serum | CBA (fixed), IHC validation | 127 | 0 | NA | NA | |
| 2015 | de Witte[36] | FEP and SSD | Serum | CBA (fixed), IHC validation | 475 | 0 | NA | NA | |
| 2016 | Ando[37] | SSD | Serum | CBA (fixed) | 59 | 6 | NA | NA | Titer range: 1:10 – 1:1000. No validation in CSF. Tumor or seizures not described. |
| 2016 | Arboleya[38] | FEP | Serum | CBA (fixed), IHC validation | 61 | 2 | 47 | 0 | Titer range: 1:20 – 1:320. 1/2 cases validated and positive in CSF. 1 case with ovarian tumor and (pseudo) seizures. |
| 2017 | Chen[39] | FEP and SCHZ | Serum | CBA (fixed), IHC | 312 | 0 | NA | NA | |
| 2017 | Endres[40] | PD | CSF | CBA (live and fixed) | 180 | 1 | NA | NA | No titer described. Case with seizures. |

**Table 1.** Continued

| Year | Author | Cohort | Sample | Testing method | Patients | Positive | Controls | Positive | Remarks regarding positive cases |
|---|---|---|---|---|---|---|---|---|---|
| 2017 | Jézéquel[41] | FEP | Serum | CBA (live) | 298 | 14 | NA | NA | No titers described. No validation in CSF. Tumor or seizures not described. |
| 2017 | Jézéquel[42] | SCHZ | Serum | CBA (live) | 48 | 9 | 104 | 3 | Titer range: 1:20 – 1:320. 5/12 cases validated but negative in CSF. No cases with tumor or seizures. |
| 2017 | Lennox[43] | FEP | Serum | CBA (live) | 228 | 7 | 105 | 0 | Titer range: 1:30 – 1:150. No validation in CSF. No cases with tumor or seizures. |
| 2018 | Hara[44] | SCHZ | Serum | CBA (fixed), IHC, ICC validation | 50 | 0 | NA | NA | |
| 2018 | Mantere[45] | FEP | Serum | | 70 | 0 | 34 | 0 | |
| 2018 | Oviedo-Salcedo[46] | SSD | CSF | CBA (fixed) | 124 | 0 | NA | NA | |
| 2018 | Scott[47] | FEP | Serum | CBA (fixed), IHC | 113 | 4 | NA | NA | No titers described. 3/4 positive in CSF. 2 cases with ovarian tumor and seizures. |

FEP = first episode psychosis, SCHZ = schizophrenia, PD = psychotic disorder, SSD = schizophrenia spectrum disorder, CBA = cell-based assay, IHC = immunohistochemistry, ICC = immunocytochemistry

*9.4.2 Different assays used to measure neuronal autoantibodies*

First off all, most studies only used serum samples for antibody detection, which seems to increase the chance of finding both false positive and false negative results and therefore studies using CSF are needed.[48] Furthermore, positive cases identified through screenings using in-house or commercial cell-based assays are often not validated using another methodology such as immunohistochemistry or immunocytochemistry on cultured neurons.[48–50]

In-house or commercial cell-based assays used for high throughput screening use a qualitative rating system. Samples are either labeled as positive or negative based on a cut-off value, while in practice some samples will show intermediate or doubtful results depending on their antibody titer. In addition to this, it has been shown that cell-based assays using live or fixed cells show different outcomes. Some authors have suggested that a cell-based assay using fixed cells is not sensitive enough to detect antibodies in patients without full-blown anti-NMDA receptor encephalitis and in cases with low antibody titers.[41,51] Others oppose this and found that results from fixed cells are more often in concordance with results from immunohistochemistry based testing.[48]

However, it is also noted that caution is warranted when comparing in-house cell-based assays as they all have their technical specificities. One research group stresses the need to further develop new imaging methods that can support in the detection of these antibodies using quantitative approaches, such as nano-tracking based imaging methods on cultured neurons, in order to prevent false results in high-throughput screenings.[41] This method is however still very time consuming, does not identify a specific antibody, is not validated in different cohorts, and it requires highly specialized techniques and is therefore not suitable as a primary screening method yet.

*9.4.3 IgG versus IgA and IgM*

In addition to immunoglobulin class G, some research groups have also examined the presence of immunoglobulin of class A and M against the GluN1 subunit of the NMDA receptor, although all identified forms of autoimmune encephalitis thus far seem to be IgG mediated.[52] Initial reports have found a prevalence of 4-10% of IgA and IgM in serum of both patients and controls and it has been suggested that these antibodies might enter the brain after

disruption of the blood-brain barrier.[32,33] However, the pathogenicity of these antibodies has never been clearly shown, they seem to target a different epitope then the IgG antibodies, and testing for their presence has no proven clinical relevance yet.[44]

*9.4.4 Antibody titer*

As depicted in table 1, several studies found low-titer antibodies in patients with schizophrenia or a psychotic disorder using cell-based assays, which were not validated using other methods or in CSF. In patients with validated anti-NMDA receptor encephalitis antibody titers are usually higher. An imperfect correlation has been found between antibody titers and severity of symptoms in patients with anti-NMDA receptor encephalitis. However, this correlation is better established for antibody titers in CSF than in serum.[48] The relevance of these low-titer non-validated antibodies in serum, detected in some cases of patients with a psychotic disorder, therefore remain uncertain.

*9.4.5 Clinical characteristics of tested patients*

There are large differences between the clinical characteristics of the patients included in studies examining the prevalence of anti-NDMA receptor antibodies in patients with a psychotic disorder, ranging from acute ill patients with neuropsychiatric symptoms to patients with schizophrenia with a long duration of illness without any neurological symptoms. As shown in Table 1 some studies included patients with seizures while in other studies neurological symptoms were one of the exclusion criteria. In addition to this, some studies only included patients participating in cohort studies, while others were able to also include incapacated patients.[53] Therefore, some studies may have already excluded severely ill patients, which might have lowered their chances of findings patients with anti-NDMA receptor antibodies.

*9.4.6 Studies using CSF*

As mentioned, only testing for these autoantibodies in serum can result in false positive results.[44] However, there are also cases (up to 15%) of anti-NMDA receptor encephalitis that only have detectable antibodies in CSF.[54] Therefore only testing serum could also result in false negative cases. Thus far only two studies were able to examine CSF of patients with a psychotic disorder for the presence of anti-neuronal antibodies. One studied screened 124 patients

and found zero positive cases.[46] The other study included 125 patients and found four cases testing positive for anti-neuronal antibodies, including three patients with anti voltage-gated potassium channel (VGKC) antibodies and one patient with anti-NMDA receptor antibodies. However, three out of the four positive cases already had neurological symptoms such as seizures when antibodies were tested.[55] In addition to this, it is not clear whether antibodies against the VGKC were detected using the radioimmunoassay for anti-VGKC antibodies or by testing for antibodies against specific targets, namely LGI1 and CASPR2. This is important, as recent studies have shown that antibodies detected using the radioimmunoassay probably have no clinical relevance.[56,57] Lastly, no data is present on the follow-up or treatment response of the positive cases.

*9.4.7 Treatment response of antibody positive patients*

The pathogenicity of low-titer neuronal autoantibodies in patients without full blown and recognizable encephalitis has been a matter of fierce debate. Treatment response of patients to immunosuppressive drugs can be a strong indication that these autoantibodies are indeed involved in the disease. Studies that investigated the response to treatment are limited, probably because treatment, such as methylprednisolone, has several potentially severe side effects and other treatments, such as intravenous immunoglobulin (IVIG) and rituximab, are very expensive.

Only one study thus far prospectively examined treatment response after screening for the presence of anti-neuronal antibodies in serum of 113 patients with a first episode psychosis. In this study both capacitous and non-capacitous, severely ill patients were included. However, prior to testing for the presence of neuronal antibodies, none of the included had symptomatology suggestive of an underlying somatic disorder. In total six patients with anti-neuronal antibodies were identified, including four patients with anti-NMDA receptor antibodies, one with anti-VGKC antibodies (without antibodies against LGI1 or CASPR2) and one with antibodies against an uncharacterized antigen. Three patients with anti-NMDA receptor antibodies also had antibodies present in CSF. Two of these patients developed severe neurological symptoms during follow-up and had an ovarian tumor. All four patients with anti-NMDA receptor antibodies

were successfully treated using immunosuppressive therapy.[47] Additional treatment response studies are currently being set up.[58]

*9.4.8 Moving beyond prevalence studies of neuronal autoantibodies*

The assays used to detect neuronal surface-protein antibodies strictly assess whether binding to the associated antigen occurs. They provide no information on potential alterations in expression, localization or function of the associated receptor.[41,59] Interestingly, two studies moved beyond measuring the prevalence of anti-neuronal antibodies in cohorts of patients with psychotic disorders and specifically examined the characteristics and pathogenicity of isolated GluN1 antibodies from patients with a psychotic disorder.[41,42] Their results suggest that anti-NMDA receptor antibodies isolated from patients with psychotic disorders differ from those of patients with classic encephalitis, which could be because these antibodies target a different epitope. The authors state that the antibodies found in patients with a psychotic disorder often occur in lower titers and might result in less severe alterations of NMDA receptor trafficking. Nano-tracking based imaging showed that antibodies isolated from these patients are capable of altering the synaptic anchoring and membrane trafficking of the NMDA-receptor.[42] However the antibodies used in these studies were isolated from serum and were not detected in CSF. Therefore replicating these findings, identifying the exact epitope of these antibodies and performing similar studies in antibodies isolated from CSF will be important next steps to take.

*9.4.9 Prevalence of autoantibodies in brain tissue of patients with schizophrenia*

Of further interest is the fact that the conformation of the NMDA receptor might differ between the human brain and transfected cells or rodent neurons. Testing for reactivity with human or primate brain tissue is therefore still of interest in schizophrenia, although such studies have been conducted in the past.[60–63]

Post-mortem examination of brain tissue from patients with anti-NMDA receptor encephalitis has shown microgliosis, IgG deposits and B-cell infiltrates.[64–66] In a study that we conducted no IgG deposits or B-cell infiltrates and a slight increase in the amount of T-cells was found in post-mortem brain tissue of patients with schizophrenia (Sneeboer et al., in press). Previous

studies examining post-mortem brain tissue have also not found evidence in support of large lymphocyte infiltrates in schizophrenia.[67] These findings plea against a role for autoimmunity in the pathogenesis of schizophrenia. However, not all brain regions have been systematically examined and certain abnormalities such as the binding of antibodies could be reversible or state-specific and therefore not detectable in post-mortem tissue.

*9.4.10 Novel neuronal autoantibodies*

In the study described in chapter 8 a screening was done for antibodies against other neuronal surface-proteins using cultured neurons and 24 candidate genes. As none of the included samples showed a synaptic staining on the cultured neurons, extending the amount of candidate proteins in the cell-based screening in this cohort does not seem fruitful. However, conducting a similar screening in a larger cohort or in a cohort consisting of patients with a first episode psychosis could be of use, as the amount of studies focusing on discovering novel neuronal autoantibodies in schizophrenia and other psychotic disorders is still limited.

*9.4.11 Next steps for research on anti-neuronal antibodies in psychosis*

Is there still reason for excitement on neuronal autoantibodies in schizophrenia? Although the subject is more complex than initial studies suggested, these antibodies may cause an effectively treatable form of psychosis in a specific subgroup of patients. To determine whether this subgroup indeed exists and how patients in this group should be diagnosed and treated further studies are needed. As a next step, large studies collecting both serum and CSF simultaneously from acutely ill patients with a psychotic disorder are needed. These samples should then be tested using a cell-based assay and immunohistochemistry for anti-NMDA receptor antibodies to minimize the chance of finding false-positive or false negative results. Patients identified via this screening with positive CSF results for both tests should then be treated using immunosuppressive therapy. As mentioned, screening for novel neuronal autoantibodies could be done simultaneously by using cultured neurons.

*9.4.12 Routine testing for anti-neuronal antibodies in psychosis*

Results thus far do not suggest that every patient with psychotic symptoms should be routinely tested for anti-NMDA receptor antibodies. However,

psychiatrists should be well aware of the clinical symptoms of anti-NDMA receptor encephalitis and testing for these antibodies is warranted, preferably in CSF, in patients with red flags as discussed in chapter 6. These include: flu-like prodromal symptoms, seizures, movement disorders, signs of severe autonomic dysfunction, altered consciousness, pronounced symptom fluctuation, admission to an ICU, catatonia, acute onset severe psychosis, severe behavioral problems and a history of an ovarian teratoma.

## 9.5 Glutamate signaling in schizophrenia

Although the studies described in this thesis suggest that the prevalence of antibodies against the GluN1 subunit of the NMDA receptor is low in patients with a first episode psychosis and schizophrenia, the suspected mechanism behind the symptomatology of anti-NMDA receptor encephalitis, namely impairment of NMDA-receptor signaling, is still highly relevant to schizophrenia and other psychotic disorders, such as catatonia. This notion is supported by previous literature, which has long suggested that hypofunction of the NMDA receptor could underlie psychosis or schizophrenia based on several lines of evidence.[68]

Previous studies have shown that psychotic symptoms can occur after altering the functioning of the NMDA receptor. One of the most important finding in this respect is the fact that NMDA-receptor antagonist such as ketamine and phencyclidine are capable of causing psychotic symptoms.[69] The symptomatology caused by these antagonists is dosage-dependent. In lower dosages these drugs result in psychotic symptoms and cognitive dysfunction but in higher dosages they result in catatonic symptoms or coma.[68,70] In addition to this, NMDA-receptor antagonists also influence neurotransmission in GABAergic, dopaminergic and pyramidal neurons.[68]

Studies in rodents have demonstrated the occurrence of schizophrenia-like behavior after modification of the NMDA receptor.[71] In addition to this, the latest genetic studies on schizophrenia found genome wide significant hits associated with glutamate signaling[72] and post-mortem studies have found alterations in the NMDA-receptor localization in schizophrenia.[73] Future studies

on the mechanisms behind anti-NMDA receptor encephalitis, and similar disorders, are therefore likely to provide valuable new insights for research on schizophrenia.

Of interest is the fact that the phenotype of schizophrenia is limited to positive, negative and in some cases catatonic symptoms without severe neurological symptoms or alterations in consciousness. These symptoms do occur in cases of anti-NMDA receptor encephalitis and after using high dosages of NMDA-receptor antagonists. It could therefore be hypothesized that there are alterations in pathways, which modulate the NMDA receptor more indirectly, in schizophrenia. These alterations might have a less severe or more fluctuating effect on glutamate signaling.[51,68]

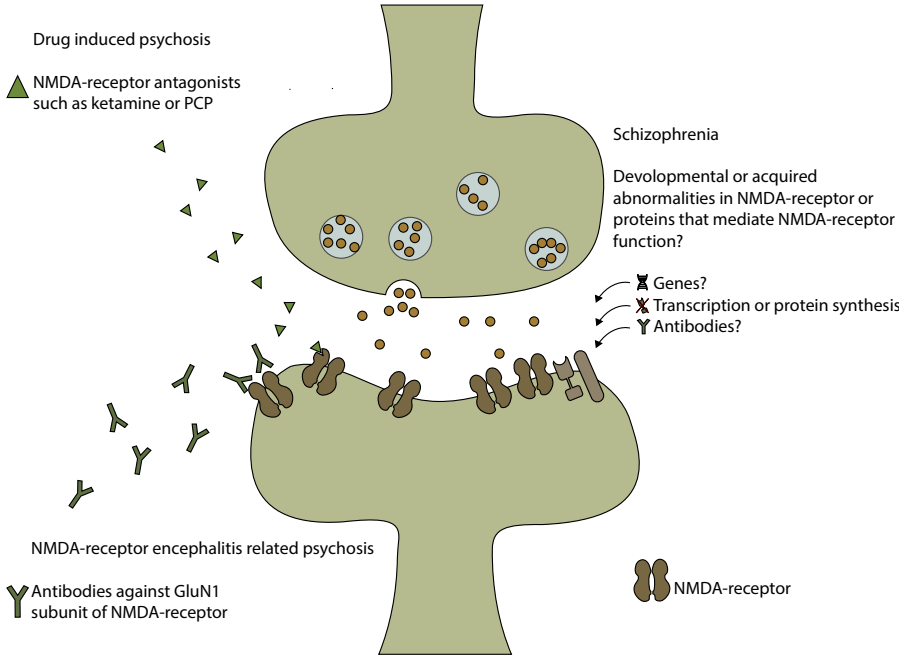


**Figure 1.** NMDA-receptor signaling disruption in psychotic disorders.

## 9.6 Considerations on autoimmunity in schizophrenia: culprit or bystander?

To asses whether schizophrenia can be classified as an autoimmune disease, we applied the modified Witebsky postulates (described in the textbox).[74] As schizophrenia is often viewed as a heterogeneous disease caused by various mechanisms, we also discuss whether autoimmunity could be involved in the pathogenesis of the disease in a subgroup of patients.

**Modified Witebsky postulates:**

1. Presence of disease-specific antibodies or auto-reactive T-lymphocytes in affected tissue
2. Ability to reproduce the disease in an animal model by transferring antibodies or auto reactive T-lymphocytes
3. Circumstantial clinical evidence such as a favorable response to immunosuppression
4. Family or personal history of autoimmune disorders or association with certain MHC variants

*9.6.1 First postulate*

The first postulate states that disease-specific antibodies or auto-reactive T-lymphocytes should be present in affected tissue. Examples include the presence of specific antibodies, directed against proteins of beta cells in the pancreas, in patients with type 1 diabetes[75] and auto-reactive T-lymphocytes found in the brain tissue of patients with multiple sclerosis.[76]

Thus far, no disease specific antibodies or auto-reactive T-cells have been identified in schizophrenia. Increased levels of some peripheral autoantibodies have been detected in a part of the patients with schizophrenia. However, at a group level these antibodies do not differentiate between patients and healthy controls.[1,77,78] Some studies suggest that pathogenic anti-neuronal surface antibodies are found in a small subgroup of patients with a psychotic disorder, however one could argue that these patients should be rediagnosed with autoimmune encephalitis and do not have schizophrenia or another psychotic disorder.[79,80]

Of further interest regarding this postulate is that a first analysis of the genome-wide significant associations discovered for schizophrenia, showed enrichment of enhancers active in B-lymphocytes.[72] However, follow-up analysis of this data did not reproduce this finding.[81] In addition to this, a study performing pathway analyses on GWAS data from various psychiatric disorders including schizophrenia, identified B-cell and T-cell activation as a pathway potentially affected in these disorders.[82] However, as described in chapter 5, the actual presence of activated B- and T-cells in brain tissue of patients with schizophrenia remains uncertain.

### *9.6.2 Second postulate*

The second postulate states that the disease should be reproducible by transferring antibodies or auto-reactive T-lymphocytes. In myasthenia gravis for example, it has been shown that injecting a rodent with serum or antibodies from a patient induces muscle weakness in the rodent.[83]

Similar experiments have been performed in schizophrenia but no distinct behavior changes were found in the animal model after injection of CSF from patients.[84] Therefore, apart from anecdotal evidence consisting of a case report describing the occurrence of psychotic symptoms after a stem cell transplant from a patient with schizophrenia,[85] there is no evidence in support of passive transference of the disorder via antibodies or lymphocytes.

### *9.6.3 Third postulate*

The third postulate states that there should be circumstantial evidence supportive of an autoimmune origin, such as a favorable response to immunosuppression.

There is some circumstantial evidence for an autoimmune origin of schizophrenia, such as altered cytokine[8] and lymphocyte[77] levels, but these findings are not undisputed and might be secondary to other factors. In addition to this, some of these abnormalities, such as elevated levels of certain cytokines, have also been described in other psychiatric disorders including major depressive disorder and bipolar disorder.[86,87]

Several studies have examined the expression of immune-related genes in post-mortem brain tissue using data generated by RNA sequencing or

microarrays. Most results do not suggest increased expression of immune-related genes or activation of immune-related pathways,[88] although the largest study to date does suggest enrichment of immune pathways among the genes differentially expressed between patients and controls.[89] Other studies examining histological or molecular markers of immune-activation in post-mortem tissue have also found heterogeneous results.[90,91]

Lastly, at a group level the effect of immunosuppressive treatment seems to be limited in schizophrenia.[88,92] However, the most potent immunosuppressant drugs have only been tested in small clinical trials thus far.[93,94]

*9.6.4 Fourth postulate*

The fourth postulate states there should be a personal or family history of autoimmune disorders in patients affected by the disease or the disease should be associated with specific MHC variants.

Various epidemiological studies have found a link between autoimmune disorders and schizophrenia. Increased prevalence rates of schizophrenia have been found in patients with autoimmune disorders and vice versa.[95–99] One study found a 50% increase in the lifetime prevalence of autoimmune disorders among people with schizophrenia and a family history of autoimmune disorders was associated with a 10% higher prevalence of schizophrenia.[96] Of interest, a negative association has been found between the occurrence of rheumatoid arthritis and schizophrenia, although this finding is not undisputed.[98] Studies examining the genetic overlap between schizophrenia and autoimmune disorders based on GWAS data, found both positive[100,101] and negative results.[102,103]



The association between schizophrenia and autoimmune disorders could be due to several factors including: shared genetic burden (although the evidence to prove this, as mentioned, is limited), shared environmental risk factors (such as stress or infections) or a common underlying disease mechanism (alterations in immune system).

Various autoimmune disorders have a strong association with specific MHC variants.[104] For schizophrenia there are known single nucleotide

polymorphisms within the MHC region that result in a small increase in the risk of developing the disorder,[72] but there are no specific MHC variants consistently associated with schizophrenia.[105]

*9.6.5 Conclusion and treatment perspective*

In conclusion, based on the current evidence schizophrenia cannot be regarded as an autoimmune disorder when applying the modified Witebsky postulates. Some postulates might apply to certain subgroups of patients but these subgroups are not well identified or characterized yet.

As mentioned the effects of immunosuppressive therapies applied in schizophrenia thus far are limited.[88] Future treatment studies using immunosuppressive therapies could try to stratify potential participants based on their genotype or other immune-related markers, in an effort to examine effectiveness in these specific subgroups, which might result in more promising outcomes.

## 9.7 Future studies on autoimmunity in schizophrenia: where to head next

Lack of replication, the influence of various confounders, small studies and differences in techniques used have complicated previous studies on the role of autoimmune mechanisms in schizophrenia. In order to further unravel the link between schizophrenia and autoimmunity collaborations between different research groups are needed in order to set up large-scale longitudinal studies with uniform measuring and analyses methods. Setting up global large-scale collaborations has proven to be an effective method in genetic studies, which also require large numbers to generate robust results. Protocols and data generated by these studies should be made available for everyone to access. In addition to this, strict inclusion criteria are needed for to prevent further broadening of the phenotype of schizophrenia, which will decrease the chance of finding disease specific abnormalities. In addition to this, data on potential confounding factors should be collected systematically. Lastly, time and effort should be spent on replicating or strengthening previous findings.

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# Chapter 10

## Nederlandse samenvatting

## Nederlandse samenvatting

Schizofrenie is een chronische psychiatrische aandoening die wordt gekenmerkt door recidiverende psychotische episodes. Tijdens een psychose verliest iemand (deels) het contact met de werkelijkheid en is er sprake van wanen, hallucinaties of andere verstoringen van het denken. Daarnaast hebben patiënten met schizofrenie vaak last van cognitieve klachten en emotionele vervlakking. Er zijn echter grote verschillen in de ernst en het beloop van de aandoening tussen patiënten.[1,2]

Er wordt geschat dat schizofrenie bij ongeveer 1% van de bevolking voorkomt, hoewel dit cijfer verschilt per regio. Ondanks uitgebreid onderzoek is de exacte oorzaak van schizofrenie helaas nog steeds onbekend. Het is duidelijk dat genetische factoren een belangrijke rol spelen bij het risico op het ontwikkelen van schizofrenie. Daarnaast zijn er meerdere ontwikkelings- en omgevingsfactoren ontdekt die dit risico ook in wisselende mate beïnvloeden.[1,2]

Sinds lange tijd wordt er in de medische literatuur gesuggereerd dat het immuunsysteem een rol zou kunnen spelen bij het ontstaan van schizofrenie. De Duitse psychiater Kraeplin schreef in 1981 al over het verband tussen koorts en psychose[3] en in 1926 beschreef de Amerikaanse psychiater Menninger een groep patiënten met psychotische klachten waarvan hij vermoedde dat het ontstaan van hun klachten samenhing met een recente griepepidemie.[4] In de daaropvolgende jaren zijn er tientallen onderzoeken uitgevoerd waarin is geprobeerd om een infectieziekte te identificeren die schizofrenie mogelijk (mede)veroorzaakt.[5–7] Hoewel die infectieziekte tot op heden nooit gevonden is, heeft dit onderzoek wel het startsein gegeven voor vervolgonderzoek naar immunologische afwijkingen bij patiënten met schizofrenie.

Die afwijkingen zijn in de afgelopen jaren wel volop gevonden in onder andere epidemiologische,[8,9] genetische[10] en laboratorium studies.[11–13] Het is echter onduidelijk of deze immunologische afwijkingen echt een rol spelen bij het ontstaan van schizofrenie of dat zij alleen voorkomen bij een deel van de patiënten of slechts een bijverschijnsel zijn op basis van bijvoorbeeld leefstijl of medicatiegebruik.

Het doel van dit proefschrift is om te onderzoeken of auto-immuniteit, een fenomeen waarbij afweercellen het eigen lichaam aanvallen, een rol speelt bij het ontstaan van schizofrenie. Dit is gedaan door middel van onderzoek naar: 1) De aanwezigheid van perifere autoantistoffen bij patiënten met schizofrenie. Aanwezigheid van deze autoantistoffen kan een marker zijn van auto-immuniteit. 2) De aanwezigheid van antistoffen tegen verschillende neurotrope pathogenen bij patiënten met schizofrenie. Aanwezigheid van deze pathogenen zou auto-immuniteit kunnen ontlokken. 3) Het functioneren van B-cellen bij patiënten met schizofrenie. Deze cellen spelen een belangrijke rol bij auto-immuniteit en produceren antistoffen. 4) De aanwezigheid van specifieke autoantistoffen gericht tegen eiwitten aanwezig op neuronen bij patiënten met schizofrenie. Aanwezigheid van deze autoantistoffen kan resulteren in directe verstoring van de neurotransmissie.

Eerdere studies hebben een hogere prevalentie van antinucleaire antistoffen gevonden in het bloed van patiënten met schizofrenie. Aan deze studies hebben echter vaak maar een klein aantal patiënten deelgenomen. Antinucleaire antistoffen bestaan uit verschillende specifieke antistoffen, gericht tegen onderdelen van de celkern. De aanwezigheid van antinucleaire antistoffen wordt gebruikt als ondersteunend diagnosticum bij een aantal auto-immuunaandoeningen. **Hoofdstuk 2** beschrijft het meten van antinucleaire antistoffen in een cohort van 368 patiënten met een schizofrenie spectrum stoornis en 283 gezonde proefpersonen. Bij deelnemers waarbij er sprake was van de aanwezigheid van antinucleaire antistoffen zijn ook 14 specifieke autoantistoffen gemeten. In deze studie is er geen associatie gevonden tussen de aanwezigheid van antinucleaire antistoffen en schizofrenie.

Het uitvoeren van een routinematig laboratoriumonderzoek bij patiënten met een psychotische stoornis kan van belang zijn bij het detecteren van relevante co-morbiditeit of bij het diagnosticeren van een psychose die veroorzaakt wordt door een onderliggende lichamelijke aandoening, zoals een infectie of auto-immuunaandoening. In **hoofdstuk 3** wordt in een retrospectieve studie onderzocht wat de toegevoegde waarde is van het uitvoeren van een routinematig laboratoriumonderzoek, bestaande uit 23 bepalingen, in een cohort van 215 patiënten met een psychotische stoornis. Het merendeel van deze patiënten is gediagnosticeerd met een eerste psychotische episode.

Dit laboratoriumonderzoek heeft niet geresulteerd in het identificeren van patiënten met een psychose door een onderliggende lichamelijke aandoening. Het meten van anti-thyroid peroxidase (anti-TPO) en antinucleaire antistoffen of meer algemene markers van ontsteking zoals C-reactief proteïne (CRP) en de erytrocytbezinkingssnelheid heeft ook niet geresulteerd in relevante veranderingen in de klinische zorg voor patiënten. Het lijkt daarom niet van meerwaarde om dergelijke onderzoeken standaard, zonder duidelijke andere aanleiding, uit te voeren bij patiënten met een psychotische stoornis.

Eerder onderzoek heeft gesuggereerd dat besmetting met pathogenen die zich in het centrale zenuwstelsel kunnen nestelen mogelijk een risicofactor is voor het ontstaan van schizofrenie. Hierbij wordt verondersteld dat een infectie met deze neurotrope pathogenen kan resulteren in directe schade of tot overactiviteit van het immuunsysteem in het brein. Door het meten van antistoffen gericht tegen deze pathogenen kan eerdere blootstelling aan deze pathogenen worden onderzocht. In **hoofdstuk 4** wordt de aanwezigheid van antistoffen gericht tegen herpes simplex virus-1 en -2 (HSV-1/HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV) en de parasiet *Toxoplasma gondii* (TG) in het bloed van 368 patiënten met een schizofrenie spectrum stoornis en 282 gezonde proefpersonen onderzocht. De resultaten van deze studie laten zien dat patiënten met schizofrenie iets vaker een infectie met VZV en CMV hebben doorgemaakt dan gezonde proefpersonen. In het voorkomen van antistoffen tegen de andere neurotrope pathogenen wordt geen verschil gevonden.

In **hoofdstuk 5** wordt in een review artikel een overzicht gegeven van studies die hebben onderzocht of B-cellen anders functioneren of in grote aantallen aanwezig bij patiënten met schizofrenie. Resultaten van eerdere genetische studies naar schizofrenie hebben namelijk gesuggereerd dat deze cellen mogelijk afwijkingen vertonen. Het aantal studies dat deze cellen heeft onderzocht bij patiënten met schizofrenie is echter beperkt. De meeste studies hebben slechts het aantal B-cellen in het bloed van patiënten gemeten, waarbij meestal geen verschillen zijn gevonden in vergelijking met gezonde proefpersonen. Er zijn weinig studies die de aanwezigheid van B-cellen in de hersenen of in de liquor hebben onderzocht en de functionele aspecten van deze cellen zijn nog nooit onderzocht bij patiënten met schizofrenie. In deze

studie wordt verder besproken op welke manier B-cellen een rol spelen bij het ontstaan van een aantal auto-immuunaandoeningen en hoe dit van belang zou kunnen zijn voor het onderzoek naar schizofrenie.

Anti-NMDA receptor encefalitis is de meest voorkomende vorm van een door antistoffen veroorzaakte auto-immuun encefalitis. De nog vrij recente ontdekking van deze aandoening is van groot belang geweest voor zowel neurologen als psychiaters. Patiënten met deze aandoening hebben namelijk zowel psychiatrische als neurologische klachten. Daarom worden zij soms, met name gedurende de eerste fase van de ziekte waarbij er vaak hoofdzakelijk psychiatrische symptomen zijn, foutief gediagnosticeerd met schizofrenie of een bipolaire stoornis. Het tijdig herkennen van deze aandoening is echter van groot belang voor het starten van effectieve behandeling met immunosuppressiva. In **hoofdstuk 6** wordt een studie beschreven waarin is onderzocht hoe vaak de antistoffen die anti-NMDA receptor encefalitis veroorzaken, voorkomen bij patiënten met een psychotische stoornis. In totaal worden er in deze studie 127 patiënten, hoofdzakelijk met een eerste psychose, onderzocht op de aanwezigheid van deze antistoffen. Deze worden echter bij geen van de onderzochte patiënten gevonden. Daarnaast worden er in deze studie 3 patiënten met anti-NMDA receptor encefalitis beschreven die alle drie hoofdzakelijk psychiatrische klachten hadden gedurende het begin van hun ziekte. Tenslotte worden er enkele adviezen gegeven over wanneer patiënten met een eerste psychose getest zouden moeten worden op de aanwezigheid van anti-NMDA receptor antistoffen.

Bevindingen over het voorkomen van anti-NMDA receptor encefalitis onder patiënten met psychotische symptomen lopen erg uiteen. Dit wordt mogelijk veroorzaakt doordat in veel onderzoeken de aanwezigheid van antistoffen tegen de NMDA-receptor slechts op een manier wordt getest, zonder dat positieve resultaten verder worden gevalideerd met behulp van een tweede test. In **hoofdstuk 7** worden de resultaten beschreven van een studie waarin bloed van 475 patiënten met schizofrenie of een eerste psychotische episode op verschillende manieren wordt onderzocht op de aanwezigheid van antistoffen tegen de NMDA receptor. Bij twee patiënten vertoonde een eerste test positieve resultaten maar deze resultaten konden niet worden bevestigd worden door middel van een tweede test. De resultaten van deze studie

suggereren niet dat een deel van de patiënten met schizofrenie eigenlijk een vorm van anti-NMDA receptor encefalitis heeft. Daarnaast onderschrijven de resultaten van deze studie het belang van het valideren van positieve resultaten in studies die grotere groepen patiënten testen op de aanwezigheid van anti-NMDA receptor antistoffen. In de meest optimale situatie zou de aanwezigheid van deze antistoffen onderzocht moeten worden in de liquor.

In **hoofdstuk 8** wordt tenslotte een studie beschreven waarin is getracht te onderzoeken of andere antistoffen, gericht tegen eiwitten die aanwezig zijn op neuronen, te detecteren zijn in het bloed van patiënten met schizofrenie. In totaal wordt er bloed van 104 patiënten met schizofrenie door middel van verschillende technieken onderzocht op de aanwezigheid van antistoffen tegen 24 verschillende eiwitten. Bij geen van de onderzochte patiënten worden deze antistoffen gevonden. De resultaten van deze studie suggereren dat antistoffen tegen neuronale eiwitten geen grote rol spelen bij het ontstaan van schizofrenie.

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# Appendix

## Dankwoord

## List of Publications

## Curriculum Vitae

## Dankwoord

In de afgelopen jaren hebben veel mensen een bijdrage geleverd aan de totstandkoming van dit proefschrift. Via deze weg wil ik jullie graag bedanken voor jullie hulp.

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## List of publications

The association between antibodies to neurotropic pathogens and bipolar disorder: Findings from the Dutch bipolar genetics (BIG) cohort and meta-analysis. Snijders GJL, van Mierlo HC, Boks MP, Begemann M, Litjens M, Ophoff RA, Kahn RS, de Witte LD. Submitted.

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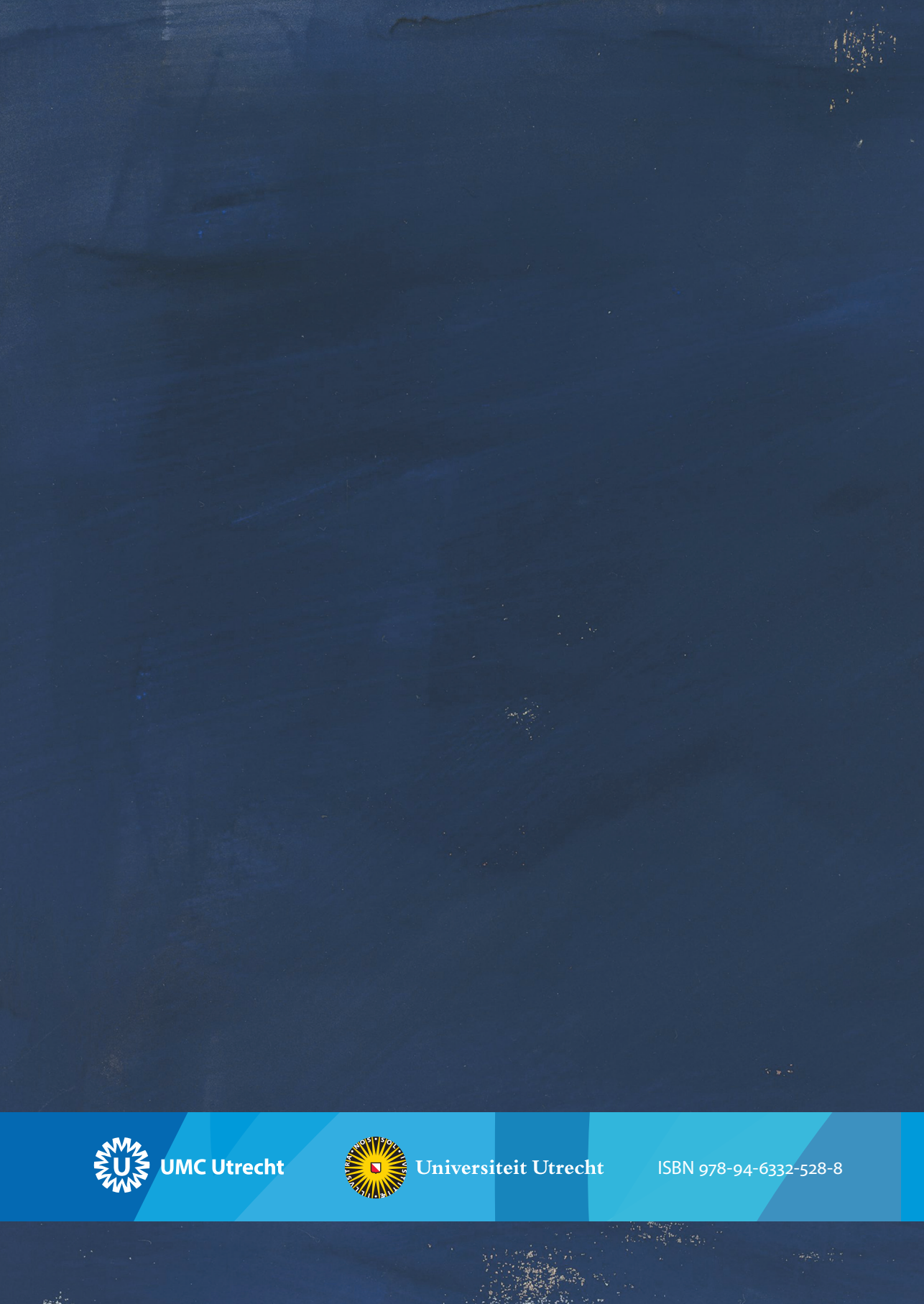
## Curriculum Vitae

Hans Christian van Mierlo was born on January 6th 1988 in Sittard, and grew up in Susteren. After graduating from high school (gymnasium, Trevianum scholengroep, Sittard) in 2006, he started studying medicine at the University of Utrecht. During his studies he visited the Hubert Kairuki Memorial University in Dar es Salaam, Tanzania for a clinical internship and the Children's Hospital of Fudan University in Shanghai, China for a research internship. After finishing medical school in 2012 he started his PhD program at the department of psychiatry of the University Medical Center Utrecht, supervised by prof. dr. R.S. Kahn and dr. L.D. de Witte. His PhD program focused on autoimmunity in schizophrenia. In 2013 he started combining research with his training in psychiatry supervised by dr. N.M.J. van Veelen until he was registered as a psychiatrist in April 2019. As of June 2019 he works as a psychiatrist at the St. Antonius hospital in Utrecht and Nieuwegein.



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