Reduced maternal levels of common viruses during pregnancy predict offspring psychosis: Potential role of enhanced maternal immune activity?

Marta Canuti, Stephen Buka, Seyed Mohammad Jazaeri Farsani, Bas B. Oude Munnink, Maarten F. Jebbink, Nico J.M. van Beveren, Lieuwe de Haan, Jill Goldstein, Larry J. Seidman, Ming T. Tsuang, Jitschak G. Storosum, Lia van der Hoek

Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center of the University of Amsterdam, Amsterdam, The Netherlands

Department of Epidemiology, Brown University, Providence, RI, USA

Antes, Institute for Mental Health Care, Rotterdam, The Netherlands

Erasmus University Medical Center, Department of Neuroscience, Rotterdam, The Netherlands

Department of Psychiatry, Academic Medical Center, Amsterdam, The Netherlands

Connors Center for Women’s Health and Gender Biology, Brigham and Women’s Hospital, Boston, MA, USA

Harvard Medical School, Boston, MA, USA

Department of Psychiatry, Harvard Medical School, Boston, MA, USA

Massachusetts Mental Health Center Public Psychiatry Division of the Beth Israel Deaconess Medical Center, Boston, MA, USA

Center for Behavioral Genomics, Department of Psychiatry, Institute for Genomic Medicine, University of California at San Diego, La Jolla, CA, USA

Harvard Institute of Psychiatric Epidemiology and Genetics, Boston, USA

Department of Psychiatry, Massachusetts General Hospital, Boston, MA, USA

Department of Psychiatry, Erasmus University Medical Center, Rotterdam, The Netherlands

Abbreviations

- Psychosis
- Schizophrenia
- Bipolar disorder
- Virus discovery
- Anelloviruses

ABSTRACT

Viral infections during the prenatal or early childhood periods are one of the environmental factors which might play an etiological role in psychoses. Several studies report higher antibody levels against viruses during pregnancy in blood of mothers of offspring with psychotic disorders, but the presence of such viruses has never been demonstrated.

The goal of this study was to investigate the potential association between viral infections during pregnancy and progeny with psychotic disorders and, for this purpose, we performed a nested case-control study involving pregnant mothers of offspring with schizophrenia or bipolar disorder with psychotic features (cases, N = 43) and pregnant women with healthy offspring (controls, N = 95). Since several potential viral candidates have been suggested in prior work, a broad-spectrum virus detection system was necessary.

A metagenomic analysis performed with the virus discovery method VIDISCA-454 revealed only common blood-associated viruses in all cohorts. However, a significantly lower viral prevalence was detected in the group of cases and in the sub-population of pregnant mothers of offspring with schizophrenia or bipolar disorder with psychotic features (cases, N = 43) and pregnant women with healthy offspring (controls, N = 95). Since several potential viral candidates have been suggested in prior work, a broad-spectrum virus detection system was necessary.

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1. Introduction

Several lines of evidence suggest that schizophrenia and bipolar disorder (BD) with psychotic symptoms are similar on a number of genetic and neurobiological characteristics and that psychotic symptoms may share a common etiology (Maier et al., 2006). Both disorders are regulated by a complex interaction of multiple genetic elements in concert with environmental factors and the overlap of clinical features might
be a consequence of common pathophysiological pathways (Tsuang, 2000; Maier et al., 2006). Recent studies reported a positive correlation between exposure to infections during the vulnerable period of gestation and the risk of developing psychoses (Buka et al., 2001a; Buka et al., 2008; Blomström et al., 2012). This hypothesis is supported by the evidence of an increased risk for schizophrenia among offspring of pregnant mothers with higher antibody levels against several pathogens (Brown et al., 2001, 2004a, 2005). Elevated levels of maternal antibodies against viruses have also been found to be associated with BD with psychotic features among offspring (S.E. Canetta et al., 2014).

However, the serological evidence has not yet been followed by direct virological evidence since no pathogen transmissible to the fetus via the placenta has thus far been detected during pregnancy in blood of mothers of offspring with the above-mentioned psychotic disorders. Here, we investigated whether maternal viremia, specific to one or a set of viruses, was associated with psychoses (schizophrenia and BD) in the offspring using a blinded, nested, case–control study.

Since a wide variety of agents have been correlated with the disorder and the involvement of an unexpected or unknown virus could not be excluded, the use of a broad spectrum approach for virus determination was necessary. Sequence independent virus identification techniques allow the simultaneous molecular detection of multiple viruses from a clinical sample without requiring any prior knowledge of its virological composition, and the possibility of combining them with next generation sequencing confer to these methods high efficiency and sensitivity (Delwart, 2007). One of these methods is VIDISCA-454, a restriction enzyme based virus discovery technique which is able to detect virtually any DNA or RNA virus from clinical samples (De Vries et al., 2011, 2012) and which was successfully used for virus discovery and metagenomic studies (Canuti et al., 2011, 2014a,b; Jazaeri Farsani et al., 2013, 2015; Oude Munnink et al., 2014, 2013). For this work VIDISCA-454 was applied on sera collected from pregnant mothers of offspring with or without schizophrenia or BD with psychotic features to evaluate the hypotheses of a viral etiology of psychoses.

2. Materials and methods

2.1. Sample collection

Participants were selected from 17,741 pregnancies enrolled between 1959 and 1966 into the Boston and Providence sites of the Collaborative Perinatal Project [CPP] (Niswander and Gordon, 1972) also known as the New England Family Study (NEFS) (Goldstein et al., 2010, 2011; Seidman et al., 2013). The pregnant women ascertainment in the CPP were largely representative of the patients receiving prenatal care. Written consent was obtained from all interviewed study participants, and subjects were compensated for participating.

The primary set of controls was selected from families participating in a larger study with no history of major psychiatric disorders (Goldstein et al., 2010, 2011), to account for the possible influence of genetically determined factors on the immune response against viral infections. Controls were NEFS adult offspring for whom parents and grandparents, as well as the parents’ siblings, were free of any known lifetime history of psychosis, bipolar, schizotypal, recurrent major depressive disorder, suicide attempts, or psychiatric hospitalizations, as described previously (Goldstein et al., 2010). Siblings of the controls were also free of any lifetime history of psychosis or bipolar disorder. From the pool of 186 potential controls (Goldstein et al., 2014), we selected 43 controls matched, as best as possible, for season of birth, maternal race/ethnicity and level of education, and child gender. Approximately six months prior to the blind matched case–control assays, we investigated a set of 52 maternal control serum samples from this same population to ascertain that the method was suitable to detect viruses in such old samples. These mothers, none of which had documented schizophrenia or other psychotic disorders, were included in the following analysis as additional controls.

Human subjects’ approval was granted by Harvard University, Brown University, Partners Healthcare system, and local psychiatric facilities. Written consent was obtained from all interviewed study participants, and subjects were compensated for participating.

2.2. Virus discovery: VIDISCA-454

To evaluate whether a virus (or a set of viruses) could be identified in association with the development of psychoses (schizophrenia and BD) a sequence independent virus discovery technique was used to perform viral metagenomics in serum samples collected from pregnant mothers of offspring with or without psychoses. Sample processing and sequence analysis were performed blindly.

VIDISCA-454 (Virus Discovery cDNA-AFLP) is a virus discovery technique that, combined with Roche 454 next generation sequencing, is able to detect virtually all DNA or RNA viruses from various clinical samples (Canuti et al., 2011, 2014a,b; Jazaeri Farsani et al., 2013, 2015; Oude Munnink et al., 2013, 2014; Tan et al., 2013; Pariani et al., 2014; Shaukat et al., 2014). Since this method is specifically designed to detect extra-cellular genetic material which is not susceptible to nucleases (viral genomes protected by a protein capsid), this system cannot be used to detect other human pathogens, such as bacteria and parasites.

Serum samples were tested as previously described with minor modifications (de Vries et al., 2011). Briefly, 110 µl of each sample was spun down to remove intact cells and 100 µl of supernatant was treated to eliminate background cellular DNA with 20 U TURBO™ DNase (Ambion). Nucleic acids were isolated as described by Boom et al. (1999). To subsequently detect RNA viruses a reverse transcription was performed with 200 U of Superscript II (Invitrogen) and non-ribosomal hexamers (Endoh et al., 2005), followed by a second strand synthesis with 5 U of Klenow fragment (3′–5′ exo–) (New England Biolabs) and 7.5 U of RNase H (New England Biolabs). After purification by phenol chloroform-extraction and ethanol precipitation, the obtained double stranded DNA was digested with 10 U of Msel (New England Biolabs) restriction enzyme. The A- and B- adaptors with multiplex identifiers, which contain specific Roche-454 primer binding sequences, were ligated to the digested fragments. After adaptor ligation, an amplicon size-selection was performed to prevent the amplification of DNA fragments smaller than 200 bp using Agencourt AMPure XP beads (Beckman Coulter), followed by 30 cycles of PCR amplification. The amplified library was then subjected to 2 consecutive purification rounds with Agencourt AMPure XP beads to completely remove excess primers and short fragments and DNA concentration was measured on a Qubit Fluorometer (Quant-it dsDNA HS Kit, Invitrogen). The libraries were pooled (35 samples per pool) and the average size of every pool was estimated with an Agilent 2100 Bioanalyzer (high sensitivity DNA Kit, Agilent Technologies) and the final concentration (copies/µl) was...
calculated using the KAPA Library Quantification Kit (KAPA Biosystems). Pools were diluted to a final concentration of 1 million copies/μl and used as input for the emulsion PCR (LIB-A emPCR Kit, Roche) and Roche-454 pyrosequencing.

2.3. Sequence analysis

All obtained reads were separated according to their multiplex identifiers. Primer sequences were trimmed from every read and reads were assembled with CodonCode Aligner software version 3.5.6. The consensus and unassembled sequences were compared to known nucleotide sequences in the GenBank database using BLAST (blastn) (Altschul et al., 1990) for viral identification.

2.4. Statistical analysis

We first examined the comparability of sociodemographic characteristics of the case and control sets through chi-square and t-tests. We then evaluated the association between exposure to any viruses or to a specific class of viruses in relation to case vs. control status. Percentages of positivity for all or classes of viruses were calculated for cases (pregnant mothers of schizophrenic offspring and/or offspring with BD with psychotic features) and compared to 3 control groups: i) matched controls, ii) additional controls and iii) total controls (matched plus additional controls). Significant differences between the groups were calculated with the Mid-P Exact test (2 tailed); values lower than 0.05 were considered significant. Conditional maximum likelihood estimates of odds ratios (CMLE OR) and relative 95% confidence intervals shown in Table 4, where the odds of any detectable virus among cases of psychotic disorders were approximately one-third that of the combined control sample (OR = 0.33; 95% CI = 0.11–0.9) and the odds associated with anelloviruses were lower than one third (OR = 0.28; 95% CI = 0.06–0.94). Similar results were evident among the sub-population of pregnant mothers of offspring with schizophrenia (Table 4).

Altogether 23.2% (32 out of 138) of samples were positive for at least one virus and the most frequently found were viruses belonging to the family Anelloviridae, detected in 16.7% (23 out of 138) of all samples, followed by the flavivirus GBVC (Hepatitis virus G) in 6.5% (9 out of 138). These 2 viruses, which together accounted for 93.8% (30 out of 32) of all positives, are commonly found in healthy individuals and, since no clear correlation with any disease has ever been definitively established, they are regarded as commensal human viruses (Bendinelli et al., 2001; Praharaj et al., 2006; Bernardin et al., 2010; Maggi and Bendinelli, 2010).

3.3. Case–control comparisons

After comparing the infection rates in the different cohorts it emerged that the percentage of samples positive for one or more viruses was significantly lower in cases (11.6%) than in controls (28.4%, p = 0.029), as shown in Table 2. Additional analyses suggested anelloviruses as the main class of viruses accounting for this difference. In fact, a lower prevalence of anelloviruses was identified in cases compared to matched controls (p = 0.07) and to additional random controls (p = 0.06), a difference that reached statistical significance upon combining the two control groups (p = 0.037) (Table 2). Furthermore, our data indicate that lower levels of detectable viruses were especially pronounced among pregnant mothers of offspring with schizophrenia (p = 0.03, Table 3), although these results should be regarded as preliminary due to small sample sizes.

The results are supported by the odds ratios and relative confidence intervals shown in Table 4, where the odds of any detectable virus among cases of psychotic disorders were approximately one-third that of the combined control sample (OR = 0.33; 95% CI = 0.11–0.9) and the odds associated with anelloviruses were lower than one third (OR = 0.28; 95% CI = 0.06–0.94). Similar results were evident among the sub-population of pregnant mothers of offspring with schizophrenia (Table 4).

4. Discussion

Schizophrenia and BD with psychotic symptoms are considered multifactorial conditions caused by a combination of multiple genetic and environmental factors (Tsuang, 2000; Maier et al., 2006). Several studies report how the exposure to infections during the perinatal or early childhood periods can be considered a potential risk factor for developing psychoses on a basis of a genetic predisposition (Rantakallio et al., 1997; Brown et al., 2001, 2004a, 2005; Buka et al., 2001a; Abrahao et al., 2005; Torrey et al., 2007; Buka et al., 2008; Blomström et al., 2012; S.E. Canetta et al., 2014).

In this study we investigated whether a potential association could exist between viral infections occurring during pregnancy and risk of psychotic disorders among offspring, by means of a nested case–control study which involved pregnant mothers of offspring with or without schizophrenia or BD with psychotic features. Serum samples collected from the studied populations were tested with a broad-spectrum virus discovery technique (VIDISCA-454) (de Vries et al., 2012, 2011; Oude

### Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1: Cases (N = 43)</th>
<th>2: Matched controls (N = 43)</th>
<th>3: Additional controls (N = 52)</th>
<th>4: Total controls (N = 95)</th>
<th>Total sample (N = 138)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
</tr>
<tr>
<td>Female offspring</td>
<td>33 (76.7)</td>
<td>39 (90.7)</td>
<td>42 (82.4)</td>
<td>81 (85.3)</td>
<td>114 (83.2)</td>
</tr>
<tr>
<td>Male's age at pregnancy (range: 15–45)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td>Family socioeconomic index at birth* (range: 10–90)</td>
<td>54.2 (19.6)</td>
<td>57.9 (18.5)</td>
<td>52.1 (20.1)</td>
<td>54.8 (19.5)</td>
<td>54.8 (19.5)</td>
</tr>
<tr>
<td>Gravida (range: 1–14)</td>
<td>3.5 (2.4)</td>
<td>3.2 (1.9)</td>
<td>3.3 (2.6)</td>
<td>3.3 (2.3)</td>
<td>3.3 (2.3)</td>
</tr>
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</table>

* A composite index of socioeconomic status was calculated on the basis of methods developed by the U.S. Census Bureau (possible range = 0–100).
from below 10% to over 90% (Ataei et al., 2012; Spandole et al., 2015). Variability in the presence of antibodies against viral pathogens during pregnancy and after birth can lead to the development of psychotic features in the offspring. The analysis revealed many common blood-associated viruses in all cohorts and no evidence was found that any specific class of viruses increased the risk of psychotic disorder among offspring.

In contrast, statistical analyses indicated that lower infection rates were identified in cases compared to the controls. The finding was especially pronounced among pregnant mothers of offspring with schizophrenia, although due to the small sample size (n = 31 cases) these results should be considered preliminary. Analyses determined that anelloviruses, the class of viruses identified most frequently in all cohorts, were the main group responsible for this finding. Anelloviridae is a recently discovered viral family that includes a wide variety of viruses, characterized by a great diversity, and which are considered apathogenic (Bendinelli et al., 2001). These viruses are highly prevalent in the global population – also due to their ability to establish persistent infections – and they are commonly identified in co-infections involving different types (Bendinelli et al., 2001). Depending also on the employed detection technique and its ability to detect all known types, the reported prevalence of anelloviruses in healthy populations varies widely between studies, with values ranging in recent reports from below 10% to over 90% (Ataei et al., 2012; Spandole et al., 2015).

We have identified an overall infection rate of 17%, comparable to what has previously been identified in pregnant women (Xin et al., 2004). It is important to keep in mind however that metagenomic methods like VIDISCA-454 are intrinsically less sensitive compared to methods like VIDISCA-454. In order to obtain a more complete picture of the infection status, we recommend combining these findings with additional metagenomic approaches.

In literature we observe a strong correlation between anelloviruses and immunity. There is, in fact, a clear increase of anelloviral levels in immunodepressed populations such as HIV-1 infected individuals (Devalle and Niel, 2004) and organ transplant recipients (Usta et al., 2002), and increased viral loads have been found to correlate with the progression of AIDS (Thom and Petrik, 2007; Li et al., 2013). Furthermore, it has been reported that anelloviral levels increase in response to post-transplant immunosuppression therapy and are lower in patients that suffer from a rejection episode, where the immunosuppression was insufficient (De Vlaminck et al., 2013). Overall, there is an inverse relationship between the ability of an individual to produce an adequate immune response and the level of anelloviruses in the bloodstream: a more competent immune system clears these viruses more efficiently. This observation led to the concept that anelloviruses might be used as a marker to evaluate the state of immunosuppression of a patient (De Vlaminck et al., 2013). Since we identified a remarkably lower prevalence of anelloviruses in serum of pregnant mothers with offspring with psychosis when compared to controls, we postulated a higher capability of these mothers to clear these infections. This hypothesis is in accordance with studies showing an association between the presence of antibodies against viral pathogens during pregnancy and affected offspring (Brown et al., 2001, 2004a, 2005; Buka et al., 2001a; Buka et al., 2008; Blomström et al., 2012; S.E. Canetta et al., 2014). Combining these findings, we suggest that an enhanced immune activity against viral infections in maternal blood might increase the risk of developing psychosis in offspring.

These results are also in line with previous studies that have reported an association between elevated maternal cytokines and the risk for developing schizophrenia (Buka et al., 2001b; Brown et al., 2004b; Goldstein et al., 2014) and investigations where elevated levels of maternal C-reactive protein were found in pregnant mothers of offspring with schizophrenia as compared to matched controls (S. Canetta et al., 2014). Furthermore, studies in animal models have demonstrated that an activation of the maternal immune system during pregnancy causes behavioral alterations in the offspring, consistent with schizophrenia (Zuckerman et al., 2003; Bauman et al., 2014) and inflammatory cytokines can alter the development of cortical neurons in vitro (Gilmore et al., 2004).

In addition, other studies have reported a significant association between risk of schizophrenia and several genetic markers related to immune function. For example, a recent large genome-wide association study found a cluster of common variant immunity genes to be among those most strongly associated with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Finally, the role of immunity in the neurodevelopmental theory of schizophrenia is not only supported by serological evidence in pregnant mothers of progeny with schizophrenia, but also by a significant association between schizophrenia and a family history of autoimmune diseases (Wright et al., 1996; Eaton et al., 2006). Future research focused on...
genetic markers involved in the immune response regulation might elucidate if hereditary factors are correlated with a more prominent immune activity in families of schizophrenic patients. Such research may potentially help integrate these findings that an increased expression of inflammatory markers has been identified both in pregnant mothers of schizophrenic individuals and in early childhood or after symptom manifestation in patients themselves (Buka et al., 2001b; Torrey et al., 2006; Dickerson et al., 2010; Muller and Schwarz, 2010; Goldstein et al., 2014; S. Canetta et al., 2014).

Finally, we have to consider that some viral infections can induce autoantibody responses with the production of self-recognizing antibodies (Ercolini and Miller, 2009), and that these antibodies can be transmitted to the fetus via the placenta (Palmeira et al., 2011). This is an important mechanism to protect the fetus from infectious diseases but placental transfer of autoantibodies may also result in serious disorders of the neonate (Palmeira et al., 2011). We speculate that an enhanced maternal immune response, possibly also involving the transfer of (auto) antibodies, may interfere with brain development of the fetus, ultimately contributing to a vulnerability for psychotic disorders. Moreover, as placental transfer of antibodies is proportional to the concentration of antibodies in the maternal blood (Palmeira et al., 2011), the hypothesis is strengthened in that a prominent activation of the immune system against viruses (correlated with a higher antibody level in serum) might be a risk factor for developing vulnerability to psychotic disorders.

We considered three potential limitations in the current work. First, the total number of affected individuals is modest, due to the cost and logistical challenges of collecting and storing maternal serum samples for a birth cohort of thousands of individuals. Second, due to limited serum availability, we include in these analyses 43 mothers of patients with schizophrenia or bipolar disorder with psychotic features from the n = 74 total cases identified. Supplemental analyses (not shown) dispelled concerns that the 58% of cases with available serum differed from the remaining cases not included in these analyses. Those included and excluded were quite similar with regard to maternal education, year of birth, race/ethnicity and family socioeconomic status. Finally, only the samples collected during the third trimester of pregnancy were available for this research and a specific viral involvement during the first two trimesters of pregnancy cannot be excluded by our investigation. Future studies should ideally include a more comprehensive set of samples – collected during the whole period of pregnancy – to determine if similar trends are visible during earlier gestational stages.

In conclusion, a maternal enhanced immune response could have been responsible for the significantly lower level of common blood-associated viruses we detected in pregnant mothers of offspring with psychotic illness as compared to controls. Our data corroborate a neurodevelopmental theory of schizophrenia and other psychotic disorders indicating that an enhanced immune response during pregnancy – possibly linked to a background of genetic liability – could be considered a risk factor for developing psychosis.

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Funders had no role in study design.

Contributors
MC, SA, LdH, MvR, J5 and LVdr are responsible for study concept and design. MC and SB drafted the manuscript and performed statistical analysis. All authors contributed to acquisition, analysis and data interpretation and critically revised the manuscript.

Conflict of interest
Authors declare no conflict of interests.

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References

Table 4
Odds estimates for maternal viremia and having offspring with psychosis.

<table>
<thead>
<tr>
<th>OR (95% CI)</th>
<th>Cases vs matched controls</th>
<th>Cases vs additional controls</th>
<th>Cases vs total controls</th>
<th>Schizophrenia vs controls</th>
<th>BD with psychosis vs controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total viruses</td>
<td>0.44 (0.12–1.41)</td>
<td>0.27 (0.08–0.8)</td>
<td>0.33 (0.11–0.9)</td>
<td>0.27 (0.06–0.96)</td>
<td>0.5 (0.1–2.5)</td>
</tr>
<tr>
<td>Anelloviruses</td>
<td>0.29 (0.06–1.11)</td>
<td>0.28 (0.06–1.04)</td>
<td>0.28 (0.06–0.94)</td>
<td>0.26 (0.06–1.18)</td>
<td>0.34 (0.04–2.8)</td>
</tr>
<tr>
<td>GBVC</td>
<td>3.11 (0.32–84.76)</td>
<td>0.71 (0.13–3.25)</td>
<td>1.11 (0.22–4.7)</td>
<td>0.49 (0.06–4.27)</td>
<td>2.97 (0.53–16.71)</td>
</tr>
</tbody>
</table>

Odds Ratios with confidence intervals excluding the null value were considered significant and are in boldface. GBVC: Hepatitis G Virus type C; BD: bipolar disorder.

a Conditional maximum likelihood estimates of odds ratios (relative 95% confidence interval, Mid-P Exact).
b Total controls (matched controls plus additional controls).

