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Neonatal Levels of Inflammatory Markers and Later Risk of Schizophrenia

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ABSTRACT

BACKGROUND: There is a long-standing interest in investigating the impact of early-life immune abnormalities on later onset of psychosis. The aim of this study was to assess inflammatory marker levels in neonatal dried blood spots and their association with later risk of schizophrenia.

METHODS: This nested case-control study included 995 cases and 980 control subjects. Cases were identified using the Danish Psychiatric Central Register. Control subjects of same age and sex were identified using the Danish Civil Registration System. Samples for the identified individuals were retrieved from the Danish Neonatal Screening Biobank. Concentrations of 17 inflammatory markers were measured in eluates from dried blood spots using a bead-based multiplex assay. Incidence rate ratios were calculated using conditional logistic regression. Principal component analysis was used to capture the overall variation in the inflammatory markers' concentrations.

RESULTS: No significant differences were found for any of the analyzed interleukins. We did not find any association with schizophrenia for any of the other examined inflammatory markers.

CONCLUSIONS: Our results suggest that persons who develop schizophrenia do not have higher or lower levels of the examined inflammatory markers at the time of birth. Our findings differ from the studies of maternal inflammatory changes during the antenatal period for which associations with schizophrenia have previously been demonstrated.

Keywords: Blood, Cytokines, Denmark, Epidemiology, Etiology, Schizophrenia

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The etiology and pathogenesis of schizophrenia remain largely unknown, but immunological factors are thought to play a role. Maternal nutrition (1), prenatal stress (2–4), obstetric complications (5), and prenatal infection (6) have all been reported as risk factors for schizophrenia. These prenatal and perinatal conditions have also been associated with imbalanced cytokine secretion (7–9).

One specific hypothesis suggests that the release of maternal cytokines in response to infectious insults mediates the adverse effects on the offspring (10). Direct support for this hypothesis is mainly derived from experimental studies of maternal immune activation in animal models (11–13). Epidemiologic evidence linking enhanced maternal/fetal expression of inflammatory markers and later development of schizophrenia is limited to a small number of investigations (14–16). Recent attempts to correlate early-life immune parameters with other neurodevelopmental disorders have also provided initial evidence for an association between altered prenatal or neonatal expression of inflammatory markers and risk of autism spectrum disorder in offspring (17–19).

Most studies have examined maternal cytokines during pregnancy (9,14,15) and after schizophrenia onset (20), leaving the neonatal period unexamined. Only one study to date has sought to investigate the association between neonatal immune changes and offspring psychosis (21). This study from Sweden used psychiatric linkages and neonatal blood spots and found decreased levels of the acute phase proteins tissue plasminogen activator, serum amyloid P, and procalcitonin to be associated with nonaffective psychosis (21).

Many cytokines and cytokine receptors are constitutively expressed during brain development in humans, suggesting essential roles for these molecules in the regulation and modulation of normal brain development (22). It is thus expected that abnormal levels of these molecules during critical periods of early brain development may adversely affect neurodevelopmental processes and contribute to a higher susceptibility for complex brain disorders of developmental origin such as schizophrenia. The development and maturation of the immune system starts early in fetal life and continues through infancy and early childhood (23). Upon birth, the neonatal immune system undergoes substantial adjustments to meet the requirements for the potentially pathogenic extrauterine environment, and failure to do so results in increased susceptibility to infectious diseases (23).

While most of the current experimental model systems assess brain and behavioral effects of prenatal exposure to infectious or inflammatory stimuli (24), negative long-term effects on adult brain functions have also been observed following neonatal exposures to such immune adversities (25–27). Interestingly, neonatal exposure to distinct classes of cytokines appears to induce differential neuronal effects in adult animals (28). We therefore opted to include a broad array

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of cytokines with the aim to explore a possible role of cytokine specificity in human association studies.

In keeping with the neurodevelopmental effects of cytokines (22), we thought it of prime importance to investigate cytokines in neonate life with respect to schizophrenia risk in later life. We measured proinflammatory and anti-inflammatory cytokines in a small amount of blood available in archived newborn dried blood spot samples from the Danish Neonatal Screening Biobank. The blood samples utilized in this study were collected 5 to 7 days after birth. Most cytokines have short half-lives and are effective locally, acting on cells in close proximity to their release. Thus, many cytokines are only detectable in peripheral circulation in response to pathology. Therefore, any changes in blood cytokine levels at this point are likely to reflect postnatal rather than prenatal immune changes.

METHODS AND MATERIALS

Participants

The study population for this nested case-control study (29) was selected from individuals born in Denmark between 1981 and 1998. Cases were identified through the Danish Psychiatric Central Register (30), at the time of their first diagnosis with schizophrenia, F20 (ICD-10). The Danish Psychiatric Central Research Register was computerized in 1969 and contains data on all admissions to Danish psychiatric inpatient and outpatient facilities (30). Cases were matched on date of birth and sex to a randomly selected population-based control subject at risk from the Danish Civil Registration register (31). The cases were diagnosed with schizophrenia in the period 2007 to August 2009. Information about gestational age was retrieved from the Danish Medical Birth Registry (32). Neonatal dried blood spot samples were retrieved from The Danish Neonatal Screening Biobank (33), where they were stored at −24°C.

Blood Collection

The dried blood specimens were capillary blood collected by heel-prick. Each sample was transferred onto specially manufactured absorbent filter paper. The blood was allowed to thoroughly saturate the paper and was air dried for several hours. All samples were sent to Statens Serum Institute where all analyses were carried out. After the neonatal screening analysis, residual blood was stored in the Danish Neonatal Screening Biobank. A previous study (34) has revealed that many of the analytes are well preserved over time, even after storage for more than 20 years. At the time the samples were taken, the national guidelines stated that the sample should be taken 5 to 7 days after birth.

Determination of Inflammatory Markers

Samples were analyzed at the Statens Serum Institute, Denmark, with a previously described (34) and validated multiplex sandwich immunoassay (Luminex xMAP; Luminex Corp, Austin, Texas) for 17 inflammatory markers in dried blood spots. Studies using this technology and dried blood spots are described in Skogstrand *et al.* (8), Natarajan *et al.* (35), Schelonka *et al.* (36), and Sood *et al.* (37). In short, two 3-mm disks were punched from each dried blood spot and extracted in 130 μL buffer (phosphate buffered saline containing .5% Tween 20, 1% bovine serum albumin, and 1 tablet complete protease inhibitor cocktail with ethylenediaminetetraacetic acid [Roche, Mannheim, Germany] dissolved per 25 mL buffer) and shaken on a microplate shaker (600 rpm) for 1 hour at room temperature. Each disk contained approximately 3 μL whole blood (38). After extraction, the liquid was aspirated, transferred to new plates, and frozen at −20°C. Before analysis, the plates with the samples were thawed and centrifuged at 3000 rpm for 10 minutes. The extracted sample (50 μL) and 50 μL of a suspension of capture antibody-conjugated beads were mixed in plate wells. After 1½ hours of incubation shaking at room temperature, the beads were washed twice and subsequently reacted for 1½ hours with a mixture (50 μL) of corresponding biotinylated detection antibodies, each diluted 1:1000. Streptavidin-phycoerythrin (50 μL) was added to the wells and the incubation was continued for additional 30 minutes. Finally, the beads were washed twice and resuspended in 125 μL of buffer and analyzed on the Luminex 100 platform (Luminex Corp). Standard curves were fitted with a five-parameter logistic equation (Logistic-5PL) using BioPlex Manager 5.0 (Bio-Rad Laboratories, Hercules, California). The following analytes were determined: interleukin (IL)-1β, IL-6, IL-8, IL-12, IL-18, tumor necrosis factor-β, soluble IL-6 receptor-α, interferon-γ (IFN-γ), epidermal growth factor, transforming growth factor-β (TGF-β), monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, C-reactive protein (CRP), intercellular adhesion molecule 1, insulin-like growth factor-binding protein-1, adiponectin, and brain-derived neurotrophic factor.

Quality Assurance and Quality Control

To minimize assay variations, batches of beads, calibration curves, and biotinylated antibodies, as well as controls, for the whole project were made initially. Calibration curves and control subjects were analyzed on each plate, and cases and control subjects to be compared were analyzed on the same plate. An internal assay marker was used to discover outliers and samples with rheumafactor or other disturbing element for immunoassays, as described before (39). All samples, calibration curves, and control subjects were measured in duplicates.

Analytical Approach

A priori, we selected the following eight markers, CRP, IL-1β, IL-6, IL-8, IL-12, IL-18, IFN-γ, and TGF-β, on the basis of being some of the most common inflammatory markers involved in inflammatory and immune responses and in some cases because of their previously documented association with schizophrenia (20,40,41). In addition, we analyzed the remaining nine markers to supplement our primary analysis with a broad assessment of the immune response and its possible association with schizophrenia. A total of 42 plates were run on the Luminex system to measure these markers in our cases and control subjects. The cases and control subjects to be compared were analyzed on the same plates to avoid the influence of interassay variation on the statistical results.

Statistical Methods

Analytes falling below the lowest concentration within the working range were assigned a value equal to that concentration. The concentrations of the inflammatory markers were explored graphically by plotting the cases and control subjects for each plate (data not shown). Owing to their initial distribution, the levels of the markers were log transformed. To account for variation between the individual plates, we decided to stratify by plate.

The log-transformed measurements for each marker were analyzed as a continuous variable using conditional logistic regression. In addition, each standardized inflammatory marker concentration was also split into quartiles, with the first quartile as the reference group.

Gestational age was split into the following categories: very preterm (less than 32 weeks of gestation), preterm (32 to 36 weeks of gestation), and term (37 or more weeks of gestation). This categorization is the same as the one used by Skogstrand *et al.* (8).

An additional method to present the data is to use principal component analysis (PCA). We performed a principal component analysis to capture the main variation across the eight markers studied. The number of components extracted in a principal component analysis is equal to the number of observed variables being analyzed. However, in most analyses, only the first few components account for meaningful amounts of variance, so only these first few components are retained, interpreted, and used in subsequent analyses. Principal component analysis was used to visually explore whether two clusters would be visible, representing specific inflammatory mechanisms. For this analysis, each analyte was log transformed to stabilize the variance and the mean value for each plate subtracted.

SAS software version 9.3 (SAS Institute, Cary, North Carolina) was used in all statistical analyses.

Approval

The study did not require informed consent from participants according to Danish legislation (Act of Processing Personal Data), and the study did not involve any contact with study participants. The study was approved by the Danish Data Protection Agency, the Danish Research Ethics Committee, and the Steering Committee for the Danish Neonatal Screening Biobank.

RESULTS

The study population consisted of 995 cases and 980 control subjects born between 1981 and 1998, and the cases had an average age of 20.2 years at first diagnosis for schizophrenia. This average age is fairly young and can be attributed to the period the cases were born and the short period of follow-up (2007–2009).

Table 1 features the distribution of the inflammatory marker concentrations in filter eluates by cases and control subjects. The percentage of samples analyzed that were below the

Table 1. Medians and Range (Minimum-Maximum) of 17 Inflammatory Markers in Cases Compared with Those of Control Subjects

		Control Subjects (n = 980)				Cases (n = 995)			
	Working Range	Median	Range	Below the Level of Detection n (%)	Missing n (%)	Median	Range	Below the Level of Detection n (%)	Missing n (%)
IL-1β	8–4000	68	<8–601	145 (14.80)	125 (12.76)	69	<8–911	153 (15.38)	140 (14.07)
IL-6	8–4000	145	<8–981	30 (3.06)	133 (13.57)	140	<8–1471	32 (3.22)	155 (15.58)
IL-8	8–4000	20	<8–1855	331 (33.78)	133 (13.57)	26	<8–>4000	330 (33.17)	154 (15.48)
IL-12	8–4000	146	<8–644	14 (1.43)	162 (16.53)	145	<8–>4000	6 (.60)	181 (18.19)
IL-18	20–10000	3346.5	<20–>10000	2 (.20)	140 (14.29)	3372.5	<20–>10000	1 (.10)	161 (16.18)
CRP (μg/mL)	.006–	.44	<.006–113.87	1 (.10)	135 (13.78)	.47	<.006–60.34	2 (.20)	157 (15.78)
TGF-β	156–80000	4565	<156–23226	27 (2.76)	138 (14.08)	4653	<156–24752	32 (3.22)	155 (15.58)
IFN-γ	8–4000	<4	<8–86	674 (68.78)	145 (14.29)	<4	<8–85	691 (69.45)	168 (16.88)
EGF	8–4000	228	<8–980	7 (.71)	143 (14.59)	230	<8–851	10 (1.01)	161 (16.18)
TNF-β	20–10000	2451	<20–8095	5 (.51)	147 (15.00)	2403	<20–9090	6 (.60)	169 (16.98)
MCP-1	20–10000	1487	<20–9351	6 (.61)	171 (17.45)	1445	<20–5433	5 (.50)	185 (18.59)
MIP-1α	20–10000	320	<20–790	205 (25.51)	141 (14.39)	316.5	<20–2337	203 (20.40)	165 (16.58)
IL-6rα (ng/mL)	.8–400	62.07	<.8–256.79	2 (.20)	146 (14.90)	82.694	<.8–272.42	2 (.20)	163 (16.38)
ICAM-1 (ng/mL)	1–1000	44.01	<1–256.53	1 (.10)	192 (19.59)	42.832	<1–246.97	2 (.20)	214 (21.51)
BDNF (ng/mL)	.16–80	46.09	<.16–>80	6 (.61)	151 (15.41)	45.704	<.16–>80	6 (.60)	168 (16.88)
Adiponectin (μg/mL)	.006–	10.41	<.006–733.26	1 (.10)	131 (13.17)	10.25	<.006–739.47	2 (.20)	157 (15.78)
IGFBP-3 (ng/mL)	.8–400	18.56	<.8–160.49	145 (14.80)	201 (20.51)	17.478	<.8–99.36	144 (14.47)	220 (22.11)

All values are pg/mL unless otherwise indicated.

BDNF, brain-derived neurotrophic factor; CRP, C-reactive protein; EGF, epidermal growth factor; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon-γ; IGFBP-3, insulin-like growth factor-binding protein 3; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-6rα, interleukin-6 receptor-α; IL-8, interleukin-8; IL-12, interleukin-12; IL-18, interleukin-18; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; TGF-β, transforming growth factor-β; TNF-β, tumor necrosis factor-β.

Table 2. Incidence Rate Ratios for Schizophrenia, Using Log Transformed Values as a Continuous Variable

Protein	IRR (95% Confidence Interval)	*p* Value
IL-1β	.99 (.91–1.07)	.72
IL-6	.99 (.86–1.13)	.87
IL-8	1.03 (.97–1.10)	.37
IL-12	1.09 (.92–1.29)	.30
IL-18	1.08 (.90–1.28)	.40
CRP	1.01 (.94–1.09)	.72
TGF-β	.98 (.87–1.11)	.75
IFN-γ	.83 (.64–1.07)	.15
EGF	.97 (.82–1.15)	.75
TNF-β	.97 (.81–1.16)	.75
MCP-1	.99 (.82–1.20)	.95
MIP-1α	1.01 (.95–1.08)	.73
IL-6rα	.89 (.73–1.09)	.26
ICAM-1	.96 (.77–1.20)	.74
BDNF	.91 (.78–1.02)	.26
Adiponectin	1.03 (.91–1.18)	.63
IGFBP-3	.98 (.92–1.05)	.52

BDNF, brain-derived neurotrophic factor; CRP, C-reactive protein; EGF, epidermal growth factor; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon-γ; IGFBP-3, insulin-like growth factor-binding protein 3; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-6rα, interleukin-6 receptor-α; IL-8, interleukin-8; IL-12, interleukin-12; IL-18, interleukin-18; IRR, incidence rate ratio; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; TGF-β, transforming growth factor-β; TNF-β, tumor necrosis factor-β.

detection limit varied by analyte from 1% to 70%. In general, the cases and control subjects were equally distributed with respect to number of missing and number of samples below detection limit. IFN-γ had the largest number of cases and control subjects with respect to percentages below detection limit (68.78% for control subjects and 69.45% for cases).

Table 2 shows the results from the analysis where each inflammatory marker was analyzed as a continuous variable. In general, when each marker was analyzed as a continuous variable, no relation between the concentration of the inflammatory marker and schizophrenia was found. Gestational age less than 37 weeks has previously been associated as an increased risk for schizophrenia (42). An association between very preterm birth (less than 23 weeks of gestation) and neonatal cytokine levels has been demonstrated by Skogstrand *et al.* (8). After adjustment for gestational week (very preterm, preterm, term) and sex, we found similar results (Table S1 in Supplement 1).

Table 3 displays the results from the analysis where each inflammatory marker was split into quartiles. Analysis comparing the first quartile with the other quartiles revealed patterns similar to the analysis of the markers as a continuous variable; that is, no significant relationship with schizophrenia was revealed. Table 3 also features the nine markers included in our supplementary analysis. These results mirror the results found for the eight a priori selected markers in the primary analysis, revealing no association with schizophrenia. Nevertheless, the marker soluble IL-6 receptor-α was found to be statistically significant for the fourth quartile. The group in the fourth quartile had a .76-fold (95% confidence interval: .58–1.00) decreased risk of schizophrenia compared with the group in the first quartile. Of note, this result is not corrected for multiple testing and should therefore be interpreted with caution. After adjustment for gestational week (very preterm, preterm, term) and sex, we found similar results (Table S2 in Supplement 1).

Figure 1 displays the principal component 1 along the x axis and the principal component 2 along the y axis. The principal

Table 3. Incidence Rate Ratios for Schizophrenia, Using Log Transformed Standardized Values by Quartiles[a]

	1.Quartile	2.Quartile	3.Quartile	4.Quartile
IL-1β	1.00 (reference)	.95 (.73–1.25)	.91 (.70–1.18)	.96 (.74–1.24)
IL-6	1.00 (reference)	.96 (.74–1.26)	.99 (.76–1.29)	.90 (.69–1.18)
IL-8	1.00 (reference)	.85 (.61–1.18)	1.09 (.83–1.42)	1.08 (.84–1.38)
IL-12	1.00 (reference)	1.08 (.82–1.42)	1.16 (.88–1.52)	1.13 (.86–1.48)
IL-18	1.00 (reference)	.90 (.68–1.17)	1.05 (.80–1.37)	1.11 (.85–1.46)
CRP	1.00 (reference)	1.01 (.78–1.33)	.98 (.75–1.29)	1.07 (.82–1.40)
TGF-β	1.00 (reference)	.97 (.74–1.27)	1.10 (.84–1.44)	.96 (.74–1.26)
EGF	1.00 (reference)	1.16 (.89–1.52)	1.08 (.83–1.42)	1.09 (.83–1.43)
TNF-β	1.00 (reference)	1.02 (.78–1.33)	1.23 (.94–1.61)	.87 (.66–1.14)
MCP-1	1.00 (reference)	.91 (.69–1.19)	.89 (.68–1.18)	.87 (.66–1.15)
MIP-1α	1.00 (reference)	.98 (.74–1.30)	1.07 (.82–1.39)	.91 (.70–1.19)
IL-6rα	1.00 (reference)	.85 (.65–1.11)	1.08 (.83–1.42)	.76 (.58–1.00)
ICAM-1	1.00 (reference)	.93 (.70–1.22)	.82 (.62–1.09)	1.00 (.75–1.32)
BDNF	1.00 (reference)	.87 (.66–1.14)	1.04 (.79–1.36)	.90 (.69–1.18)
Adiponectin	1.00 (reference)	1.05 (.81–1.38)	1.06 (.81–1.39)	1.09 (.83–1.42)
IGFBP-3	1.00 (reference)	.97 (.73–1.30)	.97 (.74–1.28)	.85 (.65–1.12)

BDNF, brain-derived neurotrophic factor; CRP, C-reactive protein; EGF, epidermal growth factor; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon-γ; IGFBP-3, insulin-like growth factor-binding protein 3; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-6rα, interleukin-6 receptor-α; IL-8, interleukin-8; IL-12, interleukin-12; IL-18, interleukin-18; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; TGF-β, transforming growth factor-β; TNF-β, tumor necrosis factor-β.

[a]IFN-γ had too many observations below working range to be split into quartiles.

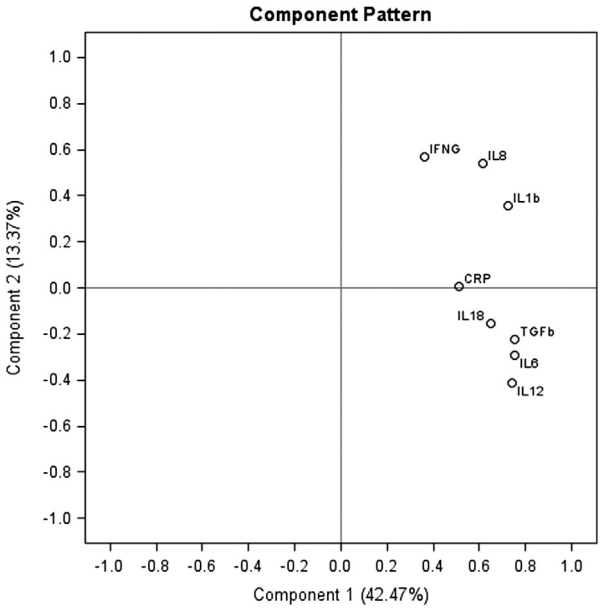

Figure 1. Scatterplot of first versus second factor loadings derived from principal component analysis of eight cytokines. CRP, C-reactive protein; IFNG, interferon-γ; IL1b, interleukin-1β; IL6, interleukin-6; IL8, interleukin-8; IL12, interleukin-12; IL18, interleukin-18; TGFb, transforming growth factor-β.

component analysis included the eight a priori selected markers and identified two principal components, which together accounted for 57% of the variance. The first component explained 43% of the total variance. This component was determined by loadings from all eight markers, suggesting a generic pattern of co-expression of anti-inflammatory and proinflammatory cytokines. The second component correlated positively with IL-8 and IL-1β and negatively with IL-18, TGF-β, IL-6, and IL-12 and explained a further 14% of the variance. Each of the eight components from the PCA was analyzed in a conditional logistic regression model, and no association was found between any of the components and schizophrenia (Table S3 in Supplement 1).

DISCUSSION

To the best of our knowledge, this is the largest study of inflammatory marker levels in neonates and their subsequent risk of schizophrenia. The cases and control subjects were derived from a large, population-based national birth cohort. We investigated eight inflammatory markers that have previously been related to schizophrenia etiology (9,43,44), and none of these markers were associated with schizophrenia. In a supplementary analysis, we explored additional nine neonatal markers, and again we found no association with schizophrenia. Overall, our findings differ from earlier studies that have utilized maternal sera (9,14,15) and from those reporting an association between cytokine level expression and schizophrenia using blood from adult patients (20). To date, only one study has investigated nine acute-phase proteins in neonatal blood and their association with nonaffective psychosis (21). That study included 196 cases and 502 control subjects and found significantly decreased levels of tissue plasminogen activator, serum amyloid P, and procalcitonin (21). Unfortunately, no comparison with these findings is possible, as these markers were not included in our panel.

Brown *et al.* (15), Ellman *et al.* (9), and Buka *et al.* (14) studied maternal cytokine levels during the prenatal stage. These authors demonstrated that increased maternal levels of the inflammatory cytokines IL-8 and tumor necrosis factor-α were associated with increased risk of schizophrenia in the offspring. The results of these studies were based on archived maternal serum, which, in turn, may mirror (infection-mediated) inflammatory changes during the antenatal period. Altogether, these data indicate an important role of prenatal cytokine disturbance in relation to later development of schizophrenia, whereas our findings suggest that neonatal blood cytokine levels may play less of a role in determining schizophrenia risk.

The discrepancy between the negative findings reported here and the positive findings reported before (9,14,15) could therefore be related to the precise timing of developmental immune insults (45). Indeed, prospective epidemiologic studies suggest that infectious or inflammatory insults may have a more extensive impact on increasing risk of developmental neuropsychiatric disorders as compared with late prenatal (or neonatal) insults (46,47). Hence, it is possible that early fetal, placental, and/or maternal immune perturbations participate in the pathogenesis of schizophrenia, whereas immune changes during the neonatal period may play only a minor role in this context.

Interestingly, our findings for CRP are similar to those reported by Gardner *et al.* (21), suggesting that neonatal CRP levels are not associated with later schizophrenia risk. It is important to mention that the assayed inflammatory markers in our sample likely reflect basal levels, as only a minority of neonates may have suffered from an acute infection at the time the sample was drawn. This could explain why we find no association between CRP and schizophrenia.

Although we did not find an association between the principal components and schizophrenia, the principal component analysis showed that the inflammatory markers clustered into immunologically plausible patterns. The first component with high loadings from all cytokines can be interpreted as an expression of a general pattern of co-expression of anti-inflammatory and proinflammatory cytokines (48). The clustering of IL-8 together with IL-1β on the second component can be interpreted as an altered IL-1-like immune response dimension (48). Although our PCA analysis does not fully validate our cytokine measurements, we do believe that the identified clusters support an immunological plausible pattern of neonatal cytokine secretion in our samples (49).

Our analysis did not change the results when adjusting for gestational week, indicating that our results were not confounded by gestational age. Gardner *et al.* (21) also did not find gestational week to affect their risk estimates. For further reassurance for the quality of our measurements, we investigated the association between very preterm birth and CRP levels below the 10th centile (odds ratio 7.50; 95% confidence interval 2.81–20.02). This association was significant and is similar to the one previously found by Skogstrand *et al.* (8).

This replication helped us to evaluate the quality of the measurements in our data. Again, this result is not a direct validation of our measurements, but we find it reasonable to conclude that our finding of no association with schizophrenia is further strengthened by this finding.

Validation of the schizophrenia diagnosis in the Psychiatric Central Register indicates that schizophrenia is diagnosed with reasonable accuracy. A study assessing 300 randomly drawn patient case records in the Psychiatric Central Register found that 97.5% of the patients diagnosed with schizophrenia in 2009 fulfilled the diagnostic criteria of the ICD-10 (50).

We do not have any clinical information about the infant at the time the blood sample was drawn. This missing information could have provided clues to the interpretation of our results, as a range of biological processes such as stress, fever, and infection are all known to influence cytokine production. We have reason to believe that the majority of samples were drawn from healthy babies in a resting state, thus reflecting basal cytokine levels. Data on cytokine levels in the first week of life in healthy, noninfected infants are very limited (51–54).

Sample handling and storage can greatly impact the reliability and reproducibility of inflammatory markers in dried blood spots. The samples used for this project were stored between 15 and 32 years at −24°C. Previous works have shown that some analytes in old dried blood spots were only detectable in relatively low concentrations compared with measurements on newer dried blood spots (34). In our study, we only compared samples from cases and control subjects that were drawn at about the same time. The samples were stored together under the same conditions for the same amount of time. We, therefore, assume that the share of degradation of the inflammatory markers is the same. It is possible though that the amount of degradation of analytes may be a cause of our negative results. Optimization and validation of the assay was done before this study. The use of the Luminex instrument and the multiplex technology has its limitations. First and foremost, it has to do with which subfractions of cytokines an assay detects and what the biological relevance of possible undetected cytokine pools may be.

Parturition is associated with upregulation in the expression of a host of inflammatory mediators, which include cytokines, growth factors, and lipid mediators that influence the growth and function of the immune and vascular compartments (55). This upregulation is of maternal origin, and whether this has a direct effect on the fetus is unknown. Cytokine levels play a significant role in parturition, during which time they rise abruptly. Such a rapid increase is especially observed in relation to preterm births. However, this inflammatory response has a short duration. Both cases and control subjects had been through the same parturition process, so this should not affect our results. Adjusting for Caesarean section birth in the study by Gardner *et al*. (21) did not affect the risk estimates for the different acute phase proteins.

The study of a restricted set of cytokines in our study may lead to misspecification of effects, given that cytokines are pleiotropic; can act in antagonistic, synergistic, or redundant manner with other cytokines; and have numerous possible functions in the body. We thus believe that it might be important to repeat this original analytical approach as new markers become available. The main limitation of this study is that we only have one sample from each subject; thus, it is not possible to assess patterns of longitudinal change in the neonate. A snapshot approach (a single blood sample) has its limitations in terms of giving a true reflection of the cytokines due to the high complexity of the interplay between various cytokines. Only changes taking place around the time of sampling would thus be reflected in these measurements.

Conclusion

In this large-scale nested case-control study, we examined neonatal inflammatory marker levels, which are indicators of the child's own immune response. Conceptually this work presents an example of the utility of the use of the Danish Neonatal Screening Biobank on a large scale. Our study did not provide evidence for an association between neonatal inflammatory marker levels and schizophrenia.

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ARTICLE INFORMATION

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