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Neonatal chemokine levels and risk of autism spectrum disorders: Findings from a Danish historic birth cohort follow-up study

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ABSTRACT

A potential role of chemokines in the pathophysiology of Autism Spectrum Disorders (ASDs) has been previously suggested. In a recent study we examined levels of three inflammatory chemokines (MCP-1, MIP-1α and RANTES) in samples of amniotic fluid of children diagnosed later in life with ASD and controls frequency-matched to cases on gender and year of birth. In this follow-up study, levels of the same chemokines were analyzed postnatally in dried blood spot samples from the same subjects utilizing the Danish Newborn Screening Biobank. Crude estimates showed decreased levels of RANTES. In the adjusted estimates, no differences were found in levels of the three examined chemokines in ASD cases compared to controls. Our findings may cautiously suggest an altered cell-mediated immunity during the early neonatal period in ASD. Further research is needed to examine the relationship between maternal/fetal and neonatal chemokine levels and their role in ASD.

1. Introduction

Autism spectrum disorders (ASDs) are complex group of neurodevelopmental disorders, behaviorally defined and characterized by qualitative impairments in social interaction, communication and stereotyped behavior [1]. Several epidemiological studies have indicated a distinct trend of increasing ASD prevalence rates [2–4]. Whilst some recent reports showed strikingly high prevalence estimates up to 2.6% [3], the most recent estimate is calculated to be around 1.1% [5]. The numbers from Denmark show a parallel trend as well, with recent estimates ranging from 62 to 82 per 10,000 [6].

The pathophysiology of ASD is complex with both genetic and environmental components [1]. Currently, the most optimistic estimates of identified genetic causes for ASD are at about 30% of the total ASD cases [7]. While these causes can be grouped into cytogenetically visible chromosomal abnormalities, copy number variants, and single-gene disorders, surprisingly, none of the individual causes accounts for more than 1–2% of ASD cases [8]. Furthermore, a recent large-scale twin study suggested that although genetic factors may play an important role in the etiology of ASD, their magnitude may be lower than the earlier estimates, what emphasizes the importance of environmental factors [9].

Mounting evidence has suggested a pivotal role of immune dysfunction in ASD [10]. Both neuroinflammatory changes and dysfunctional peripheral immune responses have been repeatedly reported in individuals with ASD [10]. However, convergence toward a unified immunologic pathway is still lacking.

Current evidence suggests an important role of cytokines on the neurodevelopmental trajectory in autistic offspring [11]. However, identifying which specific cytokines may play the pivotal role in the development of ASD is not an easy task [12]. According to Dammann and O'Shea [12], the complexity in identifying specific cytokines in charge of perinatal brain damage is mainly due to the biology of the cytokines themselves, where there is a high degree of overlap in their functions, their target cells and their sources of secretion.

Chemokines represent a family of cytokines with a diverse range of physiological and pathological functions including immune system development, inflammation and cancer metastasis [13]. A potential role of chemokines in ASD has been suggested

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and discrepant levels of chemokines (from different biologic samples like brain tissue, cerebrospinal fluid, plasma and amniotic fluid) were also found associated with the disease and several behavioral impairments in individuals diagnosed with ASD [14,15].

The aim of this study was to examine levels of chemokines during early neonatal period through utilizing biologic materials from a national screening program in Denmark along with data retrieved from nation-wide health registers.

2. Materials and methods

In this study, levels of three selected inflammatory chemokines (Monocyte Chemotactic Protein-1 [MCP-1], Macrophage Inflammatory Protein-1α [MIP-1α] and Regulated upon Activation Normal T-Cell Expressed and Secreted [RANTES]) were examined in neonatal dried blood spot samples (n-DBSS). For this purpose, a 1:2 case control study design was adopted. Both cases and controls were retrieved from a historic birth cohort based on second trimester amniotic fluid sample collection (mostly around the gestational week 15) kept and maintained at Statens Serum Institute in Copenhagen, Denmark [15,16]. All ASD cases born between 1982 and 2000 were identified in the Historic Birth Cohort utilizing the Danish Psychiatric Central Register and using International Classification of Diseases (ICD)-8 codes (299·*xx*) until 1993 followed by ICD-10 codes (DF84·*xx*) [17]. Controls were non-ASD individuals in the cohort, frequency-matched with cases at a 2:1 ratio by gender and year of birth.

Using a unique identifier (CPR number) assigned for each citizen in Denmark, background information, psychiatric and somatic comorbidities and obstetric history of the study population were retrieved from different Danish nation-wide health registers including the Danish Psychiatric Central Register [17], The Danish National Hospital Register [18] and The Danish Medical Birth Registry [19]. Data in the Danish Psychiatric Central Register are computerized and have been collected systematically from all psychiatric wards and hospitals in Denmark since 1969. Similarly, the Danish National Hospital Register has been computerized for more than 30 years and systematically collects data on all somatic hospital admissions in Denmark. Data from the Danish Medical Birth Registry include information on all live births, stillbirths and infant deaths in Denmark [17–19].

All n-DBSS for cases and controls were identified in the Danish Newborn Screening Biobank (DNSB) using the CPR number. The DNSB is a collection of screening n-DBSS since 1982 [20]. In Denmark, neonatal screening is a mandatory offer to all new parents, and the screening program consists of tests for different congenital and inherited disorders such as phenylketonuria, congenital hypothyroidism and biotinidase deficiency [21]. The DNSB enjoys almost universal coverage and more than 70,000 samples are stored every year at Statens Serum Institute (including samples from Denmark, Greenland and the Faroe Islands) [20].

Measurements of the selected chemokines were performed with an in-house assay panel using the multiplex flow cytometric assay system Luminex MultiAnalyte Profiling Technology [22]. Analytes were extracted from two 3.2-mm punches of n-DBSS filter paper in extraction buffer and working range for each chemokine was assessed from the precision profile and defined as the concentration range in which the coefficient of variation was below 20%. Working range for MCP-1 and MIP-1α was 19.5–10,000 pg/ml and for RANTES 625–160,000 pg/ml. For more specific details regarding laboratory methodology see Skogstrand et al. [23].

2.1. Statistical analyses

Case-control background characteristics differences were assessed using Mantel–Haenszel chi square tests and Mantel–Haenszel estimates of the odds ratios (ORs) controlling for variables used for frequency matching (gender and year of birth) and reported as odds ratios (ORs) with 95% confidence intervals (CIs) and Mantel–Haenszel chi square tests *P* values. Comparisons of chemokine levels were analyzed for continuous measures (tobit regression) and for categories (logistic regression) based on cut-off points derived from the biomarker distributions of controls in order not to overlook any tail effects.

Levels of MCP-1 and MIP-1α were analyzed using tobit regression models to account for measurements falling outside the working range (RANTES was not analyzed using tobit regression as more than 50% of measurements were above WR. The tobit model produces correct estimates with truncated data by breaking the likelihood function up into two parts. The first part of the likelihood function applies to all cases (within and outside the WR). The second part applies only to measurements within the working range and is essentially an ordinary regression model. In our tobit model, values obtained below WR were calculated as (Lower value of WR −1), for those above WR, values were calculated as (Upper value of WR +1). The distributions of the analytes were skewed, but log-transformed measurements turned out to comply with the assumption of a (truncated) normal distribution. Assumptions of residuals normal distribution and homoskedasticity were evaluated using normal quantile plots and residuals against fitted values plots, respectively.

In addition, chemokine levels were analyzed for categories. Two cut-off points were introduced at the 10th and 90th percentiles based on the chemokine levels in the n-DBSS of controls. Analyte levels were categorized a priori as *elevated* or *decreased* if significantly falling more in the upper or the lower 10th percentile of the controls' distribution respectively. Associations were analyzed using logistic regression models and reported as odds ratios (ORs) with 95% confidence intervals (CIs).

We calculated both crude and adjusted odds ratios. Gender and year of birth were included as covariates in both crude and adjusted estimates. In the adjusted analyses, we accounted for inter-assay variation (by including Luminex plate number as covariate in the model). Besides, potential confounders significantly associated with the outcome were included as covariates in the adjusted model and they included: (i) being co-diagnosed with any congenital malformation, (ii) other childhood psychiatric co-morbidity (for ICD-8 and ICD-10 codes used to identify congenital malformation and psychiatric comorbidity diagnoses see Appendix A), along with previously identified risk factors of ASD: (iii) mother's and (iv) father's age, (v) birth weight, (vi) gestational age, (vii) APGAR score and (viii) parity [1,24]. Furthermore, we analyzed the data stratified on gender to examine gender variation in chemokine levels and only including individuals born after 1993 where ICD-10 (a more reliable diagnostic standard for ASD) was introduced in Denmark.

To account for the heterogeneity within ASD diagnoses and the associated comorbid disorders [25], a secondary analysis was conducted on individuals born between 1990 and 1999 comparing infantile autism (IA) cases to cases with other childhood psychiatric disorder (OCPD) and controls with no psychiatric comorbidities (NPC). The additional control group (OCPD) consisted of all controls that had at least one childhood psychiatric disorder other than ASD. The reason for choosing infantile autism cases with birth years 1990–1999 was to maximize the overlap with a recent validation study, in which almost all infantile autism cases born during this period and registered in the Danish Psychiatric Central Register were individually validated based on their hospital records [26]. Logistic regression analyses were carried out analogous to our primary analyses above, comparing infantile autism and other childhood psychiatric disorder to controls with no psychiatric comorbidities.