Amniotic fluid chemokines and autism spectrum disorders: An exploratory study utilizing a Danish Historic Birth Cohort

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ABSTRACT

Introduction: Elevated levels of chemokines have been reported in plasma and brain tissue of individuals with Autism Spectrum Disorders (ASD). The aim of this study was to examine chemokine levels in amniotic fluid (AF) samples of individuals diagnosed with ASD and their controls.

Material and methods: A Danish Historic Birth Cohort (HBC) kept at Statens Serum Institute, Copenhagen was utilized. Using data from Danish nation-wide health registers, a case-control study design of 414 cases and 820 controls was adopted. Levels of MCP-1, MIP-1α and RANTES were analyzed using Luminex xMAP technology. Case-control differences were assessed as dichotomized at below the 10th percentile or above the 90th percentile cut-off points derived from the control biomarker distributions (logistic regression) or continuous measures (tobit regression).

Results and conclusion: AF volume for 331 cases and 698 controls was sufficient for Luminex analysis. Levels of MCP-1 were elevated in cases compared to controls. Logistic regression analyses, performed on individuals diagnosed using ICD-10 only, showed increased risk for ASD with elevated MCP-1 (elevated 90th percentile adjusted OR: 2.32 [95% CI: 1.17–4.61]) compared to controls. An increased risk for infantile autism with elevated MCP-1 was also found (adjusted OR: 2.28 [95% CI: 1.16–4.48]). Elevated levels of MCP-1 may decipher an etiologic immunologic dysfunction or play rather an indirect role in the pathophysiology of ASD. Further studies to confirm its role and to identify the potential pathways through which MCP-1 may contribute to the development of ASD are necessary.

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1. Introduction

Since the term was first coined by the Swiss psychiatrist Eugen Bleuler a hundred years ago (Stotz-Ingenlath, 2000), autism has evolved from being an enigmatic disability to represent a challenging public health issue with substantial cost of care (Ganz, 2006). By definition, Autism Spectrum Disorders (ASD) refers to a cluster of heterogeneous neurodevelopmental disorders characterized by a triad of qualitative impairments in social interaction, communicative and repetitive stereotypic behavior (Newschaffer et al., 2007). Despite the numerous research lines trying to disentangle the etiology of this group of disorders, no definitive biologic screening or diagnostic tools have been universally accepted, and the diagnostic standards are still based on behavioral criteria (American Psychiatric Association, 2000).

Prevalence of autism has been escalating over time. Most recent estimates from the US indicate that ASD prevalence could be as high as 1% (Rice, 2007). In Denmark a parallel trend has been observed with the most recent estimates being around 0.74% (Parner et al., 2008).

Chemokines represent a family of cytokines comprised of four subgroups based on their cysteine motif (C, CC, CXC, and CX3C) and have the capacity to control the attraction of leukocytes to different tissue targets (Epstein and Luster, 1998). Recently, a crucial role of chemokines in neuroinflammation has been postulated.
where they can function as messengers for the communication between neurons and neuroglia (Biber et al., 2008). Hence, they may actually serve as intermediate players in the well established linkage between inflammation and autism (Goines and Van de Water, 2010).

While elevated levels of chemokines have been reported in plasma and brain tissue of individuals with ASD (Ashwood et al., 2011; Vargas et al., 2005), to our knowledge, chemokine levels during pregnancy have not yet been investigated. Given the importance of the intrauterine milieu for the inception of neurodevelopmental disorders (Schlotz and Phillips, 2009), the aim of this study was to examine levels of three inducible (inflammatory) CC chemokines: Monocyte Chemotactic Protein-1 (MCP-1), Macrophage Inflammatory Protein-1α (MIP-1α) and Regulated upon Activation Normal T-Cell Expressed and Secreted (RANTES) of individuals diagnosed with ASD later in life and their controls utilizing a historic birth cohort.

2. Methods

To examine if differential levels of amniotic fluid chemokines exist in individuals diagnosed later in life with ASD compared to controls, a case-control study design was adopted. Study subjects were retrieved from a Historic Birth Cohort (HBC) kept at Statens Serum Institute (SSI) in Copenhagen, Denmark.

The HBC consists of amniotic fluid and maternal serum samples of more than 100,000 pregnant women who underwent screening or diagnostic amniocentesis and/or phlebotomy and had their biologic samples stored at SSI from 1980 to 2004 (Nørgaard-Pedersen, 2005; Nørgaard-Pedersen et al., 1985, 1984). In general, after screening or diagnostic analysis was performed (for example, measuring Alpha-Fetoprotein levels), residual amniotic fluid samples collected in the HBC were centrifuged, and the supernatants were kept at minus 20°C following the routine procedure for storing and handling biologic materials at SSI (Nørgaard-Pedersen and Hougaard, 2007).

ASD cases in the HBC were identified based on the International Classification of Diseases, 8th Danish Revision (ICD-8) codes 299.xx (Sundhedsstyrelsen, 1986) up to 1993, and ICD-10 codes DF84.xx (Sundhedsstyrelsen, 2008) since 1994. The controls, however, were non-ASD individuals retrieved from the HBC and frequency-matched with cases on gender and year of birth (i.e. selected in proportion to the distribution of gender and year of birth of cases).

In this study, only singleton ASD cases born between 1982 and 2000 were included to maximize overlapping with the Danish Newborn Screening Biobank (Nørgaard-Pedersen and Hougaard, 2007).

Psychiatric diagnoses in the HBC were identified utilizing the Danish Psychiatric Central Register (DPCR). DPCR data are computerized and have been collected systematically from all psychiatric wards and hospitals in Denmark since 1969 (Munk-Jørgensen and Mortensen, 1997). Furthermore, primary diagnoses in the Danish National Hospital Register (DNHR) were utilized to identify cases of congenital malformation and to complement diagnoses of mental retardation and childhood psychiatric co-morbidity in the DPCR. DNHR has been computerized for more than 30 years and systematically collects data on all somatic hospital admissions in Denmark. Finally, the data regarding the obstetric history of the subjects were retrieved from the Danish Medical Birth Registry (MBR) which includes data on all live births, stillbirths and infant deaths in Denmark (Knudsen and Olsen, 1998).

The chemokine measurements were performed at SSI Luminex Laboratory, Copenhagen, Denmark, according to Skogstrand et al. on 50-μl of amniotic fluid using an in-house multiplex immunoassay (Skogstrand et al., 2005). Working Range (WR) for each analyte was assessed from the precision profile and defined as the concentration range in which the Coefficient of Variation (CV) was below 25%. For the three selected chemokines, WR’s were: MCP-1: 31–500 pg/ml, MIP-1α: 125–4000 pg/ml, and RANTES: 8–1000 pg/ml.

The study was approved by the Danish Data Protection Agency (Record No. 2009-41-3173) and The Danish Ethical Committee of Midtjylland Region (Record No. M-20090066).

2.1. Statistical analysis

Chemokine levels were analyzed on categorical and continuous scales using logistic and tobit regression models. In the logistic regression models, two cut-off points were introduced at the 10th and 90th percentiles, where applicable. Percentiles for each analyte were calculated based on distribution of the analyte in controls. For MCP-1, as the 90th percentile was above WR, it was replaced with the upper value of its WR. For RANTES, however, the 10th percentile fell below the WR, and thus it was replaced with the lower value of WR. Associations between the chemokine levels and ASD were reported using odds ratios (OR) with 95% confidence intervals (CI), and chemokine levels were expressed as elevated or decreased if falling in the upper or the lower 10th percentile of the controls’ distribution, respectively.

On a continuous scale, tobit regression was chosen as it is appropriate for the truncated data with many analyte measurements falling above or below WR. The distributions of the analyzed chemokines were skewed, however, log-transformed measurements of MCP-1 and MIP-1α and inverse-transformed measurements of RANTES turned out to comply well with the assumption of a truncated normal distribution. 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). Assumptions of normal distribution of residuals and homoskedasticity were evaluated using normal quantile plots and residual against fitted values plots, respectively.

Crude and adjusted odds ratios, comparing chemokine levels in ASD cases and controls were calculated in our primary analysis. This was performed in two levels to overcome potential classification bias due to different diagnostic tools (ICD-8 and ICD-10) used over the cohort follow-up period. First, all individuals in the study were analyzed. Subsequently, a subgroup comprised of individuals born after 1993 where only ICD-10 was used as the diagnostic tool, was analyzed. Variables used for frequency matching (gender and year of birth) were included as covariates in both crude and adjusted estimates. In the adjusted analyses, however, gestational week of amniocentesis, being diagnosed with any congenital malformation diagnosis or other childhood psychiatric co-morbidity along with previously identified potential confounders (mother’s and father’s age, birth weight, gestational age, APGAR score and parity) were included (Larsson et al., 2005; Newschaffer et al., 2007). Furthermore, the data were analyzed by gender and after excluding congenital malformation diagnoses.

For individuals born in 1990–1999, a secondary analysis on infantile autism cases was conducted with an additional control group comprising a subset of controls, who had at least one childhood psychiatric disorder other than ASD. Therefore, it was possible to compare Infantile Autism (IA) cases to non-psychiatric comorbidity (NPC) controls and cases of other childhood psychiatric disorder (OCPD). The reason for choosing birth years 1990–1999 in the secondary analysis was to maximize the overlap with a recent validation study in which almost all IA cases born during this period and registered in the DPCR were individually validated based on their hospital records (Lauritsen et al., 2010). Logistic regression analyses were performed comparable to our main analyses above, comparing IA to NPC controls, OCPD to NPC controls, and IA to OCPD.
Since the study is exploratory, we did not adjust for multiple testing. Throughout the paper ‘significant’ indicates nominal significance at the five percent level. IBM PASW Statistics 18.0 (SPSS Inc., 2009) was used for data management and the statistical analyses were performed using STATA Statistical Software 11.2 (StataCorp LP, 2009).

3. Results

A flow chart of case-control selection process is presented in Fig. 1. Total of 414 ASD cases and 820 frequency-matched controls were included in the study. ASD cases comprised 94 infantile autism (IA), 126 Asperger Syndrome (AS), and 194 other ASD (O-ASD) diagnoses; average age of ASD diagnosis (defined by the first admission date registered in the DPCR) was 9.63 years. This ranged from 7.84 years for IA cases up to 11.13 years for AS. Background information for the study population is presented in Table 1.

ASD cases were more likely to have a mother older than 35 years (OR: 1.28 [95% CI: 1.01–1.63]), to be diagnosed with congenital malformation (OR: 2.78 [95% CI: 1.96–3.96]), to be born as a first child (OR: 1.32 [95% CI: 1.03–1.69]), and to have an Apgar score below seven, recorded at five minutes after birth (OR: 2.46 [95% CI: 1.01–5.99]). In addition, ASD cases were more significantly co-diagnosed with a number of psychiatric and neurodevelopmental disorders (Table 1). Out of the study population, 80% of cases (n = 331) and 85% of controls (n = 698) had sufficient amniotic fluid volume to be included in the multiplex cytokine analysis. Individuals excluded due to insufficient amniotic volume did not differ significantly from those included, except for diagnoses of congenital malformation (the included controls were less likely to be diagnosed with a congenital malformation diagnosis compared to those excluded) (OR: 0.47 [95% CI: 0.27–0.83]).

In the primary analysis, no overall difference in chemokine levels were found in ASD cases compared to controls. However, analyzing individuals born post-1993 showed significantly elevated levels of MCP-1 in ASD cases (ICD-10 diagnosed) compared to controls (crude OR: 1.74 [95% CI: 1.05–2.87], adjusted OR: 2.32 [95% CI: 1.17–4.61]), with P values 0.03 and 0.02, respectively. Excluding individuals with congenital malformation co-morbidities did not alter the direction of the overall estimates (adjusted OR: 2.34 [95% CI: 1.10–4.96]). Stratifying on gender, however, did not show significant difference between males and female ASD cases compared to their counterpart controls and tests of homogeneity for the three chemokines were statistically insignificant. Tables 2 and 3 present the crude and the adjusted ORs with 95% CIs for the three analyzed chemokines.

Exploring the analytes on a continuous scale did not yield statistically significant differences between cases and controls. Crude and adjusted tobit regression model estimates did not suggest that any of ASD regression coefficients for three examined chemokines was significantly different from zero (Table 4).

In the secondary analyses of individuals born 1990–99 (Table 5), IA cases had significantly elevated levels of MCP-1 compared to controls with no psychiatric comorbidities (NPC) (adjusted OR: 2.28 [95% CI: 1.16–4.48]). No significant difference in levels MIP1-α or RANTES were found. In OCPD cases, however, levels of chemokines did not differ significantly from controls without psychiatric disorders. Finally, no significant difference in chemokine levels was detected between IA and OCPD cases and this was largely due to the limited statistical power.

4. Discussion

In this study, maternal amniotic fluid samples for three inducible CC chemokines (MCP-1, MIP-1α, and RANTES) were analyzed. Levels of MCP-1 were significantly elevated in ICD-10 diagnosed ASD cases compared to frequency-matched controls. No significant difference was found in the levels of MIP-1α and RANTES. Results were comparable when excluding ASD cases with congenital

![Fig. 1. Flow chart of Autism Spectrum Disorders (ASD) cases and controls selection process in the Historic Birth Cohort (HBC).](image-url)
to identify depression diagnoses.

- congenital malformation diagnoses.
- Mental Retardation (MR) diagnoses.
- Attention Deficit/Hyperactivity Disorder (ADHD) diagnoses.
- schizophrenia diagnoses.

Crude estimates of chemokine levels falling below 10th or above 90th percentile in ASD cases compared to controls.

Table 2
Characteristics of the study population.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ASD cases (%)</th>
<th>ASD controls (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>79 (19.1)</td>
<td>160 (19.5)</td>
</tr>
<tr>
<td>Male</td>
<td>335 (80.9)</td>
<td>660 (80.5)</td>
</tr>
<tr>
<td>Mothers age, y &lt;30</td>
<td>109 (26.3)</td>
<td>226 (27.6)</td>
</tr>
<tr>
<td>30–35</td>
<td>120 (29.0)</td>
<td>283 (34.5)</td>
</tr>
<tr>
<td>&gt;35†</td>
<td>185 (44.7)</td>
<td>311 (37.9)</td>
</tr>
<tr>
<td>Fathers age, y&lt;30</td>
<td>73 (17.6)</td>
<td>181 (22.1)</td>
</tr>
<tr>
<td>30–35</td>
<td>130 (31.4)</td>
<td>261 (31.8)</td>
</tr>
<tr>
<td>&gt;36–40</td>
<td>112 (27.1)</td>
<td>220 (26.8)</td>
</tr>
<tr>
<td>&gt;40</td>
<td>92 (22.2)</td>
<td>154 (18.8)</td>
</tr>
<tr>
<td>Gestational age, days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preterm (&lt;260)</td>
<td>43 (10.4)</td>
<td>87 (10.6)</td>
</tr>
<tr>
<td>Term (260–294)</td>
<td>368 (88.9)</td>
<td>716 (87.3)</td>
</tr>
<tr>
<td>Post-term (&gt;295)</td>
<td>3 (0.7)</td>
<td>9 (1.1)</td>
</tr>
<tr>
<td>Birth weight (BW), grams&lt;2500</td>
<td>25 (6.0)</td>
<td>47 (5.7)</td>
</tr>
<tr>
<td>Normal BW (2500–4000)</td>
<td>328 (79.2)</td>
<td>626 (76.3)</td>
</tr>
<tr>
<td>Macrocosmic (&gt;4000)</td>
<td>59 (14.3)</td>
<td>144 (17.6)</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Child</td>
<td>156 (37.7)</td>
<td>258 (31.5)</td>
</tr>
<tr>
<td>2nd Child</td>
<td>258 (62.3)</td>
<td>561 (68.4)</td>
</tr>
<tr>
<td>APGAR score&gt;7</td>
<td>398 (96.1)</td>
<td>804 (98.1)</td>
</tr>
<tr>
<td>&lt;7</td>
<td>11 (2.7)</td>
<td>9 (1.1)</td>
</tr>
<tr>
<td>Congenital malformation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congenital malformation present</td>
<td>83 (20.1)</td>
<td>68 (8.3)</td>
</tr>
<tr>
<td>Neurodevelopment and psychiatric co-morbidities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mental retardation</td>
<td>88 (21.3)</td>
<td>10 (1.2)</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td>12 (2.9)</td>
<td>6 (0.7)</td>
</tr>
<tr>
<td>Attention deficit/hyperactivity disorder (ADHD)</td>
<td>74 (19.7)</td>
<td>26 (3.2)</td>
</tr>
<tr>
<td>Depression</td>
<td>28 (6.8)</td>
<td>10 (1.2)</td>
</tr>
</tbody>
</table>

a Percentages do not add to 100% because of missing data.
b P value <0.05.
c ICD-8 codes 740.x–759.x, and ICD-10 codes Q00.x–Q99.x were used to identify congenital malformation diagnoses.
d ICD-8 codes 310.x–315.x, and ICD-10 codes D70.x–D79.x were used to identify Mental Retardation (MR) diagnoses.
e ICD-8 codes 295.x, and ICD-10 codes D20.x–D31.x were used to identify schizophrenia diagnoses.
f ICD-10 codes D90.xx were used to identify Attention Deficit/Hyperactivity Disorder (ADHD) diagnoses.
g ICD-8 codes 296.x, and ICD-10 codes F30.x–F34.x, F38.x, F39.x were used to identify depression diagnosises.

Differential levels of chemokines have been previously reported in plasma and brain tissue of ASD individuals (Ashwood et al., 2011; Vargas et al., 2005). However, to our knowledge, chemokine levels in AF samples have not yet been investigated in relationship to autism. Chemokines play an important physiological role in placental development, maintaining pregnancy and parturition (Kayislı et al., 2002). Increased levels of a neutrophil attractant chemokine (IL-8) were reported to be associated with other neurodevelopmental disorder, namely schizophrenia (Brown et al., 2004), which is hypothesized to share a common etiologic pathway with autism during pregnancy (Meyer et al., 2011).

It is unclear whether the elevated amniotic fluid levels of MCP-1 reported in this study represent an etiologic immunologic dysfunction in ASD or an epiphenomenon of a global neurodevelopmental pathology (Heuer et al., 2008).

The role of MCP-1 in neuroinflammation has been well established using the animal model of Experimental Autoimmune Encephalomyelitis (EAE) where a positive correlation between the expression of MCP-1 and the degree of inflammation in the CNS is reported (Mahad and Ransohoff, 2003). In humans, increased expression of MCP-1 has been reported in different neuroinflammatory diseases such as Multiple Sclerosis, Alzheimer Disease and traumatic brain damage (Conductier et al., 2010). Given the potential critical role of neuroinflammation in the development of ASD (Pardo et al., 2005), the elevated levels reported in this study may reflect a neuroinflammatory state in the fetal brain which eventually leads to the clinical phenotype. Upregulated levels of MCP-1 may also reflect a more profound dysfunction of a fetal brain origin. MCP-1 is believed to play an important role in the maturation of cerebellar Purkinje cells, and may serve as a useful marker of abnormal neuronal development (Zhen Meng et al., 1999). So, in theory, a genetic predisposition and/or an

Table 2
Crude estimates of chemokine levels falling below 10th or above 90th percentile in ASD cases compared to controls.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Autism spectrum disorders</th>
<th>ICD-8 and ICD-10&lt;sub&gt;a&lt;/sub&gt;</th>
<th>NCM (n = 915)</th>
<th>ICD-10&lt;sub&gt;b&lt;/sub&gt;</th>
<th>NCM (n = 373)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.71 (0.44–1.16)</td>
<td>0.66 (0.38–1.14)</td>
<td>0.66 (0.34–1.29)</td>
<td>0.61 (0.28–1.32)</td>
</tr>
<tr>
<td></td>
<td>90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.19 (0.87–1.63)</td>
<td>1.22 (0.87–1.72)</td>
<td>1.74 (1.05–2.87)</td>
<td>1.61 (0.92–2.83)</td>
</tr>
<tr>
<td>MIP-1x</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70 (0.43–1.14)</td>
<td>0.66 (0.39–1.14)</td>
<td>0.73 (0.40–1.34)</td>
<td>0.73 (0.37–1.43)</td>
</tr>
<tr>
<td></td>
<td>90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.26 (0.83–1.90)</td>
<td>1.21 (0.77–1.91)</td>
<td>1.24 (0.64–2.39)</td>
<td>1.12 (0.53–2.38)</td>
</tr>
<tr>
<td>RANTES&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83 (0.63–1.10)</td>
<td>0.84 (0.62–1.14)</td>
<td>0.78 (0.52–1.17)</td>
<td>0.81 (0.52–1.26)</td>
</tr>
<tr>
<td></td>
<td>90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.24 (0.82–1.88)</td>
<td>1.34 (0.86–2.09)</td>
<td>1.55 (0.75–3.22)</td>
<td>1.76 (0.81–3.83)</td>
</tr>
</tbody>
</table>

NMC: No congenital malformation.

<sup>a</sup> Total number of ASD cases = 331, NCM ASD cases = 267. Includes all individuals born 1982–2000 where both ICD-8 and ICD-10 were used.

<sup>b</sup> Total number of ASD cases = 145, NCM ASD cases = 110. Includes individuals born after 1993 where only ICD-10 was used.

<sup>c</sup> Upper value of working range (WR) is reported instead of 90th percentiles cut-off point.

<sup>d</sup> Lower value of working range (WR) is reported instead of 10th percentiles cut-off point.
Environmental insult during pregnancy may be result in an overexpression of MCP-1 in fetal brain. This overexpression may intervene with normal neurodevelopment and ultimately lead to autism (Hesselgesser and Horuk, 1999).

The ability of MCP-1 to induce blood–brain barrier breakdown (Stamatovic et al., 2005) and the localization of MCP-1 in nerve terminals in the posterior pituitary gland may explain its presence in the fetal circulation (Rostène et al., 2007) and eventually in the posterior pituitary gland. Interestingly, the role of MCP-1 in preterm delivery and autism in epidemiological studies (Newschaffer et al., 2007), the consistently reported associations between preterm delivery and autism in epidemiological studies (Newschaffer et al., 2007), the consistently reported associations between preterm delivery and autism.

Additionally, MCP-1 plays an important role in response to different viral and bacterial infections (Epstein and Luster, 1998). Given the fact that maternal infections have been etiologically linked to autism (Atladottir et al., 2010; Nahmias et al., 2006), one may postulate that elevated MCP-1 levels may be induced by a maternal viral or bacterial infection or, in broader terms, a maternal immune activation that eventually leads to the clinical phenotype later in life (Ashdown et al., 2006).

As an epiphenomenon, elevated levels of MCP-1 can be approached in concert with other obstetric complications. Elevated levels of MCP-1 have been repeatedly reported in preterm labor and premature rupture of membranes (PROM) (Gomez-Lopez et al., 2010; Holst et al., 2007). Interestingly, the role of MCP-1 in preterm labor was proposed to take place regardless of the presence of intra-amniotic infection (IAI) (Esplin et al., 2005). Given the consistently reported associations between preterm delivery and autism in epidemiological studies (Newschaffer et al., 2007), it is also possible that the connection between MCP-1 and autism is rather an indirect one.

The lack of significant differential levels of MCP-1, when included in ICD-8 (code 299.00) to ICD-10 (code C08.0) from the period where ICD-8 was used till the end of 1993, after which ICD-10 was implemented (Lauritsen et al., 2010). In the ICD classification, the definition of infantile autism has changed from psychosis proto-infantalis in ICD-8 (code 299.00) to childhood autism.
in ICD-10 (code F84.0). The other ASD diagnoses, however, were listed in more specific diagnostic entities in ICD-10. The transition from ICD-8 to ICD-10 is considered to have improved considerably the definition of the diagnostic entities of ASD (Lauritsen et al., 2004). So most likely, some individuals with ASD were not diagnosed as such before 1994, but might have been among the controls instead which explains the lack of significant difference to some extent. This is supported by the significant findings of the secondary analysis where infantile autism cases from pre- and post-1993 are compared to controls with no psychiatric comorbidities.

This study has the typical limitations of register based studies. All psychiatric and somatic diagnoses were retrieved from nationwide registers (DPCR and DNHR), and no information on autistic clinical symptomatology was available to validate the diagnoses. The secondary analysis based on recently validated infantile autism cases (with a validity ratio of 94% for the period 1990–1999) suggests that relying on the DPCR data may not necessarily be an important limitation (Lauritsen et al., 2010). For DNHR, however, only primary diagnoses were included as they were reported to have the highest validity (Andersen et al., 1999). Clinical diagnoses in the DNHR have validity ratios that range from 75% and up to 98% for some cardiac congenital malformations (Agergaard et al., 2011; Andersen, 1999).

The changes in concentrations of chemokines measured in stored amniotic fluid samples due to lengthy storage periods could also bias our estimates of association (Kugler et al., 2011). The samples analyzed were stored over a period of 20 years, and some individuals did not have enough amniotic volume for analyses. However, estimates from analyzing the last 6 years in the cohort, frequency matching, and adjusting for year of birth in our regression models, along with consistent storage policies at SSI should minimize this potential bias (Nørgaard-Pedersen and Hougaard, 2007).

Despite the limitations encountered, this study had the advantage of obtaining all covariates independently of exposure which minimizes bias from differential misclassification and selection bias. Having a unique identifier (CPR number) assigned for each citizen in Denmark (Pedersen et al., 2006) made it feasible for us to identify the corresponding dried blood spots in the Danish Newborn Screening Biobank at SSI for further examination of the same analytes neonatally, and therefore, will allow us to examine the pattern of the selected chemokines in the same individuals postnatally.

In conclusion, this study showed significantly increased amniotic fluid levels of MCP-1 in ASD cases compared to controls. This was also seen in IA cases when compared to controls with no psychiatric comorbidities. Given the biologic plausibility and the functional diversity of MCP-1, future studies confirming its role and exploring the different pathways through which MCP-1 may contribute to the development of ASD should be conducted.

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