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Perinatal exposure to potential endocrine disrupting chemicals and autism spectrum disorder: From Norwegian birth cohort to zebrafish studies

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ABSTRACT

Background: The etiology of autism spectrum disorder (ASD) is multifactorial, involving genetic and environmental contributors such as endocrine-disrupting chemicals (EDCs).
Objective: To evaluate the association between perinatal exposure to 27 potential EDCs and ASD among Norwegian children, and to further examine the neurodevelopmental toxicity of associated chemicals using zebrafish embryos and larvae.
Method: 1,199 mothers enrolled in the prospective birth-cohort (HUMIS, 2002–2009) study. Breastmilk levels of 27 chemicals were measured: polychlorinated biphenyls, organochlorine pesticides, polybrominated diphenyl ethers, and perfluoroalkyl substances as a proxy for perinatal exposure. We employed multivariable logistic regression to determine association, utilized elastic net logistic regression as variable selection method, and conducted an *in vivo* study with zebrafish larvae to confirm the neurodevelopmental effect.
Results: A total of 20 children had specialist confirmed diagnosis of autism among 1,199 mother–child pairs in this study. β-Hexachlorocyclohexane (β-HCH) was the only chemical associated with ASD, after adjusting for 26 other chemicals. Mothers with the highest levels of β-HCH in their milk had a significant increased risk of having a child with ASD (OR = 1.82, 95 % CI: 1.20, 2.77 for an interquartile range increase in ln-transformed β-HCH concentration). The median concentration of β-HCH in breast milk was 4.37 ng/g lipid (interquartile range: 2.92–6.47), and the estimated daily intake (EDI) for Norwegian children through breastfeeding was 0.03 μg/kg of body weight. The neurodevelopmental and social behavioral effects of β-HCH were established in zebrafish embryos and larvae across various concentrations, with further analysis suggesting that perturbation of dopaminergic neuron development may underlie the neurotoxicity associated with β-HCH.
Conclusions: Prenatal exposure to β-HCH was associated with an increased risk of specialist-confirmed diagnoses of ASD among Norwegian children, and the EDI surpasses the established threshold. Zebrafish experiments confirm β-HCH neurotoxicity, suggesting dopaminergic neuron disruption as a potential underlying mechanism.

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1. Introduction

Autism spectrum disorder (ASD) refers to a wide range of neurodevelopmental conditions characterized by varying degree of impaired social behavior, communication and language difficulties, repetitive behaviors, and limited range of interests and activities (American Psychiatric Association, 2013). The economic burden of ASD is considerable, estimated at US $ 2.2–2.4 million per individual in the United States and in the United Kingdom, due to the need for special education services and loss of parental productivity (Buescher et al., 2014).

The prevalence of ASD in children up to 11 years is 0.7 % in Norway based on the Norwegian Patient Register (Surén et al., 2012), slightly lower than in the US (1.7 %) (Baio et al., 2018), and in the UK (1.2 %) (Baird et al., 2006) and has increased substantially in recent times. However, it is not yet clear whether the increase is due to improved awareness, better ascertainment, broadening diagnostic criteria, or a true increase in incidence (Baird et al., 2006). Genetic and environmental factors play a role in ASD's etiology, but the exact causes are still poorly understood. The prevalence of ASD is approximately four times higher in boys than in girls (Werling and Geschwind, 2013), and this could point to both genetic and hormonal factors. Known risk factors for ASD include advanced parental age, preterm birth and short interpregnancy interval, and suspected factors include metabolic conditions, non-optimal nutrients status, and endocrine disrupting chemicals (EDCs) (Lyall et al., 2017a).

Exposure to EDCs may interfere with neuronal connectivity (Stamou et al., 2013), possibly through alteration of GABAergic, glutamatergic, serotonergic and dopaminergic system, oxidative stress, and may cause epigenetic alterations (Quaak et al., 2013). Early-life exposure to EDCs has been associated with ASD in humans (Braun et al., 2014; Lyall et al., 2017b; Marijke et al., 2012). Breast milk concentration of Persistent organic pollutants (POPs) reflect maternal body burden, and thus gestational exposure, as well as postnatal exposure through breastfeeding (Cerrillo et al., 2005).

The aim of this study was to investigate the association between breast milk concentration of four classes of persistent chemicals (Polychlorinated biphenyls (PCBs), Organochlorine Pesticides (OCPs), Perfluoroalkyl substances (PFASs), and Polybrominated diphenyl ethers (PBDEs)) and the risk of ASD. Furthermore, the objective was to conduct tailored *in vivo* experimental studies using zebrafish (Danio rerio) to evaluate the effects of the chemicals identified in the epidemiologic study. Brain development was assessed by proliferation assay and whole mount in situ hybridization, and effects on touch response, locomotion and social behavior were quantified in larvae.

2. Materials and methods

2.1. Epidemiological study

Study population.

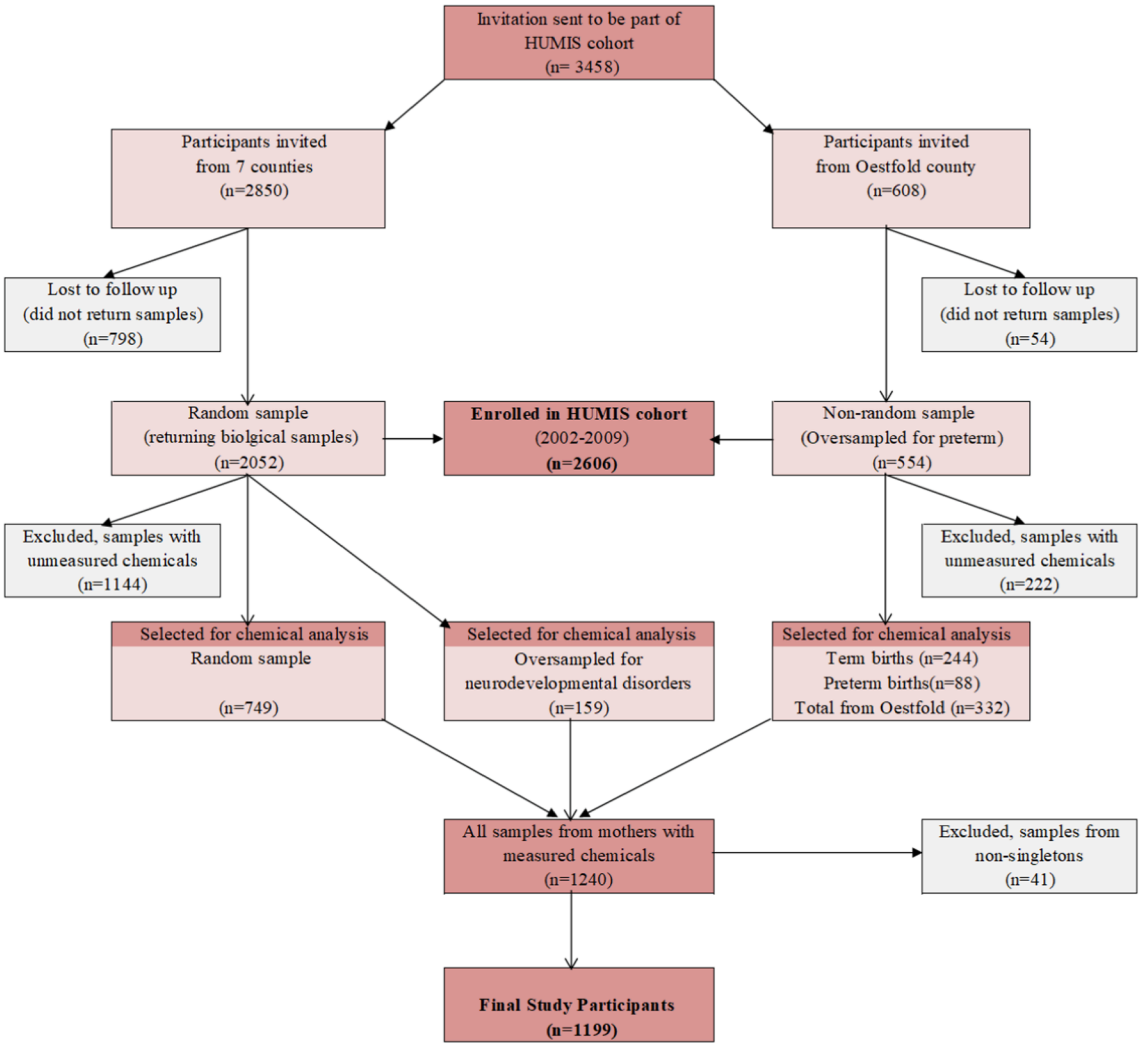

Fig. 1. Flow chart showing selection of the study participants from the HUMIS cohort.

The Norwegian Human Milk Study (HUMIS) is a prospective seven-center population-based birth cohort of 2606 mother-infant pairs established in 2002–2009, recruited postpartum (Lenters et al., 2019). In one center, for every mother of a preterm infant, two mothers of term infants were consecutively enrolled. The current study is based on the subset of 1199 mothers for whom breast milk samples have been analyzed for chemicals in previous projects (see Fig. 1). Twins/triplets (n = 41) were excluded. Three-fourths of the final study subset (883/1199) were randomly selected while 13 % were oversampled for neurodevelopmental disorders (ADHD, ASD, and cognitive delay) and 7 % for preterm. Milk was collected when the infants were around one month (median 33 days, IQR, 25–43 days), following a WHO milk collection protocol. Further details have been published elsewhere (Eggesbø et al., 2011, Eggesbø et al., 2009). The study was approved by the Norwegian Data Inspectorate (ref. 2002/1398), and Regional Ethics Committee for Medical Research (ref. S-02122). Informed consent was also obtained from all participating women prior to enrolment.

Sample/milk Collection.

Mothers were asked to collect 25 ml of breast milk samples every morning for 8 consecutive days from the child was 2 weeks until 2 months of age, in line with the WHO recommendations for milk collection, but milk sampled otherwise was also accepted (WHO, 2007). The milk was stored in a 250 ml container and kept in the freezer. Date and time of collection was recorded, as well as whether a breast pump had been used. When the container had been filled, participants mailed it by regular mail, except in the county of Østfold where the milk samples were collected by study personnel and kept frozen during transport to the Norwegian Institute of Public Health (NIPH).

Four laboratories took part in the chemical analyses as previously described (Forns et al., 2015, Polder et al., 2009, Thomsen et al., 2010, Forns et al., 2016, Cechova et al., 2017, Čechová et al., 2017, Lenters et al., 2019) (**Table S1**): the Department of Environmental Exposure and Epidemiology, Norwegian Institute of Public Health, the Department of Environmental Sciences, Norwegian University of Life Sciences, the Institute for Environmental Studies, Faculty of Earth and Life Sciences, VU University in the Netherlands, and the Research Centre for Toxic Compounds in the Environment, Masaryk University in the Czech Republic. Details about chemical analysis is described elsewhere (Lenters et al., 2019).

Chemical analysis of breast milk.

The milk biobank in the HUMIS study has been used in multiple projects throughout the years funding different chemicals and outcomes. This has resulted in a wide range of toxicants measured in different subsets (Polder et al., 2009). We restricted our analysis to 27 chemicals measured in at least 854 samples (range 854–1194), with concentrations above limit of detection (LOD) in at least half of the samples: fourteen PCBs, five OCPs, two PFASs, and six PBDEs (see Supplementary Table S1).

Exposure.

Breast milk concentrations of measured chemicals (14 PCBs, 5 OCPs, 2 PFASs, and 6 PBDEs) were used as a proxy for prenatal exposure due to positive correlation between levels measured in breast milk and in umbilical cord for persistent chemicals (Verner et al., 2013, Waliszewski et al., 2001).

For three chemicals, 7–28 % were below LOD and a value between zero and LOD was imputed. Values below the sample- and chemical-specific limit of detection were singly imputed using maximum likelihood estimation (Lubin et al., 2004), following a log-normal distribution and conditional on maternal age, parity, pre-pregnancy body mass index (BMI), and child's birth year. Among the 27 potential EDCS, 24 exposures had < 2 % below the LOD, and the remaining exposures had 7 % (PFOA), 10 % (PBDE-28), and 28 % (PBDE-154) of values below the LOD (Table S1). Postnatal concentrations of lipophilic Persistent organic pollutants POPs were calculated using a two-compartment pharmacokinetic model (Stigum et al., 2015) as a secondary analysis, which has previously been described in detail (Forns et al., 2015), and validated (Forns et al., 2018).

Outcome variable.

ASD cases were identified from the Norwegian Patient Registry which contains individual data since 2008 on specialist-confirmed diagnoses from government-owned hospitals and outpatient clinics based on International Classification of Diseases (ICD-10). Details about the outcome variable is described in the supplementary text. Linkage was performed in 2017 when children were 13 years of age (median 12.7, IQR, 11.8–13.4 years).

Covariates.

Information on potential confounders was obtained from questionnaires administered at 1, 6, 12, and 24 months postpartum, and from the Medical Birth Registry of Norway (child's sex, gestational age, birth weight, and maternal smoking during pregnancy).

Statistical analysis.

Multiple imputation by chained equations was used for all exposures below LOD (<2–28 %) and missing covariate data (≤3.2 %) up to the full sample size of 1199 (Van Buuren and Groothuis-Oudshoorn, 2011).

We defined two adjustment models based on a directed acyclic graph (Figure S1). In the first model (**M1**), we adjusted for child age at linkage (continuous), maternal age (continuous), and maternal education (≤12, >12 years). In the second model (M2) we further adjusted for covariates related to oversampling, and covariates for which evidence of an association with both the exposure and outcome is weaker: parity (primiparous, multiparous), small for gestational age (<10th percentile of sex-specific Norwegian standards (Skjaerven et al., 2000)), preterm birth (<37 weeks of gestation), smoking during pregnancy, pre-pregnancy BMI (continuous), single mothers (yes/no) and maternal fatty fish consumption (servings/year) (**M2**). The models were tested after log transformation (ln) of the exposure variables to reduce the influence of extreme values.

Multi-pollutant variable selection and estimation of measure of association.

Due to the high number of correlated exposures and potential for multicollinearity (Schisterman et al., 2017), a penalized elastic net logistic regression was used for variable selection (Zou and Hastie, 2005), which can handle correlated co-exposures and reduces the proportion of false positive results (Agier et al., 2016; Lenters et al., 2018). Details about elastic net regression method is described in the supplementary text.

As a sensitivity analysis, we also 1) tested untransformed exposure variables 2) tested estimated postnatal body burdens (Stigum et al., 2015) 3) explored potential effect modification by child sex, maternal education and parity (primiparous vs multiparous) 4) ran complete case analyses. For PFASs, we evaluated effect modification by breastfeeding duration as an indirect measure of postnatal exposure, since our pharmacokinetic model does not calculate postnatal estimates for chemicals with non-lipophilic properties.

We used Stata (version 16.0; StataCorp LP, College Station, Texas) to model postnatal estimates and R software (version 3.3.2, including the packages mice, glmnet, and selective Inference; R Foundation for Statistical Computing, Vienna, Austria) for all other statistical analyses.

2.2. *In vivo study (Zebrafish)*

Chemical exposure of zebrafish embryos.

Experimental studies explored the neurodevelopmental toxicity of β-HCH, with alpha-HCH and BDE-28 as negative controls for the cell proliferation assays. For Zebrafish husbandry see Supplements. Eggs from the AB wild type strain, collected via natural spawning, were exposed to β-HCH (Sigma 33376), α-HCH (Sigma 33856), BDE-28 (Sigma 05364) and DMSO (Sigma D8418) 3 h post fertilization (hpf). The lowest concentration we used (2.9 ng/mL β-HCH, equivalent to 0.01 uM) is approximately ten times higher than the average concentration of β-HCH in milk in our human study population, but below the maximum concentrations. We also tested a wide range of higher concentrations.

The negative control chemicals were tested in the highest concentration being equivalent to the ratio of the β-HCH concentration used (29 ng/mL), to the mean concentration in the human milk samples. The lowest concentrations are equivalent to the mean concentrations observed in our milk samples. DMSO vehicle (VEH) was added to an equivalent concentration for all dilutions and controls.

β-HCH uptake analysis in larvae.

To assess the actual β-HCH concentration achieved in the larvae, β-HCH was analyzed at the Laboratory of Environmental Toxicology at NMBU (Polder et al. (2014)), in 1 ml sample of medium, and in a sample of up to 100 larvae, taken at 3 or 7 dpf.

Whole-mount in situ hybridization analysis and hypothalamus measurement.

Treatment with 5 mM L-Dopa (Sigma 333786) started simultaneously with β-HCH exposure. Whole-mount in situ hybridization (WISH) analysis and hypothalamus measurement was performed on 3 dpf embryos, as previously described (Filippi et al., 2010, Schindelin et al., 2012). Experiments were repeated with the same exposure doses of β-HCH; 29 ng/mL, 290 ng/mL and 29 μg/mL, starting 3 hpf and thereafter refreshing the β-HCH solution daily from 3 dpf until readout at 7 dpf (i.e., four additional doses).

Shoaling assays.

Shoaling behaviour, e.g., the distance between larva, was assessed by VideoTrack System for Zebrafish™ (Version 2.3.1.0, ViewPoint, France) in groups of 5 larvae at 7 dpf, and analysed with Ethovision XT software (Version 14, Noldus, The Netherlands). Statistical analyses were performed with one-way ANOVA with Dunnett's post-hoc test.

Cell proliferation scoring in zebrafish.

To assess hyperproliferation of cells in the optic tectum, BrdU immunohistochemistry was performed at 5 dpf. Larvae were bathed in 10 mM BrdU (Sigma) solution in E3 with 15 % DMSO, incubated for twenty minutes on ice to allow for BrdU uptake, then placed for five minutes at 28 °C to label proliferating cells, and fixed in 4 % PFA. Details regarding subsequent immunohistochemistry are found in the supplementary information.

All experiments were performed in compliance with the European Community Council Directive of November 2010 for Care and Use of Laboratory Animals (Directive 2010/63/EU) and ARRIVE guidelines and with approval from The Norwegian Food Safety Authority (FOTS permit ID 15469 and 23935). For more details regarding the experiments see Suppl Text 2.

3. Results

3.1. Epidemiologic study

Table 1 displays the characteristics of the mother–child pairs. A total of 20 children (1.7 %) had been diagnosed with ASD according to the Norwegian patient registry (2017); 17 boys (85 %) and 3 girls (15 %) before they had reached a median age of 13 years (11.1–13.4). The median concentration of β-HCH (ng/g lipid) in breast milk was 4.37, IQR (2.92–6.47), 5th to 95th percentile (1.39–12.31), and range (0.002 to 280.8).

The distributions of the 27 measured chemicals are displayed in Fig. 2, while the numerical values and detection frequencies are shown in Table S1. β-HCH is weakly ($0.2 \leq r_p < 0.4$) to moderately ($0.4 \leq r_p < 0.6$) correlated with OCPs and PCBs, while the correlation with BDEs and PFASs is very low (Details in the supplementary Figure S2).

We estimated daily intake of β-HCH among Norwegian children via breastfeeding to be 0.03 *μg/kg of bw/day*, assuming a consumption rate of 125 g milk/kg of body weight per day and 3.5 % lipid composition of breast milk (van den Berg et al., 2017). The calculation is shown on the supplementary text.

Associations with ASD.

In the single-pollutant analysis, exposure to β-HCH, perfluorooctane sulfonate (PFOS) and some mono-*ortho*-substituted dioxin-like PCBs (PCB-105, PCB-156, PCB-167, PCB-189) were associated with increased risk of ASD, but β-HCH was the only chemical selected by elastic net. The risk of ASD nearly doubled for children exposed to ln-transformed β-HCH concentrations in the highest quartile compared to the lowest quartile (OR = 1.83, 95 % CI: 1.18, 2.85). The association estimates from the minimally adjusted and further adjusted models were similar in magnitude and precision. see Fig. 3 and Table S2. The multipollutant elastic net regression model selected β-HCH consistently in > 50 % of the models across imputed datasets (Figure S3, post-selection inference p = 0.001) ruling out chance finding, and no other chemical was selected in any models.

Sensitivity analysis of the exposure variables for potential effect modification for β-HCH by parity showed borderline significance when potential effect modifiers (sex, maternal education and parity) were in the model, indicating that the risk of ASD from β-HCH exposure is higher among multiparous women than primiparous women (Table S3).

Associations for modelled postnatal exposures to the toxicants did not reveal any added risks tied to the exposure during the breastfeeding period, nor was there effect modification by duration of breastfeeding, an indication that the critical window might be during the prenatal period (data not shown).

Table 1
Characteristics of study participants (n (%)[a] or median [IQR]) by ASD case status (HUMIS, Norway).

Characteristic	Cases n = 20	Total n = 1199
Age at start of pregnancy (years)		
16–26	5 (25)	298 (25)
27–33	13 (65)	645 (54)
34–44	2 (10)	193 (16)
Maternal education (years completed)		
≤12	7 (35)	267 (22)
>12	12 (60)	915 (76)
Pre-pregnancy body mass index (kg/m^2)		
Underweight (<18.5)	1 (5)	43 (4)
Normal weight (18.5–24.9)	9 (45)	723 (60)
Overweight (25–29.9)	7 (35)	281 (23)
Obese (≥30)	3 (15)	118 (10)
Parity		
1 (first child)	10 (50)	475 (40)
2	8 (40)	455 (38)
3–5	2 (10)	248 (21)
Single mother[b]	0 (0)	29 (2.4 %)
Smoked during pregnancy	1 (5)	132 (11)
Fatty fish consumption during pregnancy, (servings/year)	23 [7–67]	24 [10–48]
Child age at registry linkage (years)	12.5 [11.1–13.4]	12.7 [11.8–13.4]
Sex (boy)	17 (85)	655 (55)
Small-for-gestational age (<10th P)	1 (5)	135 (11)
Preterm (<37 weeks of gestation)	2 (10)	104 (9)
Breastfeeding, (months)	16 [12–17]	13 [9–16]

ASD, Autism Spectrum Disorder; IQR, interquartile range.
There were missing values for education (n = 63), body mass index (34), parity (20), marital status (8), smoking (12), fish consumption (39), child sex (2), and breastfeeding (20).
[a] *Percentages may not add up to 100 due to rounding or missing values.*
[b] *Single,* versus *married or living together with partner in perinatal period.*

3.2. In vivo study (Zebrafish)

Zebrafish embryos exposed from 3 hpf to β-HCH concentrations ranging from 2.9 ng/mL to 29 μg/mL, were less able to maintain an upright posture at 3 dpf, at all concentrations tested **(**Fig. 4**A)**. Touch-evoked response was significantly affected from 290 ng/mL concentration (about 20 times higher than the 95-percentile observed in infants) and upwards, based on the observation that larvae displayed a delay in typical escape behavior after being touched gently on the tail with a blunt needle (Fig. 4**B**). A significant increase in inter-individual distance

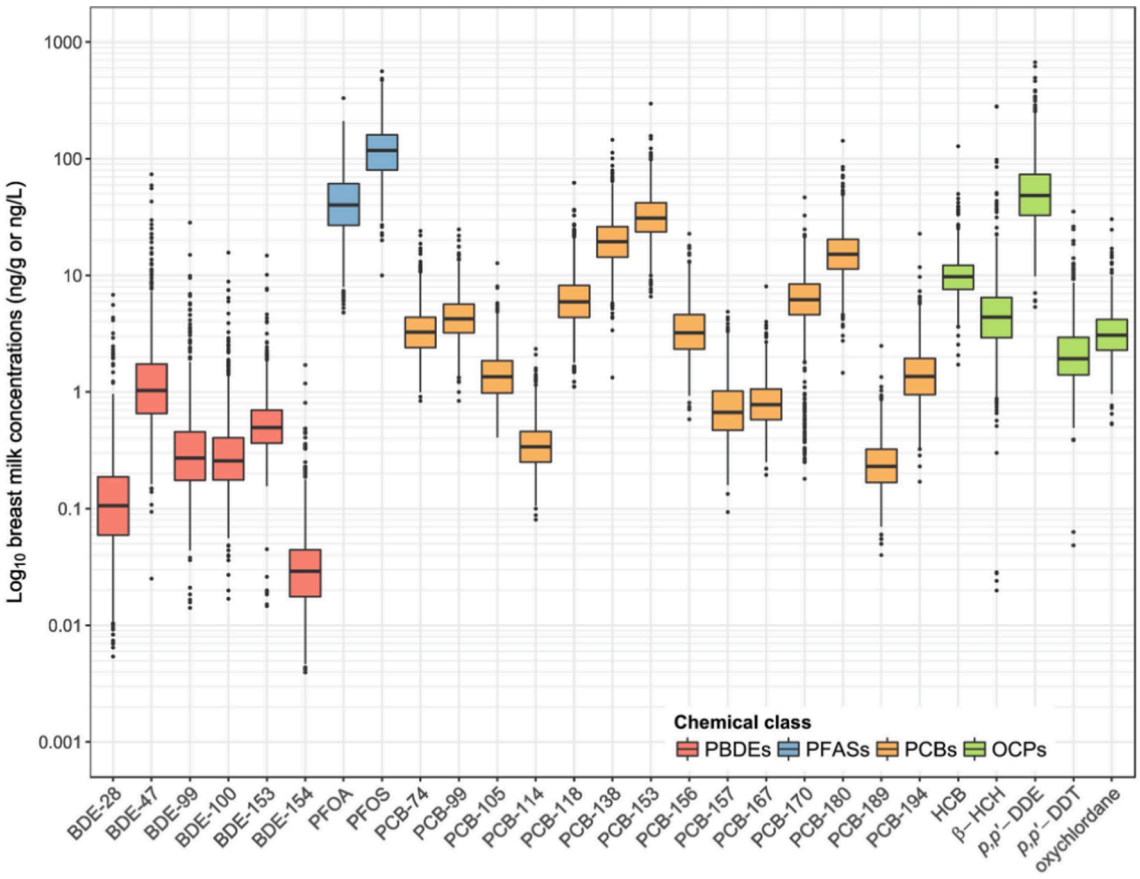

Fig. 2. Boxplot distributions of the measured breast milk concentration of the 27 chemicals. Horizontal lines correspond to medians, and boxes to the 25th–75th percentiles; whiskers extend to data within the interquartile range times 1.5, data beyond this are plotted as dots. Wet weight concentrations are presented for PFASs (ng/L) and lipid adjusted concentrations for all other toxicants (ng/g). (See supplementary material **Table S1** for numerical values.) This figure was reproduced from Lenters et al., 2019., under a Creative Commons license (CC BY 4.0.).Abbreviations: (P)BDE, (poly)brominated diphenyl ether; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; HCB, hexachlorobenzene; HCH, hexachlorocyclohexane; OCPs, organochlorine pesticides; PFASs, poly- and perfluoroalkyl substances; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate.

(the average distance between one larva and its four cognates) was observed in 7 dpf larvae exposed to 29 ng/mL β-HCH, but not in larvae exposed to 290 ng/mL or 29 μg/mL β-HCH (Fig. 4**C**).

During early development, the zebrafish optic tectum is hypothesized to perform part of the visual processing that in mammals occurs in the visual cortex (Orger., (2016)) and hyper-proliferation in this region may therefore correspond to the hyperproliferation observed in the human neonates that develop ASD (Courchesne et al., 2003, Schumann et al., 2010). We observed an increase in proliferating cells in the tectum of larvae exposed to β-HCH for 5 days, compared to vehicle-treated control larvae (Fig. 4**D-E**). Treatment with α-HCH or BDE-28 did not alter the number of proliferative cells observed in the tectum of 5 dpf larvae (Fig. 4**D**).

Exposure to β-HCH led to a reduction in size of the hypothalamus in all but the highest concentration (29 μg/mL; Fig. 5**A-B**), and addition of 5 mM of the dopamine precursor L-Dopa rescued posture irregularities. The addition of 5 mM L-Dopa restored hypothalamus size to normal but had an adverse effect on the size of untreated control larvae.

Quantitative analyses of β-HCH in larvae and medium showed that β-HCH was taken up by larvae in a dose-dependent manner. Furthermore, larval β-HCH concentrations were highest at 3 dpf for all exposure groups (**Figure S4**). At 7 dpf, and after receiving multiple doses of β-HCH, the level of β-HCH was reduced compared to 3 dpf, but still increased in a dose-dependent manner compared to control larvae.

4. Discussion

4.1. Principal findings

In this study, a significant association was found between the organochlorine pesticide residue β-HCH and ASD. Infants exposed to the highest quartile of β-HCH had doubling the odds of autism compared to those exposed to the lowest quartile. Furthermore, the neurotoxicity of β-HCH was confirmed through zebrafish experiments involving both acute and chronic exposures. However, we didn't find significant association between ASD and perinatal exposure to PCBs, PBDEs, and PFASs.

Norwegian children's average intake of β-HCH through breastfeeding slightly surpasses the threshold set by the Dutch National Institute for Public Health and the Environment (RIVM) (Tolerable daily intake = 0.02 μg/kg of bw/day)(Baars et al., 2001). However, there are no internationally recognized health-based guidance values for β-HCH at present (European Food Safety Authority et al., 2018). Low concentrations of EDCs may be enough to elicit a response, and cause lasting developmental effects (Bergman et al., 2013).

4.2. β-HCH

Epidemiologic findings. To our knowledge, this is one of the largest prospective cohort studies assessing the association of β-HCH and 26 other POPs, with specialist confirmed diagnosis of autism spectrum disorders (n = 1199), using single pollutant analysis and multi-pollutant analysis (elastic net regression as a variable selection method). A a small

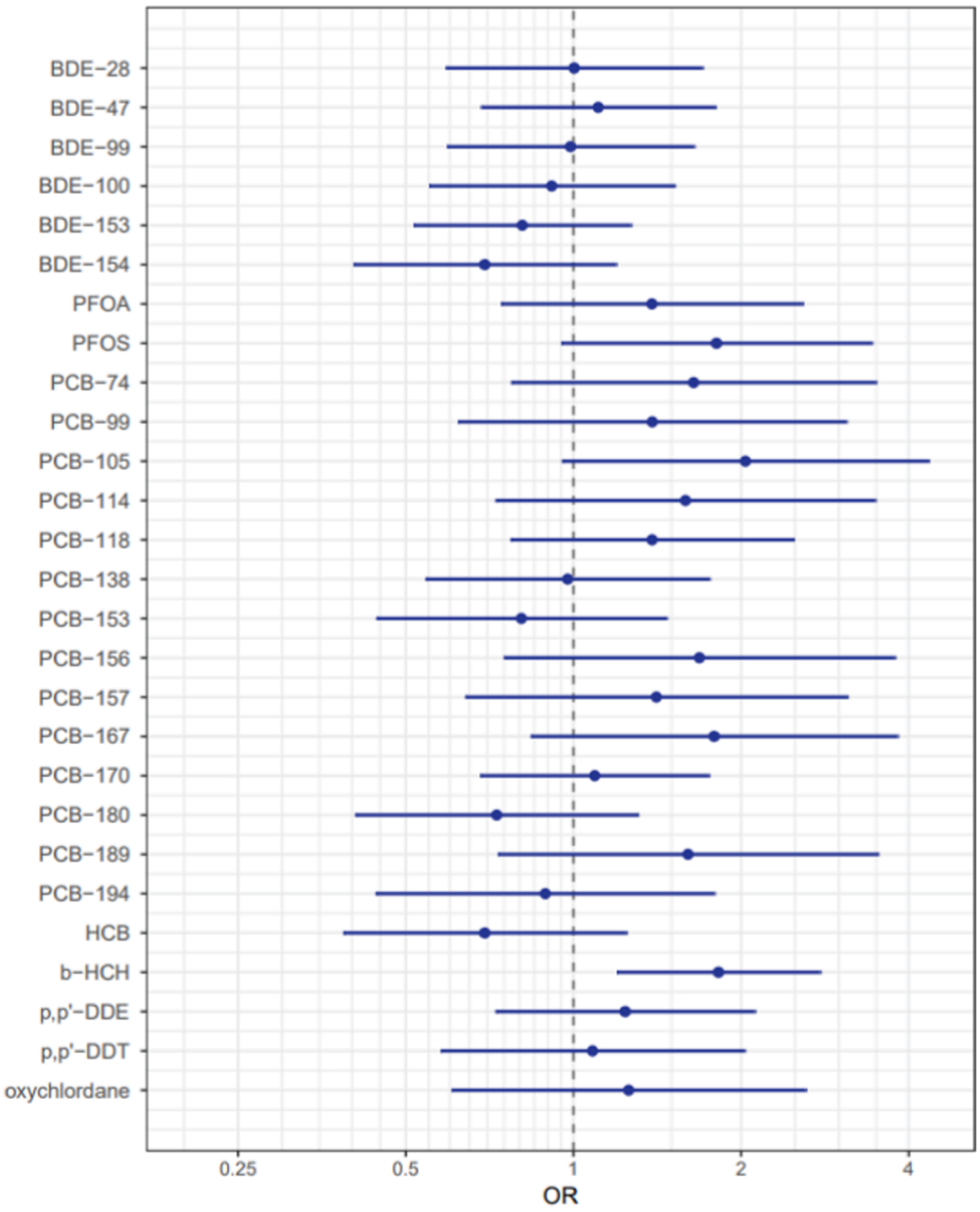

Fig. 3. Odds ratios (OR) and 95% confidence intervals for autism spectrum disorders (ASD) per interquartile ratio increase in ln-transformed measured exposure breast milk concentrations from HUMIS cohort, Norway. Coefficients from single-exposure logistic regression models are presented. Models were adjusted for child age at linkage, maternal age, and maternal education (M1). Coefficients were similar upon further adjustment for parity, small for gestational age, preterm birth, smoking during pregnancy, pre-pregnancy BMI, cohabitation or marital status, and maternal fatty fish consumption around pregnancy (M2). (See supplementary material **Table S2** for numerical values.).

cross-sectional survey in Greece observed almost twice as high mean serum concentration of β-HCH in children with ASD (n = 39) compared to controls (n = 18) (Makris et al., 2019). This compares to our results where the mean β-HCH concentration was three-fold higher in breast milk samples from mothers of ASD cases compared to non-ASD children. In contrast to our study, the prospective Health Outcomes and Measures of the Environment (HOME) Study (2003–2006), found fewer autistic behavior patterns among 4- and 5- year-old children born to 175 Ohio women with detectable versus non-detectable serum concentration of β-HCH (Braun et al., 2014). However, 73 % of the cohort had maternal serum β-HCH level below LOD, compared to only 0.3 % in our study. Furthermore, the diagnosis of ASD-related behaviors was based on lower Social Responsiveness Scale (SRS) completed by the mothers, which is less precise than specialist-confirmed diagnoses in our study.

The neurodegenerative property of β-HCH has also been reported following occupational and residential exposure. Case-control studies in Texas and the Faroe Islands have reported a positive association between β-HCH and Parkinson's disease (PD), dementia and Alzheimer's disease (Richardson et al., 2009, Petersen et al., 2008).

A sensitivity analysis examining potential effect modification of β-HCH by parity revealed borderline significance. The adjusted model suggested a higher ASD risk from β-HCH exposure in multiparous women compared to primiparous women. No additional risks were identified in relation to postnatal chemical exposure during

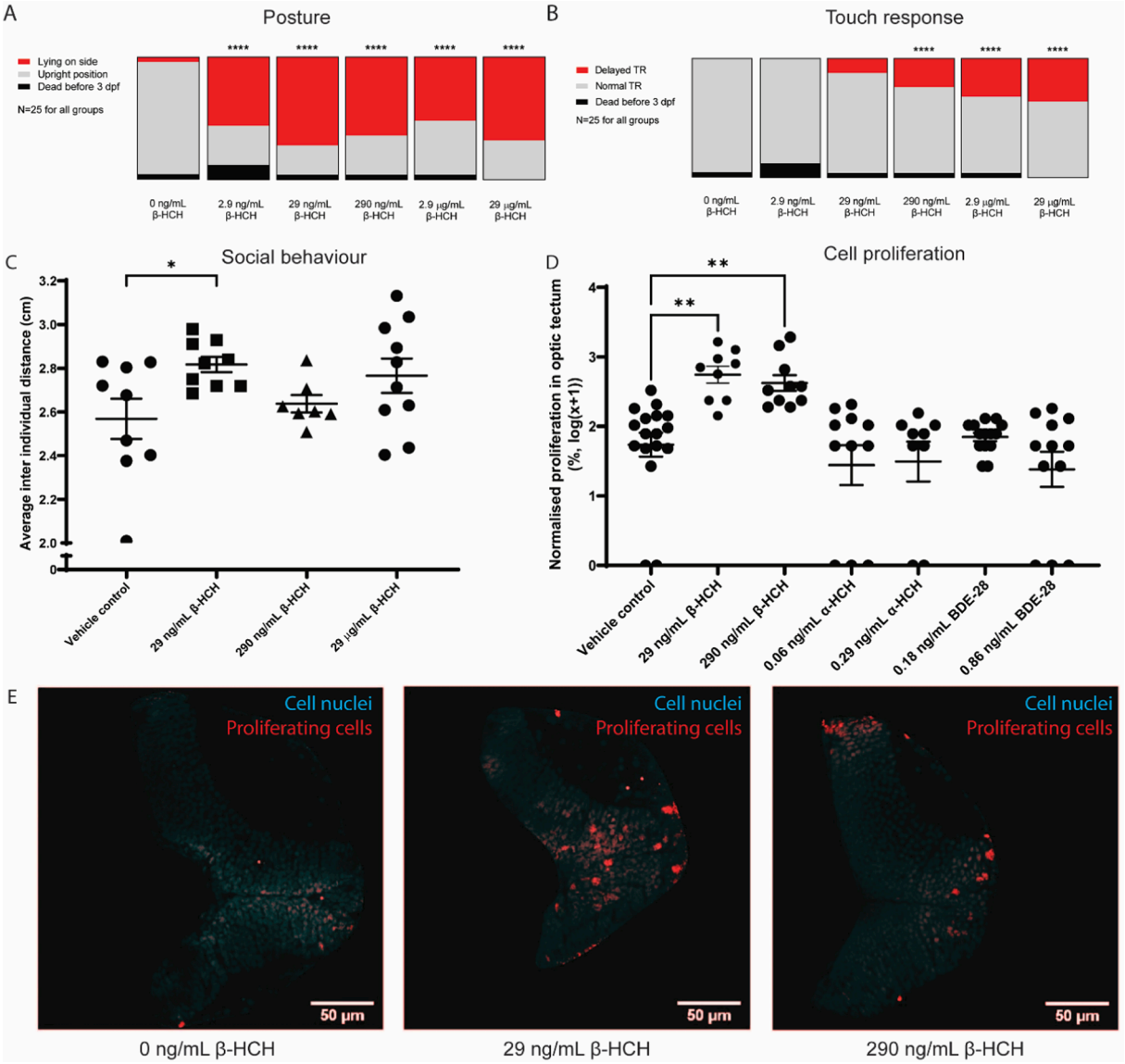

Fig. 4. Effects of β-HCH on early developmental stage zebrafish larvae. **A)** Distribution of larval posture at 3 days post fertilization after exposure to β-HCH in early developmental stages. **B)** Distribution of larval touch response at 3 days post fertilization after exposure to β-HCH. Significance was calculated using Chi-square test. **C)** Effect of β-HCH on shoaling behavior in 7 days post fertilization larvae. 0 ng/mL control: N = 9; 29 ng/mL: N = 9; 290 ng/mL: N = 7; 29 µg/mL: N = 10. Error bars represent S.E.M., significance was calculated using one-way ANOVA with Dunnett's post-test. **D)** Effect of β-HCH on cell proliferation in the optic tectum of 5 days post fertilization larvae. BrdU signal labelling proliferating cells was normalised to the mean of vehicle treated larvae and log transformed. 0 ng/mL control: N = 17; 29 ng/mL: N = 9; 290 ng/mL: N = 10. 0.06 ng/mL α-HCH: N = 11; 0.29 ng/mL α-HCH: N = 9; 0.18 ng/mL BDE-28: N = 14, 0.86 ng/mL BDE-28: N = 11. Error bars represent S.E.M., significance was calculated using a Kruskal-Wallis H test with Dunn's post-test. **E)** Representative images of BrdU staining in optic tectum of 5 days post fertilization larvae after β-HCH treatment. Red: BrdU-labeled proliferating cells, cyan: nuclear staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

breastfeeding, nor was there an effect modification by breastfeeding duration. This suggests that the critical exposure window might be the prenatal period.

Findings from zebrafish study. In the *in vivo* study, exposure to β-HCH resulted in an increase in the average distance between free-swimming individual larvae, indicating a reduction in social interaction, a behavioral pattern with similarities to ASD children. Furthermore, significantly more proliferative cells were observed in the optic tectum, which at this stage of zebrafish development corresponds to the brain region affected in children with ASD (Nevin et al., 2010) and is seen as a marker of ASD (Courchesne et al., 2003, Schumann et al., 2010). These effects were not observed when larvae were treated with α-HCH isoform, or BDE-28, at comparable concentrations. Our findings correspond to previous studies, where exposure to β-HCH, but not α-HCH, elicited neurological defects in rats, such as ataxia and significantly delayed tail nerve conduction (Dorsey, 2005).

The neurodevelopmental and socio-behavioral effects were observed in concentrations as low as 2.9 ng/ml of β-HCH in the larva, which corresponds to the milk concentrations in the mothers of the infants most highly exposed, even though direct comparisons can't be made due to different susceptibility across species. The lowest concentration used; 2.9 ng/mL β-HCH, equivalent to 0.01 uM, is approximately ten times higher than the average concentration of β-HCH, but below the maximum concentration in milk from participating mothers. We also tested a wide range of higher concentrations, since the efficiency of uptake of β-HCH from the water is not known, and to account for the large differences in exposure period between zebrafish larvae and human infants (days versus months).

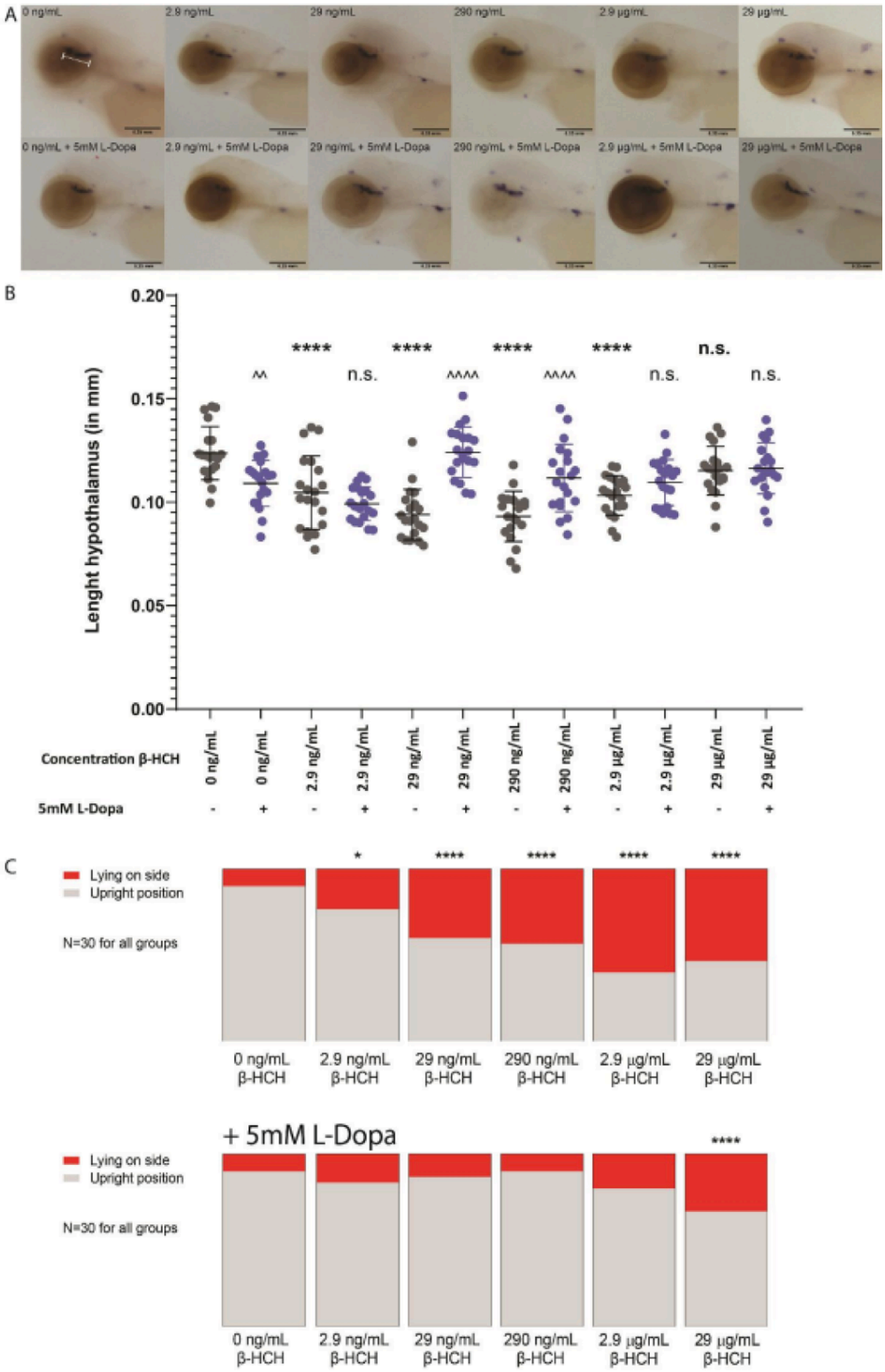

Fig. 5. (A-C). Whole-mount in situ hybridization analysis with TH1 probe in a 3 days post fertilization embryo, exposure to β-HCH, additional L-Dopa treatment, and distribution of larval posture. **A)** Whole-mount in situ hybridization analysis with TH1 probe in a 3 days post fertilization embryo. The hypothalamus is indicated in the upper left panel by a white bar. Upper panels are representative larvae treated with β-HCH at the indicated concentrations. Lower panels are representative images of larvae treated 5 mM L-Dopa in addition to indicated concentrations of β-HCH. Scale bars are 0.25 mm. **B)** Length of the hypothalamus of 3 days post fertilization embryos exposed to indicated concentrations of β-HCH, with (blue) or without (grey) additional L-Dopa treatment. Error bars represent S.E.M., significance was calculated using one-way ANOVA with Dunnett's post-test. The '*' symbol is used to indicate significance of difference compared to the 0 ng/mL group. The '^' is used to indicate significance of differences between groups treated with the same concentration of β-HCH, with or without 5 mM L-Dopa. **C)** Distribution of larval posture at 3 days post fertilization after exposure to β-HCH in early developmental stages, without (upper panel) or with (lower panel) simultaneous exposure to 5 mM L-Dopa. Significance was calculated using Chi-square test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The slow touch-evoked response at 290 ng/mL of β-HCH, and reduced ability of larvae to maintain an upright position at 2.9 ng/mL of β-HCH, was reminiscent of the phenotype observed in *nipsnap1* mutant zebrafish larvae and associated with a reduced number of dopaminergic neurons (Princely Abudu et al., 2019). Early exposure of embryos to β-HCH, using a probe targeting dopaminergic neurons, showed a reduction in hypothalamus size. Exposure of control larvae to 5 mM L-Dopa caused a reduction of the hypothalamus size, indicating potential neurotoxicity due to oxidative stress from high cytosolic dopamine levels (Mosharov et al., 2009). Overall, the L-Dopa experiments suggest a role for dopaminergic neurons in mediating the effect elicited by β-HCH.

Possible explanation and implication of the findings.

Alterations in function of the dopaminergic system have been implicated with autism (Bowton et al., 2014, Pavăl., (2017), DiCarlo et al., 2019). High rate of PD among ASD cases also suggests a shared neurobiology (degeneration of dopaminergic neuron) in both Parkinson's disease and autism spectrum disorder (Starkstein et al., 2015). Furthermore, several epidemiologic studies indicate association between elevated level of β-HCH and PD (Richardson et al., 2009, Petersen et al., 2008, Corrigan et al., 2000). However, the effect of β-HCH is likely more complex and may affect early brain development via multiple modes of action, not only restricted to dopaminergic neurons. The exact mechanism by which β-HCH contributes to ASD pathophysiology is not yet known. Also, a U-shaped response was observed in several experiments, suggesting that compensatory mechanisms are activated at higher concentrations of β-HCH. Such non-monotonic dose responses have been observed in many prior studies with endocrine-disrupting chemicals (Vandenberg et al., 2012). We have not identified the exact mechanism by which β-HCH induces such a response, but non-selectivity at higher doses (leading to involvement of multiple vs single receptors), and receptor down regulation in response to high-dose treatment are among the known mechanisms leading to this kind of response with other chemicals.

It is alarming that β-HCH, a pesticide residue banned decades ago, still is detected in breast milk. β-HCH is an isomer of lindane (γ-HCH), and a byproduct of its production. It was used in forestry and agriculture until banned in 1992 in Norway (Økland et al., 2005) and in 2008 in the EU, but still used as a second line treatment option for head lice and scabies in USA (except for the state of California) (Humphreys et al., 2008) and in most developing countries. β-HCH was also a component of the pesticide hexachlorocyclohexane that was banned in most countries in the 1970 s (EFSA, 2005). Exposure is expected to remain substantial also in years ahead (Økland et al., 2005) owing to its long half-life of 7 years (Jung et al., 1997), large (5–7 million tons) stockpiles buried in uncontrolled dumps "mega-sites" around the world (Vijgen et al., 2019, Vijgen et al., 2022), and its transboundary pollution via air and rivers (Vijgen et al., 2019).

4.3. Other findings

PCBs and OCPs.

In contrast to our study, maternal serum levels of PCBs, DDE and *trans*-nonachlor were positively associated with risk of ASD and intellectual disability in the EMA study, a population-based case–control (n = 545/ n = 418) study in California (Lyall et al., 2017b). However, the EMA study's findings are based on single pollutant analysis, while our research employed elastic net to select predictors among multiple correlated exposures (Lenters et al., 2018, Agier et al., 2016). We also noted associations for some mono-*ortho*-substituted dioxin-like PCBs in the single-pollutant analyses like the EMA study as PCBs and OCPs are highly correlated. The EMA study did not examine β-HCH due to its low detection frequency (Lyall et al., 2017b). Nonetheless, the possibility of confounding results from any correlation between β-HCH and PCBs remains.

PBDEs.

Our study found no connection between PBDEs in breast milk and autism risk. Nevertheless, the HOME study demonstrated a positive association between PBDE-28 and autism in children (Braun et al., 2014). Conversely, the EMA study in California showed negative association between PBDE and the risk of ASD in boys (Lyall et al., 2017c). The US population has ten times higher PBDE levels than Norway, limiting comparability.

PFASs.

Our study also investigated the relationship between PFAS and ASD in the HUMIS cohort. While we observed non-significant positive associations between PFOA and PFOS with ASD, elastic net regression did not select them as predictors. However, other studies have reported mixed findings regarding the relationship between PFAS and ASD. Skogheim et al. (2021) showed a positive link between PFOA and ASD but inverse associations for other PFAS in a larger Norwegian case-control study (n = 400 cases/980 controls). The Danish National Birth Cohort nested case-control study analyzing 16 PFASs in maternal plasma for children with ASD did not report significant association (Liew et al., 2015). In contrast, the HOME study found a negative association between PFOA and ASD-related behavior in young children, while the EMA study by Lyall et al. (2018) demonstrated positive associations between prenatal maternal serum PFAS concentrations and ASD. Nevertheless, strong evidence suggests the involvement of PFOAs in ASD development among PFAS.

4.4. Strengths and limitations of the study

Our study has multiple strengths. Firstly, we employed a multi-pollutant analysis examining association between ASD and mixture of PCBs, OCPs, PBDEs, and PFASs, which reduces confounding bias from correlated co-exposures (Agier et al., 2016). Secondly, the inclusion of a follow-up experimental study to confirm the neurotoxicity of β-HCH was both unique and vital in excluding potential biases. In addition, the prospective cohort design and the use of causal graphs ascertains the temporal sequence of exposure and outcome. Furthermore, a positive correlation between concentration of persistent chemicals measured in breast milk and umbilical cord has been documented, making the exposure a suitable proxy for both prenatal and postnatal exposures (Verner et al., 2013, Waliszewski et al., 2001). For several PFAS including PFOA and PFOS, breastfeeding is also found to be significant postnatal exposure route in infants and positive correlation between breast milk levels, cord blood and maternal plasma levels are well documented (Cariou et al., 2015, Papadopoulou et al., 2016, Criswell et al., 2023). The outcome (ASD) was based on a specialist diagnosis through linkage to the Norwegian birth registry, which has good validity (Surén et al., 2014). Postnatal exposure levels were calculated using a validated pharmacokinetic model (Stigum et al., 2015). Children with neurodevelopmental disorders were oversampled increasing statistical power. Moreover, another advantage of this study is the use of human milk. Due to its high fat percentage, less than 2 % of the 24 chemicals measured in breast milk were below detection, reducing the risk of misclassification bias. Also, large volumes can be collected with no risk for the baby or the mother. The concentration in breast milk reflects maternal body burden and thus also gestational exposure due to the stability of POPs. Postnatal exposure on the other hand, varies with the duration of breastfeeding in addition to the concentration in milk and therefore we modeled postnatal exposure up to 24 months of age (Stigum et al., 2015). No additional risk was observed tied to postnatal exposure, which may indicate that the critical window for the adverse effect of β-HCH is confined to the prenatal period. A previous study by our group also demonstrated a positive association between β-HCH and ADHD indicating that neurodevelopmental effects of β-HCH may not be limited to ASD (Lenters et al., 2019).

There were some limitations to our study. We cannot rule out the possibility of residual confounding bias or the amplification of bias due to unmeasured or inaccurately measured covariates, an incorrect

specification of the causal structure (Pearl., (2011)), or chance finding. Estimates from M1 reflect the total effect estimates of chemical exposures and ASD, but we cannot exclude selection bias due to the oversampling of preterm babies (Thapar et al., 2013, Sciberras et al., 2017). Estimates from M2 takes care of potential selection bias in accordance with collapsibility theory for logistic models (Greenland and Pearl, 2011) but reflect the direct effect. We thus ran two models with and without adjustment for preterm birth and small for gestational age, which yielded similar results both in effect estimates and precision, thus no obvious selection bias could be observed. The exclusion of other potential EDCs such as heavy metals, pyrethroids, organophosphate pesticides, and non-persistent organic pollutants in our study is unlikely to result in significant confounding bias, given the difference in exposure sources and pharmacokinetics compared to POPs included in our study. Another limitation to the study is that it is difficult to determine how the tested concentration of β-HCH relates to the concentrations human fetuses and neonates are exposed to, due to interspecies variation. For example, the higher amounts of lipids found in zebrafish larvae may act in a protective manner by sequestering β-HCH, similarly as was observed between different fish species exposed to γ-HCH. In attempt to overcome this limitation, zebrafish larvae were exposed to a wide range of concentrations of β-HCH. This study did not investigate the mixture effect of individual chemicals on ASD.

5. Conclusions

Perinatal exposure to β-HCH was associated with a marked increased risk of ASD, controlling for confounding by 26 correlated chemicals. An *in vivo* study on zebrafish larvae confirmed the neurodevelopmental toxicity of β-HCH in low concentrations and implicated a potential role of the dopaminergic neurons. The neurotoxicity of β-HCH at low concentrations has important public health implications. The lack of studies that simultaneously assess a wide array of highly correlated persistent chemicals is of concern. Future studies on persistent chemicals and ASD should include assessment of β-HCH despite low background levels.

CRediT authorship contribution statement

Anteneh Assefa Desalegn: Methodology, Software, Investigation, Formal analysis, Investigation, Writing - original draft, and Writing - review & editing. **Wietske van der Ent:** Methodology, Software, Investigation, Formal analysis, Investigation, Writing - original draft, and Writing - review & editing. **Virissa Lenters:** Methodology, Software, Investigation, Formal analysis, Investigation, Writing - original draft, and Writing - review & editing. **Nina Iszatt:** Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Supervision. **Hein Stigum:** Methodology, Software, Validation, Formal analysis, Writing - review & editing, Supervision. **Jan Ludvig Lyche:** Methodology, Investigation, Resources, Data Curation, and Writing - review & editing. **Vidar Berg:** Methodology, Investigation, Resources, Data Curation, and Writing - review & editing. **Karolina J. Kirstein-Smardzewska:** Methodology, Investigation, Resources, Data Curation, and Writing - review & editing. **Camila Vicencio Esguerra:** Investigation, Validation, Resources, Formal analysis, Writing - review & editing, Supervision, Project administration, and Funding acquisition. **Merete Eggesbø:** Conceptualization, Methodology, Investigation, Validation, Resources, Formal analysis, Writing - review & editing, Supervision, Project administration, and Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Role of the Funder/Sponsor

The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Access to Data

Dr. Merete Eggesbø had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data Sharing Statement

The consent given by the participants does not allow for storage of data on an individual level in repositories or journals. Researchers who want access to datasets for replication should submit an application to (datatilgang@fhi.no). Access to datasets requires approval from the Regional Committee for Medical and Health Research Ethics in Norway.

Appendix A. Supplementary data

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