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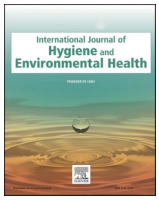

A pathway-based genetic score for inflammation: An indicator of vulnerability to phthalate-induced adverse neurodevelopment outcomes

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ABSTRACT

Introduction: Phthalates, chemical additives used to enhance plastic products' flexibility, are easily released into the environment, and can harm the brain development through various mechanisms including inflammation. Genetic variation influencing an individual's susceptibility to inflammation may play a role in the effects of phthalate exposure on neurodevelopment however there is no summary measure developed for genetic susceptibility to inflammation.

Methods: We developed a genetic pathway function score for inflammation ($gPFS^{in}$), based on the transcriptional activity of the inflammatory response pathway in the brain and other tissues. Using the Barwon Infant Study (a birth cohort of $n = 1074$), we examined the connection between $gPFS^{in}$ and key neurodevelopmental outcomes, along with the interplay between prenatal phthalate levels, children's genetic susceptibility to inflammation ($gPFS^{in}$), and adverse neurodevelopmental outcomes.

Results: Regression techniques revealed consistent associations between $gPFS^{in}$-phthalate combinations and key neurodevelopmental outcomes. A high $gPFS^{in}$ score was associated with an increased risk of doctor-diagnosed Autism Spectrum Disorder (ASD) and Attention-Deficit/Hyperactivity Disorder (ADHD) by age 11.5 years, with adjusted odds ratios of 2.15($p = 0.039$) and 2.42($p = 0.005$), respectively. Furthermore, individuals with both high $gPFS^{in}$ and prenatal phthalate exposure exhibited more neurodevelopmental problems. This included associations of high $gPFS^{in}$ and bis(2-ethylhexyl) phthalate (DEHP) levels with parent-reported ASD traits and doctor-diagnosed ASD. The attributable proportions due to this interaction were 0.39 ($p = 0.045$) and 0.37 ($p = 0.037$), respectively.

Conclusion: These findings contribute to the evidence linking gestational phthalate exposure and inflammation to adverse neurodevelopment and underscoring increased risks in children with higher genetic susceptibility to inflammation.

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1. Introduction

The immune system is widely recognized for its innate ability to trigger inflammation as a protective response to tissue injuries and exposure to harmful agents (Ferrero-Miliani et al., 2007; Medzhitov, 2010). Inflammation plays a vital role in maintaining the body's overall well-being, facilitating tissue protection, and aiding repair (Nathan and Ding, 2010). Acute inflammation involves a temporary increase in inflammatory activity, which subsides once the triggers are successfully eliminated or brought under control, thereby limiting further damage to tissues (Furman et al., 2019). In stark contrast, chronic inflammation is characterized by persistent, low-grade immune system activation, unrelated to infections, and is driven by a complex interplay of biological, psychological, social, and environmental factors that obstruct the resolution of acute inflammation (Furman et al., 2019; Han et al., 2021; Fullerton and Gilroy, 2016).

Chronic inflammation carries detrimental effects on the body's tissues and plays a significant role in development of various diseases, including cardiovascular, autoimmune, and metabolic conditions (Furman et al., 2019; Esteban-Cornejo et al., 2016; Pearson et al., 2003). Additionally, it has been associated with neurodegenerative diseases (Voltas et al., 2017; Depino, 2013). Furthermore, inflammation is linked to adverse cognitive outcomes in both infants and the aging population (Esteban-Cornejo et al., 2016; Koyama et al., 2013) and has been associated with structural and functional brain damage in infants (Leviton et al., 2013; Kuban et al., 2015). Animal studies suggest that immune dysregulations can have a substantial impact on neural plasticity, neurogenesis, brain structure, and memory, even influencing prenatal brain development (Willette et al., 2011; Vlasova et al., 2021). In humans, activation of the maternal immune system during pregnancy has been linked to abnormal brain development (Knuesel et al., 2014) and an increased risk of Autism Spectrum Disorder (ASD; Lee et al., 2015; Volk et al., 2020) in the progeny. Moreover, dysregulation of maternal cytokines and chemokines during mid-pregnancy is increasingly recognized as an etiological risk factor for developing ASD, psychiatric conditions, as well as other neurodevelopmental impairments (Jones et al., 2017; Abdallah et al., 2013; Dozmorov et al., 2018).

Plastic product chemicals, such as phthalates and bisphenols, are well-recognized sources of immunological and inflammatory triggers (Jøhnk et al., 2020; Eisner et al., 2022). Exposure to these chemical compounds has been linked to alterations of both pro-inflammatory cytokine (e.g., IL-1β, IL-6, TNFα, IFNγ, IL-12) and anti-inflammatory cytokine (e.g., IL-4, IL-10, IL-1 receptor antagonist and IL-13) levels (Schjenken et al., 2021; Kelley et al., 2019). Recent findings from the Barwon Infant Study (BIS) have revealed that prenatal exposure to phthalates is linked to changes in levels of inflammatory and regulatory cytokines and chemokines in cord blood (Eisner et al., 2022). A significant concern is that pregnant women may experience continuous exposure to phthalate pollutants, primarily because these pollutants are present in many sources, including food products such as plastic food packaging, high-fat milk, and canned foods (Sugeng et al., 2020; Fisher et al., 2019), as well as personal care items such as air fresheners, perfumes, hair treatments, and cleaning products (Philippat et al., 2015; Chan et al., 2021). Moreover, in BIS phthalate exposure during pregnancy has been associated with adverse neurodevelopmental outcomes in offspring, including ASD, attention deficit hyperactivity disorder (ADHD), and lower cognitive function (Ponsonby et al., 2020; Tanner et al., 2022).

Considering that the regulation of inflammation in an individual is somewhat affected by numerous genetic variations (Gonzalez-Jaramillo et al., 2019; Owens et al., 2001), it is pertinent to use genetic scores to enhance our understanding of the genetic predisposition and/or susceptibility to inflammation. These scores provide a concise summary of the expected functional consequences of a multitude of potential genetic variations. Rothman's component cause model proposes that two contributing factors may interact causally to generate an outcome (Rothman and Greenland, 2005).In other words, it posits that the presence of both factors is necessary for the outcome to occur, and their combined effect leads to the generation of the outcome. This model emphasizes the idea that causation is not solely determined by one factor but involves the interplay of multiple elements working together. When evaluating the impact of prenatal exposure to phthalates on adverse neurodevelopmental outcomes, the interaction between genetic predisposition to inflammation and phthalate exposure could be a vital element in the causal pathway leading to adverse neurodevelopmental effects. Thus genetic susceptibility to inflammation may play a key role in determining who is more vulnerable to the effects of phthalate exposure and therefore to adverse neurodevelopment. An Australian pre-birth cohort, the BIS cohort, presents an ideal opportunity for exploring potential links between exposure to plastic chemicals during gestation, genetic predisposition to inflammation in BIS children, and the subsequent emergence of multiple adverse neurodevelopmental outcomes (Vuillermin et al., 2015).

In this study, we have three primary objectives. First, we aim to create a genetic pathway functional score for inflammation (gPFS^{in}) that focuses on genetic pathways associated with cytokines and chemokines, following a methodology similar to Tanner et al. (2022). The choice to construct the score using the genetic pathway associated with cytokines and chemokines is grounded in the presence of diverse markers for these substances in BIS, spanning different time points. This facilitates the validation of the score against these biomarkers. Notably, we go beyond solely utilizing single nucleotide polymorphism data; we incorporate transcriptomic data sourced from the Genotype Tissue Expression (GTEx) database (Lonsdale et al., 2013). This comprehensive approach allows us to capture a functional pathway that reflects inflammatory activity rather than assessing only disease risk *per se*. We assess the predictive validity of this genetic score against the inflammatory immune markers in BIS children's cord blood and at age 4 specifically against proinflammatory cytokines, high-sensitivity C-reactive protein (hsCRP) and glycoprotein acetyls (GlycA) markers. Second, we investigate the relationship between the gPFS^{in} and key neurodevelopmental outcomes, including ASD traits and ADHD symptoms at 4 years of age and doctor-diagnosed ASD and ADHD by 11.5 years of age. Lastly, we explore how prenatal phthalate exposures interact with gPFS^{in} in the context of adverse neurodevelopmental outcomes. Specifically, we assess whether the apparent effect of prenatal plastic chemical exposure on adverse neurodevelopment varies based on gPFS^{in} scores.

2. Materials and methods

2.1. Study design

The Barwon Infant Study (BIS) is a longitudinal cohort comprised of 1074 mother-infant pairs, including 10 sets of twins, selected through antenatal sampling within the Barwon region of Victoria, Australia (Vuillermin et al., 2015). The demographic composition of this population closely resembles that of the broader Australian population, with a noteworthy distinction being a lower representation of families from non-English-speaking backgrounds (Vuillermin et al., 2015). The primary purpose of this cohort is to explore the early-life origins of non-communicable diseases (Vuillermin et al., 2015). Recruitment efforts primarily targeted women in the 15–28 weeks of completed pregnancy range over the years spanning from 2010 to 2013. Specific inclusion criteria were established to exclude cases involving (a) births occurring before the 32-week gestational mark, (b) diagnosis of genetic diseases or major congenital malformations, and (c) presence of serious illnesses that could be identified within the first few days after birth (Vuillermin et al., 2015). Ethical considerations governing this study were approved by the Barwon Health Human Research Ethics Committee (HREC 10/24), with families providing informed written consent. Characteristics of the BIS cohort are summarized in Table 1.

Table 1
Key Characteristics of the BIS cohort.

Demographic	Cohort sample (N = 1074)	Cohort sample mean (SD), median [IQR], n (%), geometic mean {GSD}	Participants with any neurodevelopment data (N = 890)	Participants with any neurodevelopment data: mean (SD), median [IQR], n (%), geometric mean {GSD}
Maternal age at conception (years)	1074	31.33 (4.79)	890	31.80 (4.49)
Paternal age at conception (years)	1024	33.49 (5.85)	848	33.75 (5.56)
Maternal education (university vs. other)	1068	548 (51.3%)	887	493 (55.6%)
SEIFA disadvantage (low tertile vs. other)	1061	357 (33.6%)	880	276 (31.4%)
Maternal pre-pregnancy BMI (kg/m2)	927	25.38 (5.46)	779	25.42 (5.50)
Maternal smoked during pregnancy (any vs. none)	1063	169 (15.9%)	884	112 (12.7%)
Maternal alcohol consumption during pregnancy (any vs. none)	989	521 (52.7%)	849	439 (51.7%)
Multiparity (any vs. none)	1073	593 (55.3%)	889	490 (55.1%)
Gestational age at urine collection (weeks)	847	36.27 (0.71)	756	36.26 (0.68)
Lone parent during pregnancy (yes vs. no)	1071	43 (4.0%)	890	25 (2.8%)
Maternal PSS score during pregnancy	810	18.66 (6.99)	669	18.20 (6.70)
Prematurity (<37 vs. 37 or more weeks)	1074	47 (4.4%)	890	38 (4.3%)
Time in labour (hrs)	1071	6.07 (5.65)	889	6.04 (5.72)
Mode of delivery (Caesarean vs. other)	1074	335 (31.2%)	890	285 (32.0%)
Gestational age at birth (weeks)	1074	39.44 (1.52)	890	39.44 (1.50)
Birth weight (kg)	1072	3.53 (0.52)	890	3.54 (0.51)
Child assigned sex at birth (male vs. female)	1074	555 (51.7%)	890	455 (51.1%)
Apgar score at 1 min	1059	9.00 [8.00, 9.00]	876	9.00 [8.00, 9.00]
Resuscitation at birth (any vs. none)	1074	9 (0.8%)	890	7 (0.8%)
Multiple Birth (yes vs. no)	1074	10 (0.9%)	890	6 (0.7%)
DEP daily intake (μg/kg bw/day)	847	1.58 {3.80}	756	1.60 {3.82}
DBPs daily intake (μg/kg bw/day)	847	1.88 {1.97}	756	1.90 {1.97}
DEHP daily intake (μg/kg bw/day)	847	1.62 {2.08}	756	1.63 {2.08}
Σ phthalates (μg/kg bw/day)	847	6.25 {2.21}	756	6.33 {2.24}
DEP daily intake top quintile (≥4.459)	847	171 (20.2%)	756	157 (20.8%)
DBPs daily intake top quintile (≥3.362)	847	170 (20.1%)	756	155 (20.5%)
DEHP daily intake top quintile (≥2.614)	847	170 (20.1%)	756	150 (19.8%)
Σ phthalates phthalate daily intake top quintile (≥11.043)	847	170 (20.1%)	756	154 (20.4%)
Parent reported ASD traits at age 4 (yes vs. no)	791	39 (4.9%)		
SDQ hyperactivity/inattention (score above 3)	791	371 (46.9%)		
Child's age at 4 year assessment (years)	791	4.16 (0.26)		
ASD diagnosis by age 11.5 (yes vs. no)	868	64 (7.4%)		
ADHD diagnosis by age 11.5 (yes vs. no)	868	76 (8.8%)		
Child's age at health screen assessment (years)	868	9.04 (0.74)		

Note: SD, standard deviation; IQR, interquartile range; GM, geometric mean; GSD, geometric standard deviation; SEIFA, Socio-Economic Indexes for Areas; BMI, Body mass index; PSS, Perceived Stress Scale; DEHP, Di-(2-ethylhexyl) phthalate; DEP, Diethyl phthalate; DBPs, sum of DiBP (Diisobutyl phthalate) and DnBP (Di-n-butyl phthalate); Σ phthalates, sum of DEP, DBPs and DEHP; SDQ, Strengths and Difficulties Questionnaire; ASD, autism spectrum disorder; ADHD, Attention-deficit/hyperactivity disorder.

2.2. General study measures

At the 28-week gestational milestone, participants completed questionnaire sessions with the objective of acquiring detailed sociodemographic, clinical, and health-related information (Vuillermin et al., 2015). This encompassed self-reported particulars such as maternal age at conception, educational background, pre-existing psychosocial conditions, pre-pregnancy weight and pregnancy-related

health conditions. The verification of perinatal factors including the child's sex, gestation length, and mode of birth, relied on reviewing hospital records (Vuillermin et al., 2015).

2.3. Phthalate measurement

A single spot urine sample was collected from the mothers at 36 weeks of pregnancy. Specific details on urine sampling were recorded, including date, time, storage conditions, as well as container type. Phthalate metabolite levels in 842 mothers were evaluated using the single spot sample. Analyses were conducted by the Queensland Alliance for Environmental Health Science (QAEHS) using high-performance liquid chromatography-tandem mass spectroscopy. A detailed explanation of their methodologies can be found in Heffernan et al. (2020). Here, we focused on five specific phthalate metabolites: monoethyl phthalate (MEP), monoisobutyl phthalate (MiBP), monon-butyl phthalate (MnBP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP). Previous research had demonstrated that for each of these phthalate metabolites, repeated spot urine specimens collected during the third trimester exhibited an intra-class correlation coefficient exceeding 0.4, in at least one study (Suzuki et al., 2009; Adibi et al., 2008). This correlation indicates that a single spot test during the third trimester provides a reliable measurement of phthalate levels.

2.4. Child neurodevelopmental outcomes

At the age of 4, parents were asked to provide information about any noticed traits of ASD in their child Ponsonby et al. (2020). Additionally at age 4, the preschool version of the Strengths and Difficulties Questionnaire (SDQ) was administered, with a specific focus on hyperactivity and inattention symptoms, quantified in the hyperactivity/inattention scale of the SDQ. Our use of a quantitative measure of hyperactivity/impulsivity at this earlier age stems from the rarity of clinical ADHD diagnoses at the age of 4 within the BIS sample. At the 9 years follow-up review, parent reported child ASD and ADHD was assessed by a telephone health screen interview. Parent reported diagnoses were verified by reviewing the child's medical records to confirm whether the child had been diagnosed with ASD and ADHD by their doctor against the DSM-5 criteria. Verification occurred after the 9-year review and was completed at the mean age of 11.5 years (SD = 0.8).

2.5. Genotyping

DNA samples were extracted from cord blood. The QIAamp 96 DNA QIAcube HT kit (QIAGEN, Hilden, Germany) was used in adherence to the manufacturer's guidelines to facilitate this process. Subsequently, these samples were stored at a temperature of −80 °C to ensure their optimal preservation. A comprehensive whole-genome genotyping procedure was then carried out, using the Illumina Global Screening Array (Illumina, San Diego, CA, USA). Genotype imputation was conducted through the Sanger Imputation Server at the Wellcome Sanger Institute in Hinxton, UK, using the Haplotype Reference Consortium panel (McCarthy et al., 2016). All aspects of genotype calling, quality control, and imputation were consistently performed on the entire cohort as a single batch (Mansell et al., 2020). Infants were then excluded if their initial genotyping results failed to register for more than 5% of single-nucleotidepolymorphisms (SNPs) ($n = 4$). Moreover, specific SNPs were disqualified from the analysis if they met any of the following criteria: (i) genotyping failure in over 5% of infants (3% of SNPs), (ii) the SNP was considered rare, with a minor allele frequency (MAF) of < 0.01 or a MAF differing by more than 0.2 from the reference population, or (iii) SNPs did not adhere to Hardy–Weinberg equilibrium (Mansell et al., 2020).

2.6. Pro-inflammatory cytokine quantification

2.6.1. Cord blood immune profile

Umbilical cord blood was collected using a syringe and divided into two containers: a 6 ml serum clotting tube (BD Vacutainer) and a 50 ml Falcon tube containing RPMI-1640 and 10 IU/ml preservative-free heparin (DBL Heparin Injection BP, Gibco, Life Technologies). The serum underwent centrifugation within 3 h of collection and was subsequently cryopreserved at −80 °C. Whole blood samples were kept at room temperature on a roller and processed within 18 h of collection. Among the BIS cohort, 43 infant samples (5%) exhibited maternal contamination in cord blood, as identified through DNA methylation profiling techniques (Morin et al., 2017). A binary variable was used to classify cord blood contamination status as 'not contaminated' with less than four CpG indicators, and 'contaminated' with four or more. This variable was then used as a covariate in the analysis (Pham et al., 2022a).

The analysis of circulating cytokines and chemokines utilized the Bio-Plex Pro Human Cytokine 27-plex Assay M500KCAF0Y and Bio-Plex R 200 software. Thawed cord blood serum samples, along with 8 standards (in duplicate) and inter-plate controls, were processed in assay plates. The assay protocol involved the addition of magnetic beads, detection antibodies, and streptavidin-phycoerythrin dye, interspersed with washing and incubation phases. Standard curves were generated from these dilutions, targeting a recovery rate between 70 and 130%. Inflammatory cytokines, including IFN-γ, TNF-α, IL-6, and IL-1β, with over 70% of samples above the limit of detection (LOD), were included in the analysis. For monocyte activation, LPS (1 ng/ml, L4391, Sigma Aldrich) was introduced to 150 ml of IP5 medium containing 1×10^5 monocytes and incubated for 24 h. Post-incubation, levels of IFN-γ, IL-1β, IL-6 and TNF-α in the media were quantified using the Bio-Plex assay, following the manufacturer's guidelines.

2.6.2. Age 4 immune profile

Blood samples were collected in tubes containing preservative-free sodium heparin. To assess cytokine production by immune cells under conditions akin to *in vivo* scenarios, monocyte-derived cytokine production was measured following stimulation of whole blood. Within a 2-h window from collection, a blood aliquot was diluted in a 1:2 ratio with RPMI 1640 and dispensed into plate strips with eight wells each. These wells contained 20 ml of RPMI (control), LPS (100 ng/ml, for TLR4 stimulation), or PGN (μg/ml, for TLR2 stimulation). A total of 180 ml of this diluted blood was added to each well, and the cells were stimulated for 24 h at 37 °C in a 5% CO_2 environment. To counteract evaporation, additional plate strips with water were incubated alongside the experimental strips. After incubation, the strips were centrifuged, blood cells were pelleted, and the supernatant was divided into two 50 ml aliquots, which were stored at −80 °C for subsequent cytokine analysis.

Cytokine levels were quantified using the Bio-Rad Bio-Plex Pro™ cytokine assay kit and detection software, with slight adjustments to the manufacturer's protocol. Standards were prepared in a dilution series using provided diluents. Thawed samples were added to plates containing eight standards in duplicate (including blanks) and inter-plate controls. The assay involved addition of magnetic beads, detection antibodies, and streptavidin-phycoerythrin dye, with washing and incubation steps. Plate analysis was conducted using the xPONENT MAGPIX® instrument (Bio-Rad®). Standard curves, derived from these dilutions, were optimized to achieve a recovery rate within the range of 70–130%. Final cytokine concentrations (IL-1β, IL-6, TNF-α, IFN-γ, and IL-12p70) in pg/ml were calculated using the Bio-Plex Manager™ Software, utilizing the established standard curves.

2.7. Other inflammatory markers

At birth and the age of 4, plasma samples were collected from children in the BIS group and subjected to analysis for high-sensitivity C-

reactive protein (hsCRP) and glycoprotein acetyls (GlycA). Following established procedures (Collier et al., 2019, 2022; O'Connor et al., 2020), hsCRP (measured in micrograms per milliliter, μg/ml) was quantified using the enzyme-linked immunosorbent assay (ELISA) technique with the Human C-Reactive Protein/CRP DuoSet ELISA DY1707 kit from R&D Systems, Minneapolis, Minnesota, United States. GlycA (measured in millimoles per liter, mmol/L) was quantified through high-throughput proton NMR metabolomics (Nightingale Health platform, Helsinki, Finland) as previously detailed (Collier et al., 2019, 2022; O'Connor et al., 2020). In all analyses, both measures were log (base 10) transformed.

2.8. Genetic pathway function score for inflammation

To quantify the genetic predisposition to inflammation for each BIS infant, we generated a genetic pathway function score (gPFS^{in}) through a structured approach following the steps detailed in Tanner et al. (2022). First, we constructed a fundamental pathway embodying the human inflammation response mapping the signaling pathways of cytokines and chemokines, comprising 9 pro-inflammatory and 7 anti-inflammatory genes exerting opposing actions (Table 4). The construction of this inflammatory pathway is based on data from the reactome pathway knowledge base (Jassal et al., 2020), with the stable identifier for this pathway being R-HSA-1280215.7. Notably, this fundamental pathway incorporates the Janus kinase/signal transduction and transcription activation (JAK/STAT) genes pathway, which is believed to be intricately linked to various functions within the immune system (Hu et al., 2023).

Next, we identify SNPs associated with the transcript expression (referred herein as activity of these genes) using the Genotype Tissue Expression (GTEx) database (Lonsdale et al., 2013). From this database, we select the SNP most profoundly associated with the expression of each inflammation gene using the expression quantitative trait loci (eQTLs) in all tissue types. These chosen SNPs, totaling 16 (with one SNP corresponding to each gene, detailed in Table 4), demonstrate a high degree of consistency in their effects across different tissue types, including the brain. Our analysis then delves into decoding the impact of each SNP on inflammation, focusing on two factors: (i) whether the SNP amplifies or diminishes the expression of its target gene, and (ii) whether the target gene intensifies or reduces inflammation. This discernment allows us to comprehensively interpret how a pro-inflammatory SNP, for instance, might intensify the expression of a pro-inflammatory gene or quell the expression of an anti-inflammatory gene.

Finally, we calculate the score for each participant. This score quantifies the number of pro-inflammatory alleles they carry within this inflammation pathway. Essentially, this score symbolizes the cumulative imbalance within the pathway at the transcriptional level, with a higher score indicating a greater genetic predisposition towards inflammation. If the N selected Single Nucleotide Polymorphisms (SNPs) are arranged such that SNPs 1 through N_{pro} are pro-inflammatory, and SNPs $N_{pro}+1$ through N are anti-inflammatory, the score is computed as follows:

$$gPFS_i^{in} = \frac{1}{2}\left(\sum_{j=1}^{N_{pro}} C_j + \sum_{j=N_{pro}+1}^{N} (2 - C_j)\right) \quad (1)$$

Here gPFS$_i^{in}$ is the score for individual i, j is the current SNP out of a total of $N = 16$ SNPs across the pathway (one for each gene), and C is the allele count for SNP j (0, 1, or 2). For each SNP, the count of pro-inflammatory alleles at that locus is added; for a pro-inflammatory SNP this is simply the number of effect alleles present. On the other hand, for an anti-inflammatory SNP it becomes 2 minus the number of effect alleles. The score is then divided by 2 to range from 0 to 16 units, reflecting the number of genes in the pathway, rather than the total number of alleles in the pathway.

2.9. Statistical analysis

Phthalate measurements are adjusted for potential batch effects and urine dilution. To address batch effects, fractional variation between batch-specific and overall geometric means of quality control measures is considered (Engel et al., 2018). For urine dilution, urine-specific gravity is measured and corrected using the Levine Fahy equation (Levineand Fahy, 1946). Additionally, the time of day for urine sample collection is adjusted for using the residual approach (Mortamais et al., 2012). In cases where phthalate metabolite levels fall below the Limit of Detection (LOD), values are imputed using LOD/$\sqrt{2}$ (Hornung and Reed, 1990). To calculate the daily intake of each phthalate compound from third-trimester maternal urine, we followed the method outlined in Rocha et al. (2017). This involves evaluating several parameters, including maternal prenatal weight, average daily urine volume, fractional excretion of the compound, and the compound-to-metabolite molecular weight ratio. Daily intake levels are crucial for determining parent compound exposure and for correcting for potential dilution due to maternal weight and fractional urine excretion. Specifically, MEP, a urinary metabolite of diethyl phthalate (DEP), is used to determine DEP daily intake, MnBP for di-n-butyl phthalate (DnBP), MiBP for diisobutyl phthalate (DiBP), and MEHHP, MEOHP, and MECPP for bis (2-ethylhexyl) phthalate (DEHP). Dibutyl phthalates (DBPs) are calculated by combining DiBP and DnBP, while DEHP, DEP, DiBP, and DnBP daily intakes are combined to form a composite phthalate daily intake measure (Σ Phthalates).

To assess the predictive strength of the constructed genetic score, we employ both linear and logistic regressions to examine the associations between the gPFS^{in} and various inflammatory cytokines, hsCRP and GlycA immune markers measured at birth and at age 4. Further, where we stratify the BIS cohort in subsequent analyses, the notation G^{lo} represents the set of children in the lower four quintiles of the distribution and the notation (G^{hi}) represents children in the upper fifth quintile (i.e. those with a score 7.5 or greater). We use logistic regression analyses to explore the connections between gPFS^{in} and ASD traits and the SDQ hyperactivity/inattention score at age 4 as well as doctor-diagnosed ASD and ADHD at age 11.5 years. Since the SDQ hyperactivity/inattention scale is a continuous variable, we stratify the scale using the clinical cutoff (Bryant et al., 2020) to create a binary variable for the logistic regression and investigate the additive interactions. For additional context, refer to our previous work in Ponsonby et al. (2020), which investigated associations between the continuous SDQ hyperactivity/inattention scale, oxidative stress genes and prenatal exposure to phthalates.

Further, we investigate how gPFS^{in} and prenatal phthalate exposure interact by: (i) evaluating the risk of adverse neurodevelopment associated with being in the top quintile for both gPFS^{in} and phthalate levels compared to being in the lower four quintiles for both, and (ii) examining the potential additive interaction. We report the attributable proportion (AP), a measure of interaction on the additive scale that utilizes risk ratios to quantify the proportion of disease risk in the doubly exposed group attributable to the interaction (Mathur and VanderWeele, 2018; VanderWeele, 2013). To calculate AP values, we employed the R functions developed and described in Mathur and VanderWeele (2018) for assessing additive interactions. In scenario (i), G^{hi} or P^{hi} identifies children in the top quintile for gPFS^{in} or phthalates, while G^{lo} or P^{lo} categorizes children in the lower four quintiles for gPFS^{in} or phthalates, respectively. It is important to highlight that we adopted a top-category approach to maintain consistency with our previous studies (Ponsonby et al. (2020); Tanner et al. (2022)). Additionally, this approach is supported by the prenatal phthalate distributions observed in this birth cohort, which reveal widespread exposure with highly variable chemical levels, exhibiting over a 1000-fold variation (Sugeng et al., 2020), adopting a median approach will result in inaccurate representation of this dataset.

Our regression models are adjusted for post-conceptional age at testing and sex. Where the models are minimally adjusted, we calculate E-values for the findings to evaluate the potential impact of unmeasured confounding (Mathur et al., 2018; VanderWeele and Ding, 2017). The E-value quantifies the minimum magnitude of association, expressed on the risk ratio scale, that an unmeasured confounder must have with both the exposure and the outcome, after adjusting for measured covariates, to completely account for a given exposure-outcome relationship. The cytokine models were additionally adjusted for various exposure factors such as the time-of-day blood was collected, season collected, days blood sample stored and whether the child was sick during blood sample collection. The regression models for GlycA and hsCRP were minimally adjusted, in line with previous analyses of this cohort (Pham et al., 2022b), accounting for the child's sex and age, exposure factors (time of day blood was collected, days blood sample stored), and whether the child was sick during blood sample collection. To determine statistical significance, we apply a threshold of p-value < 0.05. All statistical analyses are conducted using R 4.0.0.

3. Results

3.1. Study population

Phthalate metabolites were analyzed in 842 women, with detectable levels of DEHP, DEP, DiBP, and DnBP metabolites found in nearly all participants (98–100%, refer to Table 2). In the study sample, the average maternal age was 32 years, while the average paternal age was 34 years. Any maternal smoking during pregnancy was not common (12.7%). Among the infants in the study population, 51% were male. The mean gestational age at urine collection was 36 weeks and the mean gestation length was 39 weeks. The geometric mean (geometric SD) for prenatal DEP daily intake was 1.60 (3.82) μg/kg body-weight/day; prenatal DBPs daily intake was 1.90 (1.97) μg/kg body-weight/day; prenatal DEHP daily intake was 1.63 (2.08) μg/kg body-weight/day; and the combined sum of prenatal DEP, DBPs and DEHP daily intake was 6.33 (2.24) μg/kg body-weight/day (Table 1). Previous studies conducted in Europe, Canada, and Japan among pregnant or reproductive-age women (de Renzy-Martin et al., 2014; Myridakis et al., 2015; Saravanabhavan et al., 2014; Suzuki et al., 2009) reported daily intake estimates comparable to those observed in this cohort (Sugeng et al., 2020). Further, summary statistics for the immune biomarkers are provided in Table 3 The distribution of the genetic pathway function score for inflammation estimated for the BIS cohort is illustrated in Fig. 1.

3.2. The genetic score gPFS ^{in} and immune response

In general, the gPFS^{in} exhibits a positive association with the inflammatory immune markers both in cord blood and at the age of 4, as evidenced by its association with various pro-inflammatory cytokines, hsCRP and GlycA (5). For instance, the associations with each T helper type 1 (Th1) pro-inflammatory cytokines show consistent direction at both time points and with the stimulated LPS and PGN Th1 pro-inflammatory cytokines (see Table 5.). Notably, for non-stimulated cytokines, statistically significant associations are observed between the gPFS^{in}, IFN-γ (at birth), TNF-α (at age 4), as well as IL-12 (at age 4). Also, the gPFS^{in} exhibits similar associations with the stimulated Th1 pro-inflammatory cytokines, including IFN-γ (at birth), TNF-α (at birth), IL-12 (at age 4), and IL-β (at age 4). The association of gPFS^{in} with hsCRP is notably stronger, especially when comparing the top quantile to the rest of the genetic pathway score function (hsCRP at age 4: $\beta = 0.519$, 95% CI (0.027, 1.01), p = 0.038). In contrast, the association of gPFS^{in} with Glycoprotein acetyls is not statistically significant (GlycA at age 4: $\beta = 0.077$, 95% CI (−0.410, 0.565), p = 0.755).

3.3. The genetic score gPFS ^{in} and neurodevelopment outcomes

We use regression analyses to explore the relationships between the genetic pathway function score for inflammation and various neurodevelopmental measures within the BIS cohort, such as doctor-diagnosed ASD, parent-reported ASD traits, the SDQ hyperactivity/inattention binary variable as well as doctor-diagnosed ADHD. In all our analyses, we consistently adjust for age at testing and sex. In general, we find a positive and direct association between gPFS^{in} and all the neurodevelopmental measurements we are examining. Notably, a high gPFS^{in} score (the top quintile), indicating a reduced ability to counter inflammation, is linked to a higher parent-reported ASD traits at age 4, a doctor-diagnosis of ASD and of ADHD at age 11.5 years. The adjusted odds ratios (AOR) for these associations are 3.13 (95% CI: 1.26, 7.78; p = 0.008), 2.15 (95% CI: 1.03, 4.47; p = 0.039) and 2.42 (95% CI: 1.29, 4.55; p = 0.005), respectively. For a comprehensive list, refer to Table 6. We examined sex-specific associations and observed that the genetic score exhibited stronger associations with neurodevelopmental outcomes in boys compared to girls.

3.4. Gene-environment interplay and neurodevelopmental outcomes

To investigate the combined impact of genetic and environmental factors, we stratified both gPFS^{in} and maternal prenatal phthalate levels into two categories: high (top quintile) and low (bottom four quintiles). We established a reference group, $G^{lo}P^{lo}$, consisting of individuals with low levels in both gPFS^{in} and phthalates. A clear trend emerged, indicating that individuals in the $G^{hi}P^{hi}$ group—characterized by exposure to top quintile levels of phthalates and gPFS^{in} scores of 7.5 or higher—are at a significantly elevated risk of adverse neurodevelopmental outcomes. Additionally, the trend suggests that individuals in the $G^{lo}P^{hi}$ and $G^{hi}P^{lo}$ categories represent other at-risk groups, albeit with a moderate risk magnitude compared to the doubly exposed $G^{hi}P^{hi}$ group. For example, individuals with both high gPFS^{in} and high DEHP exposure consistently exhibited more neurodevelopmental problems across all adverse outcomes. This includes associations of high gPFS^{in} and high DEHP levels with parent-reported ASD traits at age 4, doctor-diagnosed ASD and doctor-diagnosed ADHD at age 11.5 years, with adjusted odds ratios (AOR) of 6.38 (95% CI: 1.12, 36.33; p = 0.019), 1.92 (95% CI:

Table 2
Phthalate metabolites, parent compound names and abbreviations.

Urinary metabolite				Parent compound	
Abbreviation	Full name	LOD (μg/L)	% Below LOD	Abbreviation	Full name
MEP	Monoethyl phthalate	0.34	0.6	DEP	Diethyl phthalate
MnBP	Mono-n-butyl phthalate	4.52	1.4	DnBP	Di-n-butyl phthalate
MiBP	Monoisobutyl phthalate	3.89	1.9	DiBP	Diisobutyl phthalate
				ΣDBPs	Sum of dibutyl phthalates
MEHHP	Mono-(2-ethyl-5-hydroxyhexyl) phthalate	0.13	0.0	DEHP	Di-(2-ethylhexyl) phthalate
MECPP	Mono-(5-carboxy-2-ethylpentyl) phthalate	0.03	0.0		
MEOHP	Mono-(2-ethyl-5-oxohexyl) phthalate	0.10	0.0		

Note: DBPs = DnBP + DiBP; LOD = limit of detection.

Table 3
BIS immune biomarkers distribution.

Immune Marker	Cohort sample (N = 1074)	Cohort sample: GM {GSD}	Participants with Any Neurodevelopment Data (N = 890)	Participants with Any Neurodevelopment Data: GM {GSD}
Cord Blood				
IFN-γ (pg/ml)	429	4.56 {2.38}	386	4.53 {2.40}
TNF-α (pg/ml)	429	4.07 {3.13}	386	4.01 {3.14}
IL-6 (pg/ml)	429	7.17 {4.96}	386	7.17 {5.02}
IL-1β (pg/ml)	429	0.62 {2.47}	386	0.61 {2.49}
IFN-γ, stimulated, 1.0 ng/ml LPS (pg/ml)	217	496.37 {1.82}	202	495.76 {1.82}
TNF-α, stimulated 1.0 ng/ml LPS (pg/ml)	217	5884.71 {2.01}	202	5856.02 {2.02}
IL-6, stimulated 1.0 ng/ml LPS (pg/ml)	217	897.31 {3.11}	202	898.84 {3.09}
IL-1β, stimulated 1.0 ng/ml LPS (pg/ml)	217	81.96 {4.55}	202	83.37 {4.57}
GlycA (mmol/L)	909	0.73 {1.23}	761	0.73 {1.22}
hsCRP (μg/ml)	916	0.05 {4.64}	766	0.05 {4.56}
4 Year				
TNF-α, unstimulated (pg/ml)	284	26.68 {2.18}	284	26.68 {2.18}
TNF-α, stimulated 100 ng/ml LPS (pg/ml)	275	3390.29 {1.80}	275	3390.29 {1.80}
TNF-α, stimulated 10 µg/ml PGN (pg/ml)	277	1141.24 {1.93}	277	1141.24 {1.93}
IL-12, unstimulated (pg/ml)	284	0.64 {1.34}	284	0.64 {1.34}
IL-12, stimulated 100 ng/ml LPS (pg/ml)	275	8.75 {1.66}	275	8.75 {1.66}
IL-12, stimulated 10 µg/ml PGN (pg/ml)	277	4.32 {1.29}	277	4.32 {1.29}
IL-6, unstimulated (pg/ml)	284	1.26 {4.45}	284	1.26 {4.45}
IL-6, stimulated 100 ng/ml LPS (pg/ml)	275	1467.91 {1.76}	275	1467.91 {1.76}
IL-6, stimulated 10 µg/ml PGN (pg/ml)	277	1297.84 {1.79}	277	1297.84 {1.79}
IL-1β, unstimulated (pg/ml)	284	0.62 {4.68}	284	0.62 {4.68}
IL-1β, stimulated 100 ng/ml LPS (pg/ml)	242	2514.05 {2.07}	242	2514.05 {2.07}
IL-1β, stimulated 10 µg/ml PGN (pg/ml)	271	504.97 {2.76}	271	504.97 {2.76}
GlycA (mmol/L)	510	1.14 {1.13}	510	1.14 {1.13}
hsCRP (μg/ml)	511	0.17 {6.50}	511	0.17 {6.50}

Note: GlycA, Glycoprotein acetylation; hsCRP, high-sensitivity C-reactive protein; LPS, lipopolysaccharide; PGN, peptidoglycan: GM, Geometric mean: GSD, Geometric standard deviation. Participants with any neurodevelopment data are those with any of the variables related to SDQ hyperactivity/inattention, ASD traits, ASD diganosis, and ADHD diagnosis.

1.10, 3.34; p = 0.021) and 2.07 (95% CI: 1.19, 3.86; p = 0.022), respectively. In comparison, the reference group ($G^{lo}P^{lo}$) has an AOR of 1.0 by definition for all interactions.

Another example is the association between the $gPFS^{in}$ and maternal Σphthalate levels with ASD diagnosis age 11.5 years. In this case the AOR is 1.0 for the reference $G^{lo}P^{lo}$ category; 1.44 (95% CI: 1.03, 2.01; p = 0.031) for $G^{lo}P^{hi}$; 2.53 (95% CI: 0.99, 6.49; p = 0.052) for $G^{hi}P^{lo}$; and 2.31 (95% CI: 1.29, 4.15; p = 0.004) for $G^{hi}P^{hi}$. The sample size, when stratified by sex, is small and lacks sufficient outcome representation across the categories. As a result, our sex-specific analyses of the gene-environment interactions and health outcomes has not yielded reliable measurements, and our regression models failed to converge. This study has been underpowered by sex stratification and further work is needed.

Furthermore, we also explored the additive interaction effects between $gPFS^{in}$ and prenatal phthalate exposure and their relationship with adverse neurodevelopmental outcomes in the BIS cohort. In this analysis, the attributable proportion (AP) metric emerged as a robust tool, revealing significant additive interactions related to various associations with ASD traits and ASD diagnoses. For instance, when examining DEHP and its association with ASD traits at age 4 and ASD diagnosis at age 11.5 years, the AP 0.77 (95% CI: 0.17, 1.71; p = 0.024) and 0.37 (95% CI: 0.06, 0.86; p = 0.037), respectively, highlighting a substantial disease proportion in the doubly exposed group ($G^{hi}P^{hi}$) attributed to this interaction.

3.5. Additional analyses

Given the numerous exposures at various stages throughout the life course up to age nine and the wide range of neurodevelopmental outcomes, conducting comprehensive confounder detection and evaluation analyses was not feasible (Ponsonby et al., 2022). Additionally, rigorous assessment would be necessary to determine whether certain factors were true confounders or inherently linked to the issues under investigation. For instance, maternal smoking during pregnancy, which is commonly adjusted for in perinatal epidemiology, could function as a co-exposure in this context. It might exacerbate the adverse effects of phthalates through shared inflammation-related pathways, and controlling for it could potentially introduce collider bias. Instead, the E-value approach was employed as an alternative (Mathur et al., 2018; VanderWeele and Ding, 2017).

To explain the observed association between the genetic pathway function score for inflammation and a doctor-diagnosed ASD (Table 6), an unmeasured confounder would need to be associated by a risk ratio of at least 4-fold with both the $gPFS^{in}$ and ASD diagnosis, beyond the effects of measured confounders. Similarly, to account for the association between a high $gPFS^{in}$ and a 2.42-fold increased likelihood of doctor-diagnosed ADHD, a potential confounder would need to elevate the $gPFS^{in}$ profile by 5-fold or more and increase the likelihood of ADHD diagnosis by 5-fold or more. As shown in Table 6, none of the potential confounders demonstrate these characteristics, making it unlikely that the findings are attributable to unmeasured confounding. Further, to

Table 4
The list of the constructed fundamental inflammation response pathway's genes, and related SNPs used to create the gPFS^{in} score for the BIS sample, refer to equation (1). The SNP relation to gene column refers to the relation in terms of gene transcript expression.

Gene	SNP rsIDs	SNP relation to gene	Inferred Effect of SNP	Relation to Cytokines & Chemokines Signaling Pathways
JAK1	rs11208536	increase	Anti-inflammation	ILs-2,4,10,7, IFNα, IFNβ, IFNγ, CSF3 (G-CSF)
MAP3K7	rs75723654	reduce	Anti-inflammation	IL-1β
SHC1	rs11590058	reduce	Anti-inflammation	IL-3,GM-CSF
JAK2	rs75310079	increase	Anti-inflammation	ILs-3,4,13, CSF3
SOCS3	rs62063841	reduce	Anti-inflammation	IL-6
SMAD3	rs452164	increase	Anti-inflammation	Transforming growth factor (TGF-β)
SMAD7	rs357904	reduce	Anti-inflammation	TGF-β
IRAK2	rs149495345	increase	Pro-inflammation	IL-1β
TRAF6	rs554949	increase	Pro-inflammation	IL-1β
STAT1	rs11689350	increase	Pro-inflammation	IL-6, IFNγ, G-CSF, PDGF
STAT6	rs73118440	reduce	Pro-inflammation	ILs-4,13
STAT2	rs143285063	increase	Pro-inflammation	IFNα, IFNβ
TRAF3	rs1956161	increase	Pro-inflammation	TNFR2 non-canonical NF-kb pathway
MAP3K14	rs3744410	increase	Pro-inflammation	TNFR2 non-canonical NF-kb pathway
BIRC2	rs76001245	increase	Pro-inflammation	TNFR2 non-canonical NF-kb pathway
SMAD2	rs11662343	reduce	Pro-inflammation	TGF-β

JAK: Janus Kinase; *MAP3K*: Mitogen-Activated Protein Kinase Kinase Kinase; *SHC*: SHC Adaptor Protein; *SOCS*: Suppressor of Cytokine Signaling; *SMAD*: SMAD Family Member; *IRAK*: Interleukin-1 Receptor-Associated Kinase; *TRAF*: TNF Receptor-Associated Factor; *STAT*: Signal Transducer and Activator of Transcription; *BIRC*: Baculoviral IAP Repeat Containing. ILs: Interleukins; IFN: Interferon; CSF: granulocyte colony-stimulating factor; PDGF: Platelet-derived growth factor.

account for the association between any of the Gene × phthalates exposure in the doubly exposed top quintile groups and the increased likelihood of doctor-diagnosed ASD as well as doctor-diagnosed ADHD (Table 7), a potential confounder would need to elevate the Gene × phthalates profile by 2-fold or more and increase the likelihood of ADHD or ASD diagnosis by 2-fold or more.

4. Discussion

In our investigation within the BIS birth cohort, we utilized a genetic pathway function score, referred to as gPFS^{in}, to gauge an individual's genetic predisposition towards inflammation. The aim of this study was to capture genetic contribution to inflammation at the level of the inflammatory response pathway. This score reflected the combined effect of 16 eQTL SNPs across the 16 genes within the specific immune pathway. In contrast, SNPs documented in the GWAS Catalog were associated individually with inflammation, following rigorous correction for multiple testing over millions of SNPs. Consequently, this

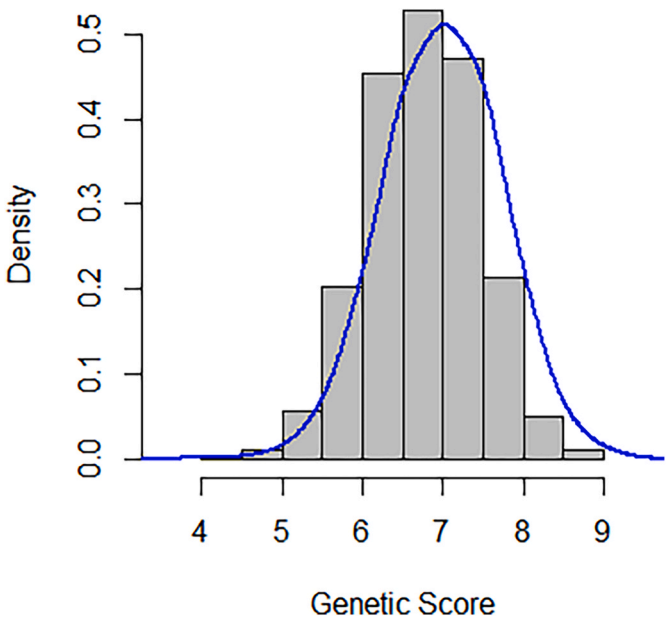

Fig. 1. The density distribution of the BIS cohort genetic function score for inflammation (gPFS^{in}). Each additional pro-inflammatory allele corresponds to a 0.5 increase in score. Higher values indicates more genetic vulnerability to inflammation.

method might not encompass the collective effects of functionally related SNPs. Despite the pleiotropic roles of the genes within the score, extending beyond inflammation, the connection with immune markers in BIS suggests that the aggregate score effectively encompasses the shared or combined function of these genes specifically within the mechanisms of inflammatory response.

We identified an association between higher genetic scores (indicating vulnerability to inflammation) and the occurrence of autistic traits, ASD, and ADHD behaviors within the BIS cohort. Furthermore, as we explored the combined effects of genes and the environment, specific patterns emerged across different categories, reflecting combinations of genetic scores and prenatal phthalate exposure levels (Table 2). Notably, these patterns were most pronounced in individuals with both high prenatal phthalate exposure and high gPFS^{in}, and these trends were consistent across all assessed outcomes, including autistic traits, ASD, and inattention/hyperactivity in children.

Our findings align with prior evidence of a connection between inflammation and adverse neurodevelopmental outcomes (Knuesel et al., 2014; Lee et al., 2015; Volk et al., 2020; Jones et al., 2017; Abdallah et al., 2013; Dozmorov et al., 2018; Zengeler and Lukens, 2021). Although the exact mechanisms through which maternal immune system activation impact fetal neurodevelopment are not yet fully comprehended, existing studies suggest their substantial involvement in the normal development of the central nervous system (Knuesel et al., 2014). Specifically, chemokines have been shown to regulate the movement, growth, and specialization of neuronal cells, playing a role in the communication between neurons and microglia (Knuesel et al., 2014; Bilbo et al., 2018). Consequently, they could serve as intermediaries in the well-documented link between inflammation and autism. Increases in specific brain-active cytokines and/or chemokines in maternal blood could potentially reach the fetal brain and influence brain development at various stages (Abdallah et al., 2013; Zengeler and Lukens, 2021; Bilbo et al., 2018; Deverman and Patterson, 2009). For instance, research indicates that chemokines like IL−8 and MCP−1 may guide the migration and specialization of neural stem/progenitor cells during early neurodevelopment (Pardo et al., 2005; Zengeler and Lukens, 2021).

Furthermore, cytokines have been associated with neurogenesis, as

Table 5
The associations between the gPFS^{in} and proinflammatory cytokines, high-sensitivity C-reactive protein as well as Glycoprotein acetyls immune markers measured at birth and at age 4.

Th1 Proinflammatory Cytokines								
	Cord Blood				Year 4			
	gPFS^{in} (Per 2 additional pro-inflammation alleles)		gPFS^{in} (Top quintile vs. rest)		gPFS^{in} (Per 2 additional pro-inflammation alleles)		gPFS^{in} (Top quintile vs. rest)	
	β (95% CI)	P value	β (95% CI)	P value	β (95% CI)	P value	β (95% CI)	P value
IFN-γ	**0.52 (0.02,1.02)**	**0.037**	**0.59 (0.16,1.03)**	**0.006**	–	–	–	–
TNF-α	−0.01(-0.33, 0.30)	0.929	−0.38(-0.85,0.08)	0.105	**0.94 (0.01,1.88)**	**0.046**	0.81 (−0.03,1.66)	0.060
IL-12	–	–	–	–	**0.20 (0.09, 1.02)**	**0.048**	**0.55 (0.25, 0.75)**	**0.003**
Other Classic Proinflammatory immune markers								
IL-6	0.20 (−0.40,0.80)	0.512	0.21 (−0.30,0.73)	0.418	0.35 (−1.32,0.62)	0.478	**0.54 (0.23,0.82)**	**0.004**
IL-1β	0.05 (−0.25,0.36)	0.713	0.05 (−0.39, 0.50)	0.816	0.11 (−0.65,0.88)	0.763	0.34 (−0.50,0.92)	0.095
GlycA	0.16 (−0.30,0.63)	0.487	0.06 (−0.32,0.45)	0.751	0.14 (−0.42,0.72)	0.614	0.07 (−0.41,0.56)	0.755
hsCRP	**0.51 (0.04,0.97)**	**0.030**	0.02 (−0.35, 0.40)	0.888	0.25 (−0.32,0.83)	0.383	**0.51 (0.04,0.97)**	**0.030**
Stimulated Th1 Proinflammatory Cytokines								
	Cord Blood (LPS stimulated)				Year 4 (LPS stimulated)			
	gPFS^{in} (Per 2 additional pro-inflammation alleles)		gPFS^{in} (Top quintile vs. rest)		gPFS^{in} (Per 2 additional pro-inflammation alleles)		gPFS^{in} (Top quintile vs. rest)	
	β (95% CI)	P value	β (95% CI)	P value	β (95% CI)	P value	β (95% CI)	P value
IFN-γ	0.53(-0.29,1.36)	0.205	**0.76 (0.03,1.56)**	**0.021**	–	–	–	–
TNF-α	**0.86 (0.13,1.58)**	**0.020**	**1.04 (0.01, 2.08)**	**0.048**	−0.34(-0.92,0.23)	0.245	0.21(-0.64,1.07)	0.621
IL-12	–	–	–	–	−0.31(-0.90,0.27)	0.29	−0.36 (−1.28,0.55)	0.435
Other Stimulated Classic Proinflammatory immune markers								
IL-6	0.13 (−0.45,0.72)	0.653	0.14 (−0.65,0.95)	0.718	−0.32 (−0.87,0.22)	0.248	0.10 (−0.71,0.91)	0.810
IL-1β	0.29 (−0.29,0.88)	0.328	0.33(-0.45,1.12)	0.410	0.33 (−0.25,0.92)	0.268	**1.11 (0.25,1.95)**	**0.011**
Stimulated Th1 Proinflammatory Cytokines								
	Year 4 (PGN stimulated)							
	gPFS^{in} (Per 2 additional pro-inflammation alleles)		gPFS^{in} (Top quintile vs. rest)					
	β (95% CI)	P value	β (95% CI)	P value				
IFN-γ	–	–	–	–				
TNF-α	0.27(-0.58,1.13)	0.526	−0.05(-0.81,0.70)	0.886				
IL-12	**1.18 (0.34, 2.03)**	**0.005**	**0.87 (0.12, 1.62)**	**0.022**				
Other Stimulated Classic Proinflammatory immune markers								
	gPFS^{in} (Per 2 additional pro-inflammation alleles)		gPFS^{in} (Top quintile vs. rest)					
	β (95% CI)	P value	β (95% CI)	P value				
IL-6	0.04 (−0.83,0.92)	0.918	−0.34(-1.14,0.45)	0.393				
IL-1β	**1.01 (0.20,1.42)**	**0.008**	0.33 (−0.42,1.10)	0.390				

NB:The β value represents the regression coefficient. Th1, T helper type 1. The cytokines models were adjusted for the child's sex and age, as well as various exposure factors (e.g., time of the day blood was collected). The regression models for hsCRP and GlycA were adjusted similarly. "-" indicates too few above detection limit in category.

Table 6
The main effect of the genetic pathway function score for inflammation response, gPFS^{in}, on ASD and ADHD outcomes.

	ASD Traits Age 4		ASD Diagnosis age 11.5		SDQ hyperactivity/ inattention Age 4		ADHD Diagnosis age 11.5	
	AOR (95% CI)	P value	AOR (95% CI)	P value	AOR (95% CI)	P value	AOR (95% CI)	P value
gPFS^{in} (Per 2 additional pro-inflammation alleles)	1.34 (0.77,2.31)	0.294	1.58 (0.76,3.25)	0.215	1.53 (0.75,3.10)	0.237	**2.28 (1.21,4.29)**	**0.010**
gPFS^{in} (Top quintile vs. rest)	**3.13 (1.26,7.78)**	**0.008**	**2.15 (1.03,4.47)**	**0.039**	**2.37 (1.18, 4.77)**	**0.015**	**2.42 (1.29, 4.55)**	**0.005**

NB: AOR = adjusted odds ratio; SDQ, Strengths and Difficulties Questionnaire; ASD, autism spectrum disorder; ADHD, Attention-deficit/hyperactivity disorder; Model adjusted for child's sex and post-conceptional age at testing. The AORs indicate the outcome likelihood after model adjustments.

well as the movement, growth, specialization, and synaptic maturation and refinement of neuronal and glial cells. Considering the potential significant role of neuroinflammation in the development of ASD and ADHD (Pardo et al., 2005; Zengeler and Lukens, 2021; Bilbo et al., 2018; Deverman and Patterson, 2009), it is plausible that a combination of genetic predisposition and an environmental insult during pregnancy may lead to adverse neurodevelopment in offspring.

5. Limitations

This study has several limitations that warrant consideration when interpreting its outcomes. To begin with, the evaluation of autistic traits relied on parental reports. Notably, significant associations were observed between autistic traits, diminished prosocial behavior, and increased peer problems on the SDQ subscales (Croft et al., 2015). The measurement of phthalates and the assessment of their environmental impacts were based on a single spot urine sample at the 36-week gestational mark. Nevertheless, prior research has shown that phthalate exposure during the third trimester tends to exhibit moderate stability (Suzuki et al., 2009) when assessed repeatedly, and efforts were made to account for factors such as the time of day and maternal weight, which are recognized contributors to variability. Also, we acknowledge that the gene-environment interaction models in our analysis are minimally adjusted for child's and post-conceptional age at testing. Other

Table 7
The interaction in terms of additive effects between the genetic pathway function score for inflammation ($gPFS^{in}$) and prenatal phthalate levels in relation to neurodevelopmental outcomes.

		$G^{lo}P^{hi}$		$G^{hi}P^{lo}$		$G^{hi}P^{hi}$		Additive Interaction	
ASD traits at age 4	Phthalate	AOR (95% CI)	P value	AOR (95% CI)	P value	AOR (95% CI)	P value	AP (95% CI)	P value
	DEP	1.19(0.74,1.93)	0.458	1.19 (0.77,1.82)	0.424	1.25(0.70,2.25)	0.436	0.15(-0.15, 0.46)	0.166
	DBPs	1.58(0.79,3.11)	0.191	2.12 (0.74,6.08)	0.159	**70.43 (1.72,188.59)**	**0.024**	0.20(-0.09,0.49)	0.089
	DEHP	**1.62 (1.04,2.51)**	**0.031**	1.37 (0.59,3.16)	0.451	**6.38(1.12,36.33)**	**0.019**	**0.39(0.06,0.84)**	**0.045**
	Σ phthalates	**1.37 (1.00,1.87)**	**0.045**	1.28 (0.54,3.02)	0.571	2.06(0.77,5.45)	0.144	**0.48(0.04,0.98)**	**0.032**
ASD diagnosis by age 11.5 years	DEP	**1.55 (1.02,2.35)**	**0.038**	2.08 (0.94,4.60)	0.069	1.50(0.89,2.54)	0.127	**0.32(0.06,0.79)**	**0.047**
	DBPs	1.48(0.66,2.52)	0.444	2.22 (0.63,6.69)	0.231	1.96(0.48,5.64)	0.361	0.24(-0.22,0.62)	0.178
	DEHP	1.01(0.70,1.46)	0.942	1.54 (0.63,3.74)	0.335	**1.92(1.10,3.34)**	**0.021**	**0.37(0.06,0.86)**	**0.037**
	Σ phthalates	**1.44 (1.03,2.01)**	**0.031**	2.53 (0.99,6.47)	0.052	**2.31(1.29,4.15)**	**0.004**	0.33(-0.55,-1.22)	0.228
SDQ hyperactivity/inattention at age 4	DEP	1.03(0.67,1.56)	0.883	0.71 (0.47,1.08)	0.116	0.97(0.41,2.31)	0.954	−0.10 (-0.51,0.31)	0.317
	DBPs	1.09(0.60,1.96)	0.761	1.03 (0.44,2.45)	0.929	–	–	−0.11 (-0.54,0.32)	0.308
	DEHP	**1.54 (1.02,2.32)**	**0.037**	1.36 (0.64,2.85)	0.414	**8.27(1.06,64.11)**	**0.043**	0.11(-0.21,0.44)	0.252
	Σ phthalates	1.17(0.89,1.55)	0.246	0.88 (0.47,1.64)	0.697	1.55(0.55,4.35)	0.403	0.06(-0.14,0.27)	0.260
ADHD diagnosis by age 11.5 years	DEP	**1.69 (1.05,2.71)**	**0.029**	1.01 (0.63,1.58)	0.981	1.22(0.02,5.89)	0.916	0.02(-0.13,0.14)	0.486
	DBPs	1.39(0.56,3.44)	0.474	1.41 (0.72,2.76)	0.305	–	–	0.166(0.66,0.92)	0.348
	DEHP	1.78(0.90,3.50)	0.094	1.61 (0.58,4.50)	0.357	**2.07(1.19,3.86)**	**0.022**	0.46(-0.44,1.36)	0.159
	Σ phthalates	**1.57 (1.02,2.40)**	**0.036**	1.29 (0.68,2.41)	0.437	**1.85(1.01,3.41)**	**0.046**	0.12(-0.30,0.55)	0.279

NB: All combinations of G and P are compared with a reference group $G^{lo}P^{lo}$; "-" indicates insufficient outcome numbers in category. AOR = adjusted odds ratio. G = $gPFS^{in}$; P = the specific phthalate; ^{hi} = top quintile; ^{lo} = bottom four quintiles; DEHP, Di-(2-ethylhexyl) phthalate; DEP, Diethyl phthalate; DBPs, sum of DiBP (Diisobutyl phthalate) and DnBP (Di-n-butyl phthalate); Σ phthalates, sum of DEP, DBPs & DEHP daily intakes; SDQ, Strengths and Difficulties Questionnaire; ASD, autism spectrum disorder; ADHD, Attention-deficit/hyperactivity disorder; AP, attributable proportion; Model adjusted for child's sex and post-conceptional age at testing. The AORs indicate the outcome likelihood after model adjustments.

confounders such as socio-demographic factors may influence our estimates and potentially impact the observed associations. We also highlight the need for future studies to replicate our findings using more comprehensive adjustment strategies to account for these potential influences. In this investigation, only three parent phthalate compounds were considered for their potential to induce inflammation. It is crucial to acknowledge the need for additional research to delve into a wider array of factors linked to inflammation. This could involve examining various elements such as other plastic chemicals and heavy metals, maternal smoking, inhaled pollutants, and dietary factors with anti-inflammatory properties.

Moving forward, our intention is to explore the impact of multiple environmental exposures along with a multi-genetic scores approach. Specifically, we will investigate the effects of the environment while taking into account oxidative stress, as highlighted in Tanner et al. (2022), as well as susceptibility to inflammation indicated by $gPFS^{in}$. This is because inflammation is only one of the proposed mechanisms linking prenatal phthalate exposure to adverse childhood development. For example, Tanner et al. (2022) demonstrated an association between phthalate exposure and adverse neurodevelopment in BIS's children. They observed a consistent pattern of heightened neurodevelopmental problems in individuals who had both a genetic susceptibility to oxidative stress and prenatal exposure to phthalates. Moreover, conducting longitudinal studies involving multiple measurements, including postnatal assessments of child phthalate levels, is essential. This would offer a more comprehensive understanding of how the timing of elevated phthalate exposures may impact neurodevelopment.

6. Conclusion

It has been well established that maternal immune system activation as well as higher early-life inflammation have adverse neurodevelopmental outcomes in offspring (e.g., Solek et al., 2018; Knuesel et al., 2014; Pham et al., 2022a; Rossignol and Frye, 2014). In this study, we developed a genetic pathway function score focusing on inflammation ($gPFS^{in}$), which captures the transcriptional activity across the inflammation response pathway. This score has emerged as a genetic indicator of inflammation linked to adverse neurodevelopmental outcomes. Within this Australian birth cohort, a prior investigation revealed a connection between maternal exposure to phthalates and an altered inflammatory state (Eisner et al., 2022). In this study, we present findings indicating that the negative impact on neurodevelopment due to prenatal phthalate exposure varies based on an individual's genetic susceptibility to inflammation. Additionally, a discernible pattern is evident, demonstrating an association between an elevated $gPFS^{in}$ score and adverse neurodevelopment. Future research investigating the origins of these neurodevelopmental conditions is poised to gain significant insights by exploring both environmental and genetic factors within the context of common biological mechanisms. This study underscores the contemporary challenge posed by chemical exposure during pregnancy, emphasizing the pressing need for continuous and timely attention from public health initiatives.

CRediT authorship contribution statement

Ahmed Elagali: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alex Eisner:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis. **Samuel Tanner:** Writing – review & editing, Formal analysis, Conceptualization. **Katherine Drummond:** Writing – review & editing, Methodology, Conceptualization. **Christos Symeonides:** Writing – original draft, Conceptualization. **Chloe Love:** Writing – original draft, Conceptualization. **Mimi LK. Tang:** Writing – review & editing, Data curation, Conceptualization. **Toby Mansell:** Writing – review & editing, Data curation, Conceptualization. **David Burgner:** Writing – review & editing, Data curation, Conceptualization. **Fiona Collier:** Writing – review & editing, Data curation, Conceptualization. **Peter D. Sly:** Writing – review & editing, Conceptualization. **Martin O'Hely:** Writing – review & editing, Conceptualization. **Sarah Dunlop:** Writing – review & editing, Conceptualization. **Peter Vuillermin:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **Anne-Louise Ponsonby:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Data availability

Barwon Infant Study (BIS) data requests are considered on scientific and ethical grounds by the BIS Steering Committee. If approved, data are provided under collaborative research agreements.

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