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Increased maternal non-oxidative energy metabolism mediates association between prenatal di-(2-ethylhexyl) phthalate (DEHP) exposure and offspring autism spectrum disorder symptoms in early life: A birth cohort study

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ABSTRACT

Prenatal phthalate exposure has previously been linked to the development of autism spectrum disorder (ASD). However, the underlying biological mechanisms remain unclear. We investigated whether maternal and child central carbon metabolism is involved as part of the Barwon Infant Study (BIS), a population-based birth cohort of 1,074 Australian children. We estimated phthalate daily intakes using third-trimester urinary phthalate metabolite concentrations and other relevant indices. The metabolome of maternal serum in the third trimester, cord serum at birth and child plasma at 1 year were measured by nuclear magnetic resonance. We used the Small Molecule Pathway Database and principal component analysis to construct composite metabolite scores reflecting metabolic pathways. ASD symptoms at 2 and 4 years were measured in 596 and 674 children by subscales of the Child Behavior Checklist and the Strengths and Difficulties Questionnaire, respectively. Multivariable linear regression analyses demonstrated (i) prospective associations between higher prenatal di-(2-ethylhexyl) phthalate (DEHP) levels and upregulation of maternal non-oxidative energy metabolism pathways, and (ii) prospective associations between upregulation of these pathways and increased offspring ASD symptoms at 2 and 4 years of age. Counterfactual mediation analyses indicated that part of the mechanism by which higher prenatal DEHP exposure influences the development of ASD symptoms in early childhood is through a maternal metabolic shift in pregnancy towards non-oxidative energy pathways, which are inefficient compared to oxidative metabolism. These results highlight the importance of the prenatal period and suggest that further investigation of maternal energy metabolism as a molecular mediator of the adverse impact of prenatal environmental exposures such as phthalates is warranted.

1. Introduction

Autism spectrum disorder (ASD) is characterized by impaired social interaction and communication, as well as perseverative and repetitive behaviors (Jonsson et al., 2017). The etiology of ASD is not yet fully understood. However, both genetic predisposition and an adverse early environment appear important (Hallmayer et al., 2011). The ASD phenotype is observable from an early age. Differences in the brains of ASD children compared to controls have been detected *in utero* and at 3 months of age using MRI and EEG, respectively Bosl et al. (2018), Ortug et al. (2022). Therefore, it is important to look at ASD symptom development in early childhood. Previously, we have reported that ASD

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symptoms at 2 years obtained from the caregiver-completed Child Behavior Checklist (CBCL) predicts later doctor-diagnosed ASD in our setting (area under the curve = 0.92) (Pham et al., 2022). ASD prevalence has risen substantially in the last two decades and is now 1–2.5 % (Atladottir et al., 2015). This increase is unlikely to be explained by diagnostic changes and genetics alone (Hansen et al., 2015; Herbert et al., 2006). Thus, there is growing concern over the impact of early environmental agents that have been epidemiologically associated with ASD (Bölte et al., 2019; Rossignol et al., 2014), and there is a need to understand common molecular mechanisms by which they contribute to ASD development.

Of the environmental candidates, exposure to manufactured chemicals, such as phthalates, is of particular concern given increasing evidence of links to adverse early neurodevelopment (Bennett et al., 2016). Phthalates are plasticizers used to increase the flexibility of plastics. However, as they are not bonded to the polymer, leaching occurs (Hahladakis et al., 2018). They can be ingested, inhaled or dermally absorbed and major sources of exposure include food processing and packaging materials and personal care products (Sugeng et al., 2020). Almost all pregnant people in Western populations have detectable levels of phthalates in their urine (Sugeng et al., 2020; Shin et al., 2020). Higher prenatal phthalate exposure has been associated with the development of ASD and ASD symptoms in many (Larsson et al., 2009; Miodovnik et al., 2011; Oulhote et al., 2020; Haggerty et al., 2021; Day et al., 2021; Alampi et al., 2021; Patti et al., 2021; Ponsonby et al., 2020; Kim et al., 2021) but not all (Braun et al., 2014; Shin et al., 2018; Radke et al., 2020) studies. In the Barwon Infant Study (BIS), we reported that a higher combined level of four phthalates prenatally is associated with an increased likelihood of offspring ASD and ASD traits at 4 years (OR 1.55, 95 % CI 1.00, 2.40; OR 1.51, 95 % CI 1.20, 2.01, respectively) (Ponsonby et al., 2020). Given the global increase in phthalate production (Prasad, 2021) and a lack of success of individual-level avoidance trials thus far (Sathyanarayana et al., 2013), there is an urgent need to obtain a higher level of causal evidence of (i) the neurodevelopmental consequences of phthalates, (ii) the underlying mechanisms, and (iii) the extent to which the prenatal period is a key window of exposure. One method for achieving this is by investigating potential molecular mediators.

Both higher prenatal phthalate exposure and ASD have been associated with alterations in central carbon metabolism (referred to as 'energy metabolism' herein), that is, either impaired oxidative metabolism or increased non-oxidative metabolism. Under normal conditions, oxidative processes within the mitochondrion maximize energy generation (Fig. 1, pathway 1) (Berg et al., 2002). Under adverse conditions, like hypoxia (Koziel and Jarmuszkiewicz, 2017; Chicco et al., 2018) or mitochondrial dysfunction (Liu et al., 2018), activity may be diverted to less energy efficient non-oxidative processes outside the mitochondrion (Fig. 1, pathway 2) (Berg et al., 2002). Elevated pyruvate, lactate, acetate and alanine blood levels indicate a metabolic shift towards non-oxidative processes with an 18-fold reduction in ATP energy production per glucose molecule compared to oxidative energy metabolism (Berg et al., 2002). An example of a non-oxidative metabolic shift, previously linked to ASD, is the Warburg Effect (Vallee and Vallee,

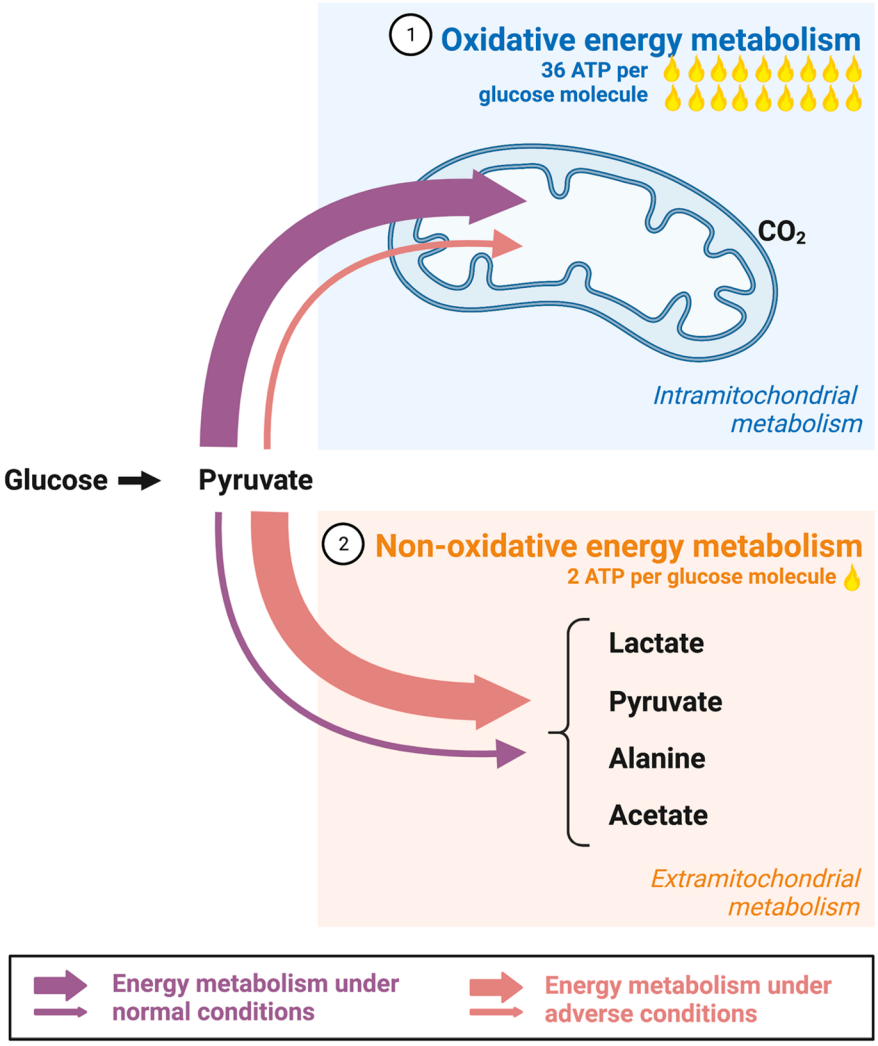

Fig. 1. The metabolic shift towards inefficient non-oxidative energy metabolism. *Normal energy metabolism typically maximizes the energy-generating potential of the mitochondrion through the oxidization of pyruvate (pathway 1). Metabolic shifts in central carbon metabolism can occur under adverse conditions, like hypoxia or mitochondrial dysfunction (Koziel and Jarmuszkiewicz, 2017; Chicco et al., 2018; Liu et al., 2018), due to toxic substance exposure or other factors. This results in the upregulation of non-oxidative pathways, whereby carbon metabolic intermediates (pyruvate, lactate, alanine, acetate) are diverted away from the mitochondrion (pathway 2) at the cost of further oxidative energy production (pathway 1). Elevated levels of these carbon metabolic intermediates in the blood or urine thus indicate a metabolic shift towards inefficient non-oxidative energy metabolism. Figure created with BioRender.com.*

2018; Warburg, 1956). It involves the conversion of a large portion of glucose to pyruvate to lactate regardless of oxygen levels.

Higher prenatal phthalate exposure has been associated with metabolic changes suggestive of reduced energy output from maternal oxidative metabolism (Maitre et al., 2018; Zhou et al., 2018) and increased non-oxidative lipid metabolism in the offspring (Kupsco et al., 2021; Perng et al., 2017; Vafeiadi et al., 2018). Non-oxidative carbon metabolites – pyruvate, lactate, acetate and alanine – have not, to our knowledge, been previously examined in children exposed to phthalates *in utero*.

Preclinical studies have shown that prenatal maternal metabolic abnormalities, such as hyperglycemia and other obesity-related changes, adversely affect offspring brain development *in utero* (Namba et al., 2021; Rash et al., 2018; Fernandes et al., 2021). In epidemiological studies, inefficient maternal energy metabolism in pregnancy, as indicated by elevated metabolic markers of non-oxidative metabolism, has been associated with an increased occurrence of offspring ASD (Hollowood et al., 2018; Lyall et al., 2014; Egorova et al., 2020; Kim et al., 2021). In individuals diagnosed with ASD, energy metabolism abnormalities are common (Cheng et al., 2017; Dhillon et al., 2011). Prevalence estimates range from 30 to 50 % for biomarkers of inefficient energy metabolism (Frye et al., 2021), including the elevation of serum carbon intermediates: pyruvate (Hassan et al., 2019), lactate (Correia et al., 2006), and alanine (Orozco et al., 2019). In fact, it is estimated that 5 % of individuals with ASD have classically defined mitochondrial disease (Frye et al., 2021) compared to 0.01 % of the general population (Skladal et al., 2003).

A modern causal Inference technique, molecular mediation, is increasingly being employed to understand the biological mechanisms by which prenatal phthalate exposure influences adverse health outcomes (Ferguson et al., 2017; England-Mason et al., 2020). However, the role of altered energy metabolism as an underlying mechanism for the link between higher prenatal phthalate levels and offspring ASD has not, to our knowledge, been evaluated. Here, we aimed to investigate (i) how phthalate exposure *in utero* associates with the mother and child's energy metabolic profiles, (ii) how energy metabolic profiles associate with subsequent ASD symptoms in early childhood, and (iii) if the mother and/or child's energy metabolic profiles mediate the association between prenatal phthalate exposure and ASD symptoms.

2. Methods

2.1. Cohort sample

From June 2010 to June 2013, a birth cohort of 1,074 mother–infant pairs (10 sets of twins) was recruited using an unselected antenatal sampling frame in the Barwon region of Victoria, Australia (Vuillermin et al., 2015). Eligibility criteria, population characteristics and measurement details of the Barwon Infant Study (BIS) have been provided previously (Vuillermin et al., 2015). The study was approved by the Barwon Health Human Research Ethics Committee (HREC 10/24) and families provided written informed consent.

2.2. Prenatal phthalate exposure

Phthalate metabolite levels in the third trimester were measured in 842 women using a single spot urine specimen collected at 36 weeks. High-performance liquid chromatography/tandem mass spectroscopy with direct injection was performed by the Queensland Alliance for Environmental Health Science as has been outlined previously (Ponsonby et al., 2020). Repeated spot urine specimens in Trimester 3 have been used in two studies to evaluate the reliability of single spot specimens for capturing third-trimester phthalate exposure (Adibi et al., 2008; Suzuki et al., 2009). Intraclass correlation coefficients (ICC) for monoethyl phthalate (MEP; ICC = 0.30–0.47), monoisobutyl phthalate (MiBP; ICC = 0.54), mono-*n*-butyl phthalate (MnBP; ICC = 0.62–0.70), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP; ICC = 0.36–0.43), and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP; ICC = 0.34–0.41) indicate moderate reliability (Adibi et al., 2008; Suzuki et al., 2009).

Urinary phthalate metabolite measurements were corrected for batch, specific gravity and time of day of sample collection (Ponsonby et al., 2020). Phthalate estimated daily intake was then calculated accounting for maternal prenatal weight, fractional excretion of the compound, and compound-to-metabolite molecular weight ratio (Ponsonby et al., 2020; Gao et al., 2017). The metabolites MEHHP, MEOHP and mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP) were used to calculate di-(2-ethylhexyl) phthalate (DEHP) daily intake; MEP for diethyl phthalate (DEP); MiBP for diisobutyl phthalate (DiBP), and MnBP for di-*n*-butyl phthalate (DnBP; see Table 1S for abbreviations). Due to their similarity, DiBP and DnBP daily intakes were summed to make a combined daily intake measure that we will refer to as 'DBPs' herein. DEHP, DEP, DiBP and DnBP daily intakes were added to make an overall sum phthalate daily intake measure. Findings expanded on our previous work (Ponsonby et al., 2020; Tanner et al., 2022) and considered the same phthalate measures which were logarithm base 2 transformed for analyses.

2.3. Metabolomic profiling

Metabolomic analysis was performed on non-fasting (i) maternal serum samples collected at 28 weeks of gestation, (ii) umbilical cord serum samples, and (iii) children's plasma samples collected at 1 year of age. All samples were sent to Nightingale Health (Helsinki, Finland) and the method of quantification used was nuclear magnetic resonance (NMR) (Emwas et al., 2019). Platform details can be found elsewhere (Soininen et al., 2009). Low-molecular-weight metabolites were quantified according to Nightingale's 2016 (maternal, child) and 2019 (cord) bioinformatics protocols (Soininen et al., 2009; Soininen et al., 2015). Analyses were restricted to metabolites related to central carbon metabolism (amino acids, n = 9; ketone bodies, n = 3; glycolysis-related, n = 5).

2.4. – Autism spectrum disorder: Symptoms

The DSM-5-oriented autism spectrum problems subscale of the Child Behavior Checklist for Ages 1.5–5 (CBCL-ASP) (Achenbach, 2013) and the peer relationship problems subscale of the Strengths and Difficulties Questionnaire for Ages 2–4 (SDQ-peer) (Goodman, 1997) completed by the child's caregiver at 2–3 years and 4 years, respectively, were used as measures of ASD symptom severity. Subscale scores were calculated by summing the responses to behavioral statements (0 = not true, 1 = somewhat true, 2 = very true). CBCL-ASP was based on 12 items (range 0–24) and SDQ-peer was based on 5 items (range 0–10).

In this cohort, CBCL-ASP predicted subsequent doctor-diagnosed autism with an area under the curve (AUC) of 0.92 (Pham et al., 2022). In the literature, the CBCL/1.5–5 pervasive developmental problems scale (CBCL-PDP), and SDQ-peer have moderate to high accuracy in distinguishing preschoolers with ASD from those who are typically developing (AUC 0.92 (Chericoni et al., 2021) and 0.82 (Salayev and Sanne, 2017), respectively). CBCL-ASP has replaced CBCL-PDP to reflect the DSM-5.

Using questionnaire measures of ASD symptoms in early childhood avoids some of the issues encountered with ASD diagnosis. Despite the ASD phenotype being present from an early age, the most frequent age of diagnosis in Australia is 5.9 years (Bent et al., 2015) and health care presentation and diagnosis can be influenced by demographic factors, thereby introducing selection bias (Bent et al., 2015; Nowell et al., 2015).

2.5. Statistical methods

Pathway scores were constructed using the Small Molecule Pathway

Database (SMPDB), an online database containing more than 30,000 small molecule pathways in humans (Jewison et al., 2014). For each central carbon metabolism pathway that contained at least three of the 17 metabolites, a principal component analysis was run on the concentrations of the metabolites in that pathway and the first principal component (PC1) was used as a composite measure. Separate multivariable linear regression models were used to estimate the associations for BIS children between (i) the phthalate measures and each of the individual and composite metabolite measures at the three timepoints (28 weeks of gestation, birth, 1 year), and (ii) the metabolite measures and each of the ASD-symptom outcomes.

Two models were run for each analysis using all available data: one with a minimal set of adjustment factors limited to child's assigned sex at birth and process factors including gestational age at urine collection, gestational age at serum collection (prenatal timepoint only), maternal contamination of cord serum (birth timepoint only), child's age at plasma collection (1-year timepoint only), and child's age at behavior ratings; and another with a more extensive set of adjustment factors. For the latter, both prior knowledge and data-adaptive methods were used to (i) identify disease determinants that are independent of exposure and (ii) help separate confounders from factors that are antecedents or mediators of the putative exposure-disease associations (Ponsonby, 2021). This data-adaptive approach is useful in low-knowledge settings where initial directed acyclic graphs may be incomplete (Ponsonby, 2021). Focus was given to factors previously identified as being associated with ASD (Pham et al., 2022). Additional potential confounders were individually added to the model and the change in the estimate of the exposure-outcome association was assessed (**Table 2S**).

Metabolite pathway scores (potential mediator, *M*) that were (i) associated with any of the phthalate measures (exposure, *E*) in the regression of *M* on *E*, and (ii) with any of the ASD-symptom outcomes (outcome, *O*) in the regression of *O* on *M*, were each tested as causal mediators of the *E*-*O* relationship in counterfactual mediation analyses. The total effect of prenatal phthalate levels on ASD symptoms was decomposed into two components: the natural direct effect (the portion of the effect that was not mediated by metabolite level) and the natural indirect effect (the portion of the effect that was mediated by metabolite level; Fig. 1S). The counterfactual framework, which enables causal interpretation, is based on four assumptions: (i)-(iii) no unmeasured confounding of the *M*−*O*, *E*-*M* and *E*-*O* relationships, and (iv) no *M*−*O* confounder that is affected by *E*.

Sex-specific and sex-interaction analyses were conducted. To check that the time of day of maternal serum collection did not influence the main results, sinusoidal and cosinusoidal functions of sample collection time were included as adjustment factors in the regression models. To assess if associations between phthalate levels and metabolomic profile differed based on the time interval between maternal blood and urine collection, an interaction term was added to the model. Statistical analyses were performed using Stata version 15.1 (StataCorp, USA) and R version 4.1.0 (R Foundation for Statistical Computing). Mediation analyses were implemented using the *mediation* R package (Tingley et al., 2014).

3. Results

All or almost all (98 %-100 %) of the 842 women with phthalate measurements in the inception cohort had detectable levels of DEHP, DEP, DiBP and DnBP metabolites which varied markedly, that is, more than 1000-fold (**Table 3S**). In the study mediation sample (n = 720 children; participant flowchart in Fig. 2; cohort and study sample characteristics in Table 1), the geometric mean for DEHP daily intake was 1.6 μg/kg bodyweight/day (geometric SD 2.1) which is well below the current tolerable daily intake of 50 μg/kg bodyweight/day in Europe (European Food Safety Authority, 2005). For the CBCL-ASP at 2 years and the SDQ-peer at 4 years, 11 % and 9 % of children had a score of 4 or more, respectively. Summary statistics for the metabolites are provided in Table 1 (prenatal) and **Table 4S** (birth and 1-year). Further details regarding the measurement of potential confounders are provided in

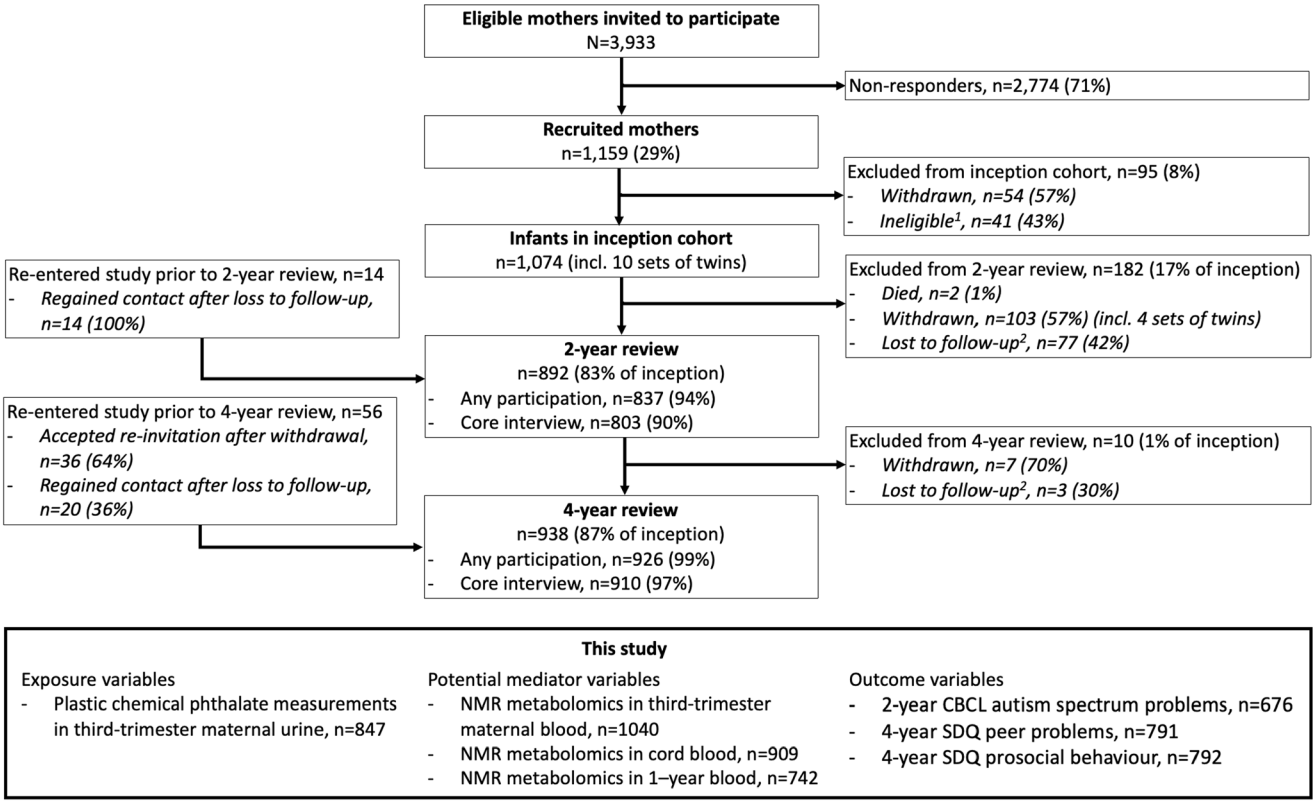

Fig. 2. Barwon Infant Study participant flowchart. Participants included in each analysis were those with complete data on the variables of interest.

Table 1
Participant characteristics.

	Cohort sample (N = 1074)		Mediation sample (N = 720)	
	N	Mean (SD) or % [n] or Median [IQR] or GM {GSD}	N	Mean (SD) or % [n] or Median [IQR] or GM {GSD}
Parent and household factors				
Mother's age at conception (years)[1]	1074	31.3 (4.8)	720	32.1 (4.3)
Mother is university-educated	1068	51.3 % [548]	717	58.0 % [416]
Neither parent born in Australia	1067	2.8 % [30]	717	2.8 % [20]
SEIFA IRSD in lowest tertile	1074	33.2 % [357]	720	29.3 % [211]
Prenatal factors				
Mother's weight at 28-week interview (kg)	1074	81.5 (15.5)	720	80.4 (14.8)
Mother smoked at any point during pregnancy	1061	15.7 % [167]	720	11.0 % [79]
Mother consumed any alcohol in pregnancy	989	52.7 % [521]	701	51.5 % [361]
Mother supplemented fish oil throughout pregnancy	608	16.1 % [98]	467	16.9 % [79]
Mother had gestational diabetes mellitus	908	4.8 % [44]	608	3.9 % [24]
Mother had pre-eclampsia	1039	3.4 % [35]	700	2.4 % [17]
Mother had hypertension	1031	2.6 % [27]	700	2.4 % [17]
Mother's Perceived Stress Scale score at 28-week interview	810	18.7 (7.0)	559	17.6 (6.6)
Mother's UVR in Trimester 1 (standard erythemal doses)	1074	2.0 (1.3)	720	2.1 (1.3)
Gestational age at blood collection (weeks)	1047	28.3 (1.3)	720	28.2 (1.2)
Gestational age at urine collection (weeks)	847	36.3 (0.7)	720	36.2 (0.7)
Time interval between blood and urine collection (weeks)	831	8.0 (1.3)	720	8.0 (1.2)
Maternal plastic chemical exposure (μ g/kg body weight/day)				
DEHP daily intake	847	1.6 {2.1}	720	1.6 {2.1}
DEP daily intake	847	1.6 {3.8}	720	1.6 {3.8}
DBPs (DiBP + DnBP) daily intake	847	1.9 {2.0}	720	1.9 {2.0}
Sum of DEP, DBPs & DEHP daily intakes	847	6.3 {2.2}	720	6.3 {2.2}
Birth factors				
Child's assigned sex at birth is female[2]	1074	48.3 % [519]	720	48.1 % [346]
Gestational age at birth (weeks)	1074	39.4 (1.5)	720	39.6 (1.2)
Child birthweight (kg)	1072	3.5 (0.5)	720	3.6 (0.5)
Caesarean birth	1074	31.2 % [335]	720	30.4 % [219]
Apgar score at 1 min	1059	9 [8–9]	709	9 [8–9]
Mother is multiparous	1074	55.3 % [594]	720	56.1 % [404]
Maternal contamination of cord serum	936	4.6 % [43]	638	4.9 % [31]
Air pollutant exposure: $PM_{2.5}$ ($\mu g/m^3$)	1074	7.7 (0.9)	720	7.7 (1.0)
Postnatal factors				
Mother's Perceived Stress Scale score at 6 months	894	18.0 (7.6)	674	17.7 (7.6)
UVR at 9 months of age (standard erythemal doses)	1074	2.1 (1.3)	720	2.0 (1.3)
Child's age at blood collection (years)	743	1.1 (0.1)	591	1.1 (0.1)
Child's weight at 1 year of age (kg)	874	10.1 (1.2)	684	10.1 (1.2)
Childcare prior to 2 years of age	1004	54.9 % [551]	720	60.6 % [436]
Child ASD diagnosis and symptoms				
CBCL/1.5–5 autism spectrum problems subscale (raw score)	676	1 [0–2]	596	1 [0–2]
Child's age at CBCL/1.5–5 assessment (years)	676	2.5 (0.1)	596	2.5 (0.1)
SDQ P2-4 peer relationship problems subscale	791	1 [0–2]	674	1 [0–2]
Child's age at SDQ P2-4 assessment (years)	791	4.2 (0.3)	674	4.1 (0.2)
Verified doctor-diagnosed ASD by year 4 follow-up	791	1.1 % [9]	671	1.0 % [7]

NB. SD, standard deviation; IQR, interquartile range; GM, geometric mean; GSD, geometric standard deviation; SEIFA, Socio-Economic Indexes for Areas; IRSD, Index of Relative Socio-economic Disadvantage; UVR, ultraviolet radiation; DEHP, Di-(2-ethylhexyl) phthalate; DiBP, Diisobutyl phthalate; DnBP, Di-*n*-butyl phthalate; DEP, Diethyl phthalate; CBCL, Child Behavior Checklist; SDQ, Strengths and Difficulties Questionnaire; ASD, autism spectrum disorder.
[1] For births registered in 2010 to 2013 in Australia, the median age of mothers ranged from 30.6 to 30.8 years (Australian Bureau of Statistics, 2021).
[2] There were no known intersex children in the cohort.

Table 5S.

3.1. Prenatal phthalates and prenatal maternal metabolomics

3.1.1. Higher DEHP exposure associated with higher pyruvate, lactate and alanine levels

Higher DEHP exposure was associated with higher levels of pyruvate and lactate and, to a lesser extent, alanine (Fig. 3/**2S**). A doubling in the daily intake of DEHP during pregnancy was associated with an estimated mean increase of 2.4 μmol/L (95 % CI 0.0, 4.7), 38.7 μmol/L (95 % CI 6.1, 71.3) and 2.8 μmol/L (95 % CI −0.2, 5.8) in pyruvate, lactate and alanine, respectively (**Table 6S**). Similar trends were evident for lactate and alanine when considering other phthalate measures (**Fig. 3S**).

3.1.2. Higher DEHP exposure associated with higher non-oxidative energy pathway scores

In non-oxidative pyruvate metabolism, pyruvate is converted to

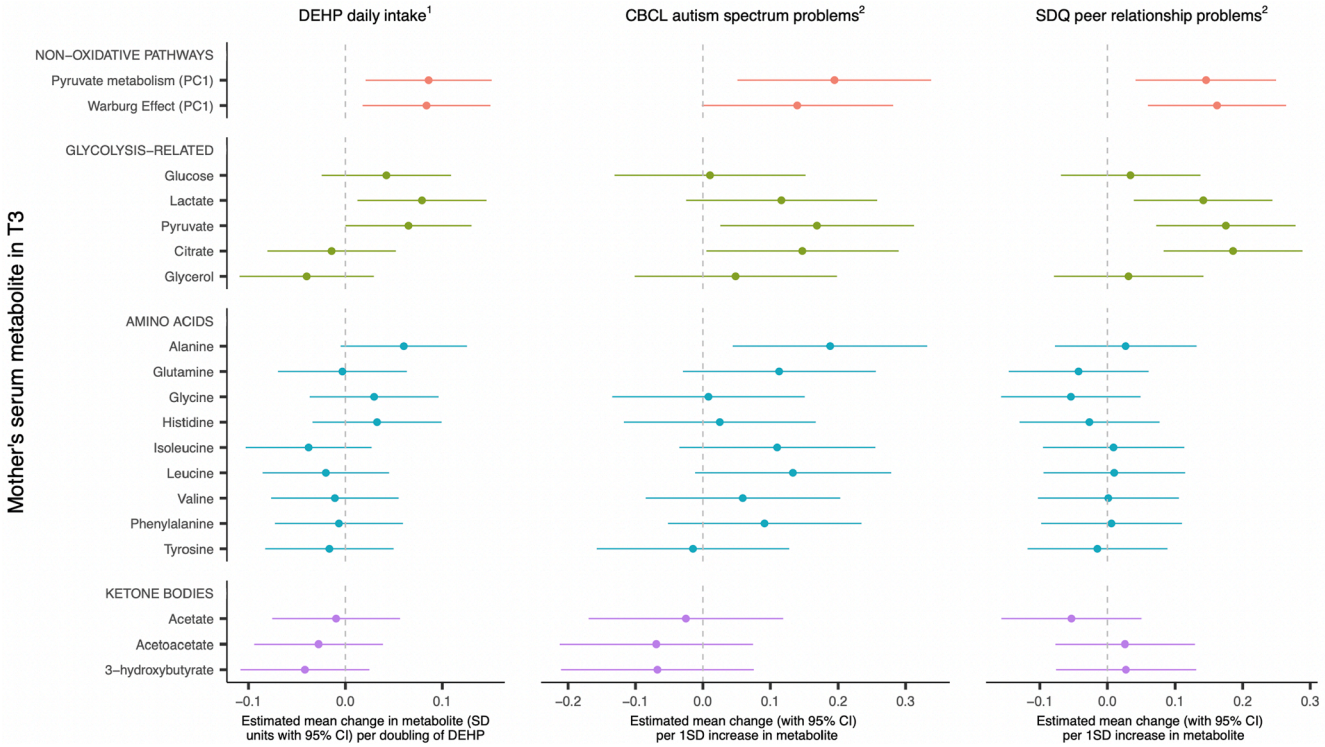

Fig. 3. Higher prenatal DEHP exposure is associated with upregulation of prenatal maternal non-oxidative energy metabolism pathways. Upregulation of prenatal maternal non-oxidative energy metabolism pathways is associated with more offspring ASD symptoms. *DEHP, di-(2-ethylhexyl) phthalate; CBCL, Child Behavior Checklist; SDQ, Strengths and Difficulties Questionnaire; T3, Trimester 3; [1] Model adjusted for child's sex, gestational age at blood collection, gestational age at urine collection, mother's age at conception, any maternal smoking in pregnancy, maternal diet in pregnancy; [2] Model adjusted for child's sex, gestational age at blood collection, child's age at assessment of ASD symptoms, socioeconomic disadvantage, maternal multiparity.*

lactate, acetate and alanine. In the Warburg Effect, glucose is converted to pyruvate which is then converted to lactate. Our metabolite pathway scores captured a substantial proportion of the overall variability, that is, 45 % for the Non-Oxidative Pyruvate Metabolism Score (NOPMS) and 57 % for the Warburg Effect Metabolism Score (WEMS). A doubling in the daily intake of DEHP during pregnancy was associated with an estimated mean increase of 0.09 SD units (95 % CI 0.02, 0.15) and 0.08 SD units (95 % CI 0.02, 0.15) in the NOPMS and WEMS, respectively (Fig. 3/**2S**). Similar but weaker patterns were observed for the sum phthalate measure (Fig. 3S). However, for DEP and the DBPs, the data had low compatibility with any association being present which suggests the findings for the sum phthalate measure were primarily driven by DEHP (Fig. 3S).

3.2. Prenatal maternal metabolomics and offspring ASD symptoms

3.2.1. Higher pyruvate, lactate, citrate and alanine levels associated with higher ASD symptom scores

For a 1 SD increase in pyruvate, citrate and alanine, the estimated mean change in CBCL-ASP at 2 years was 0.17 points (95 % CI 0.03, 0.31), 0.15 points (95 % CI 0.01, 0.29), and 0.19 points (95 % CI 0.04, 0.33), respectively (Fig. 3/**2S**). For a 1 SD increase in pyruvate, citrate and lactate, the estimated mean change in SDQ-peer at 4 years was 0.18 points (95 % CI 0.07, 0.28), 0.19 points (95 % CI 0.08, 0.29), and 0.14 points (95 % CI 0.04, 0.24), respectively (Fig. 3/**2S**). See **Table 6S** for unscaled regression estimates.

3.2.2. Higher non-oxidative energy pathway scores associated with higher ASD symptom scores

The estimated mean change in CBCL-ASP score at 2 years for a 1 SD increase in NOPMS and WEMS was 0.19 points (95 % CI 0.05, 0.34) and 0.14 points (95 % CI 0.00, 0.27), respectively (Fig. 3/**2S**). Per 1 SD increase in NOPMS and WEMS, the estimated mean change in SDQ-peer score at 4 years was 0.15 points (95 % CI 0.04, 0.25) and 0.16 points (95 % CI 0.06, 0.26), respectively (Fig. 3/**2S**).

3.3. Metabolomics in cord blood and child's blood at 1 year

Unlike the prenatal period, there were no metabolite measures at birth or one year that were associated with both prenatal phthalate exposure and ASD symptoms (**Fig. 4S-7S**).

3.4. Mediation analyses

3.4.1. Increased maternal non-oxidative energy metabolism in pregnancy partially mediated relationship between prenatal DEHP exposure and ASD symptoms

The indirect effect estimates indicated that elevations in maternal NOPMS and WEMS each partially mediated the association between higher prenatal DEHP exposure and increased ASD symptoms in early childhood (Fig. 4**a, 4b, 4d**), although the evidence was less convincing for WEMS and CBCL-ASP (Fig. 4**c**). That is, provided the assumptions of the analysis were upheld, higher levels of prenatal DEHP exposure upregulated maternal non-oxidative energy metabolism during pregnancy, which in turn increased the likelihood of offspring ASD symptoms. The estimated proportion of the effect of prenatal DEHP on ASD symptoms mediated by prenatal maternal non-oxidative energy metabolism ranged from 7 to 15 % (Fig. 4**a, 4b, 4d**).

3.5. Additional analyses

Urine was collected later than blood for most mothers in the sample with the blood-to-urine collection time interval ranging from −1 to 13 weeks. A term for the multiplicative interaction between DEHP levels

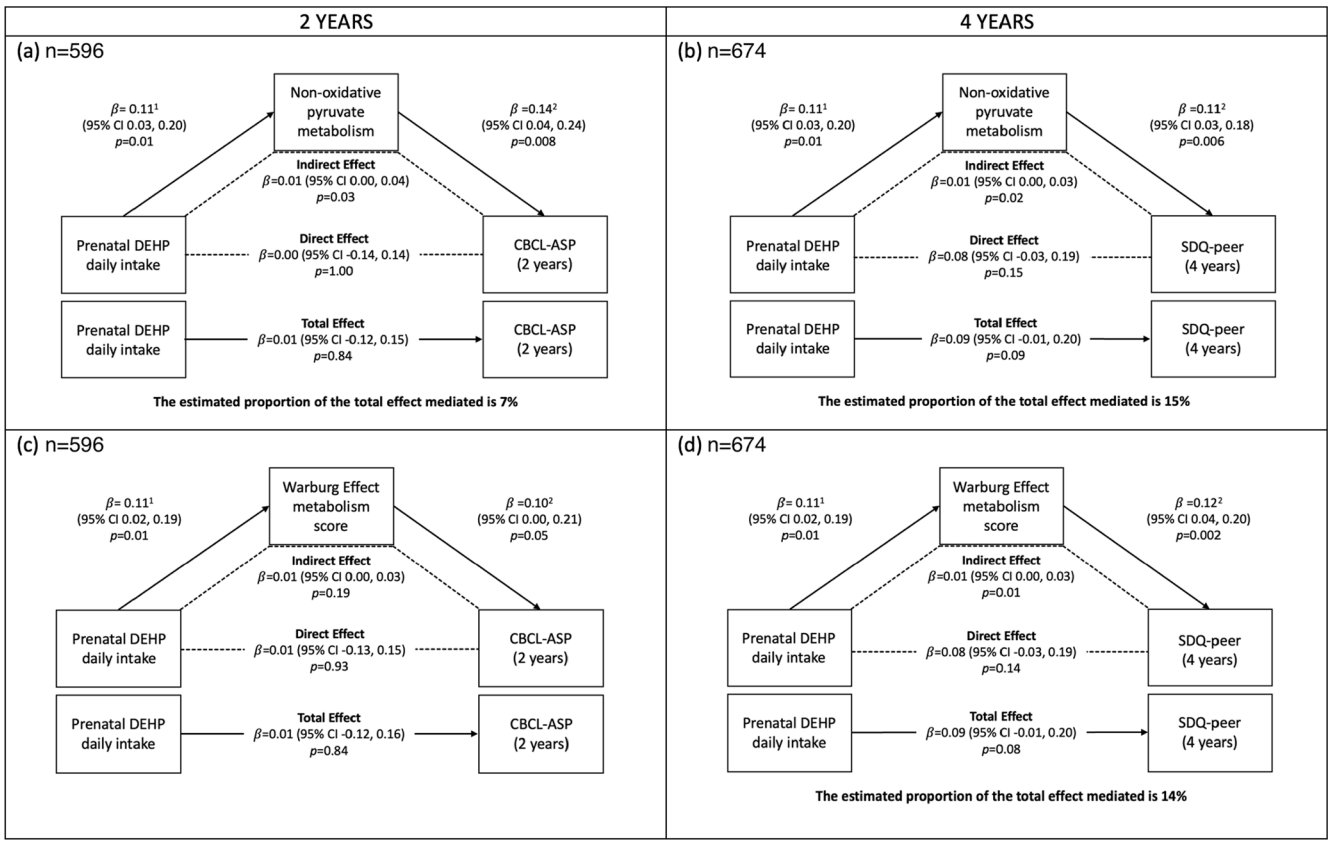

Fig. 4. Prenatal maternal non-oxidative pyruvate metabolism (a and b) and Warburg Effect metabolism (d) partially mediate the relationship between prenatal maternal DEHP exposure and offspring ASD symptoms in early childhood. *Models with metabolism pathway score as the dependent variable are adjusted for child's sex, gestational age at blood collection, gestational age at urine collection, mother's age at conception, any maternal smoking in pregnancy, and maternal diet during pregnancy; models with an ASD symptoms measure as the dependent variable are adjusted for child's sex, gestational age at blood collection, child's age at ASD symptom questionnaire, socioeconomic disadvantage, and maternal multiparity; An estimate of the proportion of the total effect mediated is only provided for models where $p < 0.05$ for the indirect effect; DEHP, di-(2-ethylhexyl) phthalate; CBCL-ASP, Child Behavior Checklist autism spectrum problems subscale; SDQ-peer, Strengths and Difficulties Questionnaire peer relationship problems subscale; [1] Estimated increase in metabolite (mmol/L) per doubling of prenatal DEHP daily intake; [2] Estimated increase in ASD symptom score per mmol/L increase in metabolite measure.*

and this time interval was added to the model, and it did not suggest that the association between DEHP and non-oxidative energy metabolism pathways differed by time interval. Including sinusoidal and cosinusoidal functions of time of day of maternal serum collection as adjustment factors in the regression models did not materially change the findings. There was some evidence of an interaction between sex and the sum phthalate daily intake measure, suggesting the association between phthalate exposure and increased non-oxidative energy metabolism is stronger in mothers carrying male compared to female fetuses (**Fig. 8S**). Larger sample sizes are needed in future work to further interrogate sex differences.

4. Discussion

Using counterfactual mediation analyses in a causal framework, this study is the first to report that a metabolic shift in maternal energy metabolism in pregnancy partially underlies the association between higher prenatal DEHP exposure and increased offspring ASD symptoms in early childhood. This shift is towards extra-mitochondrial non-oxidative pathways – non-oxidative pyruvate metabolism and the Warburg Effect – that are far less efficient at generating energy than intramitochondrial oxidative phosphorylation.

In this study, higher prenatal DEHP exposure was associated with elevated lactate, pyruvate, NOPMS and WEMS in maternal serum during pregnancy. Similar positive associations between phthalate levels and non-oxidative metabolites (lactate and pyruvate) have been reported in prenatal maternal urine samples (Maitre et al., 2018; Zhou et al., 2018). DEHP exposure also causes an accumulation of lactate, suggestive of increased non-oxidative metabolism, in various cell types *in vitro* (cardiomyocytes (Posnack et al., 2012), adipocytes (Chiang et al., 2016; Ellero-Simatos et al., 2011) and Sertoli cells (Moss et al., 1988)) and muscle tissue *in vivo* (Martinelli et al., 2006), and is linked to disrupted regulation of enzymes along central carbon metabolism pathways (**Fig. 9S**).

DEHP metabolites and other endocrine disrupting chemicals have been shown to bind to and alter the activity of regulators of non-oxidative metabolism, such as, the mitochondrial pyruvate carrier complex and peroxisome proliferator-activated receptors, and therefore could directly cause upregulation of non-oxidative pathways (**Fig. 9S**) (Kratochvil et al., 2019; Chen et al., 2018). DEHP has also been shown to disrupt mitochondrial function (Chen et al., 2020; Li et al., 2014), which increases the need for energy metabolism through non-oxidative pathways. Consistent with the findings here, disrupted mitochondrial function in mothers during pregnancy has previously been linked to increased offspring ASD risk (Frye et al., 2021). Further adding to the biological plausibility of our findings, a DEHP-induced shift to non-oxidative metabolism would lead to excess reactive oxygen species (ROS) (Yang et al., 2014; St-Pierre et al., 2002; Kakimoto et al., 2015; Schönfeld and Wojtczak, 2008; Liu et al., 2011), increasing the risk of oxidative stress. Recent studies have found higher oxidative stress indices in pregnant mothers is associated with offspring ASD and ASD symptoms (Hollowood et al., 2018; Rommel et al., 2020).

This is the first report that elevated non-oxidative energy metabolism during pregnancy is associated with increased offspring ASD symptoms at both 2 and 4 years. A shift toward non-oxidative metabolism provides a possible unifying mechanism for a variety of prenatal environmental exposures reported to increase the risk of offspring ASD. For instance, studies of pregnant women with conditions known to increase the risk of offspring ASD diagnosis (e.g. gestational diabetes (Xiang, 2017), obesity (Connolly et al., 2016), psychological stress (Hermann et al., 2019) and preeclampsia (Walker et al., 2015)) incidentally report an elevation of non-oxidative energy metabolites during pregnancy (Nagalakshmi et al., 2016). Furthermore, women with deviations in central carbon metabolism during pregnancy after exposure to high levels of air pollution are more likely to have offspring with ASD (Kim et al., 2021). Similarly, mothers exposed to chemicals known to increase non-oxidative metabolites (e.g. valproate (Huo et al., 2014) and dexamethasone (Ottens et al., 2015)) are also more likely to have a child diagnosed with ASD (Christensen et al., 2013). Rodent studies have demonstrated that manipulations of prenatal maternal energy metabolism can result in offspring structural brain abnormalities that are a hallmark of ASD (Namba et al., 2021; Rash et al., 2018; Fernandes et al., 2021). While there has been a focus in the literature on the child's metabolism in relation to ASD (Cheng et al., 2017; Dhillon et al., 2011), this study suggests that prenatal maternal metabolism plays a role in the etiology of the disorder.

Strengths of the study include comprehensive prenatal environmental measures, unique serial carbon metabolomic indices and previously validated ASD symptom scales (Chericoni et al., 2021; Salayev and Sanne, 2017) in a large study sample of over 500 children. Pathway scores allowed us to evaluate the composite impact of related metabolites in a biologically defined pathway. The highly dimensioned cohort enabled a thorough examination of potential confounders. This is important because, as previously mentioned, causal interpretations of mediation results within the counterfactual framework rely on strong assumptions of no unmeasured confounding of the exposure-outcome, exposure-mediator and mediator-outcome relationships. For example, we were able to control for maternal diet which reduced the likelihood of the association between phthalate exposure and non-oxidative metabolite levels in pregnancy being the result of positive confounding by dietary patterns. While past studies have demonstrated an association between prenatal phthalate exposure and ASD or ASD symptoms (Larsson et al., 2009; Miodovnik et al., 2011; Oulhote et al., 2020; Haggerty et al., 2021; Day et al., 2021; Alampi et al., 2021; Patti et al., 2021; Ponsonby et al., 2020), they were unable to rule out that higher prenatal phthalate levels were a proxy for higher postnatal phthalate levels and that postnatal, as opposed to prenatal, exposure is contributing to greater ASD symptoms in childhood. Here, a finding that a prenatal metabolic mediator partially underlies the DEHP-ASD symptom association strengthens the argument that the prenatal period, specifically Trimester 3, is a sensitive window for the adverse effect of phthalate exposure. Our findings are in a sample where mean DEHP exposure (1.6 μg/kg bw/day) was a fraction of the current tolerable daily intake in the European Union (50 μg/kg bw/day) (European Food Safety Authority, 2005), suggesting estimates of safe levels require urgent revision. Specific guidelines and safety limits for pregnant populations don't currently exist and should also be considered.

As discussed above, DEHP might have upregulated non-oxidative energy metabolism by various mechanisms and further functional work should be conducted, such as directly assessing maternal mitochondrial function in pregnancy. For example, a mitochondrial respiration assay on prenatal maternal live blood cells could be conducted using a Seahorse analyzer for a comprehensive bioenergetic assessment (Frye et al., 2021; Little et al., 2020). Serial measures of non-oxidative energy metabolism and oxidative stress may clarify the interplay between these two mechanisms. Only a single spot urine sample was collected from the mother during pregnancy. However, past work has found repeated phthalate measures in the third trimester to be moderately reproducible (Adibi et al., 2008; Suzuki et al., 2009), and we have further accounted for some of the features (time of day, maternal weight) associated with variability (Ponsonby et al., 2020; Gao et al., 2017). Maternal blood lactate and pyruvate levels are also relatively consistent across Trimester 3 (Bauer et al., 2019; Wang et al., 2016). Further, random error in measurement introduced by single urine and blood samples would tend to bias estimates towards the null. Therefore, estimates of the proportion mediated, which ranged from 7 to 15 %, are possibly underestimates of the true parameters in the population. The use of single samples as opposed to serial samples over time also means that we were unable to identify time points or windows in which the unborn child was most susceptible and so, again, the effects here might be underestimated. For most mothers, phthalate urinary metabolites were measured later in Trimester 3 than the maternal serum metabolites. Our sensitivity analysis did not reveal evidence of the association between phthalate levels and pathway scores differing by time interval between serum and urine measurement. Further, reverse causation seems unlikely. The relationship between phthalate metabolism and non-oxidative energy metabolism is complex and understudied. A plausible consequence of increased non-oxidative energy metabolism, if any, would be inefficient detoxification that lowers levels of phthalate metabolites in the urine. This is opposite to what was observed in this study. Another weakness is that serum and plasma were non-fasting samples. However, the sensitivity analysis which assessed the impact of time of day of maternal blood collection showed it had little effect on the associations found for the non-oxidative pathway scores. Finally, the sample was not large enough to adequately interrogate differences by sex.

5. Conclusion

Our results highlight the important role of the prenatal environment in ASD causation and suggest that one of the mechanisms by which prenatal exposure to DEHP influences offspring ASD symptom development is through factors related to a metabolic shift in maternal energy metabolism in pregnancy. A shift towards non-oxidative metabolism, which is inefficient compared to oxidative metabolism, may be a common biological response to a variety of prenatal environmental exposures that also increase the risk of offspring ASD and, if so, this could be a future target of prenatal intervention. Further research to build on these findings is now required.

CRediT authorship contribution statement

Sarah Thomson: Conceptualization, Methodology, Software, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Katherine Drummond:** Writing – original draft, Writing – review & editing, Visualization. **Martin O'Hely:** Conceptualization, Methodology, Writing – review & editing. **Christos Symeonides:** Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition. **Chitra Chandran:** Conceptualization, Methodology. **Toby Mansell:** Conceptualization, Writing – review & editing. **Richard Saffery:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Peter Sly:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Jochen Mueller:** Methodology, Investigation, Resources, Writing – review & editing. **Peter Vuillermin:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Anne-Louise Ponsonby:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, 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

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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Informed consent statement

Informed consent was obtained from all participating families.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107678.

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