Inflammation-related epigenetic risk and child and adolescent mental health: A prospective study from pregnancy to mid-adolescence

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Abstract

In 785 mother-child (50% male) pairs from a longitudinal epidemiological birth cohort, we investigated associations between inflammation-related epigenetic polygenic risk scores (i-ePGS), environmental exposures, cognitive function and child and adolescent internalising and externalising problems. We examined prenatal and postnatal effects. For externalising problems, one prenatal effect was found: i-ePGS at birth associated with higher externalising problems (ages 7-15) indirectly through lower cognitive function (age 7). For internalising problems, we identified two effects. For a prenatal effect, i-ePGS at birth associated with higher internalising symptoms via continuity in i-ePGS at age 7. For a postnatal effect, higher postnatal adversity exposure (birth through age 7) associated with higher internalising problems (ages 7-15) via higher i-ePGS (age 7). Hence, externalising problems were related mainly to prenatal effects involving lower cognitive function, whereas internalising problems appeared related to both prenatal and postnatal effects. The present study supports a link between i-ePGS and child and adolescent mental health.

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Conflict of Interest
The authors declare that they have no conflict of interest.

Keywords

ALSPAC, inflammation, attention deficit hyperactivity; conduct disorder, oppositional defiant disorder; depression; anxiety; DNA methylation; polyepigenetic score; prenatal adversity; postnatal adversity

Introduction

Children who experience adversities (e.g. poverty, caregiver psychopathology, low social support, harsh parenting) are at risk of developing persisting stress-related psychiatric disorders that can span externalising and internalising problems (Barker, Walton, & Cecil, 2017). Research has examined multiple possible mediating mechanisms for these associations; stress physiology has received the bulk of this attention (O'connor, Moynihan, & Caserta, 2014). In the current study we consider an alternative mechanism, namely, inflammation (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). The "hidden wounds hypothesis" (Danese & Baldwin, 2017; Miller & Chen, 2013) proposes that early psychosocial adversity **translates** into biological risk for mental illness by affecting the regulation of the immune system at sensitive stages of brain development (Danese & Lewis, 2016; Nusslock & Miller, 2016). Recent evidence suggests that DNA methylation, a type of epigenetic modification that regulates gene expression, may be a biological mechanism by which adversity can result in low-grade inflammation (McDade et al., 2017). However, little is known about how adversity in different developmental periods may affect immune-related DNA methylation which could affect neurodevelopmental and mental health problems. This study examines the concerted influences of pre- and postnatal adversity, an immune-related DNA methylation biomarker, and cognitive function (a proxy for brain function) on the development of mental health problems from childhood to adolescence.

Prenatal adversity, inflammation and cognitive function

During the prenatal period, larger immune molecules (e.g. cytokines, antibodies) may have access to the developing fetal brain via the blood brain barrier (BBB; Goasdoué, Miller, Colditz, & Bjorkman, 2016). There is good evidence in animal studies, and increasing evidence in humans, that inflammation in the mother during pregnancy can affect several highly orchestrated, and hence vulnerable, aspects of fetal brain development (Knuesel et al., 2014). A possible mechanism for brain and behavioural abnormalities following maternal immune activation includes the downstream effects of cytokines and other mediators of inflammation (e.g. glucocorticoids) on neural plasticity. Here, cytokines and antibodies interact with various neural and non-neural cell types (e.g. microglia) and play an important role in brain developmental processes, such as synapse modelling, neurogenesis and neurotransmission that underlie cognitive and behavioral development (Danese & Lewis, 2016).

In humans, maternal immune activation is associated with increased serum levels of pro-inflammatory cytokines (Andersson et al., 2016; Coussons-Read, Okun, & Nettles, 2007). Higher levels of inflammatory makers, such as C-reactive protein (CRP) at birth (cord blood) – a sensitive marker of low-grade chronic inflammation – can signpost a range of

obstetric risks including amniotic fluid infection (Yoon et al., 2000), neonatal sepsis (Joram et al., 2006) and predict neurologic impairment in preterm infants (Kaukola et al., 2006). **Furthermore, a study reported that placental indicators of inflammation in preterm newborns associated with variation in messenger RNA expression of genes that, in turn, predicted cognitive impairment at 10 years of age (Tilley et al., 2017).** As animal models show that maternal immune activation can associate with prenatal adversity (Babri, Doosti, & Salari, 2014), these data may be consistent with a wide-range of studies that have highlighted the relationship of prenatal adversity with subsequent impaired child cognitive function (Barker, Kirkham, Ng, & Jensen, 2013; Jensen, Dumontheil, & Barker, 2014). Of note, variation in cognitive function has been linked to both internalising and externalising problems (Rock, Roiser, Riedel, & Blackwell, 2014; Séguin, Nagin, Assaad, & Tremblay, 2004).

Inflammation and cognitive function

After birth, peripheral inflammation can induce neuroinflammation (Dantzer et al., 2008) and modulate the continued development and function of the brain (Danese & Lewis, 2016). There are several pathways that link the peripheral and the brain immune systems (Dantzer et al., 2008). Peripheral cytokines can stimulate afferent fibers of the vagus nerve, which in turn can stimulate cytokine production within the brain. Cytokines can also cross into the brain where the BBB is permeable or leaky, or engage surface receptors on astrocytes and endothelial cells that form the BBB. Animal research suggests that the immune system modulates synaptogenesis, synaptic refinement and survival, as well as myelination via its role as a modulator of oligodendrocyte function (Danese & Lewis, 2016; Nusslock & Miller, 2016). Moreover, animal research suggests that early life immune activation can bias microglia (CNS resident macrophage cells) towards an aggressive inflammatory response and reduce glucocorticoid sensitivity (Frank, Hershman, Weber, Watkins, & Maier, 2014).

In humans, adversity-related infection and low-grade chronic inflammation are associated with impaired child cognitive function and language (Jiang et al., 2017; Patrick et al., 2005), as well as an increased risk for developing depression in adolescence and adulthood (Danese et al., 2011; Danese et al., 2009; Ehrlich, Ross, Chen, & Miller, 2016; Miller, Cohen, & Ritchey, 2002) and psychosis in adulthood (Khandaker, Pearson, Zammit, Lewis, & Jones, 2014). A recent adult brain imaging study reported that an association between higher CRP levels and higher depressive symptoms was mediated by decreased functional connectivity between the ventromedial prefrontal cortex and the ventral striatum and the presupplementary motor cortex (Felger et al., 2016). Hence, animal and human research support the idea that adversity-related inflammation can shape neurodevelopment, and may also underlie behavioral and emotional disorders

The potential role of DNA methylation in adversity, inflammation and mental health

But how might exposure to adversity become biologically embedded in such a way as to systematically affect offspring inflammation and brain development? The Developmental Origins of Health and Disease (DOHaD) hypothesis posits that pre- and postnatal

environmental exposures can influence long-term vulnerability for physical and mental health problems (Wadhwa, Buss, Entringer, & Swanson, 2009). One of the mechanistic drivers of these vulnerabilities is thought to be epigenetic alterations, such as DNA methylation (Gluckman et al., 2005). With regard to prenatal effects, few published studies exist that explicitly link prenatal adversity with DNA methylation specific to immune functioning and child mental health; however, mounting evidence suggests that prenatal adversity is associated with inflammatory response of cord blood monocytes of the neonate (Sureshchandra et al., 2017). More specifically, DNA methylation in cord blood has been associated with perinatal risks for immune-related disorders (such as asthma), including caesarean section (Schlinzig, Johansson, Gunnar, Ekström, & Norman, 2009), elevated levels of maternal immune markers (DeVries, Stern, Wright, & Vercelli, 2017), birth complications (Zhang et al., 2016), maternal cigarette smoking (van Otterdijk, Binder, & Michels, 2017) and diet (Lee et al., 2013). These data are consistent with a much larger group of studies showing that prenatal maternal depression, anxiety, nutrition and toxin exposure (e.g. cigarette smoking) are associated with changes in DNA methylation in the cord blood of new-borns (Barker, Cecil, et al., 2017; Binder & Michels, 2013; Cecil et al., 2017, in press; Oberlander et al., 2008; Richmond et al., 2015), which, in turn, can increase vulnerability for neurological (Monk et al., 2016) and mental health problems (Rijlaarsdam et al., 2017).

Emerging literature also suggests that exposure to adversity after birth can continue to affect DNA methylation in the immune system. For example, individuals born with lower birth weight and shorter duration of breast feeding show higher concentrations of CRP in adulthood (McDade et al., 2014; Tzoulaki et al., 2008) – DNA methylation may be involved. Indeed, major psychosocial stressors, such as child neglect, maltreatment, housing quality and poverty associate with DNA methylation in immune system genes (Cecil et al., 2016; Essex et al., 2013). **More specifically, certain studies have examined cell cultures and shown that adversity-related DNA methylation in immune system genes can associate with the expression of both elevated and reduced plasma inflammatory biomarkers (e.g. McDade et al., 2017). Furthermore, in a study of adults (~ 20 years of age) who grew up in low income neighbourhoods, greater exposure to trauma and witnessing of community violence associated with lower levels of DNA methylation around the *IL-6* gene promoter, and in turn, a higher production of IL-6 in response to the Trier Social Stress test (Janusek, Tell, Gaylord-Harden, & Mathews, 2017)**

A recent study identified potential DNA methylation correlates of low-grade, chronic inflammation. Here, Ligthart et al. (2016) performed a epigenome-wide association of serum CRP in adults (~ 41 years of age). They reported 58 CpG sites (across 45 genes) that reliably replicated across a large European ($n = 8863$) discovery sample and African American ($n = 4111$) replication sample. The researchers further characterised the molecular and clinical relevance of the CpG sites through examining associations with gene expression and clinical outcomes. However, Ligthart et al. (2016) did not examine associations with prenatal and postnatal adversity or with mental health outcomes, which is a main aim of the present study.

The present study

Using prospective data drawn from a large population-based sample, prenatal and postnatal adversity, DNA methylation at birth, age 7 and ages 15-17, cognitive function at age 9 and internalising and externalising mental health problems spanning childhood to adolescence, the current study had three overall research aims. Firstly, to create and replicate an inflammation-related CRP epigenetic polygenic score (i-ePGS) biomarker of low-grade inflammation in a developmentally sensitive framework. Here we aimed to validate an i-ePGS biomarker by associating DNA methylation of relevant loci at age 15-17 with serum CRP at ages 15 and 17 – importantly, this is the age when the DNA methylation resource and serum CRP samples overlapped. We then aimed to create i-ePGS scores at birth and age 7 to examine the degree to which i-ePGS scores correlated over time, thereby raising the possibility of a biomarker that could index chronic, low-grade inflammation early in the life course. Second, to examine if the i-ePGS biomarker associated with cognitive function and internalising and externalising symptoms, which would represent multifinality of outcome (Cicchetti & Rogosch, 1996). More specifically, we examined whether i-ePGS would negatively affect cognitive function, which, in turn, would then increase vulnerability for both internalising and externalising problems. The third overall aim was to examine pre- and postnatal adversity-related effects. With regard to prenatal effects, we tested if adversity in utero might indirectly associate with internalising and externalising problems, by way of affecting i-ePGS (at birth) and cognitive function. With regard to postnatal effects, we similarly tested if postnatal adversity might indirectly associate with internalising and externalising problems, by way of affecting i-ePGS during childhood (age 7) and cognitive function.

Methods

Participants were drawn from the Accessible Resource for Integrated Epigenomics Studies (ARIES, www.ariesepigenomics.org.uk) (Relton et al., 2015), containing DNA methylation data for a subset of 1018 mother-offspring pairs and nested within the *Avon Longitudinal Study of Parents and Children* (ALSPAC). ALSPAC is an ongoing epidemiological study of children born from 14,541 pregnant women residing in Avon, UK, with an expected delivery date between April 1991 and December 1992 (85% of eligible population) (Fraser et al., 2013). Informed consent was obtained from all ALSPAC participants and ethical approval was obtained from the ALSPAC Law and Ethics Committee as well as Local Research Committees. The original ALSPAC sample is representative of the general population (Boyd et al., 2012). Please note that the study website contains details of all the data that is available through a fully searchable data dictionary: http://www.bris.ac.uk/alspac/researchers/dataaccess/data-dictionary/. For this study, we included youth from ARIES who had available data on symptomatology ratings (ages 7-15) as well as epigenetic data at birth (n =758, 50% male). The cohort profile of ARIES by (Relton et al., 2015) compared a selection of maternal characteristics in ARIES (n = 1018) to the rest of the ALSPAC sample. ARIES vs ALSPAC mothers were more ethnically homogenous (% white: ARIES = 100% vs ALSPAC 97.4%), slightly older (mean age: ARIES = 29.2 vs ALSPAC = 28.2), less likely to have a manual occupation (ARIES = 14% vs ALSPAC = 20.5%), and less likely to

have smoked throughout pregnancy (ARIES = 9.7% vs ALSPAC = 19.4%). Otherwise, the subsample was considered to be reasonably representative of the main ALSPAC population.

Measures

Child cognitive function was measured at age 8 by the Wechsler Intelligence Scale for Children (WISC-III UK ; Weschsler, Golombok, & Rust, 1992). The WISC was administered to the children at an ALSPAC research clinic. A short form of the measure was employed to reduce the likelihood that the children would become fatigued and that this would affect their performance on the WISC. The WISC comprises five verbal subtests (Information, Similarities, Arithmetic, Vocabulary, Comprehension) and five performance subtests (picture completion, coding, picture arrangement, block design, object assembly). We extracted the common variance between the verbal and performance tests in a latent factor of "cognitive function" in the overall model. We did so as we sought to tap the "true" score (i.e., the common variance) of "cognitive function," while accounting for potential measurement error in the verbal and performance tests, respectively (see Brown, 2014). Scores were age-normed in accordance with standard procedures. The distribution of verbal tests (M = 110.18, SD = 16.52, Skew = -0.002) and the performance tests (M = 101.81, SD = 16.99, Skew = – 0.054) were normal and within the range of age appropriate norms.

Internalising problems were assessed via mother ratings at ages 7, 10, 13 and 15 years, using the well-validated Development and Well-Being Assessment interview (DAWBA) (Goodman, Heiervang, Collishaw, & Goodman, 2011). The DAWBA was administered via a computer-based package of package of questionnaires, interviews, and rating techniques used to assess adolescent psychopathology based on DSM-IV criteria. Each question was introduced with the stem: *'over the last 6 months, and as compared with other children the same age, has s/he often....'* followed by the specific clause. The response categories were 0 = *'no more than others'*, 1 = *'a little more than others'* and 2 = *'a lot more than others'*. For depression, 12 symptoms were assessed including: *'very sad'*, *'lost interest'*, and *'lost or gained weight'*. For generalised anxiety, 7 symptoms were assessed including: *'worried about own behavior*, *'worried about health'*, and *'worried about future'*. For both anxiety and depression, scales, per age, were summed and then used to create an overall latent variable that indexed common variance in internalising problems across time.

Externalising problems (ADHD, CD, ODD) were also assessed via maternal ratings on the DAWBA at ages 7, 10, 13 and 15 years. ADHD comprised 18 symptoms including: *'often fidgets'*, *'blurted out answers before hearing questions properly'*, *'does not listen'*, and *'avoids things involving thought'*. For ODD 9 symptoms were assessed, including: *'has temper outbursts'*, *'has been touchy or easily annoyed'*, *'argued with grown-ups'* and *'taken no notice of rules/refused to do as s/he is told'*. For CD 8 symptoms were assessed, including: *'used a weapon'*, *'cruel to animals and birds'*, and *'stealing on the streets'*. For all types of externalising behaviours, scales, per age, were summed and then used to create an overall latent variable that indexed common variance in externalising problems.

Adversity exposure was assessed based on maternal reports. Risk items were organized into two developmental eras to reflect the timing of the i-ePGS scores (i) prenatal risks (18

weeks to 32 weeks) and (ii) early childhood risks (birth – age 7). For each developmental period, items were organized to create distinct but correlated risk domains: (i) *Life events* (e.g. death in family, accident, illness), (ii) *Contextual risks* (e.g. poor housing conditions, financial problems), (iii) *Parental risks* (e.g. parental psychopathology, criminal involvement and substance use), (iv) *Interpersonal risks* (e.g. intimate partner violence, family conflict), and (v) **direct victimization** (e.g. child bullied by peers or physically hurt; this measure of adversity exposure was specific to the postnatal risk composite).

These adversity scores were previously estimated and validated by Cecil et al. (2014), who used confirmatory factor analyses to assess the internal reliability of the individual risk domains and extract one adversity score for each developmental era. Model fit was good: (i) *Cumulative prenatal risk score:* X^2 (2) = .60, p = .73; (ii) *Cumulative early childhood risk score:* X^2 (5) = 14.94, p = .01; CFI = .95; TLI = .90; RMSEA = .07, 90% CIs = .03, .12. In addition, we also incorporated – at the composite level – other risks pertinent to inflammation, including low birth weight (less than 2500 grams) to the prenatal adversity, and breastfeeding (1 = 'no' for first 6 months after birth, 0 = 'yes' in the first 6 months) and body mass index (at age 7) to the postnatal adversity index. Saved factor scores were used in all analyses.

DNA methylation data. 500ng genomic DNA from blood (cord at birth; whole blood at age 7 and age 15-17yrs) was bisulfite-converted using the EZ-DNA methylation kit (Zymo Research, Orange, CA, USA). DNA methylation was quantified using the Illumina HumanMethylation450 BeadChip (HM450k; Illumina, USA) with arrays scanned using an Illumina iScan (software version 3.3.28). Samples or probes that failed quality control (>1% probes/samples with background detection p-value >= 0.05) were excluded from further analysis. Sex checks were performed using X/Y chromosome methylation. Genotype probes on the HM450k were compared between samples from the same individual and against SNP-chip data to identify and remove any sample mismatches. Samples were quantile normalised using the *dasen* function within the wateRmelon package (version 1.4.0) in R. Normalization performance was evaluated using all three testing metrics in wateRmelon (genki assessing SNP-related probes, dmrse assessing imprinted probes and seabi, assessing gender differences). Methylation levels were then indexed by beta values (corresponding to the ratio of the methylated signal divided by the sum of the methylated and unmethylated signal). Probes known to be cross-reactive or polymorphic (Chen et al., 2013; Price et al., 2013) and SNP (i.e. "rs") probes were removed. We also removed participants with non-Caucasian or missing ethnicity (based on self-reports). Cell type proportions (CD8 T lymphocytes, CD4 T lymphocytes, natural killer cells, B lymphocytes, monocytes and granulocytes) for each participant were estimated using the reference-based approach detailed in Houseman et al. (2012). These cell types were controlled for at each time point (i.e., birth, age 7 and age 15-17) in all analyses. In addition, for DNA methylation data at 15-17yrs, we also controlled for age.

Analyses

Step 1: i-ePGS and validation—CRP-related probes were identified based on a recent epigenome-wide association study by Ligthart et al. (2016). We limited our selection to

7 probes highlighted by Lightart et al. as these probes associated most strongly with the adversities and measures of cognitive function and mental health outcomes. Moreover, as can be seen in Table 1, 4 of these 7 these probes were found to associate with plasma CRP levels in both the discovery meta-analysis (9 cohorts, n =8,863) and a replication meta-analysis (4 cohorts, n = 4111). Seven out of seven of the probes associated with whole blood *cis* gene expression levels. Seven out of seven of the probes associated at least one of the cardio-metabolic phenotype of relevance to CRP (e.g. BMI, coronary heart disease, lipids, etc). We grouped the probes into a single i-ePGS. Specifically, we applied a method typically used for polygenic risk scores – that has recently been extended to epigenetic data (Shah et al., 2015) – where we multiplied the ALSPAC methylation values by their respective standardized regression betas (that is, weights) from the Ligthart et al. (2016) EWAS, and then summed these weighted methylation values together into the i-ePGS. The use of weights ensured that the probes maintained their relative magnitude of association with CRP. We then validated the i-ePGS in ALSPAC by testing whether it significantly associated with measures of CRP at ages 15 and 17, as these are the ages where the i-ePGS and CRP overlap. Once the i-ePGS at age 15-17 was characterized and validated, we then created the same i-ePGS risk measure at earlier time points, using DNA methylation data at birth and age 7. We then examined bivariate correlations amongst the inflammation-related epigenetic polygenic scores and between those scores and the measures of adversity, cognitive function, and psychopathology.

In this step we also examined, for each probe within the i-ePGS score, possible genetic influences on the level of methylation. We did so by searching the mQTLdb database (http://www.mqtldb.org). The mQTLdb database contains the results of a large-scale study based on ARIES data, characterizing genome-wide significant SNP effects on DNAm levels for all Illumina 450k probes (see Gaunt et al., 2016).

Step 2: Associations between i-ePGS, cognitive function and outcomes—
Here we examined a latent path analysis examining the inter-relations between i-ePGS, cognitive function and child externalising and internalising symptoms. In this step, we also examined the degree to which i-ePGS at birth might indirectly relate to internalising and/or externalising symptoms via an i-ePGS at age 7 and/or cognitive function at age 7.

Indirect pathways were programmed in model constraint statements in Mplus **(Muthén & Muthén, 1998-2013).** The indirect effects tested in this step assessed the extent to which i-ePGS might relate to higher internalising/externalising symptoms via cognitive function. Therefore, the indirect effects were defined by the product term of the pathways of interest (e.g., i-ePGS at birth *to* cognitive function BY cognitive function *to* internalising symptoms). Because standard errors underlying indirect effects (i.e. product terms) are known to be skewed, we bootstrapped all indirect effects 5,000 times with bias corrected 95% confidence intervals. The indirect pathways reported below are based on the bootstrapped variability around the product of non-standardized path coefficient estimates.

Step 3: Associations between adversity, i-ePGS cognitive function and outcomes—In this step, we tested the prenatal and postnatal effects by including the adversity scores (prenatal, postnatal). This enabled us to trace the inter-relationships

between environmental exposures, the i-ePGS scores (birth, age 7), latent cognitive function (age 7), and the internalising and externalising (age 7-15) scores. For the prenatal effects, we expected i-ePGS at birth to associate with internalising and/or externalising scores indirectly via cognitive function at age 7. **For postnatal effects, postnatal adversity would associate with internalising and/or externalising problems indirectly via i-ePGS and cognitive function at age 7.** Indirect effects were estimated in identical fashion as step 2.

Path analyses were conducted using Mplus version 7.11 (Muthén & Muthén, 1998-2013). Model fit was determined through the Comparative Fit Index and Tucker-Lewis Index (CFI & TLI; acceptable fit => 0.90) (Bentler & Bonett, 1980) and root mean square error of approximation (RMSEA; acceptable fit =< 0.08) (Browne & Cudeck, 1993). Maximum likelihood estimation with robust standard errors was used to estimate the model parameters, and missing data were handled through full information maximum likelihood. All analyses were conducted using SAS v9.4 (ref) and Mplus v7.11 (Muthén & Muthén, 1998-2016)

Missing data—For the 1018 families within the ARIES resource, 914 had the required methylation data available from cord blood. Twenty-five samples at birth failed quality control (>1% probes/samples with background detection p-value   0.05) and were excluded from further analysis. From the resulting cord blood sample of 889, participants with missing ethnicity were removed (n= 61). This resulted in a final total of 828 cord blood at birth. We included participants with data for the CRP, DAWBA symptoms, risk factors and DNA methylation. This resulted in a sample of 785 youth. We compared the 785 to the 828 on the study variables (i.e. prenatal risks and DAWBA symptoms) and found no significant differences.

Results

Step 1: i-ePGS score validation.

Table 1 contains the 7 probes from Ligthart et al. (2016) described above (see Table 1, **supporting information**, for total probe information). As can be seen, these probes were annotated to 9 total genes. For most genes, peripheral expression was higher than central nervous system expression; however, certain of these genes (e.g. *TUBB)* show moderate expression in the brain (see Table 2, **supporting information**). One probe *cg10636246* showed evidence of mQTL (i.e. DNA . Associations between the i-ePGS at age 15-17 and CRP levels at ages 15 and 17 were examined, as this was the age and time-span of overlapping DNAm and CRP. CRP associated with i-ePGS at age 15 $r = -0.111$, $p < 0.002$ and age 17 $r = -0.181$, $p < 0.0001$. We then created i-ePGS scores at earlier time points (i.e. birth, age 7). i-ePGS at age 15-17 auto-correlated with i-ePGS at age 7 ($r = 0.314$, $p < 0.0001$) and birth ($r = 0.175$, $p < 0.0001$). To aid in the interpretation of environmental and phenotypic association with the i-ePGS, we took the inverse value of the i-ePGS. This transformation made the i-ePGS consistent with the direction of higher CRP for potential low-grade chronic inflammation. As can be seen in Table 2, i-ePGS at birth and age 7 differentially associated with the study variables.

Step 2: Associations between i-ePGS, cognitive function and outcomes

Prior to describing the latent path model, we first highlight certain bivariate correlations between the mental health outcomes and the study variables (see Table 2). First, we found that both internalising and externalising symptoms associated negatively with cognitive function; internalising (but not externalising) problems associated with i-ePGS at age 7; and neither outcome associated with i-ePGS at birth. Second, cognitive function associated negatively with i-ePGS at birth, and the prenatal and postnatal adversity indexes. As stated earlier, i-ePGS at birth associated with a i-ePGS at age 7. We note also that cell type and sex each associated with i-ePGS (birth and age 7), and that sex associated with higher internalising (females > males) and externalising symptoms (females < males) (age 7-15).

Figure 1 contains the latent path model that showed adequate fit to the data: χ^2 (39) = 77.395, $p < 0.001$; CFI = 0.972; TLI = 0.954; RMSEA = 0.035, 90% CI = 0.024, 0.047. Only significant pathways are shown, full model estimates are available in Supplemental Information (Figure SI_1). As can be seen, the associations described above largely remained significant after accounting for the other parameters in the model as well as cell type and sex. For example, i-ePGS at birth associated with both i-ePGS age 7 and lower cognitive function. i-ePGS at age 7 associated with higher internalising, but not externalising to symptoms. Higher cognitive function associated with lower levels of both internalising and externalising symptoms. Moreover, indirect effects showed that i-ePGS at birth associated with higher internalising ($b = 0.020$, 95% CI = 0.002, 0.069) and externalising ($b = 0.029$, 95% CI = 0.003, 0.087) symptoms via lower cognitive function; and with higher internalising symptoms ($b = 0.032$, 95% CI = 0.011, 0.064) but not externalising symptoms ($b = 0.005$, 95% CI = -0.011, 0.029) via continuity of i-ePGS at age 7.

Step 3: Associations between adversities, i-ePGS, cognitive function and outcomes.

In this step we added the prenatal (18-32 wks) and postnatal (birth – age 7) adversity scores and tested the prenatal and postnatal effects. Table 2 contains the correlations. At the bivariate level, pre- and postnatal adversity associated with internalising and externalising symptoms, and cognitive function. Postnatal, but not prenatal, adversity associated with i-ePGS at age 7.

Figure 2 contains the overall latent path model, which showed adequate fit to the data: χ^2 (51) = 96.122, $p < 0.001$; CFI = 0.968; TLI = 0.947; RMSEA = 0.034, 90% CI = 0.023, 0.044. To decrease the complexity of Figure 2, only significant parameters are shown. Three results are highlighted here. First and foremost, as evidenced in the bivariate associations (and against our expectation), i-ePGS at birth did not associate with prenatal global adversity; we therefore have not included this variable in the model (see Supplemental Information Figure SI_2 for complete model estimates). Second, consistent with our expectation, postnatal adversity (birth – age 7) associated with higher i-ePGS at age 7, which, in turn, associated with internalising symptoms (age 7-15). Third, with the addition of adversity, two associations in the first model (Figure 1) changed due to the new conditional relationships. The association between higher i-ePGS at age 7 and lower cognitive function (age 7) increased in strength of association – and the association between

higher cognitive function (age 7) and lower internalising symptoms (age 7-15) decreased in strength of association.

On the one hand, these results suggest that the prenatal effects pathway does not involve the prenatal adversity index, but are confined to i-ePGS at birth, which might be a proxy for both unmeasured environmental effects during pregnancy and genetic influence (i.e. heritability) of immune system function. On the other hand, the data provides relatively straightforward support for postnatal effects, given that postnatal adversity associated with i-ePGS at age 7. Three indirect effects were identified: i-ePGS at birth associated with lower cognitive function, which, in turn, associated with higher externalising symptoms (b = 0.0.24; 95% CIs = 0.002, 0.078); higher i-ePGS at age 7, which, in turn associated with higher internalising symptoms (b = 0.030; 95% CIs = 0.003, 0.063); and postnatal adversity (birth – age 7) associated with higher i-ePGS at age 7, which, in turn, associated with higher internalising symptoms (b = 0.009; 95% CIs = 0.002, 0.022).

Discussion

In this prospective birth cohort study, we created and validated inflammation-related epigenetic risk scores (i.e. i-ePGS) and then tested prenatal and postnatal hypotheses about child and adolescent mental health. There were two indirect effects stemming from i-ePGS at birth: one to higher externalizing problems (7-15 years) via lower cognitive function (age 7), and the other to higher internalising problems (7-15 years) via higher i-ePGS at age 7. These indirect pathways suggest multifinality – that a single risk factor can associate with multiple outcomes through different developmental pathways (Cicchetti & Rogosch, 1996). We also identified one postnatal effect where adversity (birth to age 7) associated with higher internalising problems (7-15 years) via higher i-ePGS at age 7. Our findings are consistent with previous research showing associations between early life adversity, inflammation-related DNAm and vulnerability for mental health (Janusek et al., 2017; McDade et al., 2014). The present findings expand this literature in three main ways.

First, as mentioned, we were interested in the potential role of immune system-related DNA methylation in influencing cognitive function and mental health. We focused on CRP, as it is an acute-phase protein marker of systemic inflammation produced in response to increasing levels of circulating inflammatory factors (Mitchell & Aneshensel, 2017). In addition, serum CRP-related DNA methylation has recently been reported in an epigenome-wide association study by Ligthart et al. (2016), which was based on multiple adult cohorts. Based on these results, we created an i-ePGS score and (partially) replicated Ligthart et al. (2016): the i-ePGS score associated with overlapping measures of serum CRP at age 15-17. Taking this as acceptable validation, we then created i-ePGS scores at birth and age 7.

Our second main finding was in the longitudinal associations between the i-ePGS scores and study variables. In the Developmental Origins of Health and Disease hypothesis, DNA methylation is suggested as a biological mechanism that can establish early vulnerability for subsequent mental and physical health problems (Monk et al., 2016). Here, for the first time, we show a new candidate mechanism: i-ePGS. **Specifically, we found that i-ePGS at birth associated with lower cognitive function, which, in turn, associated**

with higher externalizing problems. This indirect effect is noteworthy as most research has reported direct associations between peripheral inflammation markers and internalizing, but not externalizing, problems (Danese & Baldwin, 2017). However, it is worth noting that a recent epigenome-wide association study reported variation in DNA methylation in cytokine-related genes in adults with a history of chronic childhood physical aggression (Provençal et al., 2014). Indeed, the neuroimmune network hypothesis (Nusslock & Miller, 2016) posits that peripheral inflammation can indeed associate with externalizing problems by way of affecting executive control-related processes linked to the prefrontal cortex, such as executive control, decision making, and regulating threat- and reward-related tendencies (see also Danese & Lewis, 2016; Hostinar, Nusslock, & Miller, 2017). Of interest, our measure of cognitive function (i.e. the WISC-III) was comprised of verbal subtests (e.g. vocabulary, comprehension, etc.) and performance subtests (e.g. block design, object assembly, etc.). These aspects of cognitive function have been associated with both infection/immune functioning (Jensen, Berens, & Nelson 3rd, 2017) and externalising behaviours (Barker et al., 2007; Loeber et al., 2012). Hence, the present results highlight the potential of inflammation associating with externalising problems via cognitive function.

The age 7 i-ePGS directly associated with internalizing problems, which, as stated, supports a range of research that has shown inflammation to associate with depressive symptoms, anxiety and post-traumatic stress disorder (Danese & Baldwin, 2017). The differences between this present study and those former studies are that (i) the association was not contingent on individuals being clustered by exposure to adversity (e.g. see Danese et al., 2011; Miller et al., 2002); and (ii) we examined an i-ePGS biomarker that is associated with CRP. Why would i-ePGS associate with higher internalizing problems, or lower cognitive function, for that matter? Although highly speculative, the genetic annotation of the different CpGs in the i-ePGS may help in interpreting these associations. For example, *cg18181703* is annotated to *TUBB,* a complex locus involved in the structural component of microtubules, mutations of which are reported to associate with aberrant neural development (Breuss et al., 2012). Of interest, this gene is expressed in peripheral samples, but also in the brain, bone marrow, lymphocytes and placental tissue (Fagerberg et al., 2014). *TUBB* may therefore be a gene involved in both prenatal and postnatal inflammatory effects on neural development and brain-based mental health problems. These associations, as well as the i–ePGS scores themselves, should be considered preliminary and in need of replication.

The third main finding is regarding the prenatal and postnatal adversity-related hypotheses. Against expectation, prenatal adversity did not associate with the i-ePGS at birth. On the one hand, this null result may indicate that we did not assess the correct types of immunity-related prenatal adversities (e.g. maternal obesity); or that CRP-related methylation changes do not capture the influence of prenatal adversities on the immune system. Indeed, there are many other immune markers that could be examined. **For example, interleukin-8, which can coincide or precede an increase in CRP, is shown to have specific T cell effector (i.e. proinflammatory) functions in neonates but not adults (Gibbons et al., 2014).** On the other hand, i-ePGS at birth may have a strong heritable component above and beyond environmental influence. Indeed CRP is estimated to be moderately heritable (40%; Baldwin et al., 2017; Pankow et al., 2001) and latent genetic risk can explain up to

15% of serum CRP variability (Baldwin et al., 2017). Moreover, a large-scale genome-wide association study (Dehghan et al., 2011) developed a weighted genetic risk score (similar to the weighted i-ePGS examine**d** here) that was strongly associated with CRP levels. Another genome-wide association study identified genetic pathways related to immune system functioning for several types of mental health disorders (Network & Consortium, 2015). Although we identified genetic influence (i.e. mQTL) on DNAm levels in one of the CpGs included in the i-ePGS score, for this type of analysis to be more informative we would need to also show that the SNP (*rs11667764*) is influential in cognitive function or internalising symptoms, which we were unpowered to do. However, *rs11667764,* associates with variation in putamen and thalamus volume (Hibar et al., 2015), so it may be a SNP that is worthwhile of further examination.

It is of great interest that the postnatal global adversity index (birth – age 7) associated with i-ePGS at age 7, which in turn, associated with internalizing problems (age 7-15). This finding supports the 'cumulative' adversity hypothesis (see Danese & Baldwin, 2017), where repeated exposures of stressful events can become embedded in the epigenetic functioning of the immune system (Janusek et al., 2017), which can eventually manifest as chronic, low-grade inflammation (McDade et al., 2017) and vulnerability for mental health problems. Also of note, however, is that this pathway did not include the measure of cognitive function. A recent meta-analysis has reported that the type of measure employed here may index impaired cognitive function that is concurrent to, but not predictive of, internalizing symptoms (see Scult et al., 2017) – which brings up the possibility that a different measure of brain function may be more appropriate. The neuroimmune network hypothesis, for example, highlights sensitivity to rewards and reward-related brain function in networked cortico-basal ganglia regions, as the most highly relevant for internalizing problems (Hostinar et al., 2017). Future research may want to examine dampened reward sensitivity as a risk for future internalizing problems.

Findings should be interpreted in light of a number of limitations. First, the current study was based on a modestly sized population-based sample of youth. In *the* future, it will be important to test the robustness of findings using other epidemiological cohorts. Second, the study focused exclusively i-ePGS as a proxy for CRP; additional research is needed to establish that these i-ePGS scores are indeed developmentally sensitive, by way of examining CRP associations at each age. Third, findings were based on a certain measure of cognitive function (i.e. WISC-III) that has previously been associated with both internalising and externalising problems. Therefore, research will be needed to establish the relevance of the present findings to both brain structure and function, as well as other measures of cognitive function. Fourth, despite the fact that we identified prospective associations between i-ePGS and internalising and externalising problems, it is not possible to establish causality, as associations could reflect the contribution of confounding genetic and environmental influences. Fifth, adversity can associate with inflammatory response through bidirectional interactions between the immune system and the neuroendocrine stress axes. Animal research has shown that, for instance, prolonged stress can lead to an attenuation of the anti-inflammatory properties of glucocorticoid and contribute to chronic low grade inflammation (Miller, Chen, & Parker, 2011). It will be important for future

research to examine how the neuroendocrine stress system might further inform the present results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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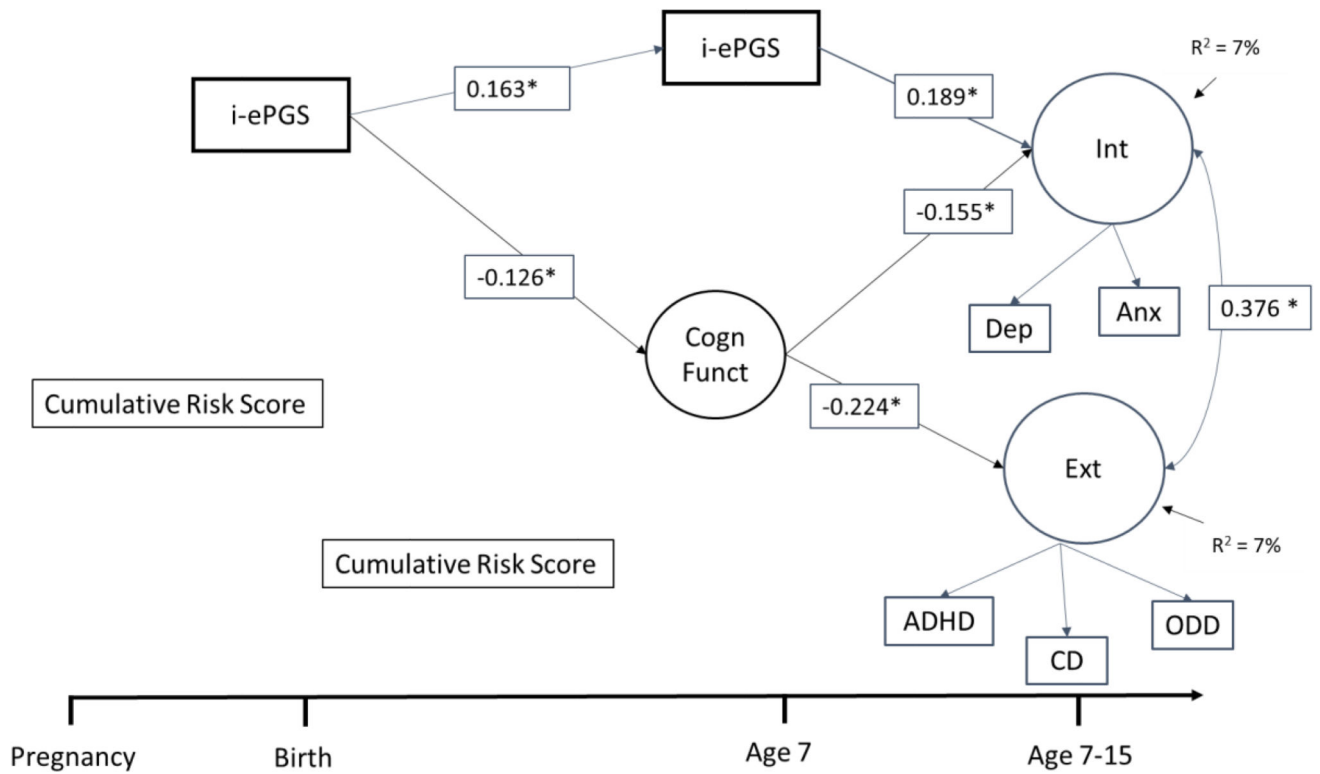
Figure 1. Latent path analytic model: Associations between i-ePGS, cognitive function and internalising and externalising symptoms
Note. i-ePGS = inflammation-related epigenetic risk score; inverse value of i-ePGS score is depicted in this graph to mirror direction of CRP (higher) based on previous DNAm immune studies (Janusek et al., 2017; Ligthart et al., 2016); only significant pathways shown; Int = internalising symptoms; Ext = externalising symptoms; Cogn Funct = cognitive function; Dep = depression symptoms; Anx = anxiety symptoms; ADHD = attention deficit hyperactivity disorder symptoms; CD = conduct disorder symptoms; ODD = oppositional defiant disorder symptoms;
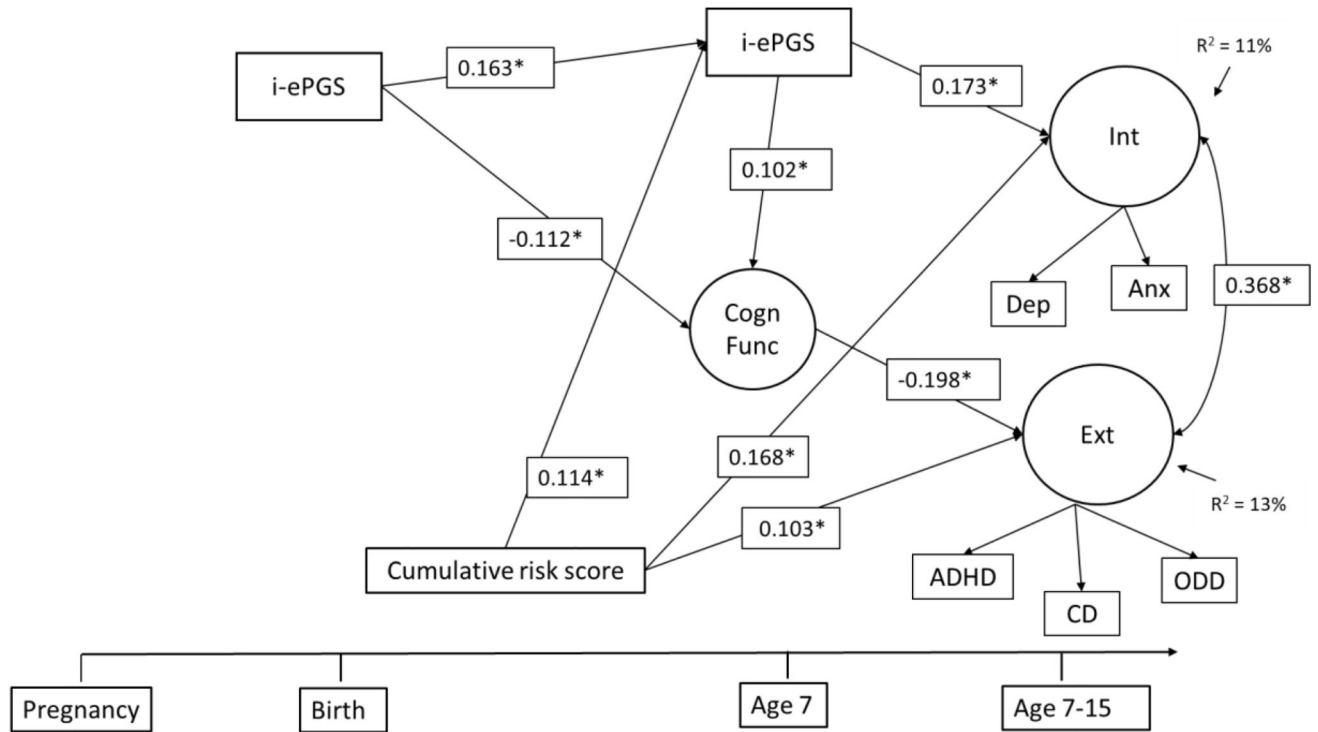
Figure 2. Latent path analytic model: Associations between cumulative risk score, i-ePGS, cognitive function and internalising and externalising symptoms
Note. i-ePGS = inflammation-related epigenetic risk score; inverse value of i-ePGS score is depicted in this graph to mirror direction of CRP (higher) based on previous DNAm immune studies (Janusek et al., 2017; Ligthart et al., 2016); only significant paths shown; * = $p <$ 0.05; Int = internalising symptoms; Ext = externalising symptoms; Cogn Funct = cognitive function; Dep = depression symptoms; Anx = anxiety symptoms; ADHD = attention deficit hyperactivity disorder symptoms; CD = conduct disorder symptoms; ODD = oppositional defiant disorder symptoms;

Table 1

DNAm sites used in the i-ePGS score: Associations with CRP and gene expression from the original study, with the addition of mQTLs

MarkerName	Gene	Methylation-CRP (discovery sample)			Methylation-expression		Expression-CRP levels		mQTL
		Effect CRP	Zscore	P value	Zscore	P value	Z-score	P value	
cg10636246	**AIM2**	**-0.0069**	**-12.37**	**3.89E-35**	**3.89E-35**		**11.01**	**3.40E-28**	*No*
cg19821297	*DNASE2*	-0.0051	-4.39	1.16E-05		1.48	0.14	*Cis - rs11667764; Chr19*
cg19821297	*FGD2*	-0.0031	-4.30	1.73E-05		-0.29	0.77	*No*
cg27023597	*HEATR6*	-0.005	-4.08	4.51E-05		1.89	0.06	*No*
cg06126421	IER3	-0.0052	-3.98	6.90E-05		-1.39	0.16	*No*
	IFI16	**-0.0069**	**-6.90**	**5.07E-12**		**6.47**	**9.90E-11**	
cg06690548	*SLC7A11*	**-0.0048**	**-6.96**	**3.34E-12**		**4.90**	**9.80E-07**	*No*
cg18181703	**SOCS3**	**-0.0053**	**-4.95**	**7.47E-07**		**3.74**	**1.80E-04**	*No*
	TUBB	-0.0052	-5.12	3.02E-07		-0.98	0.33	

Note. Bolded text = CG probes that significantly associated with serum CRP

Table 2

Bivariate correlations underlying the latent path analytic models (n = 785)

Variable	1	2	3	4	5	6	7	8	9	10	11
1. Internalising Problems (ages 7-15)	1										
2. Externalising Problems (ages 7-15)	0.390*	1									
3. Cognitive Function (age 7)	-0.151*	-0.223*	1								
4. ePGS (birth)	-0.037	0.069	-0.098*	1							
5. ePGS (age 7)	0.151*	0.036	0.025	0.201*	1						
6. ePGS (age 15-17)	0.008	-0.002	0.017	0.175*	0.314*	1					
7. Prenatal Adversity (18-32 wks)	0.145*	0.239*	-0.109	0.042	-0.041	-0.067	1				
8. Postnatal Adversity (birth - age 7)	0.215*	0.156*	-0.077	0.008	0.103*	0.005	0.179*	1			
9. Sex (1=female, 0=male)	0.104*	-0.152*	-0.028	-0.211*	-0.151*	-0.34*	-0.024	-0.004	1		
10. Cell Type (birth)	-0.024	0.043	-0.06	0.635*	0.144*	0.136*	0.015	0.003	-0.144*	1	
11. Cell Type (age 7)	0.042	-0.001	0.016	0.054	0.348*	0.138*	-0.048	-0.009	-0.049	0.080*	1

Note. Absolute value of i-ePGS score is depicted in this graph, to mirror direction of CRP (higher) based on previous DNAm immune studies (Janusek et al., 2017; Ligthart et al., 2016); non-shaded area = study variables; shaded area = control variables; i-ePGS = epigenetic risk scores; * $p < 0.05$.