Neonatal DNA methylation and childhood low prosocial behavior: An epigenome-wide association meta-analysis

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Abstract
Low prosocial behavior in childhood has been consistently linked to later psychopathology, with evidence supporting the influence of both genetic and environmental factors on its development. Although neonatal DNA methylation (DNAm) has been
INTRODUCTION

Prosocial behavior, defined as voluntary behavior intended to benefit others (e.g., helping, sharing, and comforting), provides a foundation for social competence and moral development (Eisenberg, Fabes, & Spinrad, 2006). Typically emerging early in childhood, higher levels of expressed prosociality are consistently linked with greater self-esteem, peer acceptance, and academic success in young people (Caprara, Barbaranelli, Pastorelli, Bandura, & Zimbardo, 2000; Layous, Nelson, Oberle, Schonert-Reichl, & Lyubomirsky, 2012). However, levels of prosocial expression vary considerably between individuals. Notably, at the opposite end of the prosocial continuum, young people who follow developmental trajectories denoted by persistently low levels of prosociality display elevated levels of externalizing behaviors, such as aggression and delinquency (Flynn, Ehrenreich, Beron, & Underwood, 2015; Kokko, Tremblay, Lacourse, Nagin, & Vitaro, 2006), and—albeit less consistently—internalizing problems (Nantel-Vivier, Pihl, Cote, & Tremblay, 2014).

Etiological research consistently reports both genetic and environmental contributions to prosocial development (Knafo-Noam, Vertsberger, & Israel, 2018). From twin designs, heritability estimates for observed prosocial actions range between 23 and 69%, with most studies also identifying substantial shared environment effects (Gregory, Light-Häusermann, Rijsdijk, & Eley, 2009; Knafo-Noam, Israel, & Ebstein, 2011; Knafo-Noam, Uzefovsky, Israel, Davidov, & Zahn-Waxler, 2015). These heritability estimates have also been found to prospectively associate with a range of psychological traits in childhood, its potential role in prosocial development has yet to be investigated. This study investigated prospective associations between cord blood DNA methylation at birth and low prosocial behavior within and across four longitudinal birth cohorts from the Pregnancy And Childhood Epigenetics (PACE) Consortium. We examined (a) developmental trajectories of “chronic-low” versus “typical” prosocial behavior across childhood in a case-control design (N = 2,095), and (b) continuous “low prosocial” scores at comparable cross-cohort time-points (N = 2,121). Meta-analyses were performed to examine differentially methylated positions and regions. At the cohort-specific level, three CpGs were found to associate with chronic low prosocial behavior; however, none of these associations was replicated in another cohort. Meta-analysis revealed no epigenome-wide significant CpGs or regions. Overall, we found no evidence for associations between DNA methylation patterns at birth and low prosocial behavior across childhood. Findings highlight the importance of employing multi-cohort approaches to replicate epigenetic associations and reduce the risk of false positive discoveries.

KEYWORDS
cord blood, DNA methylation, epigenome-wide association study, meta-analysis, prosocial behavior
shown to increase during childhood, while shared environmental influences diminish over time (Knafo-Noam & Plomin, 2006). However, efforts to elucidate the specific biological processes underpinning this heritability by examining associations between specific candidate genes and various domains of prosocial behavior have yielded very small meta-analytic effect sizes (Bakermans-Kranenburg & Van Uzendoom, 2014). Furthermore, no hypothesis-free study of genetic variation across the entire genome has been published for this phenotype to date (Knafo-Noam et al., 2018). At the same time, exposure to prenatal (e.g., maternal stress, androgen exposure) and postnatal adversity (e.g., parenting, family functioning, neighborhood deprivation) has been linked to less prosocial behavior in children, suggesting some etiological role for the early environment (Horn, Hungerlander, Windhager, Bugnyar, & Massen, 2018; Jambon, Madigan, Plamondon, & Jenkins, 2019; Loomans et al., 2011; Safra et al., 2016).

Although more research has begun to consider the potential interplay between genetic and environmental influences on prosocial development (Knafo-Noam et al., 2011; Sasaki et al., 2013), the underlying molecular mechanisms remain unclear. In recent years, DNA methylation (DNAm), an epigenetic process that regulates gene expression in response to both genetic and environmental signals, has emerged as a potential mechanism of interest through which the early environment, in combination with genetic predispositions, can increase biological vulnerability for psychopathology (Barker, Walton, & Cecil, 2018). Specifically, DNAm involves the addition of a methyl group to DNA base pairs—mainly, cytosine-guanine (CpG) dinucleotides (McGowan & Roth, 2015)—which can disrupt the binding of transcription factors (Zhang & Meaney, 2010). DNAm has been shown to regulate numerous neurobiological and developmental processes, with alterations in DNAm linked to psychological and psychiatric outcomes (Barker, Walton, Cecil, Rowe, et al., 2018; Cecil et al., 2014; Rijlaarsdam et al., 2017; Walton et al., 2017). It has also been suggested that DNAm may act as a noncausal biomarker indexing vulnerability for psychopathology, in a similar way to the approach that has been used in cancer risk and prognosis (Ladd-Acosta & Fallin, 2016). Thus, it may be possible to identify a consistent profile of DNAm that characterizes persistently “low prosocial” youth.

To date, no study has examined DNAm patterns associated with low prosocial behavior, although some research has been carried out on closely related phenotypes. For example, callous-unemotional (CU) traits are defined by a lack of prosocial emotions such as empathy and remorse, and represent a significant risk factor for aggressive and antisocial behaviors (Marsh, 2019). Within clinical samples of boys with conduct problems, higher CU traits have been associated with increased DNAm in OXTR gene promoter (Dadds et al., 2014) and reduced DNAm of the promoter region of the serotonin 1B receptor gene (HTR1B; Moul, Dobson-Stone, Brennan, Hawes, & Dadds, 2015). However, these approaches are limited in two main ways. First, single candidate genes are not likely to explain the majority of variance of more complex, multi-determined psychiatric traits and disorders, whose exact pathophysiology is not yet known (Salvatore & Dick, 2018). Second, these studies only examined DNAm for specific genes selected based on hypothesized biological or functional relevance, precluding their ability to detect novel biological associations.

In light of these research gaps, this study drew on four independent yet highly compatible birth cohorts to investigate epigenome-wide, prospective associations between DNAm at birth and low prosocial behavior during childhood. Focusing on methylation levels at birth (i.e., before manifestation of later behavioral or psychiatric symptoms) enabled us to separate the direction of the association, an important step for establishing whether detected epigenetic modifications may serve as potential predictive markers, as opposed to secondary effects of the “disease” process itself (Rakyan, Down, Balding, & Beck, 2011). Moreover, existing longitudinal studies comparing the stability of DNAm associations across childhood have found that DNAm patterns are (a) highly dynamic over time (Mulder et al., 2021), and (b) more strongly predictive (at birth) of neurodevelopmental and behavioral outcomes related to prosocial behavior in children, including conduct problems (Cecil et al., 2018), CU traits (Cecil et al., 2014), and social communication deficits (Rijlaarsdam, Cecil, Relton, & Barker, 2021), compared to DNAm patterns examined later in childhood. However, it is not known whether such findings at birth extend to prosocial behavior itself. First, in line with previous research (Nantel-Vivier et al., 2014), we identified developmental trajectories characterized by persistently low prosocial behavior across childhood. We then ran an epigenome-wide association study (EWAS) to identify DNAm sites within each cohort that differed between trajectories. Second, as all cohorts featured comparable time-points and identical measures of prosocial behavior, we meta-analyzed results to maximize our power to detect small effects and reduce the risk of false positives. Furthermore, we used region discovery methods to identify potentially differentially methylated regions (DMRs) associated with low prosocial behavior across cohorts. Finally, previous studies of other psychiatric traits suggest that the influence of genetic and environmental risk factors may be best observed across a continuum of severity (Thapar, Langley, O’Donovan, & Owen, 2006). Thus, we adopted a dimensional approach to complement our trajectory-based analyses, repeating all steps using continuous prosocial scores assessed at comparable time-points (6–7 years of age) within each cohort.

2 | METHODS

2.1 | Participants

Four prospective birth cohorts within the Pregnancy And Childhood Epigenetics (PACE) Consortium (Felix et al., 2018) had information on DNAm in cord blood and prosocial behavior in childhood: the Avon Longitudinal Study of Parents and Children (ALSPAC; Fraser et al., 2013), Generation R (GENR; Kooijman et al., 2016), INfancia y Medio Ambiente (INMA; Guxens et al., 2012), and the Lifestyle and environmental factors and their Influence on Newborns Allergy risk (LINA; Herberth et al., 2011). Full cohort descriptions are provided in Supporting Information Methods. In brief, all included participants...
were of European ancestry, with combined sample sizes of 2,095–
2,121 in the current study. Ethical approval for each study was
obtained by local committees and consent to use their data
was obtained for all participants.

2.2 | Measures

2.2.1 | DNA methylation

DNAm in cord blood was measured using the Illumina Infinium
HumanMethylation450K BeadChip (Illumina Inc., San Diego). Full
information on sample processing, quality control, and normalization
procedures within each cohort is described in Appendix S1 of
Supporting Information. Methylation levels were characterized by a
“beta” (β) value ranging from 0 (no methylation) to 1 (full methylation).
CpG sites with values outside the 25th percentile – 3 × interquartile
range (IQR) and the 75th percentile + 3 × IQR of the distribution
were identified as outliers and winsorized.

2.2.2 | Prosocial behavior

In all cohorts, prosocial behavior was repeatedly assessed using
maternal ratings on the “prosocial” subscale of the Strengths and Dif-
ficulties Questionnaire (SDQ), a widely used screening instrument
with established reliability and validity (Goodman, 2001). Five items
assessed the presence of the following behaviors “in the past six
months” along a three-point scale (0 = “not true”; 1 = “somewhat
true”; 2 = “certainly true”): (a) “considerate of other’s feelings”;
(b) “shares readily with other children”; (c) “helpful if someone is hurt,
upset, or ill”; (d) “kind to younger children”; and (e) “volunteers to help
others”. Prosocial behavior was assessed in ALSPAC when children
were aged 4, 7, 8, 10, 12, and 13 years, in GENR when children were
aged 6 and 9 years, in INMA when children were aged 7 and 11 years,
and in LINA when children were aged 7 and 10 years.

Latent profile analysis (LPA) was performed using Mplus version
7.11 (Muthén & Muthén, 2012) to classify ALSPAC children into
developmental trajectories based on their levels of prosocial behavior
across childhood. A type of latent variable mixture model, LPA is a
person-centered technique that identifies discrete subgroups within
a population based on a series of observed indicators—in this case,
repeated measures of the SDQ prosocial subscale at six time-points
(Oberski, 2016). We estimated two-class to five-class solutions and
used several fit indices (Bayesian Information Criterion; Akaike Infor-
mation Criterion; Lo–Mendell–Rubin test; entropy) to determine the
optimal number of classes (see Table S1, Panel A and accompanying
note for details). Based on these metrics, a three-class solution pro-
vided the best fit for these data. Within this three-class model, one
trajectory subgroup, termed “chronic-low” (n = 75, bottom 10% of
sample), identified children with consistently low levels of prosocial
behavior from age 4–13 years. The two remaining trajectories differed
significantly from this chronic-low trajectory, as well as from each
other. However, average levels of prosocial behavior among these
two trajectory groups (M = 9.0 and 7.3) fell well within the “norma-
tive” or “average” scoring band (≥6), based on the original cut-offs for
the parent-reported SDQ (Goodman, 1997). This contrasted with the
chronic-low prosocial group, where the grand mean for prosocial
behavior across all six time-points fell within the clinical threshold for
“low” prosocial behavior (M = 5.6). As the present study sought to
compare persistently low prosocial youth with more typical levels of
prosociality in a case–control approach, we combined the two
remaining trajectories, hereafter referred to as the “typical” group
(n = 673, 89.96%). The resulting dichotomous variable (i.e., “typical”
v. “chronic-low”) was found to significantly associate with several
established correlates of prosocial behavior, including empathy,
social-cognitive difficulties, and CU traits (r = −0.27–0.32, p < .001; see
Table S1, Panel B).

To enable cross-cohort comparison and meta-analysis, we sought
to establish an equivalent case–control design for all cohorts by repli-
cating the clinically significant “bottom 10%” cut-off observed from
trajectory-based analyses in ALSPAC. However, as GENR, INMA, and
LINA each had only two repeated measures of prosocial behavior,
which was insufficient to perform LPA, we instead standardized
prosocial scores for each age and then calculated an average over
both ages. Values below the 10th percentile for this average score
(i.e., “bottom 10%” cut-off) were classed as “chronic-low” prosocial
behavior across both ages, in contrast to the remainder of the sample
(i.e., typical group). Having identified these groups in children who had
available prosocial scores at both ages, our final sample within each
cohort consisted of those who also had DNAm data available at birth.

Table 1 presents the number and proportion of children in each of
these chronic-low trajectories. In all three cohorts, “grand mean”
prosocial scores in the defined chronic-low (GENR: M = 5.4;
SD = 1.7; INMA: M = 5.3; SD = 1.2; LINA: M = 5.5; SD = 1.3) and
typical groups (GENR: M = 8.8; SD = 1.3; INMA: M = 8.7; SD = 1.4;
LINA: M = 8.5; SD = 1.3) were comparable to those observed within
the two ALSPAC trajectory groups (chronic-low: M = 5.6; typical:
M = 8.2) and thus in line with clinical definitions for “low” and “aver-
age” levels of prosocial behavior, respectively.

In a complementary strategy to account for the dimensionality
of prosocial behavior and identify DNAm differences associated with the
severity (as opposed to chronicity) of low prosociality, we repeated all
analyses using continuous measures of low prosocial behavior (rather
than trajectory groups) at the closest-corresponding time-points at
age 6–7 years across all cohorts. These continuous scores were nega-
tively skewed (i.e., indicating that most children exhibit higher levels
of prosocial behavior) in all cohorts, which is a common occurrence in
general-population samples. As square root and log transformation of
the scores did not correct for this skewness, as well as the fact that
transformed scores would complicate interpretation of the results,
robust linear regression analyses were performed. Scale scores were
reversed to be consistent with trajectory coding and facilitate inter-
pretation, with higher scores representing lower prosocial behavior.
TABLE 1 Descriptive statistics across the four included cohorts (N<sub>total</sub> = 2,095–2,121)

| Cohort (sample size)<sup>a</sup> | Avon Longitudinal Study of Parents and Children (ALSPAC; n = 748) | Generation R (GENR; n = 891) | Infancia y Medio Ambiente (INMA; n = 270) | Lifestyle and environmental factors and their Influence on Newborns Allergy risk (LINA; n = 186) |
|----------------------------------|---------------------------------------------------------------|---------------------------|---------------------------------|-------------------------------------------------|
| **Child characteristics**        |                                                               |                           |                                 |                                                 |
| Sex, % female                    | 48.4                                                          | 49.6                      | 48.9                             | 48.1                                            |
| Gestational age in weeks, mean (SD) | 39.6 (1.5)                                                   | 40.2 (1.4)                | 39.8 (1.3)                       | 39.8 (1.4)                                    |
| **Child prosocial behavior**     |                                                               |                           |                                 |                                                 |
| Chronic-low prosocial trajectory, n (%) | 75 (10.0)                                              | 100 (11.2)                | 31 (11.5)                        | 20 (10.8)                                      |
| Continuous prosocial score (age 6–7 years), mean (SD) | 1.8 (1.7)                                                      | 1.7 (1.8)                 | 1.7 (1.7)                        | 1.9 (1.7)                                      |
| Age in years, mean (SD)          | 6.7 (0.1)                                                     | 6.0 (0.3)                 | 6.7 (0.4)                        | 7.1 (0.3)                                      |
| **Maternal characteristics**     |                                                               |                           |                                 |                                                 |
| Age at intake/delivery in years, mean (SD) | 29.6 (4.4)                                                  | 32.1 (3.9)                | 30.6 (4)                         | 30.8 (4.6)                                    |
| Sustained smoking during pregnancy, % | 9.7                                                          | 9.8                       | 12.6                             | 1.6                                            |
| Educational level, %             |                                                               |                           |                                 |                                                 |
| Lower (below university degree)  | 79.2                                                          | 29.8                      | 68.1                             | 36.5                                           |
| Higher (university degree and above) | 20.8                                                        | 70.3                      | 31.9                             | 63.5                                           |
| Psychopathology, mean (SD)       | 14.3, % yes<sup>b</sup>                                      | 0.1 (0.2)                 | 0.7 (0.5)                        | 2.1 (0.5)                                      |

<sup>a</sup>Reported sample sizes are drawn from prosocial trajectory analyses. For analyses of continuous “low prosocial” scores at age 6–7 years, sample sizes were as follows: N<sub>ALSPAC</sub> = 689; N<sub>GENR</sub> = 976; N<sub>INMA</sub> = 270; N<sub>LINA</sub> = 186.

<sup>b</sup>In ALSPAC, the presence of maternal anxiety/depression was coded as a binary variable (1 = yes vs. 0 = no).

2.2.3 | Covariates

We adjusted for child sex, maternal age at delivery (in years), smoking during pregnancy (binary categorization of “no smoking/quit in early pregnancy” vs. “smoked throughout pregnancy”), gestational age at delivery (in weeks), child age at the outcome assessment (in years), maternal education (binary categorization of “below university degree” vs. “university degree and above”), and technical covariates (e.g., batch or surrogate variables). We also adjusted for estimated cell type proportions calculated from a cord blood cell type reference panel (Gervin et al., 2019). Finally, we included mothers’ experience of depression and anxiety as a covariate (see Appendix S1 of Supporting Information for full details of measures used), as a caregiver experiencing these symptoms may perceive and rate their child’s behavior as more problematic than one who is not, potentially biasing observed associations between methylation and low prosociality.

2.3 | Statistical Analyses

Analyses proceeded in two main steps:

2.3.1 | Step 1: Epigenome-wide association study (EWAS) and meta-analysis

Based on a predefined analysis plan, cohort-level EWAS between DNAm at birth and prosocial trajectories during childhood was performed using linear regression models. Cohorts excluded individuals with known chromosomal abnormalities, multiple births, and one random sibling per sibling pair.

After performing quality control on cohort-specific summary statistics, we combined results in a fixed-effects inverse variance-weighted meta-analysis using the metafor R package (Viechtbauer, 2010). We excluded probes mapped to X/Y chromosomes, polymorphic CpGs (overlapping with known single-nucleotide-polymorphisms; Min, Hemani, Smith, Relton, & Suderman, 2018), and control or cross-reactive probes (targeting repetitive sequences/co-hybridizing to alternate sequences; Chen et al., 2013; McCartney et al., 2016), resulting in 364,659 autosomal CpG sites in the meta-analysis. Probes were annotated using the meffil R package (Min et al., 2018), enhanced by use of the University of California Santa Cruz Genome Browser (including data from the RefSeq and Ensembl databases). All annotations were based on genome build hg19. Genome-wide significance was defined based on a looser 450k array p-value threshold of \( p < 2.4 \times 10^{-7} \) (Saffari et al., 2018), suggestive significance at \( p < 5 \times 10^{-5} \), and nominal significance at \( p < .05 \).

We performed several additional analyses. First, we repeated analyses using “low prosocial” continuous scores at age 6–7 years, using robust linear regression in the MASS R package. Second, a candidate gene follow-up analysis was performed on results extracted from the meta-analysis for CpG sites annotated to OXTR, in order to examine associations with DNAm at this previously implicated gene. The significance threshold was set at a Bonferroni gene-level corrected \( p \)-value of \( 4.17 \times 10^{-3} \) (\( n = 12 \) CpGs).
2.3.2 | Step 2: Differentially methylated regions (DMRs) and meta-analysis

To account for the correlated structure of DNAm patterns, we next performed a regional analysis to identify regions that are differentially methylated in relation to low prosocial behavior. In contrast to site-specific EWAS analyses, which focus on individual CpG sites, regional analyses attenuate the burden of multiple testing and can also detect weaker signals that may be spread over wider regions. Regional analyses were first performed in each cohort and then meta-analyzed using the dmrff R package (Suderman et al., 2018). First, this method selects potentially DMRs by identifying genomic regions spanned by nominally significant CpG sites at most 500 bp apart. Second, EWAS summary statistics of all CpGs in the region are combined, while adjusting for correlations between sites in order to avoid inflating regional statistics. DMRs are then defined as having at least two spatially contiguous CpG sites. p-values for each region are adjusted for multiple tests using Bonferroni adjusted p < 0.05. As for the site-specific EWAS analyses, regional analyses were also repeated using “low prosocial” continuous scores at age 6–7 years.

3 | RESULTS

3.1 | Sample characteristics

In the main analyses, complete data for DNAm and the prosocial trajectories were available for 748 children (48.4% female) in ALSPAC, 891 children (49.6% female) in Generation R, 270 children (48.9% female) in INMA, and 186 children (48.1% female) in LINA. Detailed sample characteristics are presented in Table 1.

3.2 | Epigenome-wide association analysis and meta-analysis

Meta-analysis of the four cohorts (N_total = 2,095) did not identify significant associations between DNAm of 364,659 CpG sites in cord blood and chronic-low prosocial trajectories (p < 2.4 × 10^-7; Figure 1). The most significant association (β = -0.004, SE = 0.001, p = 8.37 × 10^-7) was found for cg03160045 annotated to RBX1, a gene previously associated with cognitive ability (Lee et al., 2018) and psychiatric disorders including anxiety, depression, and schizophrenia (Baselmans et al., 2019; Goes et al., 2015; Wu et al., 2020). In Table 2, we list the 24 CpG sites that were most strongly associated with prosocial trajectories at the suggestive level (p < 5 × 10^-5). The direction of effect estimates was consistent across all cohorts for 20 of these sites. There was evidence for some genomic inflation (lambda = 1.25; see Figure 1b for quantile–quantile plot).

Table S3, Panel A shows the lambda and number of cohort-level, genome-wide significant (p < 2.4 × 10^-7) hits for typical versus chronic-low trajectories. There was evidence for some genomic inflation (lambda = 1.25; see Figure 1b for quantile–quantile plot).

Table S3, Panel A shows the lambda and number of cohort-level, genome-wide significant (p < 2.4 × 10^-7) hits for typical versus chronic-low trajectories. We observed three significant cohort-level associations between DNAm and prosocial trajectories. These sites (cg22599122 [HMGB4/CSMD2] in INMA; cg03436478 [SGCE/PEG10] and cg21552290 [GABBR1] in LINA) mapped to genes associated with neurodevelopmental and mental health-related phenotypes (see Table S3 and accompanying note for full details); however, none of the three hits overlapped with other cohorts.

Similar to the main trajectory-based analyses, no genome-wide associations were observed in meta-analysis of continuous “low prosocial” scores at age 6–7 years, with the most significant association (β = 5.80, SE = 1.22, p = 1.92 × 10^-6) found for cg13899097 on the RARRES3 gene (see Table S2), which is differentially expressed in autism (Seno et al., 2011). We found seven cohort-level associations between DNAm and continuous “low prosocial” scores (Table S3,
Cord blood CpG sites most strongly associated with prosocial trajectories (typical vs. chronic-low) within meta-analysis

*Note:* Maximum N = 2,095; meta-analytic p-value < 5 × 10⁻⁵. The full model is adjusted for batch effects, estimated cell-type proportions, child sex, gestational age, maternal age in take/delivery, maternal smoking during pregnancy, maternal education, and maternal psychopathology. Chr: chromosome; Direction: direction of effect per cohort, where + and - signify positive and negative effect estimates, respectively (cohorts are listed in alphabetical order: Avon Longitudinal Study of Parents and Children [ALSPAC]; Generation R [GENR]; INFancia y Medio Ambiente [INMA]; Lifestyle and environmental factors and their [LINA]); I²: heterogeneity statistic describing variation attributable to heterogeneity across studies; high I² values suggest high heterogeneity.

| CpG site | Chr | Position | Nearest gene | Effect | SE    | p-value | Direction | I² | Heterogeneity p-value |
|----------|-----|----------|--------------|--------|-------|---------|-----------|----|---------------------|
| cg03160045 | 22  | 41,345,978 | RBX1        | -0.004 | 0.001 | 8.37E-07 |          | 42 | 0.16               |
| cg00979307 | 3   | 47,889,555 | DHX30; MIR1226 | -0.008 | 0.002 | 2.37E-06 |          | 43 | 0.15               |
| cg01402746 | 6   | 30,616,468 | C6orf136     | 0.004  | 0.001 | 2.62E-06 | +++++    | 0  | 0.66               |
| cg14931071 | 6   | 12,717,776 | PHACTR1      | -0.008 | 0.002 | 4.20E-06 |          | 54 | 0.09               |
| cg11495494 | 4   | 140,358,384 | RAB33B       | -0.004 | 0.001 | 5.71E-06 |          | 0  | 0.69               |
| cg16811988 | 3   | 42,103,138 | TRAK1        | -0.004 | 0.001 | 8.43E-06 |          | 0  | 0.72               |
| cg18278596 | 14  | 105,915,860 | MTA1         | 0.004  | 0.001 | 9.95E-06 | +++++    | 32 | 0.22               |
| cg22117637 | 2   | 232,285,495 | SGPP2        | -0.007 | 0.002 | 1.02E-05 |          | 25 | 0.26               |
| cg07572052 | 1   | 149,821,168 | HIST2H2AA4; HIST2H2AA3 | 0.014  | 0.003 | 1.23E-05 | +++++    | 1  | 0.39               |
| cg19508437 | 13  | 35,517,491 | NBEA         | -0.001 | 0.000 | 1.38E-05 |          | 0  | 0.63               |
| cg10182355 | 10  | 101,768,491 | TBC1D8       | 0.003  | 0.001 | 1.76E-05 | +++++    | 0  | 0.67               |
| cg17394129 | 12  | 71,829,267 | LGR5        | -0.011 | 0.002 | 1.83E-05 |          | 0  | 0.51               |
| cg00932677 | 4   | 187,776,068 | FAT1        | -0.008 | 0.002 | 2.17E-05 |          | 0  | 0.66               |
| cg02007434 | 10  | 98,210,804 | TLL2        | -0.010 | 0.002 | 2.32E-05 |          | 38 | 0.19               |
| cg20397078 | 7   | 28,966,605 | CRER, TRIL, CPVL | 0.004  | 0.001 | 2.39E-05 | +++++    | 0  | 0.79               |
| cg07129067 | 2   | 180,451,222 | ZNF385B      | -0.005 | 0.000 | 2.41E-05 |          | 0  | 0.62               |
| cg20313969 | 2   | 112,939,562 | FBN1        | -0.012 | 0.003 | 3.05E-05 |          | 56 | 0.08               |
| cg01825355 | 6   | 168,812,152 | SMOC2        | 0.005  | 0.001 | 3.48E-05 | +++++    | 27 | 0.25               |
| cg06850924 | 7   | 129,691,279 | ZC3HC1       | -0.002 | 0.000 | 3.48E-05 |          | 20 | 0.29               |
| cg02074728 | 5   | 50,259,086 | PARR8        | 0.003  | 0.001 | 3.52E-05 | +++++    | 0  | 0.80               |
| cg21165255 | 9   | 93,712,460 | SYK          | -0.009 | 0.002 | 3.78E-05 |          | 0  | 0.42               |
| cg14818546 | 7   | 69,296,381 | AUTS2        | -0.005 | 0.001 | 3.82E-05 |          | 0  | 0.74               |
| cg24341770 | 6   | 105,145,882 | HACE1       | -0.009 | 0.002 | 4.05E-05 |          | 0  | 0.52               |
| cg15727393 | 3   | 149,700,905 | PFN2, LOC646903 | 0.010  | 0.002 | 4.75E-05 | +++++    | 0  | 0.71               |

Panel C; three of which (cg06643156, cg09509433, and cg21144158) showed a consistent direction of effects across all cohorts. We found no evidence of genomic inflation for continuous analyses (lambda = 1.06; quantile-quantile plot in Figure S1).

In candidate gene follow-up analysis, none of the CpGs annotated to OXTR reached gene-level Bonferroni significance (Table S4). Of note, although effect sizes were trivial, most CpG sites showed the same directions of effect as previous studies examining related phenotypes (e.g., CU traits), in that higher OXTR methylation at birth was associated with lower prosociality.

### 3.3 Differentially methylated regions (DMRs) analysis

We identified no DMRs associated with “chronic-low prosocial” trajectory membership in the meta-analysis (Table 3). The top DMR associated with the chronic-low trajectory was located on chromosome 19:35,630,106–35,630,355 (β = −0.01, SE = 0.002, p = 2.5 × 10⁻⁶), nearest to genes FXDY1 and LG4. We repeated the same analyses with “low prosocial” continuous scores. Again, no DMRs reached epigenome-wide significance in the meta-analysis, with a top DMR located on chromosome 9:132,805,739–132,805,979 (β = −6.85, SE = 1.45, p = 2.3 × 10⁻⁶; nearest gene FNBP1). DMRs that were suggestively associated with the severity of low prosocial behavior (based on continuous scores) are presented in Table S5.

### 3.4 Power calculation

We performed a power calculation with a maximum effect estimate of 0.02 and alpha set at the 450k array significance threshold, p < 2.4 × 10⁻⁷. Results suggested that we had poor power (0.37) to detect the effect size observed given the current sample size (N = 2,095). Thus, it appears that larger sample sizes are needed to detect CpG sites associated with low prosocial trajectory membership.
Our power calculation also indicated that, based on the effect sizes and significance thresholds in the current study, a minimum sample size of 2,985 would be required to achieve 80% power. We did not perform this power calculation for the continuous score analysis as these power estimates would have been inflated by the large coefficient estimates of DNAm that were weighted from robust regression (the maximum effect size was 18.82).

4 | DISCUSSION

The current study examined whether DNAm patterns at birth prospectively associate with persistently low levels of prosocial behavior across childhood, using highly comparable data from four independent cohort studies. Although we identified three CpG sites from cord blood that differentiated persistently “low prosocial” children from their more typically developing peers within individual cohorts, none of these associations were replicated in another sample. Moreover, our EWAS meta-analysis (N = 2,095) did not identify any significant associations across the four cohorts at a 450k genome-wide level. This was true when examining both developmental trajectories of chronic-low versus typical prosocial behavior, as well as dimensional scores at similar time-points for all cohorts. We confirmed our findings using follow-up regional analyses to address non-independence between CpG sites, which yielded no meta-analytical DMRs following multiple testing corrections. Overall, our findings do not support an association between neonatal DNAm and low prosocial behavior in childhood.

It is important to consider the negative findings of the present study in the context of its considerable methodological strengths. First, our analyses drew on four independent large-scale cohorts with prospective designs. Second, availability of repeated measures of prosocial behavior enabled us to characterize participants based on sustained levels of low prosociality across childhood, capturing a discrete group of children at elevated risk for a range of psychiatric disorders (Flynn et al., 2015; Nantel-Vivier et al., 2014). This longitudinal approach was supplemented with dimension-based analyses (i.e., total scores) to explore associations across the entire continuum of prosocial behavior in the general population. Third, comprehensive harmonization between the four cohorts, in terms of both normalization of DNAm data and phenotypic measurement, was performed to reduce study heterogeneity and maximize comparability for replication efforts and the meta-analysis. Fourth, our analytic strategy featured both site-specific and regional analyses, which can improve power and detect functionally relevant findings across genomic regions (Michels et al., 2013).

Despite the strengths of our design and analysis, we did not identify any genome-wide associations between neonatal DNAm and childhood low prosocial behavior. We offer several potential explanations for our negative findings, which also consider a number of key study limitations. First, it appears from our power analysis that, even with the large sample size offered by these combined datasets, our analyses were underpowered to detect what appear to be reasonably small associations, particularly given the relatively low numbers of participants classified as “chronic-low prosocial.” However, our dimensional analyses involving continuous low prosocial scores across the entire sample showed similarly negative results. More generally, there may be insufficient individual variation within our data to detect differences in DNAm patterns in relation to prosocial behavior. For example, the interquartile ranges of low prosocial behavior at age 6–7 years were identical in all cohorts (median = 1; inter-quartile range: 0–3), indicating that most individuals show moderate to high levels of prosocial behavior (Figure S2). However, we found that these chronic-low and typical groups did differ on many behavioral outcomes (i.e., empathy, social-cognitive difficulties, and CU traits) that are known to associate with prosociality.

Second, the lack of associations could be the result of methodological differences across cohorts, despite the application of stringent harmonization standards to both phenotypic and DNAm data. Specifically, low prosocial behavior was assessed by the same reporting source (i.e., mothers), with the same instrument, and at comparable time-points in all samples. Nonetheless, we cannot rule out perceptual biases due to single informants or situation-specificity that may have influenced the results of this study. The use of mother reports allowed us to model prosocial behavior starting in early childhood, when child self-reports are not reliable. However, the inclusion of multiple informants (e.g., teacher report, child self-report) in future research may help to provide greater differentiation of the child’s prosocial behavior across different contexts. Moreover, differences in quality control, environmental exposures, and other unknown factors

### TABLE 3

Differentially methylated regions associated with prosocial trajectories (typical vs. chronic-low) based on a suggestive threshold (p < 5 × 10⁻⁵)

| Chr | Start   | End     | N of sites | Effect  | SE     | p-value   | Nearest gene |
|-----|---------|---------|------------|---------|--------|-----------|--------------|
| 19  | 35,630,106 | 35,630,355 | 5          | -0.008  | 0.002  | 2.50E-06  | FXYD1, LGI4  |
| 2   | 180,451,203 | 180,451,222 | 2          | -0.006  | 0.001  | 8.03E-06  | ZNF385B     |
| 11  | 3,876,513    | 3,876,808  | 7          | 0.000   | 0.000  | 1.92E-05  | STIM1       |
| 11  | 3,647,365    | 3,647,654  | 3          | -0.006  | 0.001  | 3.05E-05  | TRPC2       |
| 8   | 142,316,216  | 142,316,861 | 3          | -0.004  | 0.001  | 3.99E-05  | SLC45A4     |
| 19  | 55,996,543   | 55,996,566  | 2          | 0.000   | 0.000  | 4.37E-05  | NAT14       |

Abbreviation: Chr, chromosome.
could still contribute to cohort-specific methylation patterns and consequently dilute potential associations during meta-analysis (Joubert et al., 2016).

Third, our operationalization of prosocial behavior using a relatively brief measure may be overly broad. Our use of trajectory-based and dimensional approaches enabled us to examine associations for both a discrete group of “persistently low prosocial” children, as well as across a broader continuum of severity. Although the SDQ’s prosocial scale has been shown to robustly associate with psychosocial and functional outcomes (Meehan, Maughan, & Barker, 2019), it may be too nonspecific to reliably detect a unified underlying biological substrate. Moreover, prosocial behavior is increasingly viewed as a multi-faceted phenotype (Van Uzendoorn & Bakermans-Kranenburg, 2014) that incorporates, and interacts with, a range of motivational, cognitive, and affective processes, including altruism, perspective-taking, Theory of Mind, and empathic concern, as well as contextual factors (e.g., Imuta, Henry, Slaughter, Selcuk, & Ruffman, 2016; Preckel, Kanske, & Singer, 2018; Van der Graaff, Carlo, Crocetti, Koot, & Branje, 2018). Although it has been suggested that genetic associations with broader measures of constructs tend to be stronger (Dawson et al., 2002; Thapar et al., 2006), phenotypic heterogeneity within the measure, such as helping versus comforting (Paulus, Kuhn-Popp, Licata, Sodian, & Meinhardt, 2013), may differ in their developmental course and epigenetic patterns. Therefore, a measure capturing more granular components of this multidimensional construct may better elucidate specific associations with individual biological markers.

Fourth, we chose to focus on the potential effects of DNAm at birth (cord blood). This was informed by several previous epigenetic studies in birth cohorts, including ALSPAC and Generation R, which have found prospective associations between DNAm at birth (vs. later in childhood) and outcomes related to prosocial behavior such as CU traits (Cecil et al., 2014), conduct problems (Cecil et al., 2018), and social communication deficits (Rijlaarsdam et al., 2021). Furthermore, DNAm patterns at birth—but not in childhood—may mediate risk for psychiatric problems in later life; for example, DNAm alterations at birth may impact early neurodevelopment and downstream phenotypes that persist despite changes in DNAm patterns per se. Given our prospective study design, this focus on birth DNAm also allowed us to disentangle the directionality of observed effects (i.e., examining DNAm pre-manifestation of prosocial behavior), while also maximizing the available analytic sample. However, we cannot exclude the possibility of time-specific associations later in life. Of particular note, prosocial development is influenced by environmental processes in early childhood, most notably parental socialization (Eisenberg, Spinrad, & Knafo-Noam, 2015; Paulus, 2014), whose effects may not be reflected in DNAm at birth, but could engender changes in a child’s epigenetic profile that are only evidenced later in development. For example, Barker, Cecil, et al. (2018) found that postnatal environmental adversity was associated with age-7 DNAm, which then affected a subsequent vulnerability for internalizing problems in late childhood. It will be of interest in the future to utilize repeated measures of DNAm to explore potential associations with postnatal environmental influences and capture dynamic changes across the lifespan.

DNAm is also highly tissue-specific (Davies et al., 2012; Dempster et al., 2014) and, like other epigenetic studies, our ability to detect effects for brain-related behavior traits may therefore be limited by a reliance on blood-based samples. Although blood samples can be reflective of DNAm in other tissue-relevant samples, including the brain (Hannon, Lunnon, Schalkwyk, & Mill, 2015; Qi et al., 2018), with some epigenetic findings successfully replicated across tissues (Kaminsky et al., 2012), the extent to which these reflect changes in the brain, as well as the functional consequences of these changes for gene expression, remains unclear. Incorporating additional tissue sources (e.g., blood, brain, and saliva) may better reveal common and differential patterns of methylation in relation to low prosociality; however, this is often not feasible in population-based cohorts. Finally, the 450k array only covers 1.7% of the total number of CpGs in the human genome, and selected CpG sites are substantially biased toward promoter and gene body regions, which limits the capacity to detect potential associations between DNAm at other, unmeasured, CpGs with prosocial behavior.

Although not replicated in other cohorts, we note that one site (cg03436478), annotated to SGCE and PEG10 genes, showed significant differences in birth DNAm between chronic-low and typical prosocial trajectories in ALSPAC, as well as the same direction of effect across all cohorts. Both SGCE and PEG10, as imprinted genes, are highly expressed during embryonic development, which is a crucial period for establishing and maintaining methylation profiles (Kainz et al., 2007). With specific regard to mental health, SGCE has previously been linked to anxiety, mood disorders, and schizophrenia in genetic studies (Lam et al., 2019; Peall et al., 2013), while DNAm in the vicinity of PEG10 has been related to maternal depression during pregnancy (Liu et al., 2012) and increased risk for child maltreatment (Yang et al., 2013). Furthermore, differential methylation in these two imprinted genes has been associated with neurobehavioral outcomes in a sex-specific manner; increased methylation of SGCE/PEG10 was associated with increased risk of atypical behavior in boys, but decreased risk in girls (House et al., 2018). In this study, we did find evidence of sex differences between prosocial trajectories; specifically, boys were more prevalent in the chronic-low prosocial groups and showed lower prosocial behavior at age 6–7 years. However, due to the size of our analytic sample(s), we had insufficient power to perform epigenome-wide stratified and interaction analyses by sex. In future research with larger samples, it would be worthwhile to analyze whether DNAm was differentially associated with prosocial behavior between boys and girls.

Finally, our findings did not replicate candidate genes previously implicated in prosocial-related phenotypes, namely OXTR. One previous ALSPAC study reported prospective associations between a higher level of OXTR DNAm at birth and adolescent CU traits at age 13 (Cecil et al., 2014). Of note, while CU traits are strongly correlated with lower levels of prosocial behavior (Barker, Oliver, Viding, Salekin, & Maughan, 2011), recent factor analyses have shown that
the broader "low prosocial" phenotype denotes some unique variance over and above that shared with CU traits, and is therefore likely to be characterized by a distinct pathophysiological profile (Meehan, Hawes, Salekin, & Barker, 2019).

In summary, this study is the first to examine the association between 450k genome-wide DNAm at birth and childhood prosocial behavior, using data from four independent longitudinal birth cohorts. We also found no differentially methylated positions or regions that were associated with low prosocial behavior at an epigenome-wide significant threshold (based on examination of longitudinal trajectories and dimensional "low prosocial" scores at a single time-point). Together these findings suggest that, at least at birth and based on the current sample size, there is no robust evidence for a prospective association between birth DNAm and childhood prosocial behavior. Overall, the current study highlights the importance of multi-cohort approaches to replicate epigenetic associations and reduce the risk of false positive discoveries (Rijlaarsdam et al., 2016). Future research with larger sample sizes, more detailed phenotype definitions, and additional epigenetic time-points will help to clarify the relation between DNAm and low prosocial behavior in childhood.

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CONFLICT OF INTEREST
All named authors report no conflict of interests.

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