

Epigenome-wide Association Study of Attention-Deficit/Hyperactivity Disorder Symptoms in Adults

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ABSTRACT

BACKGROUND: Previous studies have reported associations between attention-deficit/hyperactivity disorder symptoms and DNA methylation in children. We report the first epigenome-wide association study meta-analysis of adult attention-deficit/hyperactivity disorder symptoms, based on peripheral blood DNA methylation (Infinium HumanMethylation450K array) in three population-based adult cohorts.

METHODS: An epigenome-wide association study was performed in the Netherlands Twin Register ($N = 2258$, mean age 37 years), Dunedin Multidisciplinary Health and Development Study ($N = 800$, age 38 years), and Environmental Risk Longitudinal Twin Study ($N = 1631$, age 18 years), and results were combined through meta-analysis (total sample size $N = 4689$). Region-based analyses accounting for the correlation between nearby methylation sites were also performed.

RESULTS: One epigenome-wide significant differentially methylated position was detected in the Dunedin study, but meta-analysis did not detect differentially methylated positions that were robustly associated across cohorts. In region-based analyses, six significant differentially methylation regions (DMRs) were identified in the Netherlands Twin Register, 19 in the Dunedin study, and none in the Environmental Risk Longitudinal Twin Study. Of these DMRs, 92% were associated with methylation quantitative trait loci, and 68% showed moderate to large blood-brain correlations for DNA methylation levels. DMRs included six nonoverlapping DMRs (three in the Netherlands Twin Register, three in the Dunedin study) in the major histocompatibility complex, which were associated with expression of genes in the major histocompatibility complex, including *C4A* and *C4B*, previously implicated in schizophrenia.

CONCLUSIONS: Our findings point at new candidate loci involved in immune and neuronal functions that await further replication. Our work also illustrates the need for further research to examine to what extent epigenetic associations with psychiatric traits depend on characteristics such as age, comorbidities, exposures, and genetic background.

Keywords: ADHD, CAARS, DNA methylation, Epigenetic, EWAS, Meta-analysis

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Attention-deficit/hyperactivity disorder (ADHD) is among the top-ranking psychiatric diagnoses in children and adults (1) and appears to reflect the extreme end of a continuous distribution of ADHD symptoms in the population (2–4). Genetic association studies have identified common and rare variants associated with ADHD (4–6). Numerous environmental risk factors have been reported, including prenatal and perinatal factors (7) [birth weight (8), prenatal exposure to maternal smoking (9), toxins (7), maternal fever and infections (10)] and postnatal factors [childhood maltreatment (11), current stress (11), infections (12)]. Recent studies have investigated if DNA methylation levels, which regulate gene expression, are associated with ADHD (13–25). DNA methylation may represent a

marker that captures the cumulative effects of genetic variants, stochastic effects, and environmental exposures (26,27) associated with a trait. Several prenatal exposures, including maternal famine (28), maternal folate (29), and maternal smoking (30), and later life exposures, for example, smoking (31), have been associated with stable long-term changes in DNA methylation in blood and other peripheral tissues. Importantly, DNA methylation patterns are largely tissue specific. ADHD symptom level-associated DNA methylation differences in peripheral tissues such as blood are likely to reveal epigenetic consequences of differential life conditions that correlate with ADHD (biomarker of exposures). The extent to which DNA methylation in peripheral tissues is informative

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about epigenetic mechanisms that contribute to interindividual differences in ADHD symptoms or that correlate with causal epigenetic mechanisms in other tissues is unknown. It has been reported that DNA methylation levels in blood correlate to a limited extent with methylation levels in other tissues, including the brain (32–35). One explanation for such correlations is that methylation quantitative trait locus (mQTL) effects correlate to some extent across tissues, as was recently demonstrated for *cis* mQTL effects in blood and brain (36).

Several candidate gene studies, most of limited sample size (mean 192; range, 82–426), have reported associations between ADHD symptoms in children and DNA methylation in cord blood (13), peripheral blood (14,18,20), buccal samples (22), and saliva (19,20). Other candidate approaches in small samples have reported relationships between DNA methylation, ADHD symptoms, and environmental risk factors (15,21,23). To date, three epigenome-wide association studies (EWASs) of ADHD (symptoms) in children have been published (16,19,24). The first study measured DNA methylation in saliva in a small group of boys with ADHD and control subjects (age 7–12 years, sample size = 112) and reported genes with suggestive evidence for association (19). The second study measured DNA methylation in 384 cord blood samples and found no significant differences between children who later received a diagnosis of ADHD and controls (24). The largest EWAS (817 children in a population-based study in the United Kingdom) identified 13 loci, where DNA methylation level in cord blood was significantly predictive of ADHD symptom trajectories between age 7 and 15 years (16); among those was *ST3GAL3*, one of the significant loci from the genome-wide association study (GWAS) of ADHD (4). As both DNA methylation and ADHD symptoms change with age, it is unknown if findings in children persist in adulthood. Moreover, the robustness of associations between ADHD symptoms and DNA methylation remains to be investigated in larger studies of multiple cohorts.

We report the first EWAS meta-analysis of ADHD symptoms in adults; this is also the largest EWAS of ADHD symptoms to date. We assessed the association between whole-blood DNA methylation and ADHD symptoms in three population-based cohorts: the Netherlands Twin Register (NTR), the Dunedin Multidisciplinary Health and Development Study from New Zealand, and the Environmental Risk (E-Risk) Longitudinal Twin Study from the United Kingdom. In secondary analyses, we 1) tested if CpGs with a lower, albeit nonsignificant, *p* value showed enrichment for loci previously identified in GWASs of psychiatric disease or in EWASs of psychiatric phenotypes or exposures; 2) analyzed differentially methylated regions (DMRs) to examine the evidence for small methylation differences at multiple nearby CpGs; and 3) examined the relationship between DNA methylation and RNA transcript levels in blood, the effects of mQTLs, and the correlation between DNA methylation in blood and brain to facilitate the biological interpretation of findings.

METHODS AND MATERIALS

Overview

The EWAS was performed in three cohorts (Figure 1): NTR (37) (*N* = 2232 individuals from twin families), Dunedin study (38)

(*N* = 800 unrelated individuals), and E-Risk (39) (*N* = 1631 twins). Results were combined in a meta-analysis to identify differentially methylated positions (DMPs) and to examine heterogeneity of effects across cohorts. Next, we performed secondary analyses. Genome-wide meta-analysis test statistics were used to test for enrichment of ADHD EWAS signals in loci detected in previous GWASs and EWASs of relevant traits. We performed DMR analyses in each cohort, compared results across cohorts, and performed a meta-analysis-based DMR analysis. Functional follow-up analyses were performed on top DMPs from the meta-analysis (nominal *p* value < 1.0×10^{-5}) and on significant DMRs. Further details about the analyses are provided in Supplement 1.

Ethical Permission

The study protocol was approved by the institutional ethical review boards of the participating universities. Study members gave informed consent before participating. Written informed consent was obtained from all participants.

ADHD Symptoms

The EWAS in NTR was performed on the Conners Adult ADHD Rating Scale (CAARS) index (total ADHD symptoms) (Figure S1A in Supplement 1) (40). Sensitivity analyses were performed on DSM-IV-based CAARS inattention and hyperactivity subscales, available for 1846 samples. In the Dunedin study, DSM-5 ADHD symptoms (Figure S1B in Supplement 1) were assessed based on private structured interviews as described previously (41) at age 38 years. In the E-Risk study, DSM-5 ADHD symptoms (Figure S1C in Supplement 1) were assessed based on private structured interviews as described previously (42) at age 18 years.

Peripheral Blood DNA Methylation

DNA methylation was assessed with the Infinium Human-Methylation450K BeadChip Kit (Illumina, Inc., San Diego, CA). Normalization was performed with functional normalization in NTR (43), with methylumi in the Dunedin Study, and with dasen in E-Risk (44). The following probes were removed from all cohorts: sex chromosomes; probes with a single nucleotide polymorphism (SNP) within the CpG site (at the C or G position), regardless of minor allele frequency, based on the Genome of the Netherlands reference population (45); probes with common (>5% minor allele frequency) SNPs within 10 bp of the single base extension site (46); and ambiguous mapping probes with an overlap of at least 47 bases per probe (47). Only methylation sites that were present in all cohorts were kept in the analysis, leaving 394,194 sites.

Statistical Analyses

Epigenome-wide Association Study. The association between DNA methylation levels and ADHD symptoms was tested for each site under a linear model (Dunedin) or generalized estimating equation model accounting for relatedness of twins and other family members (NTR and E-Risk); methylation β value was assessed as outcome, and the following predictors were used: ADHD symptoms, sex, smoking status, white blood cell percentages, age at blood sampling (only in NTR), cohort-specific technical covariates (i.e., sample plate

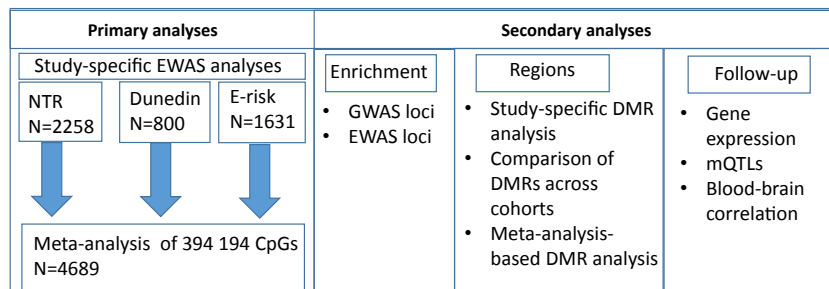


Figure 1. Summary of analyses of epigenome-wide association study (EWAS) in three cohorts: Netherlands Twin Register (NTR), Dunedin Multi-disciplinary Health and Development Study from New Zealand, and Environmental Risk (E-Risk) Longitudinal Twin Study. DMR, differentially methylated region; GWAS, genome-wide association study; mQTLs, methylation quantitative trait loci.

and array row or principal components based on control probes), and principal components based on genome-wide SNPs. Analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria). The R package Bacon was used to compute the Bayesian inflation factor (48).

Meta-analysis. A p value-based fixed-effects sample size-weighted meta-analysis was performed in METAL (49). The sample size-weighted method was chosen because of the differences in measurement scales of ADHD symptoms across studies. Statistical significance was assessed considering Bonferroni correction for the number of methylation sites tested ($\alpha = .05/394194 = 1.3 \times 10^{-7}$). False discovery rate Q values are presented in Supplemental Tables S1 through S14 in Supplement 2. The I^2 statistic provided by METAL was evaluated to assess heterogeneity.

Inattention and Hyperactivity. For top DMPs, sensitivity analyses were performed in which the association of these DMPs with inattention and hyperactivity/impulsivity subscales was tested in NTR.

Overlap With EWAS and GWAS Loci. Enrichment analysis was performed to examine whether CpGs in or near loci detected by GWASs or top-ranking CpGs from previous EWASs, on average, showed a stronger association with ADHD symptoms than other genome-wide Infinium Human-Methylation450K methylation sites. We considered the most recent GWASs for ADHD (4), major depressive disorder (50), schizophrenia (51), and autism spectrum disorder (52) and the largest available EWASs on ADHD symptoms in children (16), schizophrenia (53), smoking (31), and maternal smoking (30).

Differentially Methylated Regions. We used the python module comb-p (54) to identify regions where multiple correlated methylation sites show evidence for association with ADHD symptoms. We report significant regions (Sidák $p < .05$) with at least two methylation sites within a 500-bp window. Comb-p was applied in each of the three cohorts separately and on the meta-analysis results.

Functional Follow-up Analyses. In follow-up analyses, previously published datasets were used to test if DNA methylation level at top DMPs and at CpGs within significant DMRs were associated with whole-blood gene expression level in *cis* (55) and with whole-blood mQTLs (55) and to

examine the correlation between DNA methylation in blood and four brain regions (prefrontal cortex, entorhinal cortex, superior temporal gyrus, and cerebellum) (34).

Power Analysis. Power analyses are described in Supplement 1.

RESULTS

Meta-analysis

Demographic information of the cohorts is provided in Table 1. Genome-wide EWAS test statistics from each cohort showed no inflation (Figure S2 in Supplement 1; Table S1 in Supplement 2). One significant DMP ($\alpha = 1.3 \times 10^{-7}$) was detected in Dunedin (cg26197679, chromosome 8 intergenic) and none in NTR and E-Risk. Meta-analysis of the three cohorts ($N = 4689$) detected no significant DMPs, and meta-analysis test statistics showed no inflation (Figure S2 in Supplement 1; Table S1 in Supplement 2). Summary statistics for genome-wide methylation sites are provided in Table S2 in Supplement 2. Top DMPs with a nominal p value ($p < 1.0 \times 10^{-5}$) are presented in Table 2. Two top DMPs showed a negative relationship with ADHD symptoms (cg26197679, intergenic, and cg23144852, gene body *OPA1*), and one DMP showed a positive relationship with ADHD symptoms in all cohorts (cg10984962, gene body *AGAP1*). The effect sizes for these DMPs in the individual cohorts are presented in Table S3 in Supplement 2. In NTR, an increase of 1 SD in CAARS ADHD symptoms (3.9 points) was associated with a methylation change of -0.04% (cg26197679), -0.16% (cg23144852), and 0.10% (cg10984962).

Heterogeneity of Top DMPs

One top DMP (cg26197679) displayed large between-study heterogeneity ($I^2 = 93.6\%$, heterogeneity $p = 1.6 \times 10^{-7}$). This DMP showed the strongest association with ADHD symptoms and was epigenome-wide significant in the Dunedin study, where an increase of 1 SD in DSM-5 ADHD symptoms was associated with a methylation change of -0.62% . Although the effect size was weaker in the other cohorts, the direction of association was the same in all cohorts (Table 2). Inspection of all top-ranking sites from the individual cohorts ($p < 1.0 \times 10^{-5}$; 14 sites) revealed that all sites were characterized by substantial between-study heterogeneity (mean $I^2 = 87.2\%$; range, 71.3% – 93.8%) (Table S4 in Supplement 2). Nine sites (64.3%) showed the same direction of effect across all cohorts.

Table 1. Cohort Characteristics

Characteristic	Cohort		
	NTR (N = 2258 ^a)	Dunedin Study (N = 800)	E-Risk Study (N = 1631)
CAARS ADHD Index	7.9 (3.9)	–	–
DSM-5 Adult ADHD Symptoms	–	1.5 (2.3)	5.8 (4.3)
Age at Blood Sampling, Years	37.3 (12.9)	38 (0)	18 (0)
Sex, Female	1549 (69.0%)	392 (49.0%)	817 (50.1%)
Current Smoker ^b	438 (19.4%)	162 (20.3%)	372 (22.8%)
Former Smoker	532 (23.6%)	233 (29.1%)	–
Never Smoked	1288 (57.0%)	405 (50.6%)	–
Nonsmoker ^b	–	–	1259 (77.2%)

Values are presented as mean (SD) or *n* (%).

ADHD, attention-deficit/hyperactivity disorder; CAARS, Conners Adult ADHD Rating Scale; NTR, Netherlands Twin Register.

^aIn NTR, 2258 samples from 2232 individuals were included (for 26 individuals, two longitudinal DNA samples were included).

^bIn E-Risk, the smoking variable that was included as covariate in the analyses and that is presented in this table is current daily smoking at age 18.

Inattention and Hyperactivity

In sensitivity analyses, the association with inattention and hyperactivity/impulsivity subscales, separately, was tested in NTR for the three top DMPs from the meta-analysis. This analysis showed that the direction of effect and the strength of the association with each of the subscales was highly similar (Table S5 in Supplement 2).

Enrichment of EWAS and GWAS Loci

In testing for overlap of our EWAS meta-analysis results with findings from previous EWASs and GWASs, we failed to observe enrichment of CpGs previously associated with longitudinal ADHD trajectories in children (16) or schizophrenia in adults (53); CpGs associated with individual (31) or maternal smoking (30); and CpGs near GWAS loci for ADHD, autism spectrum disorder, major depressive disorder, or schizophrenia (Table S6 in Supplement 2). Methylation sites previously associated with schizophrenia (53) showed a small but significant depletion of signal for ADHD symptoms.

Differentially Methylated Regions

In NTR, six significant DMRs were identified, which spanned from 164 to 848 bp and included 3 to 32 CpGs (Table 3;

Table S7 in Supplement 2). One example is illustrated in Figure S3 in Supplement 1. In the Dunedin study, 19 significant DMRs were identified, spanning from 2 to 51 CpGs within regions of 18 to 1818 bp (Table 3; Table S7 in Supplement 2). In E-Risk, no significant DMRs were identified. Six distinct DMRs were detected in the major histocompatibility complex (MHC) region (chromosome 6): three in NTR and three in the Dunedin study. In line with the heterogeneity of DMP results across cohorts, none of the DMRs detected in NTR and Dunedin overlapped, and DMR analysis on the meta-analysis of the three cohorts did not detect significant DMRs.

Significant DMRs did not overlap with CpGs from the previous EWAS of ADHD trajectories in children (16) or with schizophrenia in adults (53). One of the six DMRs identified in NTR (chromosome 6: 33245460 to 3324630) contained CpGs previously associated with smoking (12 of the 32 CpGs) and maternal smoking (3 CpGs). Five of the 19 DMRs identified in the Dunedin study contained CpGs associated with smoking or maternal smoking (Table S7 in Supplement 2).

Several DMRs were located in proximity of SNPs associated with schizophrenia (51). Of the DMRs in NTR, two were located within 100 kb and one was located within 1 Mb of schizophrenia-associated SNPs, respectively (all in the MHC region). Five DMRs in the Dunedin study were located within 1 Mb of schizophrenia-associated SNPs (two on chromosome 6, two on chromosome 7, and one on chromosome 15). None of the DMRs was located within 1 Mb of significant GWAS loci for ADHD, major depressive disorder, or autism spectrum disorder.

Gene Expression in *cis*

To examine potential functional consequences of top DMPs and DMRs, we used previously published data on whole-blood DNA methylation and RNA sequencing (*n* = 2101 samples). Whereas DNA methylation levels at the ADHD symptom level top DMPs were not associated with RNA levels of genes in *cis*, methylation levels at CpGs within five of the six significant DMRs detected in NTR were associated with expression levels of 14 genes (Table 3; Table S8 in Supplement 2). At one DMR, higher methylation level correlated with lower expression; at another DMR, higher methylation level correlated with higher expression; and at three DMRs, expression of some genes correlated positively and others negatively with methylation level. Of the 19 DMRs identified in the Dunedin study, the methylation levels at CpGs within three DMRs were associated with expression levels of seven genes (Table 3; Table S9 in Supplement 2). At two DMRs, a higher methylation level correlated with lower expression, and at one DMR, a higher methylation level correlated with higher expression.

Table 2. Top DMPs From EWAS Meta-analysis

cglID	CHR	Position ^a	Gene	Location	Nearest Gene	Weight ^b	Z Score	<i>p</i> Value	Direction
cg26197679	8	142310085	–	Intergenic	LINC01300	4689	–4.9	1.1×10^{-6}	---
cg23144852	3	193405999	OPA1	Gene body	OPA1	4684	–4.8	1.7×10^{-6}	---
cg10984962	2	236462202	AGAP1	Gene body	AGAP1	4688	4.5	9.7×10^{-6}	+++

CpGs with a *p* value < 1.0×10^{-5} are shown.

CHR, chromosome; DMPs, differentially methylated positions; EWAS, epigenome-wide association study; +, positive direction of effect; –, negative direction of effect.

^aGenome build Hg19 (build 37).

^bTotal sample size in the meta-analysis.

Table 3. Significant DMRs Associated With ADHD Symptoms

CHR	Start	End	Probes (n) ^a	Region Size (bp)	Region Sidák <i>p</i> Value	Genes ^b	Gene Expression Associated With CpGs
Netherlands Twin Register							
6	31650735	31651363	19	628	4.3×10^{-10}	–	LY6G5C, DDAH2, AIF1, C6orf48, LST1, VWA7, HSPA1L, PRRC2A, MICB
11	67417958	67418406	11	448	7.6×10^{-9}	ACY3	ACY3
6	31583915	31584224	4	309	1.2×10^{-6}	AIF1	AIF1, LY6G5C, C6orf48, PRRC2A, LST1, LY6G5C, VWA7, BAG6, HSPA1L
6	33245460	33246308	32	848	3.0×10^{-5}	B3GALT4	B3GALT4, RPS18
13	113613574	113613738	3	164	4.8×10^{-5}	–	–
3	48632484	48633145	11	661	2.1×10^{-4}	COL7A1	COL7A1
Dunedin Study							
6	32063394	32065212	51	1818	2.3×10^{-17}	TNXB	SKIV2L, C4B, C4A, TNXB, TNXA
5	191127	192104	12	977	4.4×10^{-11}	LRRC14B	–
19	17931932	17932370	8	438	2.3×10^{-8}	INSL3	–
20	748992	749621	8	629	4.9×10^{-8}	C20orf54	–
6	32847441	32847846	16	405	1.3×10^{-6}	PPP1R2P1	HLA-DQA
15	75019070	75019377	10	307	4.8×10^{-6}	CYP1A1	–
4	56458183	56458623	7	440	5.1×10^{-6}	PDCL2	–
11	1769152	1769646	9	494	6.8×10^{-6}	HCCA2; LOC402778; LOC402778	–
4	186732837	186733332	8	495	8.9×10^{-6}	SORBS2	–
15	90727560	90728141	9	581	5.5×10^{-5}	SEMA4B	SEMA4B
7	1250038	1250757	10	719	6.4×10^{-5}	–	–
7	2728344	2728913	4	569	8.8×10^{-5}	AMZ1	–
8	41655673	41656080	6	407	1.5×10^{-4}	ANK1	–
12	25801455	25801622	4	167	3.5×10^{-4}	IFLTD1	–
6	3247958	3248099	2	141	2.3×10^{-3}	–	–
14	96670628	96671170	11	542	3.9×10^{-3}	BDKRB2	–
20	22795685	22795775	2	90	8.7×10^{-3}	–	–
4	187985377	187985473	2	96	.02	–	–
8	1993528	1993546	2	18	.03	MYOM2	–

More detailed information on the regions is provided in Table S7 in Supplement 2. More detailed results for the association between DNA methylation levels and gene expression in *cis* is provided in Table S8 in Supplement 2 (Netherlands Twin Register) and Table S9 in Supplement 2 (Dunedin study). No significant DMRs were found in E-Risk.

ADHD, attention-deficit/hyperactivity disorder; CHR, chromosome; DMRs, differentially methylation regions.

^aTotal number of methylation sites in the window.

^bGenes mapping to CpGs in the window.

Methylation Quantitative Trait Loci

To gain insight into genetic causes of variation underlying top DMPs and DMRs, we obtained whole-blood mQTL data ($n = 3841$ samples) (55). One of the three top DMPs from the ADHD symptom meta-analysis was associated with six independent SNPs (mQTLs) in *cis* (Table S10 in Supplement 2). The majority of DMRs (92.0%; 23) was associated with mQTLs. For ADHD-associated DMRs in NTR, on average 68% of the CpGs within a DMR (range, 36.4%–92.3%) was associated with at least one mQTL SNP. A total of 164 mQTL associations were identified for NTR DMRs (76.8% were *cis* mQTLs and 23.2% were *trans* mQTLs) (Table S11 in Supplement 2), involving 59 CpGs and 55 SNPs. For ADHD-associated DMRs in Dunedin, 323 mQTL associations were identified, involving 126 CpGs and 154 SNPs (88% *cis* and 13% *trans*) (Table S12 in Supplement 2). On average, 64.9% (range, 0%–100%) of CpGs within DMRs identified in the Dunedin study was associated with one or more mQTLs. We highlight one example: a DMR on chromosome 11,

detected in NTR and associated with the expression of ACY3, was associated with *cis* and *trans* mQTLs, and the correlation structure of DNA methylation within this DMR (Figure S2 in Supplement 2) mirrored the sharing across CpGs of *trans*-mQTLs on chromosome 6 (Figure S3 in Supplement 2). Comparing the overlap of mQTLs across cohorts revealed that two DMRs were associated with a common set of *trans* mQTLs. The DMR on chromosome 11 in NTR (associated with expression of ACY3) and the DMR on chromosome 15 in Dunedin (associated with expression of SEMA4B) were both associated in *trans* with SNPs on chromosome 6: 109626965 to 109616420 (rs9374080, rs1008084, and rs9386791).

Correlation Between DNA Methylation Level in Blood and Brain

Whereas DNA methylation levels at the ADHD symptom top DMPs did not correlate significantly between blood and brain, CpGs within 4 of the 6 DMRs (66.7%) detected in

NTR (Table S13 in Supplement 2) and 13 of the 19 DMRs (68.4%) detected in the Dunedin study (Table S14 in Supplement 2) showed significantly correlated DNA methylation levels between blood and one or multiple brain regions. For all CpGs except for one, the correlation was positive (mean $r = .50$; range, $-.42$ to $.70$). The number of CpGs per DMR that showed correlated methylation levels between blood and brain ranged from 1 to 31 (mean 5.5). The DMR with the largest number of CpGs with significant blood-brain correlations (31 CpGs) was a DMR in the MHC region detected in the Dunedin study and associated with the expression level of multiple genes, including *C4A* and *C4B*. An exemplary plot of methylation levels in blood and brain for one CpG in this region (cg01337207) is provided in Figure S5 in Supplement 1.

DISCUSSION

We performed an EWAS of ADHD symptoms in three population-based adult cohorts. Our hypothesis was that DNA methylation in blood may provide insight into epigenetic consequences of life conditions that correlate with ADHD symptoms and potentially into epigenetic mechanisms that contribute to ADHD symptoms or that correlate with causal epigenetic mechanisms in the brain. In the Dunedin study, we identified one significant DMP where a higher methylation level correlated with fewer ADHD symptoms (cg26197679, chromosome 8 intergenic). This CpG as well as other top-ranking CpGs from individual cohorts showed considerable heterogeneity, and no significant DMPs were detected in a meta-analysis of the three cohorts. In secondary region-based analyses, we tested if ADHD symptoms were associated with methylation differences at multiple nearby CpGs that individually failed to reach epigenome-wide significance. We identified six significant DMRs in NTR, 19 in the Dunedin study, and none in E-Risk. In line with the heterogeneity of DMP results, none of the DMRs overlapped across cohorts, and the meta-analysis-based DMR analysis did not detect significant DMRs. With respect to effects of differential exposures, although the EWAS signal showed no significant enrichment for CpGs previously associated with smoking, some DMRs contained CpGs previously associated with smoking (31) or maternal smoking (30), even after we adjusted for smoking. There are several possible explanations for this finding (56): 1) residual confounding effects of smoking; 2) second-hand smoking exposure, including maternal prenatal smoking; 3) exposures other than smoking; for instance, ADHD is associated with general substance use (57); and 4) these CpGs are connected to a shared underlying biology of ADHD symptoms and smoking. Importantly, the current study shows that effects of mQTLs and correlated methylation levels between blood and brain exceed effects of smoking: 92% of DMRs were associated with genetic variants, 68% of DMRs showed correlated methylation levels between blood and brain regions, and 24% of DMRs were associated with smoking. These observations suggest that interindividual differences in DNA methylation at these DMRs are not merely driven by lifestyle differences associated with ADHD symptoms, such as smoking, and that some of the methylation differences in whole blood associated with ADHD symptoms may be a marker for methylation variation in the brain.

Some top DMPs and top DMRs mapped to genes that have been previously linked to psychiatric disorders or implicated in brain biology. These are potential candidates for being involved in the underlying biology of ADHD symptoms, provided that these loci also show symptom-associated differences in epigenetic regulation in the brain. For instance, cg10984962, the third-ranking CpG from the meta-analysis, is located in *AGAP1*, which encodes a protein involved in endosomal trafficking. In neuronal cells, it plays a role in the recycling of muscarinic acetylcholine receptors (58) and was shown to influence dendritic spine morphology (59). Yet, methylation levels in blood did not correlate with methylation levels in the brain at this CpG. Six significant DMRs in distinct subregions of the MHC were identified in NTR and the Dunedin Study. The top DMR in MHC in the Dunedin study was associated with expression levels of *SKIV2L*, *C4B*, *C4A*, *TNXB*, and *TNXA*. CpGs in this DMR were not associated with smoking (31). The *C4* genes are of great interest, as they have been previously implicated in functional effects of schizophrenia-associated SNPs in the MHC on postnatal synaptic pruning (60). At many CpGs in this DMR, DNA methylation levels in blood showed moderate to strong correlations with DNA methylation levels in multiple brain regions. However, as the DMRs did not replicate across cohorts, it remains to be established whether they are relevant to ADHD symptom levels.

The lack of overlap of our findings with CpGs from a previous study, in which methylation level in cord blood significantly predicted ADHD trajectories in childhood (16), could indicate that epigenetic associations relevant to ADHD symptoms are age specific [which has already been described for genetic contributions to ADHD symptoms (61)]. The loci detected in cord blood also did not show an association with ADHD symptoms when methylation was assessed at age 7 years in the previous study (16). Age-specific epigenetic associations may also potentially explain a lack of overlap with GWAS loci. Whereas our EWAS included only adults, the GWAS of ADHD contained mainly children (4).

This is the first EWAS of ADHD symptoms in adults, the largest epigenetic study of ADHD symptoms to date, and the first study of ADHD symptoms to apply a multicohort approach. This study also has limitations. Although all cohorts included adults and applied continuous measures of ADHD symptoms reported by the same informant (self-report), there were also differences between cohorts that may have reduced power. First, CAARS was used in NTR, which is based on DSM-IV, whereas DSM-5 symptoms were assessed in the E-Risk and Dunedin study. Second, ADHD symptoms were assessed by a self-report scale in NTR and by structured interviews in the Dunedin study and E-Risk. Third, DNA methylation measurements, processing of data, and quality control were performed separately in each cohort, based on quality control and normalization pipelines that were optimized for each cohort. Fourth, cohorts varied in age and reported number of ADHD symptoms. NTR included a broad age range (mean age 37 years), whereas participants from the E-Risk study were young adults (age 18 years), and the age of participants from the Dunedin study was 38 years. The prevalence of ADHD in these cohorts, based on the instruments used in the current study, has been previously

reported: 7% in NTR (62), 3% in the Dunedin study (42), and 8% in E-Risk (43). Fifth, the cohorts were from different countries, and it is possible that epigenetic differences exist between cohorts owing to differences in genetic background or in the presence and frequency of environmental exposures.

We performed a power analysis for the three meta-analysis top DMPs, based on their effect size observed in NTR (Figure S6 in Supplement 1). The effect sizes of these three DMPs ranged from 0.09% to 0.92% explained variance. For the top DMP that showed the least heterogeneity (cg10984962; *AGAP1*; variance explained 0.28% in NTR), the required sample size to achieve 80% power is 13,508. Similar to our population-based EWAS of ADHD symptoms, a recent blood-based EWAS meta-analysis of cognitive abilities identified only a few significant sites for most cognitive measures in >6000 individuals (63).

Several limitations apply to epigenetic epidemiology studies, including ours. First, the HumanMethylation450K array captures only approximately 1.7% of all CpGs in the genome, and the removal of methylation probes that overlap with genetic polymorphisms, albeit inevitable because these polymorphisms compromise the quality of microarray-based measurements of DNA methylation, may limit the ability to detect DNA methylation-mediated SNP effects on the phenotype. Second, blood is unlikely to provide a complete picture of ADHD-related epigenetic processes because epigenetic markers are largely tissue specific. Yet, methylation changes in blood have already been associated with ADHD symptoms (16), other psychiatric conditions (53), cognition (63), and hippocampal volume (64) in earlier studies. Furthermore, we found that several of the DMRs detected in blood contain CpGs with correlated methylation levels across blood and brain. Finally, a general constraint in epigenetic studies is that trait-associated variation in DNA methylation may arise secondary to trait development (reverse causality) or may be a marker of trait-associated environmental exposures (lifestyle; medication use; infections; and early life environmental factors, such as nutrition and maternal smoking). These associations nevertheless may provide valuable insight into the underlying biological changes associated with ADHD and its risk factors. The (direction of) causality may be addressed in human studies, for example, with longitudinal study designs or Mendelian randomization (65).

In conclusion, by performing an EWAS of ADHD symptoms in three population-based adult cohorts, we found no significant sites in an EWAS meta-analysis of 4689 individuals and observed considerable heterogeneity of effects across cohorts. We found several significant cohort-specific DMPs and DMRs, with the MHC region emerging in two cohorts. The significance of these findings is unknown, but they may point at new candidate pathways awaiting further replication. Larger studies are necessary to identify methylation sites in whole blood that are robustly associated with population-based ADHD symptoms in adults. Our findings also illustrate the need for further research to examine to what extent epigenetic associations with psychiatric traits depend on characteristics such as age, sex, lifetime exposures, genetic background, symptom severity, and comorbidities.

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The HumanMethylation450K BeadChip and RNA sequencing data from the BIOS Consortium are available in the European Genome-phenome Archive, under accession code EGAD00010000887. Data from the Dunedin Multidisciplinary Health and Development are available via a managed access system (contact: ac115@duke.edu). The HumanMethylation450K BeadChip DNA methylation data from the E-Risk study are available in Gene Expression Omnibus under accession number GSE105018.

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