DNA methylation associated with persistent ADHD suggests TARBP1 as novel candidate

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ABSTRACT

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by age-inappropriate symptoms of inattention and/or hyperactivity and impulsivity. ADHD is highly prevalent in childhood and often persists into adulthood. Both genetic variants and environmental factors play a role in the onset and persistence of ADHD, and epigenetic changes, such as DNA methylation are considered as a link for their interplay. To investigate this, we studied DNA methylation in 37 candidate genes by performing targeted bisulfite sequencing of DNA isolated from whole blood of N = 91 unaffected individuals (mean age 34.2 years). Differentially methylated sites were assessed by generalized linear models testing ADHD status and ADHD symptoms, accounting for a methylation-based smoking score, age, sex, and blood cell count. DNA methylation of single sites within DRD4 and KLDR1 was associated with adult ADHD status, and multiple DNA methylation sites within TARBP1 were associated with ADHD symptoms in adulthood and childhood. Awaiting replication, findings of this pilot study point to TARBP1 as a new candidate gene for ADHD symptoms. Our work also stresses the need for research to further examine the effects of environmental factors, such as nicotine exposure, on epigenetic modifications associated with psychiatric traits.

1. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by age-inappropriate symptoms of inattention and/or hyperactivity and impulsivity (Faraone et al., 2015). The disorder has a high prevalence of 5% in children and often persists into adulthood, where the population prevalence is 2.8% (Fayyad et al., 2017; Polanczyk and Rohde, 2007; Simon et al., 2009). The clinical manifestation of adult ADHD may differ from that of childhood ADHD, i.e. by less obvious symptoms of hyperactivity and impulsivity, but represents a severe form of the disorder, given the lifelong impairment (Franke et al., 2018; Haavik et al., 2010).

Twin and adoption studies have shown that ADHD is highly heritable, with heritability estimates of approximately 80% in both children and adults (Faraone and Larsson, 2019). ADHD is a heterogeneous disorder with a complex, multifactorial background; multiple genetic and environmental factors and their interplay contribute to its pathophysiology. Recently, the first genetic risk factors for ADHD have been identified, implicating genes involved in neurotransmitter transport, neuronal growth, developmental processes, cell adhesion, and ion...
transport (Demontis et al., 2019; Rovira et al., 2020).

In addition to genetic risk factors, ADHD onset and persistence are also related to environmental factors (Faraone et al., 2005), such as a low birth weight (Lim et al., 2018), prenatal maternal stress (Humphreys et al., 2019; Palladino et al., 2019), and toxin exposure (Schwenke et al., 2018; Williams and Ross, 2007). Importantly, the environment can interact with the genome via epigenetic modifications, such as DNA methylation (Murgatroyd et al., 2009; Plazas-Mayorca and Vrana, 2011), which shows high sensitivity during early life and the prenatal period (Bauer et al., 2016). Up to now, the role of altered DNA methylation in ADHD has been assessed mainly through candidate gene studies (Hamza et al., 2019). In addition, first epigenome-wide association studies (EWAS) have been performed for ADHD diagnosis and population symptoms, most in relatively small cohorts of children (Mooney et al., 2020; Walton et al., 2017; Wilmot et al., 2016) and adolescents (Meijer et al., 2020). Little overlap between the findings of individual studies has been observed. Epigenetic studies focusing on adult ADHD are scarce. Few studies have targeted candidate genes for ADHD, such as norepinephrine transporters (Sugurardoottir et al., 2019), dopamine transporters (Wiers et al., 2018), and serotonin receptors (Perroud et al., 2016). A single EWAS has been performed each for ADHD symptoms in the adult general population (van Dongen et al., 2019) and on ADHD status (Rovira et al., 2020). Since twin studies suggest that the genetic contribution to ADHD changes from childhood and adulthood (Chang et al., 2013) and our earlier findings suggested epigenetic differences between remitted and persistent ADHD (Meijer et al., 2020), a more elaborate investigation of the association of DNA methylation with persistent ADHD in adults is warranted.

Here, we performed targeted bisulfite sequencing for 37 candidate genes to investigate differential DNA methylation between adults with ADHD and unaffected individuals. In contrast to EWAS, this approach provides information on the methylation level of all CpG sites within the target regions. We assessed the association of DNA methylation with both ADHD diagnostic status and hyperactivity/impulsivity and inattention symptoms.

2. Materials and methods

2.1. Samples

In this study, a subsample of N = 179 participants (N = 88 participants with persistent ADHD, N = 91 healthy controls) of the Dutch node of the International Multicenter persistent ADHD Collaboration (IMPACT) (IMPACT Consortium, 2020; Mostert et al., 2015) was used. Patients with ADHD were included if they had been previously diagnosed with ADHD by a psychiatrist according to DSM-IV criteria. A structured diagnostic interview for adult ADHD (DIVA) was performed to investigate current symptoms as well as symptoms during childhood (Kooij and Francken, 2010). Additionally, the ADHD-DSM-IV Self-Rating scale, a questionnaire on current symptoms, was filled in by participants (Kooij et al., 2005). Maternal smoking during pregnancy was assessed by the pregnancy, birth and child’s first week's questionnaire (Altink et al., 2009) filled in by the mothers of participants. Condition for the inclusion was a Caucasian background of parents and grandparents for all participants. Exclusion criteria for participants were psychosis, alcohol or substance use disorder in the last 6 months, current major depression, full-scale IQ estimate <70 (estimated from Block Design and Vocabulary of the Wechsler Adult Intelligence Scale-III), neurological disorders, sensorimotor disabilities, non-Caucasian ethnicity, and extended intake of drugs/medication other than psychostimulants or atomoxetine. An additional exclusion criterion for healthy comparison subjects was a current neurological or psychiatric disorder according to the Dutch version Structured Clinical Interview for DSM-IV Axis I disorders (SCID-I) (Groenestijn et al., 1999) and Axis II disorders (SCID-II) (Weertman et al., 1996). Blood samples were collected for biobanking of DNA, RNA, and serum (Mostert et al., 2015).

The subsample for this study was selected based on data availability (required: age, sex, IQ, medication, ADHD status, DIVA adulthood, DIVA childhood, self-report, smoking status; preferred: pregnancy, birth and child’s first week’s questionnaire), matching of the groups of ADHD patients and healthy controls for age, sex and IQ and preferred selection of smokers in the healthy control group and non-smokers in the ADHD patients group to minimize the difference in this variable.

This study was approved by the regional ethics committee (Centrale Commissie Mensgebonden Onderzoek: CMO Regio Arnhem-Nijmegen; Protocol Number III.04.0403). Written informed consent was obtained from all participants.

2.2. Candidate gene selection

A complete list of the included genes/regions and reasons for inclusion is summarized in Supplementary Table 1. Candidate genes for the present study were chosen in 2016 based on three criteria: (1) A selection of previously reported candidate genes was checked for evidence of association of DNA methylation with ADHD or ADHD symptoms in the general population, with related psychiatric disorders and/or with environmental risk factors for ADHD. (2) Selection was based on overlap between the top-findings of different previously published EWSAs of DNA methylation for ADHD diagnosis or ADHD symptoms in the general population (Walton et al., 2017; Wilmot et al., 2016), with mood disorder (Walker et al., 2016) and prenatal exposure to smoking (Bauer et al., 2016; Joubert et al., 2016). (3) Additional candidate genes were selected based on the preliminary analysis of a small pilot EWAS in patients with persistent and remittent ADHD and healthy controls (Meijer et al., 2020) and the preliminary analysis of adult ADHD symptoms (van Dongen et al., 2019). Two additional sources of evidence were used for the candidate gene selection: 1) Gene-based association of candidate genes with ADHD as derived from GWAS meta-analysis data of 9 ADHD GWAS cohorts (Demontis et al., 2019). 2) Association with psychiatric disorders and involvement in neuronal development or neuronal transduction as reported by QIAGEN Ingenuity Pathway Analysis (Krämer et al., 2014).

For control purposes, genomic regions informative of potential variables in the blood cell type count were included. To calculate the proportion of six blood cell subtypes (B cells, CD4+ T Cells, CD8+ T cells, granulocytes, monocytes, NK cells) in our samples, we used the methylation of characteristically demethylated CpG sites within promoter regions, as described previously by Bauer and colleagues (Bauer et al., 2016; Reinius et al., 2012). Since smoking is known to cause altered DNA methylation, and ADHD is associated with higher levels of smoking (Whalen et al., 2002), we included multiple regions of AHRR, a gene whose methylation levels are strongly associated with smoking (Elliot et al., 2014; Zeilinger et al., 2013). As the decrease in methylation at specific CpG sites is quantitative and shows partial recovery of methylation levels after quitting smoking (Elliot et al., 2014; Zeilinger et al., 2013), this data allowed the generation of a quantitative smoking estimate in our cohort.

Target genes were not covered entirely, rather, we focused on specific regions implicated in the associations with ADHD and related disorders, as reported in the literature (Supplementary Table 1) and/or specific promoter CpG islands as annotated in the UCSC Genome Browser in GRCh37/hg19 (Feb. 2009) (Kent et al., 2002). Chromosomal positions and primer sequences are summarized in Supplementary Table 2.

2.3. Targeted bisulfite sequencing

DNA was isolated from whole blood as previously described (Klein et al., 2015). Bisulfite treatment was performed on ~500 ng of DNA with the EZ DNA Methylation Kit (Zymo Research, Irvine, USA) according to the manufacturer’s protocol. Target regions were amplified with methylation-unspecific primers using TaKaRa EpiTaq HS polymerase
Filtering, quality control (Supplementary Figure 1-5), and statistical analyses were performed in R (v3.6.3) (R Core Team, 2020). Of all 41 selected candidate regions and genes, 39 were included in the final sequencing panel (DAT1 and ZDHHC14 excluded) and Region 2 (chr6:31650613-31651397), one of eight regions in sequencing panel (methylation levels of CpG sites known to be specifically unmethylated in Non-Methylated controls (Zymo Research) showed a mean methylation of 87% of the target region. Human Methylated and Non-Methylated controls (Zymo Research) showed a mean methylation level of 94.6% and 5.1% (Supplementary Figure 1). One sample was excluded from the analysis based on aberrant overall methylation.

To correct for variability in the composition of blood cell types, the proportion of six major cell types was investigated based on DNA methylation levels of CpG sites known to be specifically unmethylated in one of the blood cell types (Granulocytes: ACAD8; CD4+ T cells: CD28, CD8+ T cells: CD8A; Monocytes: KIAA0930; B cells: LILRB4; NK cells: KLRD1, Supplementary Table 1). Using the mean methylation level of three to four CpG sites per cell type, we performed a principal component analysis (Supplementary Figure 2) with the R factoextra package (v1.0.6) (Kassambara and Mundt, 2019), showing that variation in blood cell counts was homogenous across all samples. The first two principal components were used as covariates for further analysis, explaining 68.5% of the total variance.

An association between smoking and significant demethylation at multiple CpG sites within AHRR was confirmed in this study. DNA methylation at multiple CpG sites were found to be associated with smoking behavior (45/140 CpG sites p-value < 0.05, 29/140 CpG sites p-value < 0.001) (Supplementary Figure 3). Regions with multiple neighbouring significantly associated CpG sites were found in addition to previously described ones, which were not covered by Illumina 450k arrays in previous studies. Methylation levels of three previously reported CpG sites (cg05575921 (p-value $4.6\times10^{-11}$), cg14817490 (p-value $4.8\times10^{-09}$), cg25648203 (p-value $7.6\times10^{-09}$)) were summed up to a single quantitative smoking score, which was used as a covariate in subsequent analyses.

The R-package corrgram (v1.13) was used to generate a correlation matrix between different symptom scores for all participants of the cohort (Supplementary Figure 4) (Wright, 2018). The estimation of correlation of methylation with symptom scores was only performed in individuals with ADHD to exclude effects driven by status of diagnosis. The variability of symptom intensity was broader than in individuals without diagnosis (Supplementary Figure 5, Table 1). To test for the overlap of findings of the analysis with symptom scores as measured by DIVA and the self-report, enrichment was calculated using a Fisher’s exact test.

Analysis of differential methylation was performed using the MethyKit package (v1.12.0) in R (Akalin et al., 2012). Age, sex, and the methylation-based scores for smoking and cell count were used as covariates in all analyses.

As a sensitivity test, the robustness of results to changes in the smoking variable was investigated by applying the generalized linear model p-values were calculated using a Fisher’s exact test for categorical or a Wilcoxon signed-rank test for continuous data, Digital Intelligence Quotient (DQ) is used as estimate of IQ, a structured diagnostic interview for adult ADHD (DIVA) was used to evaluate symptom severity (range 0–9) during adulthood and during childhood and with the ADHD-DSM-IV Self-Rating scale (self-report), maternal smoking behaviour during pregnancy was assessed with the pregnancy, birth and child’s first week’s questionnaire, methylation-based score for smoking behaviour was calculated as the sum of methylation levels of three CpG sites within AHRR (cg05575921, cg14817490, cg25648203), methylation-based score for cell count is represented by the first two components of a principal component analysis using the mean methylation levels of three to four CpG sites of six different blood cell types (for additional information about methylation-based scores see Data Analysis and Quality Control and Supplementary Figure 2-3).

### Table 1

|                     | Healthy controls (N = 91) | ADHD patients (N = 88) | p-value |
|---------------------|---------------------------|------------------------|---------|
| Children, mean (SD) | 34 (37.4%)                | 34 (38.6%)             | n.s.    |
| Age                 | 35.4 (11.16), 19-57       | 32.95 (9.44), 18-53    | n.s.    |
| Digital Intelligence Quotient (DQ) mean (SD), range | 109.5 (14.46), 74.8-137.8 | 108.52 (15.96), 65.5-147.25 | n.s.     |
| Hyperactivity/impulsivity symptoms adult mean (SD), range | 0.42 (0.96), 0-6 | 7.33 (1.62), 3-9 | <2.2*10^{-16} |
| Hyperactivity/impulsivity symptoms adult mean (SD), range | 0.65 (1.10), 0-4 | 5.55 (2.43), 0-9 | <2.2*10^{-16} |
| Hyperactivity/impulsivity symptoms adult mean (SD), range | 0.51 (1.09), 0-6 | 7.26 (1.90), 1-9, N = 87 | <2.2*10^{-16} |
| Hyperactivity/impulsivity symptoms adult mean (SD), range | 0.67 (1.32), 0-6 | 5.64 (3.09), 0-9, N = 87 | <2.2*10^{-16} |
| Hyperactivity/impulsivity symptoms adult mean (SD), range | 0.59 (1.13), 0-5 | 6.29 (2.28), 0-9 | <2.2*10^{-16} |
| Hyperactivity/impulsivity symptoms adult mean (SD), range | 1.02 (1.63), 0-6 | 5.43 (2.37), 0-9 | <2.2*10^{-16} |
| Duration mean (months) | 71 (80.7%), 23.1 | (32.86) |                   |
| Maternal smoking during pregnancy mean (SD), range | - | - | - |
| Smoking self-report mean (SD), range | 1.05 (2.16), 0-9, N = 63 | 1.06 (1.94), 0-9, N = 78 | n.s.    |
| Smoking (self-report) mean (SD), range | 7 (7.7%) | 29 (33.0%) | 2.675*10^{-5} |
| Smoking (self-report) mean (SD), range | 174.3 (14.56), 119.2-193.2 | 161.1 (21.02), 105.5-195.5 | 2.77*10^{-6} |
| Smoking cell count mean (SD), range | -1.20 (7.25), -21.55-13.51/0.31 | 1.13 (11.08), -23.29-38.65/-0.33 (5.03), 9.42 | -16.78-10.53 |

p-values were calculated using a Fisher’s exact test for categorical or a Wilcoxon signed-rank test for continuous data, Digital Intelligence Quotient (DQ) is used as estimate of IQ, a structured diagnostic interview for adult ADHD (DIVA) was used to evaluate symptom severity (range 0–9) during adulthood and during childhood and with the ADHD-DSM-IV Self-Rating scale (self-report), maternal smoking behaviour during pregnancy was assessed with the pregnancy, birth and child’s first week’s questionnaire, methylation-based score for smoking behaviour was calculated as the sum of methylation levels of three CpG sites within AHRR (cg05575921, cg14817490, cg25648203), methylation-based score for cell count is represented by the first two components of a principal component analysis using the mean methylation levels of three to four CpG sites of six different blood cell types (for additional information about methylation-based scores see Data Analysis and Quality Control and Supplementary Figure 2-3).
Table 2
Differentially methylated CpG sites in TARBP1 associated with (a) ADHD diagnosis status, (b) inattention and hyperactivity/impulsivity symptoms in adulthood or childhood in adult ADHD patients, (c) results of replicative analysis of inattention symptoms in adulthood in non-smoking subgroup and (d) analysis of association with mothers smoking behaviour during pregnancy. q-values < 0.2 are shown (<0.05 bold), uncorrected p-values and methylation difference in percent; * age, sex, and the methylation-based scores for smoking and cell count were used as covariables in all analyses with methylation as model variable.

| chr | pos | gene | Meth diff [%] | p-value | q-value | Meth diff [%] | p-value | q-value | Meth diff [%] | p-value | q-value | Meth diff [%] | p-value | q-value | Meth diff [%] | p-value | q-value |
|-----|-----|------|--------------|---------|---------|--------------|---------|---------|--------------|---------|---------|--------------|---------|---------|--------------|---------|---------|
| 1   | 234613937 | TARBP1 | 0.46 | 8.60*10^-04 | 7.82*10^-02 | (A) |
| 1   | 234614008 | -0.33 | 1.22*10^-03 | 1.71*10^-01 | |
| 1   | 234614058 | 1.24 | 1.59*10^-04 | 3.08*10^-02 | (A) |
| 1   | 234614117 | 2.08 | 3.74*10^-05 | 9.65*10^-03 | (A) |
| 1   | 234614117 | 1.29 | 1.43*10^-03 | 9.72*10^-02 | (A) |
| 1   | 234614123 | 1.69 | 3.36*10^-07 | 5.20*10^-04 | (A) |
| 1   | 234614204 | 1.12 | 6.94*10^-04 | 7.54*10^-02 | (A) |
| 1   | 234614269 | 0.96 | 3.72*10^-05 | 9.65*10^-03 | (A) |
| 1   | 234614273 | 0.67 | 8.15*10^-04 | 7.82*10^-02 | (A) |
| 1   | 234614283 | 0.68 | 5.16*10^-04 | 7.54*10^-02 | (A) |
| 1   | 234614289 | 0.84 | 2.42*10^-01 | 1.39*10^-01 | (A) |
| 1   | 234614297 | 0.71 | 3.56*10^-05 | 5.60*10^-02 | (A) |
| 1   | 234614330 | 1.01 | 1.17*10^-03 | 9.50*10^-02 | (A) |
| 1   | 234614376 | 0.98 | 1.50*10^-04 | 3.08*10^-02 | (A) |
| 1   | 234614381 | 1.22 | 7.00*10^-04 | 7.54*10^-02 | (A) |
| 1   | 234614438 | -0.67 | 1.89*10^-03 | 1.92*10^-01 | (A) |
| 1   | 234614441 | -0.28 | 2.00*10^-03 | 1.92*10^-01 | |
| 1   | 234614499 | 0.61 | 1.17*10^-04 | 8.74*10^-02 | (C) |
| 1   | 234614566 | -0.34 | 3.06*10^-03 | 1.97*10^-01 | (A) |
| 1   | 234614569 | 0.71 | 3.67*10^-04 | 6.30*10^-02 | (A) |
| 1   | 234614609 | 1.09 | 8.08*10^-04 | 9.32*10^-02 | |
| 1   | 234614702 | 0.45 | | | |

(continued on next page)
Table 2 (continued)

| samples                        | (a) all (N = 179) | (b) ADHD-diagnosed (N = 88) | (c) ADHD-diagnosed, non-smoker (N = 59) | (d) all with information (N = 141) |
|-------------------------------|------------------|-----------------------------|----------------------------------------|-----------------------------------|
| model variable*               | methylation      | methylation                 | hyperactivity/impulsivity (DIVA)       | methylation                      |
| predicted variable            | ADHD status      | inattention (DIVA)          | inattention adult (DIVA)               | mothers smoking                   |
| chr  | pos | gene | Meth diff [%] | p-value | q-value | Meth diff [%] | p-value | q-value | Meth diff [%] | p-value | q-value | Meth diff [%] | p-value | q-value |
| 1    | 234614706  | 0.66 | 7.31*10^-04  | 7.54*10^-02  | (A) | 4.31*10^-02 |
| 1    | 234614800  | 1.38*10^-03  | 1.76*10^-01  | 1.69*10^-01  | (A) | 1.69*10^-01  |
| 1    | 234614832  | 2.23 | 1.32*10^-03  | 1.32*10^-03  | (A) | 1.32*10^-03  |
| 1    | 234614870  | 0.74 | 2.34*10^-04  | 1.53*10^-01  | (C) | 2.34*10^-04  |
| 1    | 234614893  | 0.79 | 2.55*10^-06  | 1.32*10^-03  | (A) | 2.55*10^-06  |
| 1    | 234614999  | 0.96 | 6.19*10^-05  | 3.33*10^-02  | (C) | 3.33*10^-02  |
| 1    | 234614924  | 2.23 | 3.86*10^-04  | 2.00*10^-02  | (A) | 2.00*10^-02  |
| 1    | 234614953  | 3.45 | 5.00*10^-04  | 2.00*10^-02  | (A) | 2.00*10^-02  |
| 1    | 234614963  | 3.36 | 6.37*10^-04  | 7.54*10^-02  | (A) | 6.37*10^-04  |
| 1    | 234614965  | 4.12 | 2.90*10^-04  | 1.53*10^-01  | (C) | 2.90*10^-04  |
| 1    | 234614967  | 6.19 | 2.10*10^-05  | 3.33*10^-02  | (C) | 3.33*10^-02  |

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model to non-smoking ADHD-affected individuals only (as reported by participants, \(N = 88\)).

To investigate the distribution of differentially methylated CpG sites across analyses (ADHD status, ADHD symptom severity during adulthood and childhood as measured by DIVA or self-report) within the TAR RNA Binding Protein 1 (TARBP1) locus, we performed a sliding window approach, summing up the logarithmic q-values in a window of ±10 bp surrounding each CpG site. Regional enrichment of associated sites results in peaks. CpG sites with higher significance levels contribute stronger to the peak’s height. Evidence for regulatory elements was based on data from the Integrated Regulation Track from Encyclopedia of DNA Elements (ENCODE) project, such as DNase hypersensitivity clusters, histone modifications, transcription factor binding and motifs (Davis et al., 2018; Encode Project Consortium, 2012; Gerstein et al., 2012; Wang et al., 2012, 2013).

Correlation between the smoking behavior of mothers during pregnancy with methylation changes in the offspring during adulthood was analyzed on all samples with available information (\(N = 141\)). Correlation of the smoking behavior of mothers with the smoking behavior of the participants was analyzed independently for ADHD patients (\(N = 78\)) and healthy controls (\(N = 63\)). Both correlation analyses were performed with polycor (v0.7-10) (Fox, 2019).

P-values were corrected for multiple testing by false discovery rate in all analyses (Benjamini and Hochberg, 1995). Sites with a q-value below 0.05 were considered significant and sites below a q-value of 0.2 were considered indicative to support significant CpG sites.

3. Results

3.1. Study cohort

Demographics of the study sample can be found in Table 1. Sex, age, and estimated IQ did not differ between participants with ADHD (\(N = 88\)) and those without (\(N = 91\)). ADHD symptoms were significantly increased in the ADHD subgroup, both during childhood and adulthood. Variability of symptoms as measured by the DIVA was broader in the participants with ADHD than in those without (Supplementary Figure 5). Smoking behavior was observed more frequently in participants with ADHD (Table 1); consistent with this, significantly decreased methylation levels were seen in participants with ADHD at 26 of 45 smoking-associated CpG sites within the AHRR gene (Supplementary Figure 3).

3.2. DNA methylation associated with persistent ADHD

The methylation levels of two single CpG sites were significantly associated with ADHD diagnostic status as measured by the DIVA after correction for multiple testing (Supplementary Table 3a). The methylation level of one CpG site within DRD4 was significantly associated with ADHD status (chr11:638421, q-value = 8.61×10\(^{-3}\), methylation difference = 4.75%). Supporting this result, eight indicative CpG sites within a region of 500 bp surrounding the significantly differentially methylated CpG site within DRD4 were weakly associated with ADHD status although not reaching significance (0.05 < q-value < 0.2) (Supplementary Table 3a). Surprisingly, the methylation of a CpG site within KLRD1 was also significantly associated with ADHD status (chr12:10459851, q-value = 8.61×10\(^{-3}\), methylation difference = 2.58%); other CpG sites within this gene had been included as a control for blood cell count, which was used as a covariate in this analysis.

3.3. TARBP1 methylation levels are associated with ADHD symptom severity and prenatal smoking

As severity of inattention and hyperactivity/impulsivity symptoms differed widely between participants with persistent ADHD (Supplementary Figure 5), we investigated the association of methylation with symptom severity scores during adulthood and childhood as measured by the DIVA in participants with an ADHD diagnosis (Table 2, Supplementary Table 3). Eight CpG sites were significantly associated with inattention scores in adulthood; all located in TARBP1 (Table 2, Fig. 1). Methylation of one CpG site within TARBP1 was also associated with retrospectively assessed inattention symptoms in childhood (Table 2). Robustness of these findings was tested by analysis of self-reported symptoms (Supplementary Table 4), which showed a phenotypic Spearman rank correlation of 0.65 with symptom scores as measured by DIVA for hyperactivity/impulsivity and of 0.60 for inattention symptoms (Supplementary Figure 4). TARBP1 CpG sites associated with inattention symptoms overlapped between the analysis of the DIVA-assessed and self-reported symptoms in participants with ADHD: three out of eight CpG sites with significant association with inattention as measured by the DIVA were also significantly associated with self-reported inattention. This constitutes a significant enrichment as compared to a random distribution (p-value = 3.07×10\(^{-11}\)).

Persistent changes in the DNA methylation have been shown to occur in the offspring of mothers having smoked during pregnancy (Bauer et al., 2016; Joubert et al., 2016). Since mothers’ smoking behavior is a known risk factor for ADHD (Kotimaa et al., 2003; Sen and Swaminathan, 2007), we investigated the association of DNA methylation levels with maternal smoking behavior during pregnancy in our cohort. We found one CpG site within TARBP1 to be significantly associated with maternal smoking status (q-value 4.31×10\(^{-2}\) (Table 2, Fig. 1); this site also showed indicative association with inattention during adulthood.

Despite the association with prenatal smoking exposure (Table 2) (Bauer et al., 2016; Joubert et al., 2016), methylation status of TARBP1 was neither found stably differentially methylated in older children after prenatal tobacco exposure (Joubert et al., 2016) nor associated with smoking behavior (Bauer et al., 2016; Zeilinger et al., 2013). Our analyses were corrected for smoking using a quantitative, methylation-based score. Smoking was not associated with reported smoking behavior of mothers in our cohort in healthy controls or ADHD patients (p > 0.05). However, smoking behavior and ADHD diagnostic status can hardly be separated (Supplementary Figure 3). To exclude that the associations with ADHD were partly driven by smoking, we applied the generalized linear model to the non-smoking individuals with ADHD only (\(N = 59\)). Six of the eight significant findings for inattention remained significant in the non-smokers (q-value < 0.05; Table 2). Also 11 of the 21 CpG sites with indicative association in the total sample (0.05 < q-value < 0.2) as well as several additional sites within TARBP1 showed significant or indicative association in the analysis of non-smoking individuals (Table 2).

In summary, 20 significant findings associated with inattention symptoms in adulthood as measured by DIVA or self-report in adulthood or during childhood were distributed across 17 unique CpG sites within TARBP1. For all these findings, methylation levels increased with symptom severity (Table 2, Supplementary Table 4). As shown in Fig. 1, all significant findings within TARBP1, 58 indicative CpG sites with q-values between 0.05 and 0.2 (across 45 unique CpG sites) as well as a CpG site associated with prenatal tobacco exposure were located within a single CpG island, upstream of and overlapping with the first exon of TARBP1. Analyzing the distribution of differentially methylated CpG sites in our analyses by running sum statistics, showed that the findings were not randomly distributed but formed clusters in four regions (Fig. 1). This effect is not solely driven by the significant sites, as demonstrated by a large overlap of peaks resulting from the same analysis with indicative sites only (0.05 < q-value < 0.2), confirming that these sites are not randomly distributed, but support biological importance of these regions.

4. Discussion

We performed targeted bisulfite sequencing across 37 candidate genes to investigate the association of their DNA methylation levels with ADHD diagnostic status and ADHD symptom severity in adults. In the
case-control analysis, we identified single differentially methylated CpG sites in the regulatory regions of both DRD4 and KLRD1. DNA methylation of multiple sites in TAR RNA Binding Protein 1 (TARBP1) was robustly associated with inattention symptoms in 88 individuals diagnosed with ADHD. TARBP1 was selected for this study based on the earlier observed association of DNA methylation with ADHD diagnostic status in children (Wilmot et al., 2016). Rare genetic variants in TARBP1 have been linked to schizophrenia (Walsh et al., 2008) and psoriasis (Tang et al., 2014), and hyperactivity/impulsivity symptoms (blue) with different shades of colours indicating symptom score measure (inattention DIVA for adulthood (light orange), inattention DIVA for childhood (yellow), inattention self-report (dark orange), hyperactivity/impulsivity DIVA for adulthood (light blue), hyperactivity/impulsivity DIVA for childhood (turquoise), hyperactivity/impulsivity self-report (dark blue)). The significant finding showing association with maternal smoking behaviour is in the same region (black star). q-values below 0.2 are shown with the dashed line indicating threshold of significance at 0.05. The lower panel shows summarization of the inverse logarithmic q-value for each CpG site ±10 bp for all CpG sites associated with ADHD status or symptom severity with a q-value <0.2 (grey peaks). Findings are enrichment in four clusters (chr1:234614068-234614123 (56 bp): 6 significant plus 11 indicative findings, chr1:234614269-234614297 (29 bp): 3 significant plus 8 indicative findings, chr1:234614893-234614899 (7 bp): 3 significant and 1 indicative findings, chr1:234614953-234614971 (19 bp): 4 significant and 8 findings). Sliding window summation of the inverse logarithmic q-value of indicative sites (0.05< q-value<0.2) shows enrichment in the same genomic regions (orange peaks), supporting non-random distribution. (Mooney et al., 2020). We found a specific association between TARBP1 and inattention, which was not being tested in previous EWASs. Moreover, the most significantly associated CpG-sites of our study were not tested in the ADHD symptom EWASs since these sites are not included in both the 450K and EPIC Illumina arrays.

Previous studies have also described TARBP1 DNA methylation to be associated with prenatal exposure to smoking (Bauer et al., 2016; Joubert et al., 2016; Li et al., 2018). We also found DNA methylation sites in TARBP1 to be associated with prenatal smoking exposure, which might be part of the molecular basis for increased susceptibility to ADHD after prenatal tobacco exposure (Huang et al., 2018; Minatoya et al., 2019). Association of the same methylation site with inattention symptoms in adulthood suggests a biological mechanism that links maternal smoking behavior and inattention symptom severity in persistent ADHD in adults. Together with the notion that blood DNA methylation patterns in TARBP1 are generally not correlated with brain DNA methylation (Hannon et al., 2015a, 2015b), this may suggest a biological mechanism that links maternal smoking behavior and inattention severity in adult ADHD via the peripheral immune system. However, the observed association of TARBP1 DNA methylation with ADHD symptom severity cannot be (fully) explained by maternal smoking during pregnancy, implicating additional underlying mechanisms.

All associated sites in TARBP1 are located within a CpG island in the beginning of the gene. This region is also characterized by a DNase hypersensitivity cluster, increased H3K27Ac histone marks, increased...
H3K4Me3 histone marks and surrounded by increased H3K4Me1 histone marks, all providing evidence that this is a promoter region and regulatory element (Davis et al., 2018; Encode Project Consortium, 2012). Furthermore, binding of numerous transcription factors in multiple cell lines was shown by ChIP-seq and transcription factor binding motifs are found within and in close proximity to the regions with enriched differential methylation (Davis et al., 2018; Encode Project Consortium, 2012; Gerstein et al., 2012; Wang et al., 2012, 2013), potentially altering binding affinity of transcription factors and thereby effecting the expression of TARBP1. Hence, our data provides evidence for TARBP1 as a novel candidate gene for adult ADHD, showing differential methylation levels in a presumably regulatory region. However, it should be taken into account that bisulfite sequencing cannot distinguish between DNA methylation and hydroxymethylation, which have generally opposite effects on gene expression levels (Kato and Iwamoto, 2014). Future studies should focus on this distinction, especially when studying brain tissue where hydroxymethylation is more abundant than blood. Thus, investigation of the consequences of altered methylation on TARBP1 expression and the resulting mechanisms leading to ADHD and related symptoms is necessary.

The dopaminergic neurotransmission system is one of the main candidates for a role in ADHD etiology (Faralone and Mick, 2010). Previous targeted DNA methylation studies of DRD4 in childhood ADHD have shown inconsistent results. In a cohort of 330 children with ADHD, increased methylation levels of DRD4 were found to be associated with increased levels of ADHD symptoms in a combined sample of saliva and blood cells (Dadds et al., 2016). A second study, performed in 426 individuals from the general population, covered the same DRD4 genomic region and found lower overall DRD4 DNA methylation levels in cord blood to be associated with ADHD symptom scores at age six years (van Mil et al., 2014). Lastly, a case-control study of 100 children reported hypermethylation of a single CpG site in the promoter region of DRD4 in whole blood in childhood ADHD (Xu et al., 2015). Ours is the first study to investigate ADHD diagnostic status and symptom severity in adult individuals, where we found hypermethylation of a different genomic region, the first intron, of DRD4 to be associated with ADHD status. This data indicates that altered methylation levels of DRD4 are not only associated with childhood ADHD (symptoms), but that those changes may persist into adulthood or even play a role in the persistence of ADHD.

We identified a second association of DNA methylation levels with ADHD diagnostic status in KLRD1. To our knowledge, we are the first to report on DNA methylation levels of KLRD1 (or CD94) in ADHD. This gene was included in our study to control for the differences in the proportion of NK cells between samples, and we cannot exclude that this result originates from insufficient control for cell proportion differences. KLRD1 is an antigen exclusively expressed on NK cells, and therefore DNA methylation levels are unique for these cytotoxic lymphocytes (Chang et al., 1995). More generally, these cells are involved in the innate immune system and rapidly detect and kill stressed (i.e. infected) cells (Vivier et al., 2011). Increased levels of KLRD1 DNA methylation might suggest reduced circulating levels of NK cells in adults with ADHD, although nothing is known about the role of NK cells in ADHD yet. A previous study showed a relationship between NK cell-mediated cytosis via DNA methylation to episodic memory and cortical thinning (Freytag et al., 2017); both traits are also related to ADHD (Krauel et al., 2007; Shaw et al., 2006). However, replication of the finding is essential before conclusions should be drawn. Although the KLRD1 gene is covered in the 450K and EPIC arrays, we cannot directly compare our findings with existing EWAS results, because our significant finding is located outside of the targeted KLRD1 region on the arrays.

Our study should be viewed in the context of its strengths and limitations. To our knowledge, this is the first study that investigated at broad coverage the role of differential DNA methylation in adult ADHD diagnostic status and symptoms severity, an underrepresented study population in the field of ADHD research. We assessed the robustness of our association findings for ADHD symptom severity from the DIVA adult ADHD diagnostic interview by also analyzing self-reported symptoms scores from the Adult Self-Reported ADHD Scale and the DIVA childhood symptom scores. This is considered a functional measure, since the DIVA childhood symptom score asks retrospectively about childhood symptoms and thereby will be biased: we expect that individuals with ADHD will recall answers differently than healthy controls. We also performed analyses in non-smoking participants with ADHD, separately to exclude smoking-driven changes in methylation. The consistency of the results for TARBP1 across all three approaches provides confidence in our findings. Information on diet was not available for this cohort, but it might be valuable for future studies to include this information, as dietary patterns are associated with ADHD (Howard et al., 2011; Jacob et al., 2018), and can have possible effects on DNA methylation (Kadayifci et al., 2018), including prenatal exposure to an unhealthy diet of the mother (Bijlstraasdam et al., 2017). Moreover, we did not correct for psychostimulant medication status, given the collinearity of medication use with case-control status and the uncertainty of the role of ADHD medication on DNA methylation (Mooney et al., 2020; Walton et al., 2017). Future studies may want to investigate the potential influences of ADHD medication on DNA methylation, preferably in a longitudinal study design. Using a targeted sequencing approach, we were able to achieve (1) broader coverage of prioritized genes and (2) almost complete coverage of DNA methylation sites within the selected regions (e.g. TARBP1: 97%). This allowed us to detect and analyze target sites that are usually missed by array-based approaches. However, we assessed DNA methylation levels in DNA extracted from blood cells as biomarkers of ADHD; we therefore cannot draw conclusions about mechanisms in the brain, considering ADHD as a brain disorder. Although attempts have been made to determine the correlation between DNA-methylation levels in blood and brain (Hannon, 2015), not all our covered CpG sites are included in this publicly available database. Lastly, our sample size was limited, and findings await replication in an independent sample of adult ADHD.

5. Conclusion

In conclusion, using a targeted bisulfite sequencing approach based on candidate genes from previous epigenetic studies in psychiatry, we investigated the role of DNA methylation, a measure influenced by genetics and the environment, in adult, persistent ADHD. We identified several significant associations of DNA methylation of CpG sites with ADHD diagnosis, ADHD symptom severity, and prenatal exposure to smoking. The findings of this pilot study suggest TARBP1 as a novel candidate gene for persistent ADHD.

Declaration of interest

Barbara Franke received educational speaking fees from Medice. Jan K Buitelaar has been in the past 3 years a consultant to/member of advisory board of/and/or speaker for Takeda/Shire, Roche, Medice, Angelini, Janssen, and Servier. He is not an employee of any of these companies, and not a stock shareholder of any of these companies. He has no other financial or material support, including expert testimony, patents, royalties. All other authors declare that there is no potential conflict of interest.

CRediT authorship contribution statement

Annika L. Weiß: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. Mandatory Meijer: Conceptualization, Investigation, Writing - original draft, Writing - review & editing. Bettina Budeus: Formal analysis, Visualization. Marc Pauper: Formal analysis. Marina Hakobian: Investigation, Johanne M. Groothuismink, Investigation. Yan Shi: Supervision. Kornelia Neveling: Investigation. Jan K. A. Wei et al. Neuropharmacology 184 (2021) 108370
Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2020.108370.

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