university of groningen

Emerging evidence on a potential link between DNA methylation and Attention Deficit Hyperactivity Disorder (ADHD)

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Essay

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08-01-2024

Abstract

This essay attempts to explore epigenetic mechanisms, particularly DNA methylation, in the context of Attention-Deficit/Hyperactivity Disorder (ADHD). ADHD is a neurodevelopmental condition affecting millions globally. Individuals with ADHD face significant life challenges, from academic and occupational struggles to strained interpersonal relationships. Its prevalence is estimated at 8% globally, highlighting its societal impact. Even though a great number of studies have focused on it, the underlying mechanisms are yet poorly understood. In this context, exploring epigenetic factors can provide a unique lens to comprehend the dynamic interplay between genetic predispositions and environmental influences that shape ADHD. In a thorough examination of candidate-gene studies and Epigenome-Wide Association Studies (EWAS), the essay highlights the most frequent findings, centering on genes like *VIPR2*, *DDR4*, and *DAT1*. Despite promising discoveries, the prevailing heterogeneity of the results requires further discussion, exploration of possible reasons and novel perspectives. Acknowledging the challenges, a combination of longitudinal studies and more targeted study designs, might help capturing the dynamic nature of epigenetic modifications in ADHD, paving the way for prevention, stratification, and targeted interventions.

Abbreviations

ADHD Attention-deficit hyperactivity disorder
ASD Autism spectrum disorder
BD Bipolar Disorder
BMI Body mass index
CBT Cognitive Behavioral Therapy
CpG Cytosine-guanine base pair (probe)
DMP Differentially methylated position
DMR Differentially methylated region
DNA Deoxyribonucleic acid
DNMT DNA methyltransferase
DSM-5 Diagnostic and Statistical Manual of Mental Disorders
EWAS Epigenome-wide association study
GWAS Genome-wide association study
MDD Major depressive disorder
ODD Oppositional defiance disorder
PTSD Post-traumatic stress disorder
SCZ Schizophrenia

SNP Single nucleotide polymorphism
SUD Substance use disorder

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1. Introduction

1.1 ADHD

Attention- deficit/ hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized mainly by symptoms of inattention and/or hyperactivity and impulsivity. These symptoms usually have an early-childhood onset, and they are lifelong persistent (da Silva et al., 2023). Sibley and his colleagues showed that only approximately 9.1% of adults with ADHD were able to fully recover from childhood ADHD, while ~65% presented fluctuations between remission and persistence (Sibley et al., 2021). ADHD can severely impact the quality of life of individuals as it affects multiple aspects of life. Specifically, ADHD can cause emotional dysregulation and difficulties in social skills and relationships, educational underachievement, accidents, unemployment, substance abuse and premature mortality (Faraone et al., 2021). Frequently, ADHD is accompanied by other psychiatric and/or neurodevelopmental disorders. During childhood, ADHD comorbidities include conduct disorder (CD), anxiety disorders and autism spectrum disorders (ASD). During adolescence and adulthood, among others ADHD can attribute to the development of major depressive disorder (MDD), psychosis and substance use disorder (SUD) (Cecil & Nigg, 2022).

1.2 Diagnosis

The current diagnostic procedure relies on behavioral data. According to DSM-5 (Diagnostic and Statistical Manual of Mental Disorders), for a person to be diagnosed with ADHD, they need to have presented adequate symptoms of impulsivity-hyperactivity and/or inattention before the age of 12. The symptoms must appear in at least two environmental settings such as school and home. Additionally, these symptoms should not be explainable by any other disorder (APA, 2022).

1.2.1 Diagnostic challenges

The diagnosis can be challenged by several factors. When diagnosing a child, many of these symptoms can overlap with natural childhood behavior - therefore assessing the level of severity needs to be taken with caution. Symptom severity evaluation can also be influenced by different cultural norms, as well as gender/sex behavioral expectations. What is more, during adolescence and adulthood, symptoms can have different presentations. For instance, hyperactivity often becomes more internalized, and can appear as restlessness, impatience, or jitteriness (APA, 2022).

Increasing the complexity of the diagnosis, the symptoms are not specific to ADHD and overlap with a great number of other disorders such as oppositional defiance disorder (ODD), anxiety, MDD, ASD, post- traumatic stress disorder (PTSD), bipolar disorder (BD) etc. At the same time, as already mentioned, ADHD is often comorbid with other disorders, creating greater confusion on the accuracy of diagnosis.

Additional aspects under consideration are the likelihood of a late-onset ADHD variant (Breda et al., 2021), the fluctuation of symptom severity across time (Sibley et al., 2022) and the balancing of male-to – female sex ratio during development (Franke et al., 2018).

ADHD also has an important economic impact for both patients and society - especially regarding late diagnosis. For instance, in Germany the average cost for healthcare was 4000 euros for individuals who received ADHD diagnosis as adults (Libutzki et al., 2020).

1.3 Treatment

Currently available treatments for ADHD aim to alleviate the symptoms, rather than cure them. Pharmacological treatments include stimulant (methylphenidate and amphetamines) and non-stimulant drugs (atomoxetine, guanfacine, clonidine) while non-pharmacological approaches regard psychotherapy, and psychoeducation. The efficacy of both approaches is very much dependent on the individual person. The pharmacological treatments, while in most cases effective, might provoke some side effects like anxiety, or they might present low effectiveness (~30% of patients do not respond to treatment) (Mechler et al., 2022). Cognitive Behavioral Therapy (CBT) has been proven to help patients create new behavioral habits, and gain adaptive thinking, redirect emotions, improve the integrity of self-identity, and determine cognitive errors. It is suggested that combination of CBT and pharmacological treatments can have higher efficacy (Pan et al., 2019).

1.4 Genes and Environment

In recent years there has been a constant increase of knowledge attained for ADHD, regarding the genetic and environmental mechanisms that influence it. However, this understanding reveals a highly complex and heterogenous relationship, perplexing the liability of the disorder (Sonuga-Barke et al., 2023).

1.4.1 Genes

ADHD heritability has been estimated to be as high as 74% in twin studies (Faraone & Larsson, 2019). A GWAS (Genome-wide association) study by Demontis and his team revealed 12 genome wide significant risk SNPs (single nucleotide polymorphisms) for ADHD. In a newer study from the same team, 27 variants were found, 21 of which are new. Regardless, the SNPs heritability was estimated to be around 14%, not explaining the results from twin studies. Additionally, revealing ADHD's polygenic nature, the common variants that can explain 90% of the SNP heritability were estimated to be 7300. A large number of those are also influencing other psychiatric disorders such as schizophrenia (SCZ), MDD and ASD (Demontis et al., 2023). Lastly some rare variants with stronger effects have also been described (Sonuga-Barke et al., 2023).

1.4.2 Environment

1.4.2.1 Causal versus confounding factors

An essential concept that needs to be addressed before analyzing environmental risk factors is the role they play in a disorder, as throughout development there is dynamic interconnection with genetic liability. The term "risk factor" explains a possible association/ correlation with a disease, but not a causal relationship. A causal factor might be explaining more about the mechanism of a disease, but such a distinguishment is often hard to achieve in the case of ADHD. Given the polygenic nature of it, the interplay between genes and environment can greatly complicate our understanding, especially when studies are not designed to test this. An additional burden can be the possible genetic nurturance, where the genotype of a parent affects their behavior, and therefore the environment that the child is being raised. What is more, confounding effects play a different role, as they reveal an interactive result of genotype and environment, but cannot necessarily provide an explanation.

As the distinguishment between risk factor and mechanistic factor is quite hard, in this paragraph the term "risk factor" will be used, acknowledging the possibility of overlap and/or misuse. Among others, important risk factors of ADHD as well as for other psychiatric disorders include very low birth weight (<1500 g) and very preterm birth (<32 weeks) (Anderson et al., 2021). An umbrella review conducted by Kim and their colleagues, shows significant correlation between ADHD and pre-pregnancy obesity, hypertension and gestational hypertension during pregnancy as well as maternal acetaminophen exposure, expanding the pre- and peri-natal environmental risks.

Maternal smoking during pregnancy and maternal pre-pregnancy overweight also presented a smaller effect (Kim et al., 2020). Other risk factors found by the same scientific team include childhood eczema, pre-eclampsia, childhood asthma as well as serum vitamin D (Kim et al., 2020). Additionally, exposure to lead has been found to increase the likelihood of developing ADHD, when found in high levels (Faraone et al., 2021). Exposure to several other toxicants such as artificial food dyes, the anti-epileptic drug valproate, maternal high phthalate levels, pesticides and nitric oxide exposure have been found to have some correlation with ADHD (Faraone et al., 2021). Lastly, Kennedy and his team exhibited that high levels of institutional deprivation in early-life increased four to seven times the possibility of developing ADHD later in life (Kennedy et al., 2016).

On some occasions, risk factors have been tested for their causal or confounding effect. For instance, maternal smoking (Haan et al., 2022) has not been found to have a causal relationship with ADHD, but more likely a genetic or familial confounding one. On the other side, low birth weight (Lim et al., 2018) and institutional deprivation appear to have a causal role (Kennedy et al., 2016). Other factors such as parenting (Daley et al., 2018) and psychosocial environmental circumstances (such as low income) (Thapar et al., 2013) can have a modifying role on the development of ADHD.

Deciphering how environmental influences interact with genetic liability to develop a neurodevelopmental disorder like ADHD can become intricate. Potential biomarkers of this interaction can be epigenetic modifications (Dall'Aglio et al., 2018).

2. Epigenetic modifications

Epigenetic modifications are mechanisms that can alter gene expression, without changing the DNA sequences (Dall'Aglio et al., 2018). These mechanisms are naturally occurring, for instance in cell differentiation during development. Sometimes, alterations in those mechanisms can cause unwanted gene expression that can lead to diverse defects, such as neurodevelopmental disorders (Kubota et al., 2014). Those alterations can be either congenital or can derive from environmental exposure to certain factors. Herein, studying epigenetic alterations in ADHD will assist in a better understanding of the interaction between genes and environment, which is crucial to

gain more insight of the underlying mechanisms. Identifying an alternate epigenetic profile for ADHD might not only provide information about the causation, but it can also act as a biomarker and/or therapeutic target (Cecil & Nigg, 2022; Kubota et al., 2014). In other diseases such as cancer, applications based on epigenetic profile have arisen. For instance, epigenetic assays that predict response to a specific chemotherapy for glioblastoma are already commercially available (Davalos & Esteller, 2023).

An important characteristic of epigenetic modifications is their spatio-temporal specificity. In other words, the epigenetic profile of an organism can change over time, and it is tissue specific. In the case of neurodevelopmental disorders, as the tissue of interest is the brain, acquiring accurate data from peripheral tissues can become intricate. Moreover, as ADHD progress is connected to development, possible epigenetic alterations that change with time can only be detected in longitudinal studies. At the same time, it has been found that epigenetic alterations like DNA methylation caused by stress can remain for a lifetime. There are also indications that they could even be transgenerationally inherited to the next generation (Kubota et al., 2014).

2.1 DNA methylation

DNA methylation is a well-studied epigenetic mechanism, where DNA methyltransferases add a methyl group to the fifth position of the cytosine ring of the DNA base pairs. In mammals DNA methylation is more commonly added to cytosines that are followed by guanines, also known as CpG sites (Jin et al., 2011). CpG sites are usually heavily methylated unless they are in CpG islands. CpG islands are 1000bp regions of DNA, rich in CpG, that are not methylated. Most of the gene promoters can be found in these regions, where transcription factors can bind (Moore et al., 2013). Depending on the site and the level of the methylation, gene expression can be promoted or disrupted. Genes are usually silenced when methylation occurs on the promoter of a gene, while expression is enhanced when methyl groups are added inside the gene body (Dall'Aglio et al., 2018). Other epigenetic mechanisms include histone modification and non-coding RNAs, which interact with DNA methylation, and they might also influence ADHD, but they will not be analyzed in this essay.

Considering the aforementioned, tracing DNA methylation patterns for ADHD can assist in comprehending the etiology/ mechanisms of the disorder. Additionally, as methylation is influenced by environmental circumstances, it can also help in identifying risk/mediator factors. In turn, awareness of these factors can be a tool for early detection, prevention, and successful intervention. Moreover, identifying DNA methylation patterns can be utilized as a biomarker for stratification and subtyping. What is more, methylation patterns sometimes can predict treatment response (for instance in the case of cancer). Consecutively, patients can be provided earlier with the appropriate treatment, decreasing excessive mental and financial costs that can derive from misdiagnosis, or failed treatment. Lastly, by understanding the mechanisms of ADHD, treatments for novel targets can be developed. In the following paragraphs, potential methylation markers for ADHD are being discussed, as new observations give promising and encouraging data.

Epigenetic studies regarding DNA methylation are usually approached by either studying specific candidate genes or across the epigenome (EWAS).

3. Candidate- gene studies

As candidate- gene studies need to focus on specific genes, usually those chosen are relative to the mechanism of action of the disorder. In the case of ADHD, even though its etiology is not known yet, there are some prevalent theories that can explain ADHD symptomatology.

The most dominant hypothesis is that of the dysregulation of the dopaminergic and noradrenergic neurotransmission systems, which are functioning excessively under stressful circumstances and underperform in calmer conditions (Beaulieu & Gainetdinov, 2011). Subsequently, research has focused on genes related to dopamine. *DAT1* is the gene of the dopamine transporter and has been widely investigated in the context of ADHD, along with dopamine receptor gene *DRD4,* norepinephrine transporter gene *NET,* and serotonin transporter gene *5-HTT* (Silk et al., 2022).

3.1 *DRD4*

Specifically, results regarding methylation of these genes vary. van Mil et al (van Mil et al., 2014), in a cohort of 426 children, investigated the DNA methylation signatures of

their cord blood when newborns, and tested them behaviorally after six years for ADHD symptoms. The results showed significant association between ADHD symptoms and lower methylation levels of *DRD4* and *5-HTT* gene regions (van Mil et al., 2014). Xu and their team, in a case- control study of 50 (ten females) children (mean age of eight years) with ADHD (and 50 controls), examined their blood epigenetic profile. Statistically significant higher methylation levels were found upstream of the *DRD4* for the ADHD group (Xu et al., 2015). Additional data supporting the *DRD4* gene were given by a research team lead by Dadds, that tested 330 children (mean age of 8.88 years) using blood and saliva samples. They found that increased methylation of the dopamine receptor gene was correlated with increased symptom severity as well as with symptoms of inattention (and not hyperactivity and impulsivity), in ADHD (Dadds et al., 2016). Weiß and colleagues conducted an adult study (mean age 34.2) of 88 ADHD and 91 control participants and identified a significant correlation between the case group and the methylation of *DRD4* gene (along with *KLRD1* and *TARBP1)* (Weiß et al., 2021).

3.2 *DAT1*

A different approach was chosen by Andriani and colleagues, that conducted a study in buccal samples of 30 children with ADHD, who specifically were genotyped for the *DAT* gene, and were tested at the 5'- untranslated region (UTR) of the *DAT1* gene. The results showed a significant decrease in methylation levels of the ADHD group (Adriani et al., 2018). Xu et al. (Xu et al., 2015) also tested the *DAT1* gene, but did not find significant methylation differences. In a children study of 111 ADHD patients (mean age of 9.23 years), Ding et al. wanted to test the relationship between treatment response and *DAT1* methylation in their blood. The findings suggest that methylation of the gene was associated with ameliorated symptoms of hyperactivity and OD, but not inattention, in response to stimulant treatment (methylphenidate) (Ding et al., 2017).

3.2.1 The *DAT1* potential

Several studies have identified some interesting results regarding specifically methylation patterns within the *DAT1* gene, that could be utilized as indicators of symptom severity/ progress of ADHD, given that patients have one of the two specific

genotypes. These are the DAT 9/x genotype which indicates the existence of at least one 9-repeat allele and the DAT 10/10 genotype which stands for two 10-repeat alleles. The study from Adriani that was already mentioned, found an association between hypermethylation of CpG1 position and DAT 10/10 genotype. Additionally, methylation of the CpG2 and/or CpG6 positions were correlated with the DAT 9/x genotype. The last genotype is related to recovery, while DAT 10/10 corresponds to severity of the disorder (Adriani et al., 2018). This study is suggesting a biomarker for diagnosis/ recovery progress of ADHD, at least when one of these genotypes is present. Marzilli and her colleagues recently published their own results from a study conducted on 76 school-aged children with ADHD using buccal samples. They showed that CpG1 higher methylation levels indicated increased symptom severity in children with the DAT 10/10 genotype. Moreover, they showed that for children with the DAT 9/x genotype, higher methylation of CpG6 was correlated with lower severity. For children with either of the two genotypes, lower methylation of the CpG2 position was associated with higher symptom severity (Marzilli et al., 2023). Another study led by Carpentieri and colleagues, pinpointed the potential of *DAT1* methylation patterns as a biomarker for treatment response with data collected from 60 children (6-12 years old) with ADHD. The data presented an association between methylation of the CpG1 and severe ADHD that was not improved after treatment. Methylation of CpG2 and CpG6 was correlated with improvement after therapy (Carpentieri, Lambacher, et al., 2023).

3.3 *5-HTT* and other genes

Moreover, data show that serotonin might also be involved in ADHD psychopathology as it is involved in emotional regulation mechanisms, impulse control, and cognition (Sonuga-Barke et al., 2023). In addition to (van Mil et al., 2014) findings, another study led by Park and their colleagues (Park et al., 2015) shows that higher methylation of *5-HTT* gene, in peripheral blood samples taken from 102 children with ADHD (6-15 years old), is related to increased symptoms of hyperactivity and impulsivity. Although this finding is not in accordance with van Mil's result, where the association was between symptom severity and lower methylation levels, there are several methodological

differences between the two studies that will be discussed further in the next section of the essay.

Lastly, several other genes have been correlated with ADHD symptomatology, though the results have not been replicated or derive from small sample studies (such as *NET*, *IGF2, LIME1 and SPTBN2)* (Silk et al., 2022).

4. Epigenome-wide and Genome- wide methylation studies

Most recent studies have shifted towards the use of EWAS, as methylation arrays are becoming more accessible. Like GWAS, EWAS follows a hypothesis- free strategy, and gives information about the entire epigenome (Cecil & Nigg, 2022).

Specifically, regarding psychiatric diseases, when compared to candidate gene studies, it is suggested that GWAS are becoming more and more useful and trustworthy, as they are reproducible, can identify new risk variants and can estimate polygenic risks (Duncan et al., 2019). Additionally, GWAS have shown that most psychiatric diseases do not have large effect variants, and genetic risk variants can be distributed across the genome, maybe in regions that are not expected or understood (Duncan et al., 2019). Of course, EWAS is different from GWAS, but similar rules apply to it when compared with the candidate genes approach. Even on epigenome level, EWAS provides a completer and more informative image of methylated regions in comparison to specific genes that are investigated according to a hypothesis - that can lead to false- positive results (Silk et al., 2022).

4.1 *VIPR2*

Vasoactive intestinal peptide receptor gene (*VIPR2*), also known as *VPAC2*, encodes a neuropeptide that participates in muscle relaxation, endo- and exocrine secretion and regulation of water and ion flow in lung and intestinal epithelia. It is expressed in both the central nervous system (CNS) and the peripheral tissues, and more specifically it is highly expressed in the brain, heart, ovaries, and prostate (Moody & Jensen, 2021; National Center for Biotechnology Information (NCBI), 2023).

The first epigenetic cross-sectional case-control study that related *VIPR2* with ADHD, was conducted by Wilmot and their team, where salivary samples were taken from 85 boys (43 diagnosed with ADHD, and 42 without) aged between 7-12 years old. It was

found that boys from the ADHD group had lower CpG methylation (Wilmot et al., 2016). On the same year, a longitudinal study was published by (Peter et al., 2016) that showed how malnutrition during the first year of life was associated with *VIPR2* methylation (among other genes) and decline in attention and cognition. In this study blood samples were used, and the methylation patterns were found in adults of 45 years old mean age, demonstrating malnutrition as a risk factor (Peter et al., 2016). The largest EWAS conducted by Mooney and colleagues, compared saliva samples of 391 children (7-12 years of age) with diagnosed ADHD and 213 controls. This study revealed a sex- dependent association of *VIPR2* methylation, as boys with ADHD had lower levels of methylation in comparison with the boys of the control group, while the opposite was true regarding girls (Mooney et al., 2020). Additionally, a study conducted on 14 monozygotic twins (12 male pairs) that were discordant for ADHD and had a mean age of 10.9 years, found hypermethylation of the *VIPR2* gene (probes in slightly different locations than other studies) on their blood samples (Y.-C. Chen et al., 2018). Lastly in a study unrelated to ADHD, lower methylation in two regions of the *VIPR2* gene was associated with higher maternal pre-pregnancy BMI (Body mass index) (Sharp et al., 2017), which is a risk factor for ADHD.

While at first sight there seems to be some accordance regarding the *VIPR2* gene differential methylation and ADHD, the data are not phasing towards one direction. This can be attributed to several factors regarding the methodological differences between studies. These methodological differences regard the variation in tissue samples (blood or saliva), ages, developmental stage, sample sizes, ratio of males and females, as well as sex- dependence consideration.

More specifically as ADHD progress can change in different ages (Sonuga-Barke et al., 2023), and so does DNA methylation variation from childhood to adolescence (Mulder et al., 2021), conclusions are hard to be drown. Taking this into account, with the combination of other limiting factors, such as attention and cognition assessment taking place during the studies, it is intricate to pinpoint if certain effects are causally, additively, or mediately related to the disorder. In a sense, these complications beat the purpose of the studies themselves. Of course, these findings are an important first step, pinpointing to new study targets and they are giving insight into various aspects

of the disease. For instance, the sex-dependent methylation direction of the *VIPR2* gene (Mooney et al., 2020) , is an interesting finding as increasing data show that ADHD has a diverse expression in males and females (Faheem et al., 2022), and can serve as an inspiration for other studies to follow. Additionally, findings regarding risk factors such as the effect of malnutrition during the first year of life (Peter et al., 2016), should be taken into account as part of prevention not only for ADHD, but for overall healthy development.

As a last connotation, the genetic variance of the *VIPR2* gene is associated not only with psychiatric disorders, such as schizophrenia (C.-H. Chen et al., 2022) and mood disorders (Soria et al., 2010), but also several other diseases like cancer (Asano et al., 2023), myopia (Zhao et al., 2022) and metabolic syndrome (H. Park et al., 2023). With that being said, the general contribution of this gene to several other "distinguished" pathologies cannot be overlooked when the role of a biomarker is being discussed.

4.2 Other emerging genes

While *VIPR2* has been the most consistent finding of DNA methylation studies, there are also other genes that were identified in different studies. In the study of Wilmot and colleagues (Wilmot et al., 2016), *MYT1L* hypomethylation was also identified, although it did not meet the study's confirmation standards. *MYT1L* is a gene related to several neurodevelopmental and psychological disorders such as MDD and SCZ (Coursimault et al., 2022). The EWAS conducted by Mooney and their team, showcased several other genes with differentially methylated positions (DMP), with *SLC7A8* and *MARK2* being the top-ranked (Mooney et al., 2020), but without genome-wide significance. Furthermore, another EWAS control case study that took place in 2020, tested 103 adult individuals with ADHD (and 100 controls) and their blood samples for possible differential methylation. Among others *DENND2D, PWWP2B* and *UBASH3A* genes, and a CpG site close to *PCNXL3 (*cg07143296*),* had genome-wide significance. An additional interesting finding of this work was that while smoking did not seem to have an effect on the methylation status of ADHD, it did share common epigenetic signatures with it. The fact that the genes annotated here are not common with aforementioned studies can be partially explained by the fact that DNA

methylation profile changes throughout different developmental ages (Rovira et al., 2020).

An EWAS population adult study led by van Dongen, did not result in statistically significant methylation patterns correlated to ADHD. The study consisted of three EWAS cohorts, a total sample of 4689 individuals (mean age of 37, 38 and 18 years) that had their blood tested for differential DNA methylation loci. The data were combined and analyzed through meta-analysis. Separately each study had some differentially methylated loci with higher significance scores, but no overlap was found (van Dongen et al., 2019).

4.3 Longitudinal studies

The need for longitudinal studies is paramount, especially for epigenetic studies where temporal influences can be strong. The largest longitudinal study led by Walton provided some very interesting results that opened novel discussions and pinpointed the need for more analogous studies. The cohort consisted of 817 (49% male) newborns that had methylation levels tested in their cord blood, and 892 (50% male) 7-year-old children that had their whole blood tested (overlap of 783 participants). Additionally, children were also observed from 7-15 years to see the disorder trajectory. Among the 13 probes that were detected upon birth, the highest scored DMP's were associated with *SKI, EPX, PEX2, ST3GAL3* and *ZNF544* genes. *SKI* participates in neural tube development and *EPX* and *PEX2* are associated with peroxisomal processes ((Wilmot et al., 2016) also highlighted genes that seem to be relevant with fatty acid oxidation in ADHD) (Walton et al., 2017b). *ST3GAL3* is one of the highest scored genetic loci related to ADHD that were found in the first GWAS conducted by Demontis, that was already described on the 1.4.1 paragraph (Demontis et al., 2019). Strikingly, none of the probes was found to be significantly different at the age of 7 (Walton et al., 2017b). Neumann and their team conducted a meta-analysis of EWAS studies, and even though the associated genes were different from Walton's, the same pattern remained. The genes identified on birth cord- blood (n = 2477) and were associated with ADHD were not identified later in childhood (n = 2374). Among the highest scored genes, lower methylation of *ERC2* and *CREB5* were correlated with ADHD development later in life, which participate in neurotransmitter

release and neurite outgrowth respectively (Neumann et al., 2020). Among other genes, *ERC2* different allele frequencies have been suggested for distinguishment between BD and SCZ.

5. Discussion and future directions

5.1 Discussion of the results and limitations

The goal of this endeavor was to explore the literature regarding related research on DNA methylation and ADHD. An overview of the results for both EWAS and candidate – gene approaches indicate the great heterogeneity of the results, regarding not only the variety of related genes but also the different directions of results for the same genes. This discrepancy of the results can be attributed to several factors.

5.1.1 Participants

On a first level, the sample size from study to study varies significantly, with some studies having only a few decades of participants while others have thousands. Some studies do not have controls, while others do. The ratio between males and females is not adjusted to be equal, with some studies not even including females. The ages of the participants vary a lot, and only few regard adults. The ones including children usually have a broad range of ages from 6- 15, which entails different developmental stages. Another limitation regards the different ethnic groups of the children, with some studies including only one ethnicity. For instance, the studies from (Adriani et al., 2018; Carpentieri, Cugno, et al., 2023; Marzilli et al., 2023) only included Italian children and the study from (van Mil et al., 2014) only had Dutch children.

5.1.2 Methodology

Methodological discrepancies might have also influenced the results. Not all studies accounted for sex dependent (even if they did, the unequal ratio of the participants might have shadowed such an effect) and developmental stage dependent differences. Additionally different assays were used for methylation detection that do not give the same level of high throughput data. The data analysis can also become quite intricate as thresholds should be adjusted according to the sample size and to the number of loci tested.

5.1.2.1 ADHD diagnosis and symptoms

Another important issue that needs to be addressed is the ADHD symptom and/or diagnosis assessment. Firstly, there are some studies that assessed ADHD symptoms at the same time as the experiment, creating space for biases that could lead to validity concerns. Moreover, the assessment was not always conducted in the same way, since studies use different assessment forms, others use diagnostic tools, some take information only from the parents and others include teachers (in the case of children studies). Some studies do not even assess ADHD as a disorder per se, but rather score some specific symptoms of it such as hyperactivity, impulsivity, or inattention.

5.1.2.2 Peripheral tissues

One of the most important challenges that brain related studies face regards the lack of direct access to the brain. In the case of epigenetics and ADHD, the most common peripheral tissues that are being used are blood, saliva, and buccal samples. In the case of newborn participants, cord-blood is preferred. Apart from the fact that different sampling methods unavoidably can cause disparity in the results, explaining the heterogeneity observed, they are also not equally informative about the actual methylation status. As already discussed, since epigenetics are tissue specific and cross-tissue correspondence is rare, it is important to at least maintain analogous methodology in the experiments, to have comparable results. Braun and her colleagues compared the DNA methylation levels of samples from saliva, blood, and buccal to brain tissue, and found that all types of samples are equally comparable, and it is dependent on the different CpGs and genes (Braun et al., 2019). While this is comforting, at the same time if the level of correspondence depends on the specific CpG, this might also pinpoint the need to stick with one method.

Lastly, regarding the cord-blood, issues arise given that it is a tissue that contains multipotent cells which go into apoptosis shortly after birth, they have a specific immune profile which is not met in the other tissues, and sometimes the origin of the blood can be contaminated with mother-blood (Cecil & Nigg, 2022). Regardless, cord-blood still gave some promising results that might be possible to be utilized if they are carefully assessed.

5.2 What can we learn from the results from cord-blood samples be utilized?

It is important to be mentioned that although the results from (Walton et al., 2017a) longitudinal study and (Neumann et al., 2020) meta-analysis might implicate that the methylation profile of newborns can provide biomarkers related to ADHD in later ages, it might also mean that the difference is not related to age, but to the specific tissue (both spatially and temporally). Another explanation of these data is that since environmental influences can greatly impact the methylation status of individuals, maybe the number of such alterations throughout the years creates such a variability that overshadows any possible patterns (Cecil & Nigg, 2022). A hypothesis like this can also explain the heterogeneity in the results, and the luck of DNA methylation patterns in the adult EWAS cohort that was previously mentioned (van Dongen et al., 2019).

On the other side, an additional aspect that needs to be addressed is the possibility that methylation of specific genes found on newborns are not biomarkers directly related to ADHD pathology but rather indicators of ADHD genetic liability. More specifically, the results from the two aforementioned studies, highlighted two genes *ST3GAL3 in* (Walton et al., 2017a) and *CREB5* in (Neumann et al., 2020) that have been top hits in GWAS studies related to ADHD ((Demontis et al., 2019) and (Klein et al., 2019) respectively).

Consequently, another factor that needs to be addressed regarding the methodology of the epigenetic studies, is the need to control the results for the presence of genetic effects. Literature findings support that possibly methylation levels of ADHD individuals are under genetic control (da Silva et al., 2023).

5.2 Future directions

Overall, the aim of this discussion is to pinpoint the need for carefully designed EWAS with bigger sample sizes, equal ratios of males and females, control groups, age groups carefully chosen according to distinct developmental stages and consistent methodologies. It is stressed that more investigation is required regarding sex dependent and age dependent effects. Even more importantly, longitudinal studies are a necessity, as previously discussed.

Acknowledging the possible obstacles, a suggestion for future research is to conduct longitudinal studies that take repeatedly methylation data once every year, from

newborns to adults to investigate the hypothesis regarding the environmental influence on the epigenome. Replication of cord-blood data might also help establishing specific genes as biomarkers of ADHD, even as predictors of genetic liability.

Regarding the results from candidate-gene approaches, the *DAT1* and *DRD4* genes give promising results, but the fact that they do not appear in the EWAS studies at least not in a significant level, raises some concerns. Candidate- gene studies are prone to Type I errors, producing false positives, especially given the fact that ADHD is a complex, polygenic disorder. However, it is still useful that the data are replicated from independent teams, meta-analyzed or integrated with other omics data.

As a final comment, it is meaningful to observe the broader image of ADHD as well as of other mental disorders. As it is already established in the literature, patients with ADHD more often than not have additional comorbid disorders. Its symptoms are also not specific. Moreover, the most recent GWAS by (Demontis et al., 2023) gives striking results regarding the common gene variants that are shared between ADHD and other disorders (~84-98%), especially with MDD, SCZ and ASD. The literature is still not coherent regarding genetics, epigenetics or known mechanisms of action. Taking these into account, the chance that some of these disorders might be more connected than initially believed becomes more realistic. Such a perspective could help re-establish the categories as they are known today (Sonuga-Barke et al., 2023) and pave the way for novel study designs.

A more abstract suggestion would be to develop methylation/ gene assays that calculate scores from known loci that might overlap for several disorders. Depending on the score and the participating genes it could create suggestions for disorders that need to be evaluated from a clinician. Such a tool could be utilized for early and accurate diagnosis.

6. Literature

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