Translational Epigenetics

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CHAPTER

3

Discordant monozygotic twin studies of epigenetic mechanisms in mental health

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1 Introduction

In recent years, large progress has been made in genetic studies of psychiatric disorders and mental health-related traits through very large genome-wide associations studies (GWAS) on tens to hundred thousand and even millions of individuals. Examples of successes are the discovery of 108 loci for schizophrenia,[1] 44 and 102 loci associated with depression,[2, 3] 12 loci associated with attention deficit hyperactivity disorder (ADHD),[4] and 5 loci associated with autism spectrum disorders[5] (where differences in the number of identified genes reflect differences in power of the study). Many of these studies originate from the Psychiatric Genomics Consortium () and have provided important novel insights into the biological mechanisms of these disorders and contribute to the development of polygenic scores that summarize an individual's genetic vulnerability for mental disorders.[6] It is clear that mental disorders are highly polygenic and that many more loci remain to be identified.

Besides genetic influences, mental health is also influenced by non-genetic factors. For the major psychiatric disorders, concordance rates of monozygotic (MZ) twins range from approximately 40% for major depressive disorder and schizophrenia to ~80% and ~90%, respectively, for ADHD and autism spectrum disorders.[7] The imperfect concordance of monozygotic twins who have (nearly) identical polygenic scores, illustrates the importance of studying non-genetic mechanisms and biomarkers for these disorders. Epigenetic mechanisms regulate the activity of genes and have been proposed as a candidate mechanism involved in mental health and as a possible explanation for mental health discordance of monozygotic twin pairs.[8, 9]

1.1 Psychiatric epigenetics

The field of psychiatric epigenetics studies the role of epigenetic mechanisms in mental health.[10] The epigenome may mediate long-term effects of (early) life events and environmental exposures on mental health.[11–13] This concept was first illustrated by a study of rats that showed how the quality of maternal care early in life affects lifelong stress response of the offspring: maternal care induced stable DNA

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methylation changes of the glucocorticoid receptor gene in the hippocampus of offspring changing its expression level for life.[14] Epigenetic mechanisms also mediate the effects of genetic variants on phenotypic outcomes.[15] This is illustrated by the devastating effects of rare mutations that disrupt key components of the epigenetic machinery on brain development. For example, mutations affecting the gene encoding methyl-CpG binding protein 2 (*MeCP2*), a so-called reader of DNA methylation that is required for gene silencing, cause deficits in synaptic plasticity in the hippocampus leading to impairments in memory formation and resulting in Rett syndrome, a severe genetic neurodevelopmental disorder.[16] In addition to being shaped by environmental exposures and genotype of the individual, the epigenome is also subject to stochastic variability, because maintenance of the epigenome in dividing cells is not 100% accurate.[17] In summary, individual differences in epigenomic profiles may arise from genotype, life-long environmental exposures, and stochastic influences.

To identify epigenetic mechanisms involved in mental health, early studies mainly focused on candidate genes. Nowadays, epigenome-wide association studies (EWASs) are considered the gold standard. Epigenome-wide association studies offer the advantage that they do not start from a previously selected candidate gene, and could in this respect be called "hypothesis-free," but screen for differences in epigenetic regulation across the whole genome. These studies are conducted on DNA derived from brain (usually postmortem material), and on DNA from other peripheral tissues (most frequently blood). The majority of EWA studies have focused on DNA methylation,[18] although other epigenomic mechanisms, including histone modifications and non-coding RNAs, have also been studied. Comprehensive reviews of epigenomic studies of neurodevelopmental disorders,[19] depression,[20] and schizophrenia[21] have been published elsewhere. Illumina DNA methylation arrays have become a popular technique in EWAS studies. The coverage of these arrays has increased from ~27,000 CpGs in CpG islands on the Illumina Infinium HumanMethylation27 BeadChip (Illumina 27 K array) to >450,000 CpGs including multiple CpGs within various regions of genes, intergenic CpGs, and CpGs in and outside of CpG islands on the Illumina Infinium MethylationEPIC BeadChip (Illumina 450 K array), to > 850,000 CpGs with greater coverage of enhancers on the Illumina Infinium MethylationEPIC BeadChip (Illumina EPIC array).

EWA studies may contribute to the identification of disease biomarkers and to a better understanding of disease mechanisms. This is illustrated by a study that analyzed blood DNA methylation data assessed on the Illumina EPIC array from schizophrenia patients and controls (total sample size = 7488 participants).[22] The study identified a promising blood DNA methylation signature that predicts disease status, mirrors DNA methylation differences in the prefrontal cortex, and correlates with dorsolateral prefrontal cortex hippocampal connectivity during working memory performance, which is considered a strong intermediate phenotype of schizophrenia. Other studies have shown that some of the loci identified in genome-wide association studies of schizophrenia also show DNA methylation differences in blood and brain tissue from schizophrenia patients,[23, 24] illustrating the potential of EWA studies to detect disease-associated epigenetic variation at disease-causing genes. A limitation of many EWA studies is that association studies typically cannot distinguish genetically driven epigenetic differences (i.e. epigenetic differences between cases and controls caused by differences in genotypes) versus environmentally-driven epigenetic differences. The associations may reflect the epigenome as a cause or as a consequence of the disease and designs that go beyond cross-sectional studies of unrelated cases and controls are required. Furthermore, EWA studies are vulnerable to confounding, due to e.g. differences in age, sex, lifestyle and cellular composition between patients and controls. Careful assessment of factors that may impact on epigenomic profiles is critical in EWAS.

1.2 Scope of this chapter

In this chapter, we describe epigenetic studies of mental health and related traits in discordant monozygotic twins. In this design, cases and controls are compared, where a case and control are from the same twin pair and within-family comparisons, e.g. paired *t*-tests, are employed to study the relation of an epigenetic profile and a disease with control for genetic background and (unmeasured) early environment shared by twins. MZ twins have a (nearly) identical DNA sequence, which explains why they are often so remarkably similar, but the epigenomes of MZ twins can acquire differences.[25–27] Epigenetic differences between monozygotic twins are already present at birth, may arise due to exposure to different environmental conditions and stochastic errors, and may cause different usage of the same code, which can sometimes lead to remarkable phenotypic differences.[28] The study of such phenotypically discordant persons with identical DNA sequence is regarded as a particularly strong research design to identify epigenetic mechanisms involved in mental health. A large advantage is that many other factors that can differ between unrelated disease cases and controls are controlled for in discordant MZ twin studies by design, including age, sex, birth year, DNA sequence (de novo mutations excepted), and many prenatal and postnatal environmental influences.[25]

We focus on common mental disorders and related traits that have been examined in monozygotic twins: autism spectrum disorders, externalizing problems and ADHD, schizophrenia, psychosis, bipolar disorder, and depression (Table 1). These conditions are all thought to be caused by a combination and interplay of genetic vulnerability and environmental influences, but there are also important differences, including[29]: (1) Large differences in age of onset and lifetime prevalence. Autism spectrum disorders (prevalence ~1%) and ADHD (prevalence ~5%) are typically regarded as neurodevelopmental disorders that emerge early in childhood, although adult-onset ADHD is also recognized. Schizophrenia (prevalence ~1%) and major depressive disorder (prevalence >10%) typically emerge in adolescence or adulthood. (2) These conditions show large differences in heritability (ranging from ~37% for major depressive disorder to >70% for autism, ADHD, aggression, and schizophrenia).[7] We review these disorders in order of the typical age of onset/diagnosis.

2 An overview of MZ twin studies of epigenetic mechanisms in personality behavior and mental health

2.1 Autism spectrum disorders

Autism spectrum disorders (ASD) affect ~0.6%–1.5% of the population[30–32] and are characterized by deficits in social interactions and understanding, aberrant communication and/or language development, and restricted, repetitive and stereotyped behaviors.[33] Environmental risk factors that might act through epigenetic mechanisms have been proposed, such as increased parental age, maternal health complications during pregnancy, prenatal exposure to certain medication, smoking and alcohol use, and toxic exposures.[34–39] Mendelian syndromes that are comorbid with social deficits, such as Rett, Fragile X, Prader-Willi, Angelman, and CHARGE, are associated with aberrant epigenetic regulation.[40, 41] For autism spectrum disorders, differentially methylated positions (DMPs) and regions (DMRs) have been detected in a variety of tissues: whole blood,[42] buccal,[43] lymphoblastoid cell lines,[44] and postmortem brain.[45–49] In addition to DNA methylation, epigenomic studies of ASD have examined histone marks,[50, 51] and miRNA and long noncoding RNA expression.[52–55]

Table 1 Epigenetic studies of mental health (−related traits) in twins.

Ref.	Trait	N MZ pairs	Tissue	Method
1. Autism spectrum disorders				
Nguyen, 2010[44]	ASD	3 Males discordant (age 6, 8, 16 years old) + non-autistic siblings of 2 pairs + postmortem brain tissues from autistic individuals and controls	Lymphoblastoid cell lines	8.1 K CpG island microarrays + bisulfite sequencing of top sites
Wong, 2014[94]	ASD and ASD-associated traits	50 Discordant, concordant affected, or concordant unaffected twin pairs (age 15 years)	Whole blood	Illumina 27K
Liang, 2019[56]	ASD	5 Twin pairs ASD-discordant MZ twins (age 2, 9, 12 years) + validation 30 pairs case-control	Whole blood	Illumina 450K (3 twin pairs). Validation: Pyrosequencing (2 twin pairs)
2. Externalizing problems and ADHD				
Kaminsky, 2008[57]	Risk-taking behavior	1 Discordant twin pair	Whole blood	Microarray-based
Van Dongen, 2015[59]	Aggressive behavior	20 Discordant twin pairs	Whole blood	Illumina 450K
Chen, 2018[58]	ADHD	12 MZ twin pairs (mean age 9.7 years)	Whole blood	Illumina 450K
3. Schizophrenia, bipolar disorder and psychosis				
Dempster, 2011[69]	Major psychosis (schizophrenia and bipolar disorder)	22 Discordant pairs +45 postmortem brain samples from schizophrenia and bipolar disorder patients and controls	Whole blood	Illumina 27K + EpiTyper of top hits + global methylation (LINE1) assay
Sugawara, 2011[70]	Bipolar disorder	2 Discordant male twin pairs + same tissue from 20 unrelated cases and 20 controls + postmortem cortex (BA10) from 35 unrelated cases and 35 controls	Lymphoblastoid cells	Methylated DNA enrichment (using the MethylCollector kit) followed by Affymetrix GeneChip Human Promoter 1.0R tiling array + bisulfite sequencing of top hits
Bönsch, 2012[79]	Schizophrenia	114 MZ or DZ twins discordant for schizophrenia, concordant for schizophrenia, concordant healthy, and concordant for other psychiatric disorders	Whole blood	Global methylation status of HpaII/MspI cleaving sites were measured by a modified non-radioactive elongation-assay on genomic DNA. Methylation of the promoter regions of *SOX10* and *RLN* was measured by quantitative PCR after digestion with *Hpa*II/*Msp*I

	Results	Genome-wide (GW) or candidate-gene (CG) study
	Top loci: *BCL2* and *RORA*. The expression of *RORA* and *BCL2* proteins was reduced in cerebellum and frontal cortex tissue from autistic patients	GW
	Numerous differentially methylated regions within discordant pairs and between autism cases and controls across twin pairs, and sites where DNA methylation level correlated with continuous autistic trait scores	GW
	SH2B1 is suggested as one of the candidate genes whose methylation status affects ASD and genes on neurotrophin signaling pathway	GW
	Twins showed several DNA methylation differences, including a difference at CpG islands proximal to the homeobox *DLX1* gene, which might be relevant to the stress response and risk-taking behavior	GW
	Top sites were cg21557159 (nearest gene = *RAB39*), cg08648367 (nearest gene = *SIGLEC10*), and cg14212412 (nearest gene = *PREP*)	GW
	Differentially methylated probes associated with striatum, thalamus, and cerebellum. Several candidate genes were identified, including *MEIS2* and *VIPR2* genes. The signaling pathways are related to neurotransmission	GW
	Top differentially methylated region (hypomethylated in affected twins) in the promoter of *ST6GALNAC1*	GW
	Hypermethylation of *SLC6A4* in bipolar twin of 1 discordant pair	GW
	Global DNA methylation was lower in twin pairs with schizophrenia or other psychiatric disorders compared to healthy twin pairs, and the lowest correlations for global methylation were observed in twin pairs discordant for schizophrenia	Global and CG (*RLN* and *SOX10*)

Continued

Table 1 Epigenetic studies of mental health (−related traits) in twins—cont'd

Ref.	Trait	N MZ pairs	Tissue	Method
Kinoshita, 2013[71]	Schizophrenia	3 Male discordant twin pairs + 24 unrelated cases and 23 non-psychiatric controls	Whole blood	Illumina 450K
Melka, 2015[110]	Schizophrenia	2 Discordant twin pairs + brains of 8 rats who received olanzapine +8 control rats	Whole blood	NimbleGen Human DNA Methylation 3×720 K Promoter Plus CpG Island Array
Castellani, 2015[73]	Schizophrenia	2 Female discordant twin pairs + both parents of each twin pair	Whole blood	NimbleGen Human DNA Methylation 3×720 K Promoter Plus CpG Island Array
Petronis, 2003[67]	Schizophrenia	1 Concordant twin pair, 1 discordant affected pair	Peripheral lymphocytes	Bisulfite sequencing of the 5′ region of *DRD2*
Rosa, 2008[68]	Schizophrenia and bipolar disorder	63 Concordant or discordant, female twin pairs	Whole blood and/or buccal swabs	Allele-specific methylation of the androgen receptor (AR) assessed with PCR and methylation sensitive restriction enzyme
Kuratomi, 2008[74]	Bipolar disorder	1 Discordant male twin pair + 23 unrelated cases and 18 controls	Lymphoblastoid cells	Methylation-sensitive representational difference analysis (MS-RDA) + bisulfite pyrosequencing of top genes
Fisher, 2015[75]	Childhood psychosis	24 Discordant + 20 concordant twin pairs	Buccal swabs	Illumina 450K
4. Depression				
Zhao, 2013[80]	Number of current depressive symptoms	84 Twin pairs	Whole blood	Bisulfite pyrosequencing of the *SLC6A4* promoter region
Byrne, 2013[85]	MDD	24 Discordant and concordant unaffected twin pairs	Whole blood	Illumina Infinium 450K
Dempster, 2014[94]	Adolescent depression	18 Discordant twin pairs + postmortem brain (cerebellum) from 14 MDD patients and 15 controls	Buccal swabs	Illumina 450K + bisulfite pyrosequencing of top site
Davies, 2014[86]	MDD	50 D iscordant twin pairs + 356 unrelated case-control individuals	Whole blood	Genome-wide MeDIP-Sequencing
Córdova-Palomera, 2015[95]	Depressive symptoms during the last 30 days (Brief Symptom Inventory [BSI])	17 Twin pairs	Whole blood	Illumina 450K

	Results	Genome-wide (GW) or candidate-gene (CG) study
	10,747 CpGs differed in methylation (FDR 5%) between unrelated cases and controls. 234 CpGs of these CpGs also differed in methylation in discordant pair (average methylation difference > 1%, $p < 0.05$). 92% of the 234 CpGs showed hypermethylation in schizophrenia cases	GW
	A number of pathways and networks affected by olanzapine-induced hypermethylation or hypomethylation in rat brains, also showed differential methylation in schizophrenic twins from discordant pairs (who used antipsychotic drugs)	GW
	Twin pair 1 showed 58 genes with difference(s) in DNA sequence as well as promoter methylation between co-twins. Twin pair 2 showed 13 of such genes. 1 overlapped (*PTPRN2*)	GW
	The methylation profile of the affected twin from the discordant pair was more similar to the methylation profile of the concordant affected twin than to the unaffected co-twin	CG (*DRD2*)
	Twins discordant for bipolar disorder had greater differences in X-chromosome methylation than concordant twin pair. Skewed X-chromosome inactivation may contribute to their discordance, and X-linked loci may be involved in the disorder	X-chromosome
	Two regions (*SMS* and *PPIEL* gene) with methylation differences in the discordant pair replicated with bisulfite sequencing and replicated in an independent group of unrelated cases versus controls	GW
	The top-ranked site (cg23933044), located in the promoter of *C5ORF42*, was hypomethylated at age 10 but not at age 5 in twins with psychotic symptoms and in post-mortem prefrontal cortex brain tissue from schizophrenia patients	GW
	At 10 out of 20 CpG sites, the intrapair methylation differences was significantly associated with the intrapair difference in the number of depressive symptoms, after adjusting for potential confounders. A 10% increase in methylation was associated with a 4.4 increase in depression score on average	CG (*SLC6A4*)
	52.4% of methylation sites showed greater variance in twins with MDD compared to unaffected co-twins	GW
	The top-ranked site (cg07080019) was located in the *STK32C* gene, encoding a serine/threonine kinase, which is highly expressed in the brain. Two of the 10 top-ranked sites were replicated in postmortem cerebellum samples from MDD patients	GW
	Hypermethylation at *ZBTB20* in depressed twins; finding replicated in independent cohort of unrelated case-control individuals. Increased global variation in methylation in depressed twins	GW
	Replication analysis of 2 top sites from Dempster et al.[94]. DNA methylation at cg09090376 (CpG) located near *DEPDC7* but not cg07080019 (*STK32C*)—was associated with current depressive symptoms	CG (*DEPDC7* and *STK32C*)

Continued

Table 1 Epigenetic studies of mental health (−related traits) in twins—cont'd

Ref.	Trait	N MZ pairs	Tissue	Method
Córdova-Palomera, 2015[87]	Lifetime depression or related anxiety disorder (DSM-IV)	17 Concordant affected, discordant, or concordant healthy twin pairs	Whole blood	Illumina 450K
Oh, 2015[88]	MDD	100 Discordant twin pairs + prefrontal cortex or sperm samples from 104 subjects; bipolar cases and controls	Whole blood	8.1 K human CpG island microarrays + bisulfite pyrosequencing top sites
Malki, 2016[89]	MDD	97 Discordant twin pairs	Whole blood	8.1 K human CpG island microarrays
Córdova-Palomera, 2018[93]	Lifetime depression or related anxiety spectrum disorder (DSM-IV diagnoses)	6 Discordant, 4 concordant affected, 7 concordant unaffected twin pairs	Whole blood	Illumina 450K
Palma-Guidiel, 2018[83]	Lifetime depression or related anxiety disorder (DSM-IV)	13 Concordant, 14 discordant and 21 healthy MZ twin pairs	Whole blood	Pyrosequencing *NR3C1* gene, exons 1F and 1D
Peng, 2018[84]	Continuous depression symptoms, Beck Depression Inventory-II (BDI-II)	84 Male twin pairs, 35 female twin pairs	Peripheral blood leukocytes or peripheral blood monocytes	Bisulfite pyrosequencing and Illumina 450K
Palma-Gudiel, 2019[111]	Lifetime depression or related anxiety disorder (DSM-IV)	148 MZ twin individuals	Whole blood	Illumina 450K and bisulfite pyrosequencing
Starnawska, 2019[90]	Continuous depression symptoms	362 Twin pairs	Whole blood	Illumina 450K
Zhu, 2019[91]	Lifetime history of MDD (DSM-IV)	79 Twin pairs +sorted neuronal nuclei from 58 postmortem brain tissue samples, including 29 MDD patients and 29 controls	Monocytes	Illumina EPIC
Roberson-Nay, 2020[92]	MDD	27 Discordant, 42 concordant negative (i.e. both twins MD unaffected), and 6 concordant positive (i.e. both twins MD affected) twin pairs	Whole blood	Illumina 450K

ADHD, attention-deficit/hyperactivity disorder; ASD, autism spectrum disorder; BDI-II, Beck depression inventory-II; DSM-IV, diagnostic and statistical manual for mental disorders; MDD, major depressive disorder; PCR, polymerase chain reaction; GW, genome-wide; CG, candidate gene.

Results	Genome-wide (GW) or candidate-gene (CG) study
Various differentially methylated positions in discordant pairs and depression-related variably methylated positions (VMPs): sites with a large difference in concordant affected twins	GW
Several nominally significant DNA methylation differences at individual loci. Some MDD-associated epigenetic changes overlapped across brain, blood, and sperm more often than expected by chance	GW
Network analysis pointed at a network centered around PPAR-gamma (*NR1C3*), which is a target for the depression-reducing drug pioglitazone	GW
In 13 CpG sites depressed co-twins from the discordant pairs exhibited outlying DNA methylation signatures. None of them exhibited a methylation outlier profile in the concordant and healthy pairs, and some of these loci spanned genes previously associated with neuropsychiatric phenotypes, such as *GHSR* and *KCNQ1*	GW
Exon 1D CpG-specific methylation within a glucocorticoid responsive element (*GRE*) was correlated with familial burden of anxious-depressive disorders. Right hippocampal connectivity was significantly associated with CpG-specific *GRE* methylation	CG (*NR3C1*)
Multiple CpGs showed nominal individual associations, but very few survived multiple testing. Two CpGs in the *BDNF* and *NR3C1* mediated approximately 20% of the association between childhood trauma and depressive symptoms	CG (*BDNF, NR3C1, SLC6A4, MAOA* and *MAOB*)
SLC6A4 methylation was significantly higher in women when compared to men independent of DSM-IV diagnosis. *SLC6A4* methylation was further associated with the BSI-derived somatization dimension	CG (*SLC6A4*)
Through EWAS analyses adjusted for sex, age, flow-cytometry based blood cell composition, and twin relatedness structure in the data, depression symptomatology score was associated with blood DNA methylation levels in promoter regions of neuropsin (*KLK8*) and DAZ associated protein 2 (*DAZAP2*) genes. Other top associated probes were located in gene bodies of *MAD1L1*, *SLC29A2* and *AKT1*, all genes associated before with development of depression	GW
39 DMRs and 30 differentially expressed genes (RNA-seq) were identified. Some genes were replicated in postmortem brain tissue. Network analysis revealed distinct gene modules enriched in signaling pathways related to stress responses, neuron apoptosis, insulin receptor signaling, mTOR signaling, and nerve growth factor receptor signaling, suggesting potential functional relevance to MD	GW
760 DMPs and DMRs mapped to 428 genes. Regions associated with early-onset MD were found to overlap genetic loci identified in the latest Psychiatric Genomics Consortium meta-analysis of depression	GW

To date, three studies have examined genome-wide epigenomics profiles in ASD discordant MZ twins.[42, 44, 56] In samples from the Autism Genetic Resource Exchange (AGRE) repository, Nguyen et al.[44] examined lymphoblastoid cell lines derived from peripheral blood lymphocytes collected from three ASD-discordant MZ twin-pairs (aged 6, 8, and 16 years), and the unaffected siblings of two pairs. ASD was diagnosed with the Autism Diagnostic Interview-Revised (ADI-R). The co-twins also had autistic traits, but fell below the criteria for being diagnosed as autistic. Methylation was interrogated with the 8.1 K CpG island microarray (HN Microarray Centre, University of Toronto, Toronto, ON, Canada). In total, 73 CpG islands displayed differential methylation between discordant monozygotic twins. These loci were enriched for several biological processes, including gene transcription, nervous system development, and cell death/survival. More differentially methylated loci, 201 CpG islands in total, were detected in a comparison of ASD-twins to their unaffected non-twin siblings. Two genes were selected as candidates for further analyses, based on their biological functions in apoptosis and morphogenesis/inflammation networks, respectively, *RORA* (retinoic acid-related orphan receptor A) and *BCL2* (BCL2 apoptosis regulator). Upstream CpG islands of these genes were hypermethylated in lymphoblastoid cell lines of autistic individuals compared to unaffected siblings. First, their methylation difference was confirmed by additional independent techniques: bisulfite sequencing and methylation-specific PCR. Second, gene expression analysis showed that expression of *BCL2* and *RORA* was higher in the unaffected controls compared to the autistic twins. The relevance of findings was further examined by the analysis of post-mortem brain tissue from an independent group of 5 individuals with ASD and 5 age- and sex-matched controls: the protein levels of Bcl2 and Rora were decreased in the cerebellum of individuals with ASD. A more recent independent study of ASD discordant monozygotic twins from China[56] also detected differential methylation in genes involved in the neurotrophin signal pathway, including *BCL2*.

The largest epigenetic study to date on autistic traits in MZ twins included 50 MZ twin pairs (age 15 years) from the Twins' Early Development Study (TEDS) from the United Kingdom.[42] ASD and autistic traits were measured by the Childhood Autism Symptom Test (CAST) and the study included 6 pairs discordant for ASD, 5 concordant for ASD, 28 pairs discordant for autistic traits (social autistic traits, autistic restricted and repetitive behaviors and interests, and communicative autistic traits), and 11 pairs concordant for a low CAST score (used as control group). DNA methylation in whole blood samples was measured with the Illumina 27 K array and global levels of DNA methylation were quantified using the LUminometric Methylation Assay (LUMA). Global levels of DNA methylation were highly correlated in all MZ twin pairs, and there were no differences in global DNA methylation between discordant twins. Next, within-pair methylation differences at individual CpGs were compared between ASD-discordant MZ twin pairs and concordant unaffected twin pairs, which showed that the overall distribution of within-pair differences was significantly different in discordant pairs compared to concordant pairs, with a greater number of CpGs with a large average difference in discordant pairs. The study further ranked CpGs based on both the significance (*t*-test statistic) and magnitude of the methylation difference, and reported the 50 top CpGs. With this approach, the top differentially methylated site between ASD discordant twins, cg13735974 was located in the *NFYC* (nuclear transcription factor Y subunit gamma) promoter and showed a higher methylation level in ASD-affected twins compared with their unaffected co-twins. Although the majority of sites that showed a large methylation difference in discordant pairs were twin-pair specific, several were consistent across two or more discordant twin pairs and had the same direction of effect.

A third study included five Chinese monozygotic twin pairs discordant for ASD of both sexes and different ages (2, 7, 9, 11, and 12 years old).[56] ASD was diagnosed with a semi-structured observational

instrument; the Autism Diagnostic Observation schedule-generic (ADOS). DNA methylation in whole blood was measured with the Illumina 450 K array in three twin pairs and with reduced representation bisulfite sequencing (RRBS) in two twin pairs, covering over one million CpGs. Results gave no indication for a global methylation difference in discordant pairs. The study next selected loci with large methylation differences in any of the five discordant pairs: a fold change > 2 in RRBS data or an absolute difference in methylation level (Illumina array) > 10%, and then obtained a list of genes that met this criterium in all 5 twin pairs; yielding a list of 2397 genes. These genes were enriched for multiple biological pathways, of which the neurotrophin signaling pathway was regarded as a plausible candidate pathway for ASD pathophysiology. Many genes in this pathway (85.7%) had been implicated in ASD previously by genetic studies. One gene *SH2B1* (SH2B adaptor protein 1) was considered particularly interesting because this chromosomal region has been linked by a number of genetic studies to autism, other (neuro)developmental disorders, and obesity and was analyzed in two additional independent groups: 5 ASD-concordant MZ twins (both twins affected) and 30 pairs of unrelated ASD cases and age-and-sex- matched controls. While ASD concordant twins exhibited no difference in methylation at *SH2B1,* the comparison of unrelated cases and controls showed significant replication of the methylation difference.

2.2 Externalizing problems and ADHD

Externalizing behavior and problems, such as aggression, and ADHD, have received relatively little attention in epigenomic studies, including epigenomic studies in twins.[57–59] ADHD affects around 5% of children and adults[60] and is characterized by symptoms of inattention, impulsivity, and hyperactivity. Epigenetic mechanism have been proposed as a potential mediator for the numerous proposed prenatal and perinatal environmental risk factors.[61] Genetic variants (SNPs) that influence both ADHD and methylation level at CpGs in fetal brain, adult brain, or blood have been detected.[15] Such methylation sites might mediate the effect of SNPs on ADHD (and thus be a marker of a causal mechanism underlying ADHD), or might be related to ADHD through genetic pleiotropy, where the SNP influences methylation level and ADHD independently.

A study from 2018 examined 15 ADHD discordant MZ twins at around 10 years old from the United States.[58] The study performed brain imaging on 14 MZ twin pairs and measured genome wide DNA methylation in blood samples from 12 MZ twin pairs (mean age 9.7 years). First, by analyzing brain imaging data, the study found that the twins with ADHD had smaller right caudate and right thalamic nuclei and a larger right cerebellar cortex compared to the unaffected twin. Of note, neuroanatomic differences have also been found in monozygotic twin pairs discordant for other psychiatric disorders, such as schizophrenia[62, 63] and anxiety and depression.[64] Next, genome-wide DNA methylation data were analyzed with the Illumina 450 K array and the authors hypothesized that methylation differences are likely to contribute to the pathophysiology of ADHD if the differences are of a large magnitude (but potentially only present in one or a few pairs) or if the methylation difference is present in multiple pairs. Therefore, methylation differences between discordant twins were calculated and binned by magnitude of the methylation difference. Next, all differences were grouped into 173 categories of sites according to the magnitude of the methylation difference and the number of twin pairs in which the difference was observed. The study integrated their results with data on gene expression from an independent brain transcriptome database. It was found that genes that are expressed during early development in the striatum, thalamus and cerebellum showed significant enrichment for differential

methylation in blood in discordant pairs at shores and shelves (the regions surrounding CpG islands). Thus, differentially methylated regions were enriched for genes expressed in the discordant brain areas. On the other hand, genes that are expressed in the cerebral cortex (a brain region that not differ in size between discordant twins) did not show different methylation levels between discordant twins.

In a study by ourselves and colleagues, we analyzed genome-wide DNA methylation (Illumina 450 K array) in whole blood samples from 20 adult MZ pairs who were discordant for aggressive behavior assessed with the ASEBA Adult Self-Report (ASR).[59] Paired *t*-tests were performed to identify differences in methylation at individual CpGs, after adjusting the methylation data for commonly used covariates in EWAS analyses. Note that the MZ discordant twin design does not require adjustment for sex, birth cohort, maternal characteristics or age (assuming that twins were included in the study at the same age), but that they still may differ for other important covariates, especially blood cell composition (proportions of white blood subtypes), which can confound DNA methylation analyses if cases and controls have different blood cell composition because the different white blood cell types have distinct methylation profiles.[65, 66] No genome-wide significant methylation differences were identified between discordant twins that survived multiple testing correction. In the paper, we reported the 3 top CpGs with the smallest *p*-values (p-values $< 1 \times 10^{-5}$), which may be of interest for follow-up studies. These 3 CpGs showed an average within-pair difference in DNA methylation between 0.8% and 1.8%, and were in or nearby the following genes: *RAB39* (*RAB39A,* member RAS oncogene family), *SIGLEC10* (sialic acid binding Ig like lectin 10), and *PREP* (prolyl endopeptidase). The latter encodes prolyl endopeptidase, a proteinase that cleaves small neuropeptides and peptide hormones, such as angiotensin, thyrotropin-releasing hormone, gonadotropin-releasing hormone, neurotensin, vasopressin, and oxytocin. We also described how often large DNA methylation differences ($> 15\%$ or $> 30\%$) occurred in aggression discordant MZ pairs.[59] Each aggression-discordant MZ pair had on average 24 methylation sites (range: 0–311), where the within-pair methylation difference was larger than 30%, and on average 291 sites with a difference larger than 15% (range: 39–793). However, these highly discordant methylation sites were generally twin pair-specific, as was also observed in discordant MZ twin studies of autism[42] and ADHD.[58]

2.3 Schizophrenia, psychosis, and bipolar disorder

The first study that investigated DNA methylation patterns in monozygotic twins with schizophrenia was done in 2003 by Petronis and colleagues.[67] DNA was extracted from peripheral lymphocytes from 2 adolescent MZ twin pairs: 1 discordant for schizophrenia and 1 concordant pair (both twins affected), and DNA methylation was measured in the 5′ regulatory region of the *DRD2* gene (dopamine receptor D2) mapped by direct sequencing of sodium bisulfite-treated DNA. The results indicated that the methylation profile of the affected twin from the discordant pair was more similar to the methylation profiles of the twins from concordant affected pairs than to methylation profile of the unaffected co-twin.

In 2008, Rosa and colleagues investigated allele-specific methylation patterns of the Androgen Receptor (AR) on chromosome X, which provides a measure of X-chromosome inactivation, in buccal and blood samples from 63 female MZ twin pairs. This study included pairs who were discordant or concordant for schizophrenia or bipolar disorder, and concordant unaffected pairs.[68] MZ pairs who were discordant for bipolar disorder showed greater differences in the percentage of inactivated maternal and paternal X-chromosomes compared to concordant affected twin pairs and compared to concordant unaffected twin pairs, suggesting that skewed X-chromosome inactivation may be connected to

bipolar disorder and that X-linked loci may potentially be involved in the disorder.[68] This pattern was seen in both of the investigated cell types (blood and buccal). No such pattern was observed in the twin pairs who were discordant for schizophrenia.

At least seven studies have measured DNA methylation on a genome-wide scale in MZ twins discordant for schizophrenia or bipolar disorder, by various techniques.[69–75] Kuratomi et al. investigated genome-wide DNA methylation in lymphoblastoid cells from one adult male pair discordant for bipolar disorder, using methyl-sensitive representational difference analysis (MS-RDA).[74] This technique detected 10 regions that were subsequently fine mapped using bisulfite sequencing. For four regions, bisulfite sequencing confirmed the methylation difference between twins. These four regions were selected for replication analysis in lymphoblastoid cells from an independent group of 23 unrelated patients with bipolar disorder I or II (men and women) and 18 controls. Two regions showed significant methylation differences in the unrelated case control comparison: a region upstream of *PPIEL* (peptidylprolyl isomerase *E*-like pseudogene) gene on chromosome 1 (lower methylation in bipolar cases) and a region upstream of the *SMS* (spermine synthase) gene on chromosome X (higher methylation in bipolar cases). The methylation difference in *PPIEL* was accompanied by higher RNA expression levels of *PPIEL* in bipolar cases, while variation in *SMS* methylation was not associated with *SMS* expression level.

In a study of 22 MZ pairs who were discordant for schizophrenia or bipolar disorder, genome-wide methylation was assessed in whole blood.[69] Using the LINE-1 element assay, no difference in global methylation was identified between discordant twins. Differences in methylation level at individual CpG sites were investigated with the Illumina 27 K array. The top site, defined as the site with the largest methylation difference and smallest *p*-value in the comparison of all discordant twins, was located in the promoter of *ST6GALNAC1* (ST6 *N*-acetylgalactosaminide alpha-2,6-sialyltransferase 1), a region that had previously been detected to harbor a rare duplication in schizophrenia patients. The methylation level of this CpG was on average 6% higher in affected twins compared to their unaffected co-twin, although the exact difference varied considerably across different discordant pairs (up to a methylation difference $>20\%$ in some discordant pairs). The site was followed up for replication in 45 postmortem brain samples from unrelated schizophrenia and bipolar disorder cases and controls. The site showed no significant difference in methylation level in the brain in psychosis cases, but the authors noted that 13% of cases showed substantial hypomethylation at this CpG and other sites in the region, while no such outlier values were observed in any of the controls. The authors suggested that this finding may indicate that large changes in methylation at this locus may be present in a proportion of patients, rather than in all patients.

Another study of bipolar disorder screened methylation differences across genome-wide promotors in lymphoblastoid cells from 2 discordant male MZ pairs to select candidate loci for further analysis in 20 unrelated bipolar cases and controls.[70] Three regions retrieved by methylated DNA enrichment followed by promoter tiling arrays, in the discordant twins were selected (out of a large number of potential regions) for follow-up by bisulfite sequencing, which confirmed a methylation difference for one region, located in the CpG island shore in the promoter region of the serotonin transporter gene (*SLC6A4;* Solute Carrier Family 6 Member 4), in one of the two discordant twin pairs. Two of the 5 CpGs in this region showed a significant methylation difference in lymphoblastoid cells from unrelated bipolar cases and controls (hypermethylation in cases). Methylation level of one of these 2 CpGs correlated significantly with RNA expression level of *SLC6A4* in individuals who are homozygous for the 5-HTTLPR short allele (S/S), but not in heterozygotes individuals (S/L). One CpG was also hypermethylated in postmortem prefrontal cortex tissue from bipolar patients compared to controls.

Castellani and colleagues measured DNA methylation levels in genome-wide promoter islands with the NimleGen Human DNA Methylation 3×720K CpG Island Plus RefSeq Promoter Array, which measures DNA methylation at CpG islands and gene promoters, in whole blood from 2 schizophrenia discordant pairs and their unaffected parents.[73] In total, 138 and 330 differentially methylated regions were found, respectively, in each twin pair, of which 75% and 87% of the changes in the affected twin were not present in either the mother or the father, suggesting that they represented de novo methylation changes in the affected twin. Methylation differences mapped to genes involved in pathways previously implicated in schizophrenia, such as cell death and immune cell trafficking. Comparison of the 2 twin pairs showed that only 27 regions overlapped between pairs, thus, most large differences in methylation were twin-pair specific. The overlapping regions mapped to three gene clusters, including the *HIST2H* and *HIST1H* (histone) gene clusters on chromosomes 1 and 6, and *SNORD115* and *SNORD116* on chromosome 15. The region on chromosome 6 overlapped with a region previously identified to harbor common genetic variants associated with schizophrenia.[76] For the two discordant MZ twin pairs, whole genome sequences of DNA extracted from whole blood were also analyzed,[77] which indicated that 58 genes displayed differences in DNA sequence and methylation level in one twin pair, and 13 genes showed differences in DNA sequence and methylation level in another twin pair. In one of these genes, *PTPRN2* (protein tyrosine phosphatase receptor type N2), the affected twin from both pairs carried a unique intronic sequence variant and increased methylation of the promoter region of this gene.

The first study that measured genome-wide methylation in regions other than gene promoters used the Illumina 450K array on DNA extracted from whole blood samples of 24 unrelated schizophrenia patients and 23 controls, and in 3 discordant male twin pairs.[71] The results of the study indicated that methylation differences in schizophrenia may occur in regions outside of promoters.

In children, one study examined DNA methylation in relation to discordance for psychotic symptoms,[75] with the Illumina 450K array in buccal samples at age 5 and 10 years in 24 MZ pairs who were discordant for psychotic symptoms at age 12. Average genome-wide methylation levels did not differ between discordant twins. The 13 top sites with a p-value $<5\times10^{-5}$ at age 10 were followed up in postmortem prefrontal cortex tissue from 38 schizophrenia cases and 38 controls. The top-ranked site at age 10 (cg23933044, located in the promoter of *C5ORF42,* now referred to as *CPLANE1* (ciliogenesis and planar polarity effector 1) showed a 3.4% lower methylation level in the psychotic twin at age 10 and a 2.1% lower methylation level in the brain tissue of schizophrenia patients compared to controls. Though the function of the protein encoded by *C5ORF42/CPLANE1* is largely unknown, mutations in this gene cause Joubert syndrome, a severe neurodevelopmental disorder.[78] The other 12 top sites did not show a methylation difference in brain tissue. Nine of the top sites were also followed up in 20 unaffected concordant twin pairs (no psychotic symptoms at age 12) and showed smaller methylation differences in these concordant pairs compared to the discordant twin pairs (for the other top sites, no data were available in the concordant pairs). Comparing methylation differences across the two ages, none of top sites at age 10 showed a similar methylation difference at age 5. One top site from age 5 (cg10377582, *POU6F1;* POU class 6 homeobox 1, methylation difference = −7.7%) showed a nominal methylation difference at age 10 (−4%). The findings provide suggestive evidence that childhood psychotic symptoms are associated with methylation changes in peripheral tissue that are also seen in the brains of schizophrenia patients.

Another study reported that global methylation levels in whole blood were less strongly correlated between twins (most of whom were MZ) who were discordant for schizophrenia compared to twins who were concordant for schizophrenia, concordant for other psychiatric disorders or concordant healthy (total $N=114$ twin individuals).[79]

2.4 Depression

The connection between epigenetic mechanisms and depression and stress has been addressed in a number of studies of discordant MZ twins. One of the earliest studies from 2013 measured DNA methylation level at 20 CpG sites in the promoter region of the serotonin transporter (*SLC6A4;* solute carrier family 6 member 4) gene in peripheral blood samples from 84 monozygotic twin pairs, and tested whether within-pair differences in methylation correlated with within-pair differences in depressive symptoms.[80] At 10 CpGs, larger within-pair differences in methylation were significantly associated with a larger within-pair differences in depressive symptoms, after adjusting for BMI, smoking, physical activity and alcohol consumption. The study found no evidence for an effect of the 5-HTTLPR genotype (a polymorphism in the promoter region of *SLC6A4* that has been frequently studied in candidate gene studies) on within-pair differences: differences in methylation level and depression scores were similar for twin pairs with different 5-HTTLPR genotypes for the short/long allele. The *SLC6A4* gene has also been studied in MZ twins discordant for bullying victimization.[81] This study compared methylation levels of 12 CpG sites in buccal cells from 28 discordant MZ twin pairs, and identified a higher methylation level at one CpG site at the age of 10 in twins who had been bullied compared to their non-bullied co-twins.[81] Methylation level of this CpG showed a weak correlation with cortisol response to stress (individuals with a higher methylation level showed a blunted cortisol response). The methylation difference between discordant twins was not present at the age of 5, before one twin became a victim of bullying, suggesting that methylation level of *SLC6A4* in buccal cells correlates with physiological effects of bullying victimization. The study illustrates the strength of longitudinal data from discordant MZ twins to draw inferences about possible causal effects of life events on the epigenome. Of note, *SLC6A4* has been intensively studied in genetic candidate gene and candidate gene-by-environment interaction studies for depression, however, recent very large genetic studies do not support an association of this or any other historically studied candidate genes for depression.[82] Other candidate genes that have been studied in epigenetic studies of MZ twins discordant for depression and childhood trauma are *BDNF* (brain derived neurotrophic factor), *NR3C1* (nuclear receptor subfamily 3 group C member 1), *MAOA* (monoamine oxidase A) and *MAOB* (monoamine oxidase B).[83, 84]

To date, 10 studies have examined genome-wide DNA methylation with various techniques in whole blood[85–93] or buccal samples[94] from monozygotic twins discordant for depressive symptoms. A study of 18 adolescent MZ twin pairs discordant for depression symptoms measured genome-wide methylation levels in buccal cells with the Illumina 450 K array.[94] The top 10 sites, defined as sites with the largest methylation difference between twins and lowest *p*-value, were followed up for replication in postmortem cerebellum samples from 14 major depressive disorder (MDD) cases and 15 controls. Although none of the methylation sites showed a genome-wide significant difference between cases and controls within pairs (after multiple testing correction), the methylation difference was replicated for 2 sites in brain tissue from MDD cases and controls: cg07080019, located in *STK32C* (serine/threonine kinase 32C), showed an average increase in methylation level of 7% in buccal cells from depressed twins and an increase of 2% in cerebellum tissue of MDD cases, and cg09090376 in *DEPDC7* (DEP domain containing 7) showed a decrease of 5% in buccal cells from depressed twins and an increase of 3% in cerebellum. The two CpGs were also investigated in an independent study of 17 adult MZ twin pairs to test if within-pair differences in methylation level in whole blood correlate with within-pair differences in depressive symptoms.[95] This study replicated the association between methylation at cg09090376 (*DEPDC7*) and depressive symptoms (a lower methylation level in whole blood correlated with more

depressive symptoms), while the CpG in *STK32C* did not replicate in this study.[95] Little is currently known about the function of *STK32C* and *DEPDC7* and how they might be connected to depression.

A study employing MeDIP-seq in blood samples from 50 MZ pairs discordant for MDD from the United Kingdom and Australia identified 17 genome-wide significant DMRs.[86] Four were located in genes previously implied in MDD and selected for replication in an independent cohort of 356 unrelated MDD cases and controls (also using blood samples). One DMR replicated in the independent cohort; a DMR showing increased methylation in depressed subjects, located in the coding region of *ZBTB20* (zinc finger and BTB domain containing 20) which plays a role in the development of neuron structure within the hippocampus.

In a study by ourselves and colleagues, DNA methylation was measured in genome-wide CpG islands using 8.1 K CpG island microarrays, which interrogates the methylation status of 8109 CpG-islands, in peripheral blood from 100 monozygotic twin pairs discordant for MDD from the Netherlands, Australia and the United Kingdom, and in brain prefrontal cortex, and sperm samples from independent cohorts of cases and controls.[88] Many nominally significant, but no genome-wide significant, DNA methylation differences were identified in each tissue. Some nominal MDD-associated methylation differences overlapped across brain, blood, and sperm. For instance, 58 loci were differentially methylated between MDD cases and controls in postmortem brain tissue (cortex) and in whole blood samples from discordant twins. If DNA methylation differences are consistently found in the brain and in a peripheral tissue, this suggests that peripheral tissues such as blood may be used to monitor some (but not all) disease-associated epigenetic changes occurring in the brain.

In a population-based sample from the Danish Twin Registry, genome-wide DNA methylation (Illumina 450 K array) was assessed in whole blood samples from 362 MZ pairs tested for associations with depression symptoms measured on a continuous scale.[90] In a within-twin-pair analysis DNA methylation level differences within the twin pairs were correlated with differences in depression symptoms. No individual CpGs were significant after multiple testing correction. The top-ranking CpG was cg05777061 in the promoter region of *KLK8* (kallikrein related peptidase 8), also known as the neuropsin gene. Additional top sites were reported from a between-pair analysis, and various differentially methylated regions were identified in region-based analysis.

In 79 MZ pairs from the Washington State Twin Registry who were discordant for lifetime history of MDD, genome-wide DNA methylation (Illumina EPIC array) was assessed in monocytes. In addition to region-based DNA methylation analysis, the study examined differential gene expression using RNA-sequencing.[91] After multiple testing correction, there were 39 differentially methylated regions and 30 differentially expressed genes in monocytes, which were significantly enriched for genes involved in biological processes related to neuronal function, stress response, insulin regulation, mTOR signaling, and cytokine secretion, suggesting potential relevance to MDD pathogenesis. Differentially methylated regions occurred more often than expected by chance in loci detected in GWAS of MDD and drug targets of antidepressants. DMRs and differentially expressed genes were followed up in postmortem brain samples from 29 MDD patients and 29 controls. Ten of the 39 DMRs identified in blood contained at least one CpG that replicated in brain samples and one gene (*NDUFA8;* NADH: ubiquinone oxidoreductase subunit A8) showed lower expression in MDD patients in both blood and brain samples.

An EWA study of early-onset major depression in monozygotic twins, based on whole blood DNA methylation data (Illumina EPIC array) included 27 pairs who were discordant, 42 concordant unaffected pairs, and 6 concordant affected pairs.[92] In a region-based analysis, 17 differentially methylated

regions associated with major depression, all of which had a higher methylation level in the twins with major depression and 10 variably methylated regions were detected, each of which showed a larger variance in the twins with major depression. Analyses of individual CpGs identified several hundreds of CpGs with a difference in methylation level or variance associated with major depression. Six of the differentially methylated sites and 12 variably methylated sites overlapped with loci detected in the GWAS of MDD[96]; most of this overlap was driven by differential methylation at the major histocompatibility complex (MHC) locus on chromosome 6.

Several studies of monozygotic twins have reported that depression is associated with an increase in the amount of variation (rather than mean level) of DNA methylation. The first to report this was a study of DNA methylation in whole blood (Illumina 27 K array) from 12 pairs discordant for MDD.[85] While no significant differences in (mean) methylation levels were detected in this study, affected twins showed greater variance in methylation level at 52.4% of all genome-wide sites. Similar findings were also reported in other studies of blood[86, 87] and buccal cells.[94] A study of genome-wide DNA methylation (Illumina 450K array) in whole blood from 6 discordant pairs, 4 concordant affected pairs, and 7 concordant unaffected pairs, detected 13 CpGs with a larger variance in the affected twins from discordant pairs after multiple testing correction and correcting for cellular composition. These findings were referred to as epigenetic outlier values, because typically only one of the six affected co-twins showed an extreme methylation level compared to all other twins, which increased the overall variance in the affected twins.[93] None of such outlier values were detected at these CpGs in the concordant pairs. Among the loci with epigenetic outlier values were genes that have been previously implicated in psychiatric disorders, including the ghrelin receptor (*GHSR;* growth hormone secretagogue receptor), *KCNQ1* (potassium voltage-gated channel subfamily Q member 1) and *KCND2* (potassium voltage-gated channel subfamily D member 2).

3 Discussion and conclusions

We described MZ discordant twin studies whose aim was to identify epigenetic mechanisms connected to mental health disorders and related traits. Over the years, these studies have used a range of designs and techniques. While early studies focused on candidate genes, genome-wide studies are nowadays most often performed. Increasingly study designs recognize the importance of including covariates such as cell composition, either from measured cell counts (in e.g. blood samples) or estimates of cell counts.[65] For genome-wide studies, Illumina DNA methylation arrays remain the first choice for many researchers. The number of sites that these arrays target has increased from 27,000 to over 850,000. The studies described in this chapter come with important limitations that apply to epigenetic epidemiology in general.[97] Some of the earlier twin studies that measured DNA methylation in blood did not adjust for cellular composition. Not doing so can confound DNA methylation analyses, because the proportion of subtypes of white blood cells can differ between affected individuals and controls. Second, epigenetic variation was primarily measured in peripheral tissues with most studies done in peripheral blood samples. Several twin studies performed additional analyses to connect the findings in blood or buccal cells to information from brain tissue, with some findings replicating in the brain. Third, most studies measured DNA methylation and while this is a good starting point because the role of DNA methylation in development and environmental responses is well-established, many more epigenetic mechanisms exist that are at present largely underexplored.[98, 99] Forth, many techniques including the

Illumina DNA methylation arrays, although commonly referred to as "genome-wide" cover only a small proportion of all genome-wide CpGs. Finally, the majority of discordant MZ twin studies had a small to modest sample size. Although MZ discordant twin studies can have larger power compared to case-control studies of unrelated individuals (because many sources of variation are reduced),[100] it remains possible that some studies were underpowered. Several studies described top-ranking loci based on *p*-value or magnitude of the methylation difference, but many of these loci would not survive formal correction for multiple testing. In this case, replication in an independent sample, as was done by several twin studies, is an important strength.

In analogy to large-scale GWAS meta-analysis efforts, recent EWAS studies have seen an increase in sample size through meta-analyses and formation of consortia. The most often used techniques in these meta-analyses are the Illumina 450 K and the Illumina EPIC array. The largest psychiatric EWAS studies to date are whole blood DNA methylation studies of ADHD symptoms in childhood ($N=2477$ cord blood sample from neonates, and 2374 older children)[101] and adulthood ($N=4689$),[102] aggressive behavior in children and adults ($N=15{,}324$),[103] depressive symptoms (discovery stage, $N=7948$; replication, $N=3308$),[104] and schizophrenia or first-episode psychosis ($N=4483$).[105] These studies, several orders of magnitude larger compared to discordant MZ twin studies described in this chapter, typically reported a smaller number of differentially methylated loci than the studies in discordant twins. At an epigenome-wide significance threshold of $\sim 1\times 10^{-7}$ (Bonferroni correction for ~450,000 tests), these studies detected the following number of DMPs: 9 for ADHD symptoms in childhood and DNA methylation in cord blood and 0 for ADHD symptoms in childhood and methylation in older children,[101] 0 for ADHD symptoms and DNA methylation in adulthood,[102] 13 for aggressive behavior,[103] and 3 for depressive symptoms.[104] The study of psychosis and schizophrenia identified 95 DMPs associated with first-episode psychosis (cases, $N=2379$, controls, $N=2104$) and a much larger number of DMPs (1048) associated with diagnosed schizophrenia (cases, $N=1681$, controls, $N=1583$).[105] It furthermore detected DMPs associated with a treatment-resistant subgroup of schizophrenia, potentially caused by clozapine, a medication prescribed for this sub-type. The observation that these large meta-analyses typically reported fewer loci compared to the much smaller discordant MZ twin studies described in this chapter could be related to different analysis strategies (e.g. exploratory analysis approaches and less stringent criteria applied in some discordant MZ twin studies to derive a list of differentially methylated loci, no correction for cell counts or medication use in some MZ twin studies), but could also reflect larger power of MZ twin studies due to their homogeneity (affected twins are compared to their genetically identical same-sex and same-aged co-twin, and the entire experiment is conducted at a single lab, reducing noise in the analysis).

A common lesson from large-scale epigenomic studies is that the interpretation of epigenetic associations is typically not easy. Because DNA methylation is dynamic, DNA methylation signatures associated with psychiatric conditions might be causal to the disorder or may have arisen secondary to the condition, for instance by medication.[105] Another option is that the association is caused by a third factor that influences DNA methylation and the disease independently (e.g., genetic variants, lifestyle and prenatal exposures, in particular smoking). There is a need for strong research designs in psychiatric epigenetics, and studies of discordant MZ twins can make an important complementary contribution to ongoing meta-analysis efforts in psychiatric epigenetics. The strength of large EWAS meta-analyses and analyses in smaller but special samples from discordant MZ twins could be combined, for instance through mutual look-up of top-loci. The power of future epigenetic studies in MZ twins could be increased by combining samples from multiple twin cohorts. Studies in MZ twins also offer a perfect

opportunity to validate epigenetic scores (weighted sumscores based on DNA methylation at multiple loci, calculated with weights obtained in large EWAS meta-analysis).[106–108] The ultimate test for such scores is if they can identify the affected twin from discordant MZ pairs, or if they can explain differences in symptoms or disease severity between MZ co-twins.

Several discordant MZ twin studies reported greater DNA methylation variance in the affected twin, epigenomic outlier values in affected twins, or large methylation differences affecting only one or a few pairs. Such findings might reflect rare epimutations[109] that may only be present in some but not all discordant MZ twin pairs. If such epimutations indeed contribute to psychiatric disorders, phenotypically discordant-MZ twins may represent the ultimate design to detect them.

In conclusion, monozygotic twins discordant for mental health offer a unique opportunity to study epigenetic mechanisms linked to mental health. Future studies may integrate findings from large-scale GWAS and EWAS meta-analysis efforts in unrelated individuals, with information from unique cohorts of discordant MZ twins (Table 1).

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