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Title: Twin study designs as a tool to identify new candidate genes for depression: A systematic review of DNA methylation studies

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Abstract

Monozygotic (MZ) twin studies constitute a key resource for the dissection of environmental and biological risk factors for human complex disorders. Given that epigenetic differences accumulate throughout the lifespan, the assessment of MZ twin pairs discordant for depression offers a genetically informative design to explore DNA methylation while accounting for the typical confounders of the field, shared by co-twins of a pair. In this review, we systematically evaluate all twin studies published to date assessing DNA methylation in association with depressive phenotypes. However, difficulty to recruit large numbers of MZ twin pairs fails to provide enough sample size to develop genome-wide approaches. Alternatively, region and pathway analysis revealed an enrichment for nervous system related functions; likewise, evidence supports an accumulation of methylation variability in affected subjects when compared to their co-twins. Nevertheless, longitudinal studies incorporating known risk factors for depression such as childhood trauma are required for understanding the role that DNA methylation plays in the etiology of depression.

Keywords: twin study designs; depression; DNA methylation; epigenetics

MAIN TEXT

1. Background

The study of monozygotic twins has been instrumental in virtually all complex disorders (van Dongen et al., 2012) but specially so in the field of psychiatry. Far-reaching psychiatric research has been conducted using human twins. Perhaps the first studies on this topic using scientific methodologies were conducted in the 19th century by Savage (1883) and Gill (1883), by examining a couple of cases of mania and melancholia. Twin studies initially allowed to determine the heritability of complex traits but have since evolved to aid in the search for biomarkers of disease (Polderman et al., 2015; van Dongen et al., 2012; Young et al., 2017). This approach has proven especially useful in complex disorders characterized by considerable environmental influences, as is the case for psychiatric illness.

Major Depressive Disorder (MDD) is the non-communicable disease with the highest impact worldwide (Friedrich, 2017). Unipolar depressive disorders have been predicted to be the second leading cause of disease burden worldwide by 2030 and the first in high-income countries (Mathers & Loncar, 2006). Furthermore, MDD exhibits one of the lowest heritability values (around 40-50% depending on the study) of all psychiatric disorders suggesting a major role for environmental factors in triggering the disorder (Kenneth S Kendler et al., 2018). Indeed, several twin studies have documented the risk to develop psychiatric symptoms after exposure to stressful life events and traumatic experiences, such as childhood maltreatment or bullying (Arseneault et al., 2011; K S Kendler & Halberstadt, 2013; Silberg et al., 2016).

Due to both its low heritability and the heterogeneity of its clinical manifestations, the search for genetic risk variants for depressive phenotypes has proven extremely challenging. Recently, a genome-wide meta-analysis encompassing more than 800,000 subjects from three different cohorts yielded 102 significant risk loci (Howard et al., 2018). Interestingly, this study took into account broader definitions of depression; e.g. self-reported clinical diagnosis of depression and

self-reported help-seeking for problems with nerves, anxiety, tension or depression. Likewise, the genetic architecture of depression seems to greatly overlap with that of neuroticism highlighting the fact that more dimensional approaches are required in the field (Nagel et al., 2018).

The development of animal models for psychopathological conditions remains challenging due to the complexity and inherently human nature of psychiatric symptomatology. That is why monozygotic twins emerge as quasi-experimental models to dissect the effects of the environment in genetically identical human subjects who have been raised in similar conditions (shared environment). While monozygotic (MZ) twins origin from a single zygote that splits into two individuals in the early stages of embryonic development, dizygotic (DZ) twins origin from different zygotes; thus, MZ twins share virtually 100% of their genetic sequence while DZ twins only share approximately 50%. In fact, somatic mutations occurring at the earliest stages of embryonic development have been described in MZ twins (Li et al., 2014); however, MZ twins are considered as identical for research purposes given the low frequency of those mutations. Interestingly, the simultaneous study of MZ and DZ twins allows the disentangling of the role of non-shared (or unique) environment in the etiology of several complex traits and disorders (Figure 1).

Such non-shared environmental influences start as early as during the prenatal stages of life where intrauterine factors differentially affect co-twins of the same MZ pair (e.g. unequal placental distribution). Interestingly, MZ twins can share or not their chorion and amnion depending on the timing of the twinning process (Hall, 2003). Thus, MZ twins can be classified in diamniotic dichorionic, diamniotic monochorionic and monoamniotic monochorionic, respectively. Notably, monochorionic MZ twins exhibited higher epigenetic discordance than dichorionic MZ twins, suggesting that sharing the chorion involves a higher struggle for resources (Gordon et al., 2012; Palma-Gudiel et al., 2019).

Known risk factors for psychiatric disorders such as urbanicity, the loss of a parent, or low socioeconomic status are all shared environmental influences between MZ twins who have been raised together. Likewise, MZ twins share biological factors that are typically difficult to disentangle from psychopathology, such as gender, age and genotype. Thus, the study of MZ twins discordant for disease states allows the identification of novel biomarkers and gene pathways involved in the etiology of a certain trait of interest.

Epigenetic modifications might mediate the effects of non-shared environment on differential vulnerability for complex disorders. Briefly, epigenetics refers to heritable and dynamic chemical modifications modulating gene expression without altering the genetic sequence. DNA methylation is one of the most explored epigenetic mechanisms due to its accessibility, inter-individual variability and temporal stability (Talens et al., 2010). Unlike genetic variability, DNA methylation is dynamic and tissue-specific; thus, transversal studies do not allow establishing causal mechanisms while the tissue of interest is usually inaccessible (Mill & Heijmans, 2013).

Although identical in their genetic sequences, MZ twins diverge at the epigenetic level. Notably, epigenetic differences between twins of a pair accumulate throughout the lifespan suggesting the epigenome has the capacity to respond and adapt to the surrounding environment (Fraga et al., 2005). In this context, epigenetic discordance between MZ twins emerges as a tool to better understand the etiology of depression (Bell & Spector, 2011).

MZ twin study designs offer a unique opportunity to unravel the epigenetic basis of depressive phenotypes by means of the exploration of discordant pairs. Thus, the main goals of this review are (i) to systematically assess all research papers assessing DNA methylation correlates of depression by means of MZ twin studies, and (ii) analyze the advantages and disadvantages of each of the methodologies reviewed.

2. Methodology

2.1. Selection criteria

A systematic literature search was conducted in September 2019 in the PUBMED and SCOPUS databases, and the reference lists of the papers collected therein. The search items were limited to the title and abstract. The keywords combination was: (twin*) AND (depression OR depressive) AND (epigenetic* OR methylation). See Figure 2 for a flow diagram of the literature search and selection of papers to be included in the present review. The search yielded 14 papers to be reviewed (Byrne et al., 2013; A. Córdova-Palomera et al., 2015; A Córdova-Palomera et al., 2015; Aldo Córdova-Palomera et al., 2018; Davies et al., 2014; Dempster et al., 2014; Malki et al., 2016; Oh et al., 2015; H Palma-Gudiel et al., 2019; Helena Palma-Gudiel et al., 2018; Peng et al., 2018; Starnawska et al., 2019; Zhao et al., 2013; Zhu et al., 2019).

2.2. DNA methylation assessment

All the studies retrieved differ greatly in (i) the methodology employed to assess DNA methylation and (ii) the statistical approach used to identify epigenetic biomarkers. With regard to DNA methylation assessment, some authors performed hypothesis-free genome-wide DNA methylation association studies while others focused on candidate genes functionally relevant for depressive phenotypes. These strategies are each followed by specific downstream statistical pipelines.

The genome-wide approach can be used to identify differentially methylated probes (DMPs), differentially methylated regions (DMRs), variably methylated probes (VMPs), methylation outliers and canonical gene pathways enriched for the DMPs, DMR and VMPs identified (Rakyan et al., 2011). This kind of approaches require strict correction for multiple testing; however, due to the overall limited sample sizes assessed by the papers reviewed, most of the studies simply report top signals rather than genome-wide significant probes.

Candidate gene approaches allow to explore a specific region of interest more thoroughly. Pyrosequencing of target regions yields DNA methylation values for a number of contiguous CpG sites. This method allows both the identification of specific CpG sites involved in pathogenic

pathways by means of CpG-specific analysis or the assessment of the overall methylation of the whole target region. Complementarily, some studies approach the study of candidate genes using genome-wide data and focusing exclusively in the CpG sites located within the gene that are included in the array. However, this methodology generally leads to an infra-representation of the methylation landscape of a certain gene.

2.3. Clinical assessment

As for the assessment of depressive phenotypes, the papers reviewed have followed two main approaches: (i) assessment of lifetime prevalence of depressive disorders according to DSM (n = 9), or (ii) self-report of current depressive symptomatology (n = 5). While the former allows the comparison of affected versus non-affected co-twins of discordant pairs, the latter includes subjects with less severe forms of depressive symptomatology; however, by doing so, the epigenetic differences observed between co-twins reflect more qualitative aspects of psychopathology. Both approaches are complementary and help find different types of epigenetic biomarkers.

3. Results

3.1. Genome-wide DNA methylation studies

As shown in Table 1, twin studies on DNA methylation and depression started with Byrne et al. (2013), and often include genome-wide analyses of tens or a few hundred subjects. The largest sample size analyzed to date was recently published by Starnawska et al. (2019), in a report on depressive symptoms from 724 twins from two cohorts. In many cases, authors analyze DNA methylation of peripheral tissues at the genome-wide level (mostly with Illumina DNA methylation arrays or Methylated DNA immunoprecipitation sequencing (MeDIP-Seq)).

Half of the studies reviewed (n = 7) looked out for DMPs or DMRs in association with depression. While only one of them reported genome-wide significant DMPs (Starnawska et al., 2019),

significant DMRs were identified by three independent studies (Davies et al., 2014; Starnawska et al., 2019; Zhu et al., 2019). Unfortunately, neither DMPs nor DMRs appear to replicate across studies. Nevertheless, a considerable proportion of the identified hits map to genes previously implicated in psychiatric disorders guaranteeing the use of twin studies in the field.

Similarly, network analysis by the different reports point to an enrichment of gene pathways involved in either nervous system development and function (e.g. “dopaminergic synapse”, “neuron apoptotic process”), metabolic (e.g. “lipid metabolic process”), immune (e.g. “interleukin-2-mediated signaling events”) and neuroendocrine processes (e.g. “rapid glucocorticoid signaling”), fitting with the different etiological hypotheses for depression.

3.2. Candidate gene studies

On the other hand, studies of candidate genes/loci generally do not require dedicated multiple testing correction strategies, although it is still important to conduct analysis protocols that consider the technical limits of detection. The five candidate gene studies included in this review assessed DNA methylation at: solute carrier family 6 member 4 (*SLC6A4*), monoamino oxidase A (*MAOA*) and B (*MAOB*), nuclear receptor subfamily 3 group C member 1 (*NR3C1*), brain-derived neurotrophic factor (*BDNF*), DEP domain containing 7 (*DEPDC7*), and serine/threonine kinase 32C (STK32C) genes.

SLC6A4 encodes the serotonin transporter, involved in serotonin reuptake from the synaptic cleft, which is the pharmacological target of selective serotonin reuptake inhibitors (SSRI), a widely prescribed antidepressant type. *SLC6A4* methylation has been repeatedly assessed with regard to exposure to stress and subsequent development of different psychiatric disorders (Palma-Gudiel & Fañanás, 2017). Three of the studies reviewed focused on *SLC6A4* methylation revealing its association with depressive (Peng et al., 2018; Zhao et al., 2013) and somatization (Palma-Gudiel et al., 2019) symptomatology.

Similarly, *MAOA* and *MAOB* genes encode two different variants of the monoamine oxidase enzyme, a key component of the monoamine degradation pathway. Thus, they are involved in dopaminergic, noradrenergic and serotoninergic neurotransmission, which makes candidate genes for psychiatric disorders (Ziegler & Domschke, 2018). One of the studies reviewed reported MAOA, but not MAOB, methylation to be associated with depressive symptomatology (Peng et al., 2018).

NR3C1 encodes the glucocorticoid receptor, key player in initiation and termination of the stress response. *NR3C1* methylation has been previously linked with early life stress while his role in the etiology of stress-related disorders is currently under investigation (Palma-Gudiel et al., 2015). Two of the studies reviewed assessed *NR3C1* methylation supporting its role in depressive etiology and brain connectivity (Palma-Gudiel et al., 2018; Peng et al., 2018).

BDNF encodes a neurotrophic factor that promotes neuronal differentiation, dendritic growth, brain cell survival and proliferation, among others. Genetic variability at this gene (and Val66Met polymorphism in particular) has been traditionally linked to neurodegenerative and psychiatric disorders. More recently, *BDNF* methylation has also been explored, suggesting its role on schizophrenia pathogenesis, with a less clear role in depression (Zheleznyakova et al., 2016). One of the papers reviewed reported BDNF methyhlation to be associated with depressive symptomatology (Peng et al., 2018).

Finally, while *DEPDC7* and *STK32C* functions remain unclear, they were targeted in one of the studies reviewed as CpG-specific differential methylation at both genes was the more robust finding in one of the genome-wide DNA methylation approaches (Dempster et al., 2014). Examination of both CpG sites in an independent twin sample revealed replication of the association between *DEPDC7*, but not *STK32C*, methylation and depressive symptomatology (Córdova-Palomera et al., 2015).

4. Discussion

4.1. Strengths of the twin study design to explore DNA methylation

While the use of moderate sample sizes in virtually all studies warrants the need for replication to validate the findings, it is important noting that the research community has implemented different analytical approaches to gain statistical power while accounting for different features of the methylation assays. For instance, Byrne et al. (2013) used a permutation-based approach to establish significance of their top results, while reports published by Dempster et al. (2014), Córdova-Palomera et al. (2015b) and other authors take into account both statistical significance of group differences (e.g., p-value) as well as magnitude of the changes (e.g., absolute Δβ). Noteworthy, by integrating statistical significance and magnitude, the latter approach has advantages such as filtering results from probes with small variability within the study population (analogous to monomorphic sites in the DNA sequence), as well as removing probes with methylation differences close to the technical detection limits of the study platforms.

In the same vein, a subset of studies explored the role of DNA methylation variability within the depressed group rather than searching for absolute significant DNA methylation differences between groups (Figure 3). All of them reported higher DNA methylation variability and higher number of DNA methylation outliers in depressed subjects when compared to controls (Byrne et al., 2013; Aldo Córdova-Palomera et al., 2018; Dempster et al., 2014; Oh et al., 2015). In this context, methylation outliers could operate like copy number variants of very low frequency in the population but outstanding penetrance. Thus, identified CpG probes with outlying methylation signatures only in one of the co-twins of a pair can be useful for the identification of new pathways involved in the pathogenesis of depression.

With regard to study design, the use of self-reported symptomatology by means of questionnaires, such as the Brief Symptom Inventory (BSI) or the Beck Depression Inventory (BDI), instead of categorical diagnoses based on DSM criteria yields quantitative continuous scores to be used as dependent variables of the analysis, thus increasing the statistical power of

the sample. The lack of dichotomic subgroups allows to estimate intrapair differences for both variables of interest and test how they interact (figure 3).

Downstream statistical pipelines greatly differ between the studies reviewed. Our research group has explored some of the possibilities that can only be developed in datasets encompassing not only discordant pairs but also concordant (i.e. both twins of a pair affected by the trait of interest) and healthy (none of the twins of a pair affected by the trait of interest) twin pairs. As displayed in Figure 3a, mean twin pair methylation, intrapair methylation difference and methylation outliers that are specific to the discordant pairs but not the concordant or healthy ones are just some of the strategies explored in the UB Twin Registry (Córdova-Palomera et al., 2018, 2015a, 2015b; Palma-Gudiel et al., 2019; Palma-Gudiel et al., 2018). Specifically, the use of mean intrapair methylation allowed us to detect a familial hypermethylation in the *NR3C1* gene further associated with hippocampal connectivity; such findings were circumscribed to concordant pairs of our sample contributing to explain the heterogeneity of the depressive phenotype (Helena Palma-Gudiel et al., 2018).

4.2. Limitations of studies reviewed

General limitations of human DNA methylation studies apply to the papers reviewed herein. First, observational cross-sectional studies do not allow to establish causality regarding the identified DNA methylation changes. Thus, the correlational nature of the findings implies that either DNA methylation precedes psychopathology or appears afterwards as a downstream consequence of the symptoms; this phenomenon is also known as reverse causation (Tobi et al., 2018). Similarly, pharmacological treatment for depression might also influence the DNA methylation landscape (Csoka & Szyf, 2009); thus, studies focusing on current sub-threshold symptomatology rather than categorical diagnoses can help find the genuine biomarkers of disease. All the studies analyzed DNA methylation in peripheral tissues, mainly peripheral blood; although brain tissue appears as the most relevant source to explore psychiatric disorders, it

remains inaccessible in human studies; however, neuroendocrine and immune components of the depressive phenotype are likely to be biologically programmed in peripheral tissues (Raison & Miller, 2017). Alternatively, correlation of DNA methylation levels across tissues, specifically blood and brain, can be ascertained thanks to available online tools (Hannon et al., 2015). All studies were developed in populations of European descent, calling into question the generalizability of reported findings to other ethnic groups (Zhang et al., 2011); epigenetic estimation of chronological age has been already reported to differ as a function of ethnicity (Horvath et al., 2016).

One of the challenges of epigenetic epidemiology is the technical difficulty to access specific cell types (Teschendorff & Zheng, 2017). This is reflected in the fact that multiple research reports include peripheral tissues with mixed cell types, with each cell type potentially contributing different epigenetic signatures. One of the ways of mitigating this type of confounding is by accounting for cell mixture proportions with *in silico* methods (e.g., Houseman et al. 2012), although this is not yet a widespread practice among the community.

Further limitations specific to genome-wide studies include the use of commercially available platforms such as the widely used Illumina arrays (Moran et al., 2015; Sandoval et al., 2011). Since these arrays have been enriched in cis-acting promoters and CpG islands, they lack power to detect methylation changes in other putatively relevant regions, such as trans-acting promoters or distal regulatory regions (Pidsley et al., 2016).

5. Future directions

Despite the discussed caveats, epigenetic twin studies hold promise in the field of psychiatric epigenetics. Thanks to genetically informative designs and the removal of a great number of potential confounders, twin studies provide the opportunity to test epigenetic hypotheses in psychiatric disorders in resource-limited settings. Smaller sample sizes are sufficient to yield

promising hits in proof-of-concept studies that can be later replicated in bigger more representative samples (Sugawara et al., 2011; Tsai & Bell, 2015).

As summarized in Figure 3, twin studies offer different alternative to tackle the issue. Although one of the most intuitive strategies is to measure intrapair differences for the predictors and/or outcomes of interest in twin pairs discordant for the trait under study, both concordant and healthy twin pairs can be incorporated in more nuanced analyses (Fig. 3A). Likewise, a certain complex trait can also be explored from different points of view; e.g. categorical diagnosis of major depression and depressive symptomatology scores will yield different findings. Besides the phenotypical level of analysis, the DNA methylation analysis can be either (i) targeted or hypothesis-free, (ii) CpG- or region-specific, (iii) based on absolute differences or on variability rates, among other variations in the analysis strategy (Fig. 3B).

While the focus of the present systematic review was to evaluate the usefulness of twin study designs in the search for epigenetic biomarkers of depression, the different analytical strategies described herein can be extended to other biological correlates and other diseases as well. In fact, twin studies have also shed some interesting results in fields as diverse as transcriptomics and neuroimaging (Córdova-Palomera et al., 2017; Saffari et al., 2019). Integration of gene expression, epigenetic patterns and brain connectivity in a single model will surely contribute to a better understanding of the physiopathology of depression and other complex disorders.

In order to overcome the peripheral source of biological samples aimed for DNA extraction and methylomic profiling, the developing of brain organoids from discordant twin subjects could help elucidate the epigenetic changes present in nervous tissue in association with depression and other psychiatric and neurologic disorders (Luo et al., 2016). Since exposure to stressful and traumatic events plays an essential role in the etiology of depression, incorporating environmental threats into genome-wide DNA methylation studies is mandatory to unravel the mechanistic insights of hypothesized gene – environment interactions. Preliminary candidate

gene twin studies have already revealed the usefulness of longitudinal twin designs to dissect the dynamics of DNA methylation changes after trauma (Ouellet-Morin et al., 2013).

Recent developments in the epigenetic field emphasize the utility of computing *polyepigenetic scores*, paralleling polygenic risk scores developed from genome-wide association studies, as summary measures of exposure to stress and smoking (Provençal et al., 2019; Sugden et al., 2019). Following these pioneering studies, MZ twins discordant for depression might contribute in the estimation of new epigenetic biomarkers of vulnerability for depression.

Finally, most of the papers reviewed have taken advantage of already established twin registries in Europe (UK, Denmark, The Netherlands and Spain), USA and Australia. The promising findings obtained so far highlight the importance of establishing such big cohorts of MZ twins and the need to merge available datasets to improve the power of the analysis. In this regard, Malki et al. (2016) describe several strategies to reduce noise and unwanted variance to optimize data consolidation in studies encompassing samples from independent datasets.

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FIGURE CAPTIONS

Figure 1. Path diagram for the basic univariate twin model. The phenotype of each twin is decomposed into A, C and E variance components corresponding to additive genetics (A), common (shared) environment (C) and unique (non-shared) environment (E), respectively. MZ twins are assumed to share 100% of their genetic sequence while DZ twins share an approximate 50%. Abbreviations: DZ, dizygotic; MZ, monozygotic.

Figure 2. Flow diagram summarizing the systematic search performed. Sixty-eight records were excluded through title and abstract screening including 41 reviews, 5 book chapters, 1 editorial, 2 comments, 2 study designs, 1 opinion article and 16 research papers in which either depressive phenotypes or DNA methylation were not assessed.

Figure 3. Analytical strategies to explore epigenetics of depression in twin study designs. Different approaches can be developed according to **(a)** sample subsetting and **(b)** downstream statistical analysis. **(a)** Twins of a pair can either be concordant, discordant or healthy for a disease of interest as a function of whether both, one or none of the twins of a pair is affected, respectively. While comparisons between twins of discordant pairs (right arrow) allow the calculation of intrapair differences in genetically identical individuals, comparison between different types of twin pairs according to concordance (left arrow) provide an opportunity to explore familiality of traits and different degrees of severity. **(b)** The left panel illustrates the possibility to focus the analysis in functional candidate genes (e.g. due to their involvement in serotoninergic neurotransmission) or perform hypothesis-free genome-wide approaches; right panel displays different opportunities derived from genome-wide studies, which include (i) differential vs variable methylation, (ii) exploration of individual probes vs regions, and (iii) gene pathway analysis.

Abbreviations: DMP (differentially methylated position); MWAS (methylome-wide association study); VMP (variably methylated position).

Competing interests

ACP is currently collaborating with Takeda California Inc., San Diego, CA, USA. HPG and LF declare that they have no competing interests.

Study	Sample size	Mean age (range)	Psychiatric phenotype	Tissue assessed	Region explored	Main findings
Byrne et al., 2013	48 (pertaining to 2 independent cohorts)	NA (31 – 63)	MDD diagnosis (12 discordant pairs and 12 healthy pairs) according to CIDI or SSAGA	White blood cells	Genome-wide (450k)	No probe reached Bonferroni-corrected levels of significance. The top significant DMP was located in *MAST4* gene. No canonical pathways were enriched. No significant differences in overall methylation between discordant pairs. Cases exhibited higher methylation variance than controls.
Zhao et al., 2013	168	55	Depressive symptoms (BDI-II)	Peripheral blood	*SLC6A4* gene (pyrosequencing)	Intrapair differences in *SLC6A4* methylation were associated with differences in BDI-II scores at 10 out of 20 CpG sites assayed.
Davies et al., 2014	100 (pertaining to 2 independent cohorts)	NA (23 – 73)	MDD diagnosis (50 discordant twin pairs) according to CIDI or SSAGA	Peripheral blood Replication in unrelated individuals	Genome-wide (MeDIP-sequencing)	Seventeen Bonferroni-adjusted significant DMRs were identified. Four of them were located in genes previously identified with MDD pathology: *ZBTB20*, *AGTPBP1*, *TBC1D8* and *CLSTN1* genes.
Dempster et al., 2014	36	16.8	Self-rated depression (SMFQ)	Buccal cells Replication in postmortem brain samples of unrelated individuals	Genome-wide (450k) and validation through pyrosequencing	No probe reached Bonferroni-corrected levels of significance. The top significant DMP was located in *STK32C* gene. Network analysis revealed enrichment of pathways involved in nervous system development and function. No significant differences in overall methylation between discordant pairs. Depressed twins showed higher methylation variance than their healthy co-twins.
Córdova-Palomera et al., 2015a[1,2]	34	35.5	Depressive symptoms (BSI)	Peripheral blood	CpG probes cg07080019 and cg09090376[a] (from 450k)	CpG probe cg09090376, located in *DEPDC7* gene, was found hypomethylated in association with current depressive symptomatology. No associations were found regarding cg07080019 methylation (*STK32C* gene).

Córdova-Palomera et al., 2015b[2]	34	35.5	Lifetime depression or anxiety spectrum disorders diagnosis (4 concordant, 6 discordant and 7 healthy twin pairs) according to SCID-I	Peripheral blood	Genome-wide (450k)	No probe reached Bonferroni-corrected levels of significance. The top significant DMP was located in *HOXB7* gene. 175 and 221 VMPs were identified in the discordant and concordant subgroups, respectively. Pathway analysis revealed enrichment for variable methylation at "rapid glucocorticoid signaling" in discordant and "plasma membrane" in concordant twins, among others.
Oh et al., 2015	200 (pertaining to 3 independent twin cohorts)	41.2 38.4 53.7	Depression diagnosis according to DSM-IV criteria[5] Degree of twin discordance according to personality (EPQ-R, ABV) and number of episodes	White blood cells	Genome-wide (8.1K human CpG island microarray)	No probe reached Bonferroni-corrected levels of significance. A higher number of epigenetic outliers was retrieved from the depressed group when compared to controls. Pathway analysis revealed enrichment for postsynaptic membrane and density, lipid and methionine metabolic processes.
Malki et al. 2016[3]	194 (pertaining to 3 independent twin cohorts)	41.2 38.4 53.7	Depression diagnosis according to DSM-IV criteria[5]	White blood cells	Genome-wide (8.1K human CpG island microarray)	Network analysis of CpG sites included in a blood-based MDD classifier revealed enrichment for *c-MYC* and *PPARGC1A* gene hubs.
Córdova-Palomera et al., 2018[2]	12	37	Lifetime depression or anxiety spectrum disorders diagnosis (6 discordant twin pairs) according to SCID-I	Peripheral blood	Genome-wide (450k)	Higher methylation variance in affected twins when compared with their healthy co-twins. Thirteen CpG sites exhibited outlier-like profiles in association with depression. Three of them were located within *CCDC181* gene.
Palma-Gudiel et al., 2018[2]	96	33	Lifetime depression or anxiety spectrum disorders diagnosis (13 concordant, 14 discordant and 21	Peripheral blood	*NR3C1* gene, exons 1_F and 1_D (pyrosequencing)	Higher GRE methylation at exon 1_D, but not exon 1_F, of *NR3C1* gene promoter region was described in MZ twins concordant for lifetime anxious-depressive disorders when compared with either discordant or healthy twin pairs.

			healthy twin pairs) according to SCID-I			Higher methylation at this region was associated with decreased right hippocampal connectivity.
Peng et al., 2018	238 (pertaining to 2 independent cohorts)	55 36	Depressive sympyoms (BDI-II)	Peripheral blood leukocytes (n = 168) and monocytes (n = 70)	*BDNF*, *NR3C1*, *SLC6A4*, *MAOA* and *MAOB* genes (pyrosequencing or 450k depending on the study)	Methylation at 27 CpG sites in all 5 genes was associated with depressive symptoms. Of those, 18 CpG sites (located within *BDNF*, *NR3C1*, *SLC6A4* and *MAOA* genes) remained significant after correction for multiple testing.
Palma-Gudiel et al., 2019[2]	148	35.5	Lifetime depression or anxiety spectrum disorders diagnosis according to SCID-I Psychiatric symptoms (BSI)	Peripheral blood	*SLC6A4* gene (pyrosequencing)	No associations with either MDD diagnosis or depressive symptomatology were found. Higher *SLC6A4* methylation was associated with increased number of somatization symptoms as measured by BSI.
Starnawska et al., 2019	724 (pertaining to 2 independent cohorts)	65.9 77.9	Depressive symptoms (9-symptom scale adapted from CAMDEX)	Peripheral blood	Genome-wide (450k)	Methylation at 13 DMPs ($p < 10^{-5}$) and 30 DMRs ($p < 0.05$) was associated with depressive symptomatology in a paired twin analysis. The top significant DMP was located in *KLK8* gene encoding neuropsin. Pathway analysis revealed enrichment for regulation of myelination, longevity and schizophrenia. None of them remained significant after correction for multiple testing.
Zhu et al., 2019[4]	158	38.2	Lifetime MDD diagnosis (78 discordant pairs) according to SCID-4-RV	Monocytes	Genome-wide (EPIC)	Methylation at 39 DMRs was associated with lifetime history of MDD after correction for multiple testing. Network analysis revealed enrichment for neuron apoptotic process, stress-activated signaling cascades, and insulin receptor, mTOR, and nerve growth factor receptor signaling pathways.

Abbreviations: 450K, Infinium HumanMethylation450 BeadChip; ABV, Amsterdamse Biografische Vragenlijst; BDI-II, Beck Depression Inventory-II; BSI, Brief Symptom Inventory; CAMDEX, Cambridge Mental Disorders of the Elderly Examination; CIDI, Composite International Diagnostic Interview; DMP, differentially methylated probe; DMR, differentially methylated region; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders 4th Edition criteria; EPIC, Infinium HumanMethylationEPIC BeadChip; EPQ-R, Eysenck Personality Questionnaire Revised; GRE, Glucocorticoid Responsive Element; MDD, Major Depressive Disorder; SCID-4-RV, Structured Clinical Interview for DSM-IV Research Version; SMFQ, Short Mood and Feelings Questionnaire; SSAGA, Semi-Structured Assessment for the Genetics of Alcoholism; VMP, variably methylated probe.

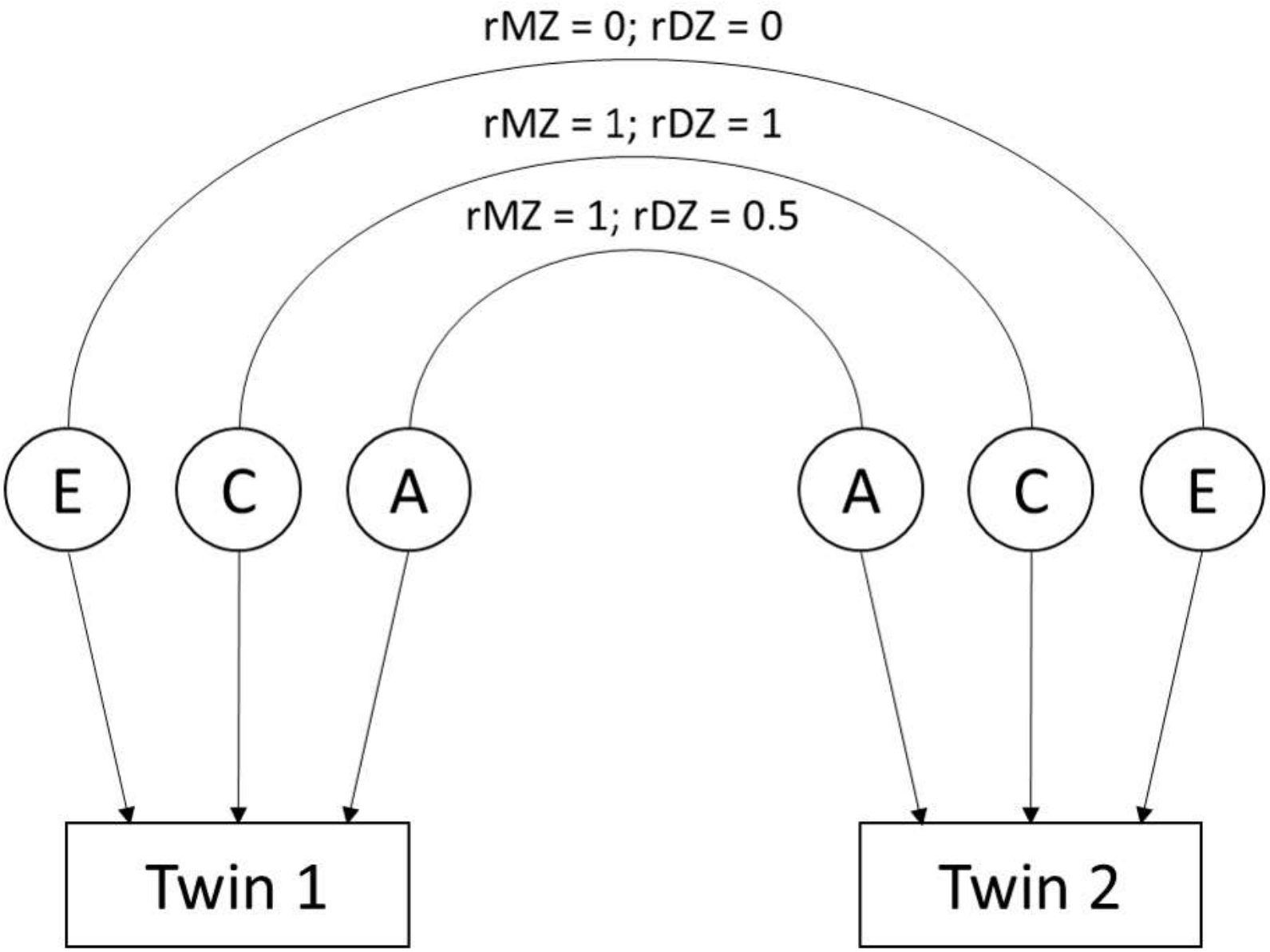
[1] Replication of Dempster et al., 2014

[2] These papers were developed using the same sample of MZ twins from the UB Twin Registry

[3] Replication of Oh et al., 2015

[4] One of the samples included in Peng et al. 2018 was already assessed by Zhao et al. 2013

[5] Depending on the study, depressive symptoms were evaluated by means of the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA), the Composite International Diagnostic Interview (CIDI), the Beck Depression Inventory (BDI) short form, the anxious-depression scale of the Young Adult Self Report (YASR) or the Schedules for Clinical Assessment in Neuropsychiatry (SCAN).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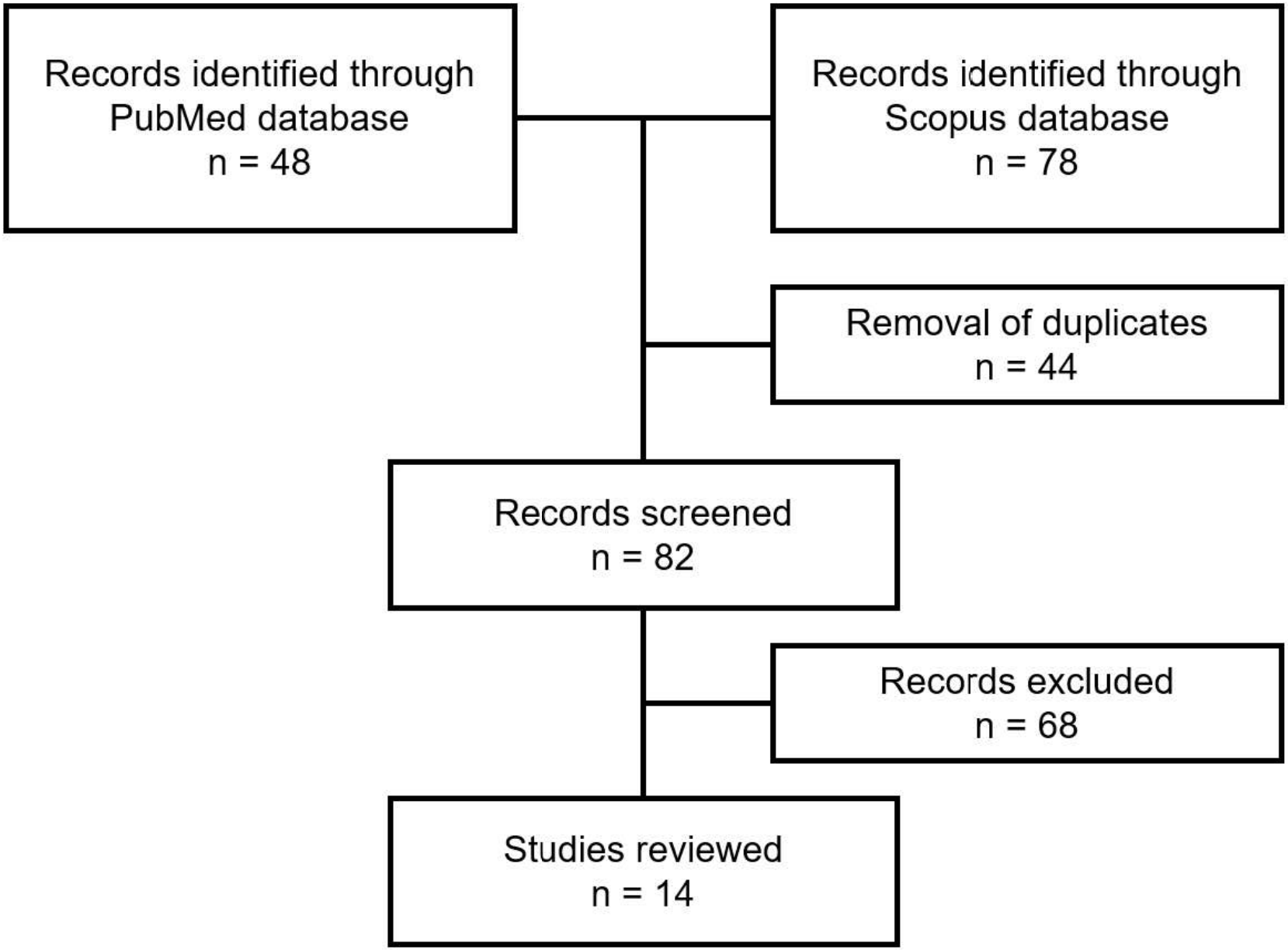
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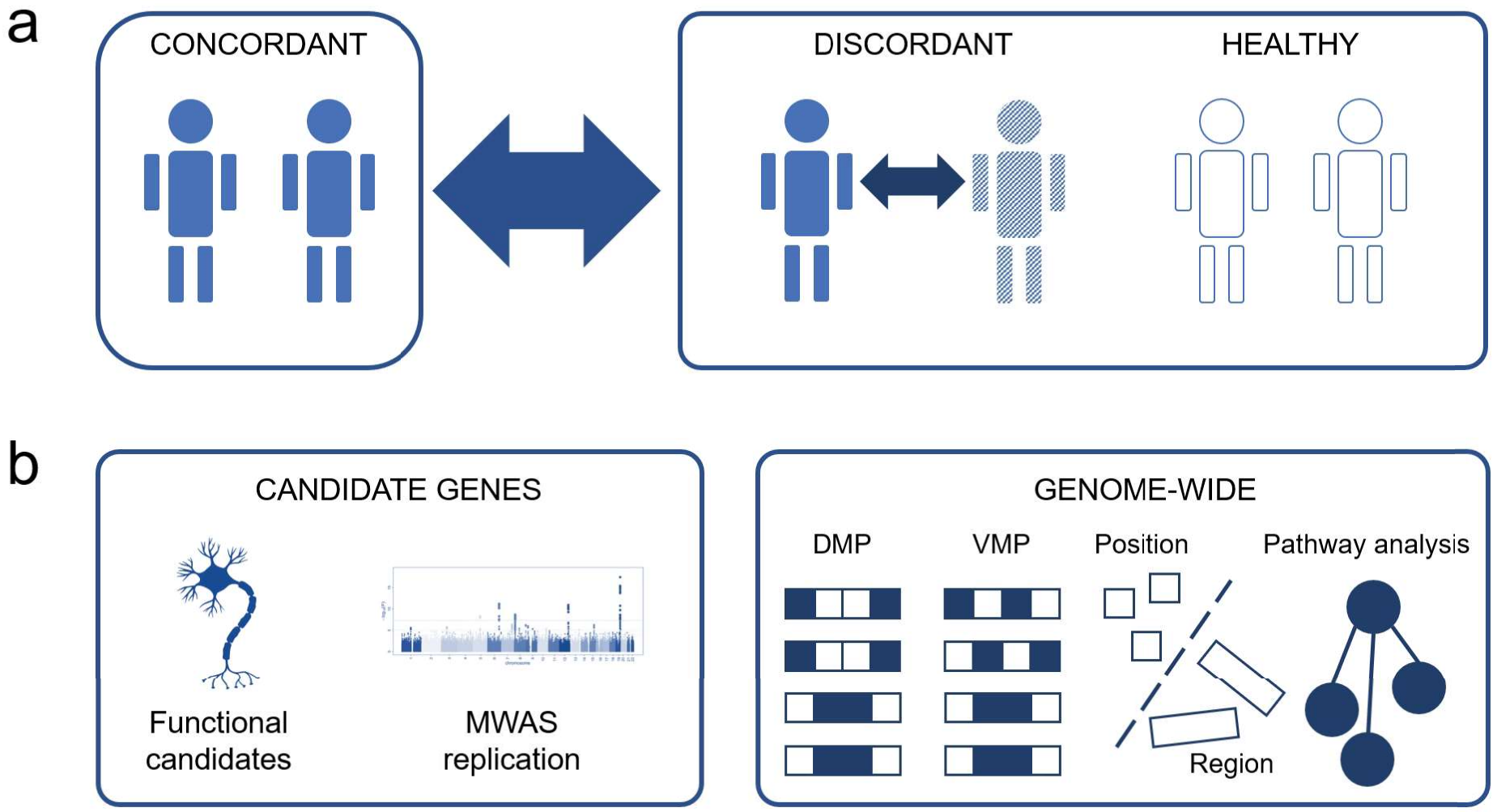
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