Current Neuropharmacology, 2022, *20,* 2267-2291

REVIEW ARTICLE

DNA Methylation in Depression and Depressive-Like Phenotype: Biomarker or Target of Pharmacological Intervention?

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ARTICLE HISTORY

Received: November 11, 2021
Revised: January 18, 2022
Accepted: January 26, 2022

DOI:
10.2174/1570159X20666220201084536

Abstract: Major depressive disorder (MDD) is a debilitating psychiatric disorder, the third leading global cause of disability. Regarding aetiopathogenetic mechanisms involved in the onset of depressive disorders, the interaction between genetic vulnerability traits and environmental factors is believed to play a major role. Although much is still to be elucidated about the mechanisms through which the environment can interact with genetic background shaping the disease risk, there is a general agreement about a key role of epigenetic marking. In this narrative review, we focused on the association between changes in DNA methylation patterns and MDD or depressive-like phenotype in animal models, as well as mechanisms of response to antidepressant drugs. We discussed studies presenting DNA methylation changes at specific genes of interest and profiling analyses in both patients and animal models of depression. Overall, we collected evidence showing that DNA methylation could not only be considered as a promising epigenetic biomarker of pathology but could also help in predicting antidepressant treatment efficacy. Finally, we discussed the hypothesis that specific changes in DNA methylation signature could play a role in aetiopathogenetic processes as well as in the induction of antidepressant effect.

Keywords: Depression, DNA methylation, antidepressant, epigenetics, animal model, biomarker.

1. INTRODUCTION

Major depressive disorder (MDD) is a debilitating psychiatric disorder characterized by persistent low mood, loss of interest or pleasure in nearly all activities, and associated with symptoms of fatigue, sleep disturbance, anxiety, and neurocognitive impairment [1]. MDD impacts not only on lives of patients but is also an economic burden for national health systems since people affected by this disorder exhibit significantly lower work performance, higher suicidal rate, social isolation, and higher alcohol and substance abuse [1-3]. MDD is the third leading global cause of disability [4], and mental health problems are dramatically increasing as a consequence of the pandemic situation [5, 6].

Despite the effort of research in trying to elucidate the aetiopathogenetic mechanisms involved in depressive disorders' onset, these remain a matter of discussion.

Nevertheless, different hypotheses converge in giving the main role to the interaction between genetic vulnerability traits and environmental factors [7, 8]. Both targeted and genome-wide association studies (GWAS) have been applied to study molecular genetic basis and polygenic risk for MDD [9-11]. Twin studies attribute approximately 40% of the variation in liability to MDD to additive genetic effects, while the identification of causal loci for MDD has been proven difficult, requiring extremely large sample sizes [12]. Although recent GWAS identified a number of risk loci reaching significance levels for depression [9-12], the biological mechanisms by which the risk variants exert their effects on depression remain largely unknown.

On the other hand, environmental conditions, especially the exposure to stressful events, are key determinants as precipitating risk factors, shaping phenotype presentation, and disease risk. Indeed, stress has been consistently proven to induce long-lasting brain modifications, including the alteration of synaptic structure and function, together with related molecular patterns, leading to behavioural changes in mood domains [13-19]. Accordingly, several studies have highlighted the role of stress exposure in developing psychiatric disorders, particularly mood disorders.

Although much is still to be elucidated about the mechanisms through which the environment can interact with the genetic background, there is a general agreement about the key role of epigenetic changes [17, 20, 21]. The word "epigenetics" was coined in the early 1940s by Conrad Waddington, who defined it as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" [22], thus ideally including all the molecular pathways translating a genotype into a particular phenotype. Over the years, the meaning of this term has been gradually refined, and today epigenetics refers to dynamic modifications of DNA without implying

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any alteration of sequence, but mitotically and/or meiotically heritable and leading to changes in gene function.

Mechanisms of epigenetic regulation include DNA methylation/demethylation, histone modifications, and non-coding RNAs. DNA methylation implies the covalent modification of DNA, with the addition of a methyl group to the 5' carbonium of cytosine, adenine, or guanine rings [20, 23]. Histone modifications are dynamic, post-translational modifications that contribute to chromatin organization and regulate gene expression. Although various modifications of histones are possible, most studies have focused on phosphorylation, acetylation, methylation, and ubiquitination [24]. Non-coding RNAs are classified in short-chain non-coding RNAs, including short interfering RNAs (siRNAs), microRNAs (miRNAs), and Piwi-interacting RNAs (piRNAs) and long non-coding RNA (lncRNAs). Non-coding RNAs are mainly (although not exclusively) involved in gene silencing but also participate in the regulation of DNA methylation and histone modifications [25].

In this narrative review, we will focus on DNA methylation, the first discovered and most studied epigenetic modification among epigenetic mechanisms. While DNA methylation has been exclusively involved in cellular development and differentiation for several years, more recent evidence highlighted a key role of this epigenetic factor in adapting to external stimuli in adult life.

Here we will discuss previous literature highlighting the association between changes in DNA methylation patterns and MDD in patients or depressive-like phenotype in animal models and response to antidepressant treatments. Compelling evidence resulting from the studies reviewed here supports the idea that DNA methylation signature has the potential to be used as an epigenetic biomarker not only for the diagnosis of depression but also for predicting antidepressant treatment efficacy. As discussed in the last section, this could be of great help for antidepressant drugs positioning, as well as for the study of molecular mechanisms involved in depression aetiopathogenesis/antidepressant effect, with the final aim to identify new targets for the development of innovative epigenetic antidepressant medications.

2. DNA METHYLATION

DNA methylation is catalysed by a family of enzymes called DNA-methyl-transferase (DNMT), which includes DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L. DNMT1 is accounted responsible for the maintenance of the levels of DNA methylation (thus ensuring cell identity during replicative phases), while DNMT3A and DNMT3B are involved in de novo methylation because they can introduce methylation into naked DNA [8, 26]. DNMT3L has an accessory function to DNMT3A and DNMT3B de novo methylation and, in humans, is expressed only in germ cells and embryonic stem cells but not in somatic cells.

DNA methylation is a dynamic and reversible process. There are two mechanisms by which methylation can be reverted: "passive" and "active" demethylation. The first one occurs by inhibition of DNMT1 in the nucleus, which causes the loss of its maintenance activity and, therefore, a decrease of pre-existing methylation. While the "active" process requires the intervention of ten-eleven translocation (TeT) enzymes, which remove the methyl group by oxidation of methylated bases [27].

DNA methylation was initially identified in CpG regions, namely CpG islands, which are stretches of DNA roughly 1000 base pairs long that have a high CpG density [8, 21, 26]. Most gene promoters (about 70%) reside within CpG islands. Since DNA methylation at promoters is typically associated with gene silencing, CpG islands in promoter regions of active genes are largely unmethylated [21]. Both direct (through the steric inhibition of binding with transcription factors) and indirect mechanisms (dependent on Methyl CpG binding Proteins, MeCPs, that recruit histone deacetylases) have been implicated in the repression of gene transcription induced by CpG island methylation [8, 21, 26]. Nevertheless, DNA can also be methylated within exons, leading to a higher level of gene expression selectively in dividing cells but not in slowly dividing and non-dividing cells [21].

Moreover, as already mentioned, methylation can occur at non-CpG sites, namely CpA, CpT, and CpC, among which CpA are more frequently methylated. Intriguingly, non-CpG methylation is enriched in human embryonic stem cells, neurons, and glial cells [23]. Unlike CpG methylation, which occurs in early development and does not increase with growth, neurons accumulate non-CpG methylation during development [21, 23]. In fact, it has been observed that non-CpG methylation increases simultaneously with synaptic development and synaptic density [28]. Intriguingly, non-CpG methylation is also highly conserved through species [23]. In brain tissue, non-CpG methylation starts during early postnatal development for both humans and mice (in the first 2 years and in the first 2-4 months, respectively) and slows down during later adolescence [28]. Methylation of non-CpG regions initially parallels the increase in synaptic density that happens from birth to 5 years in humans but continues to increase during synaptic pruning in adolescence [28].

3. ALTERATIONS OF DNA METHYLATION AT SPECIFIC GENES ASSOCIATED WITH DEPRESSION AND ANTIDEPRESSANT TREATMENT

A high majority of studies regarding the association between DNA methylation and depression/antidepressant effect are focused on the analysis of genes already known for a certain degree of association with increased risk of MDD (Fig. **1A**). In this regard, some paths, where results were consistent, had more success than others and have been followed by several research groups, while others were only preliminarily explored (see below). Since brain tissue analysis in humans is possible only post-mortem, clinical data are mainly coming from studies on peripheral tissues (primarily DNA from blood or blood cells). Therefore, despite the limits of animal models, preclinical research could be of key support in the identification of DNA methylation alterations associated with both depressive-like phenotype and antidepressant effect in the brain.

In this section, we will collect and compare data from both MDD patients and animal models of depression on DNA methylation at specific genes and promoters of interest.

3.1. Brain-derived Neurotrophic Factor (BDNF)

Brain-derived neurotrophic factor (BDNF) is the most abundant and extensively studied neurotrophin in the adult

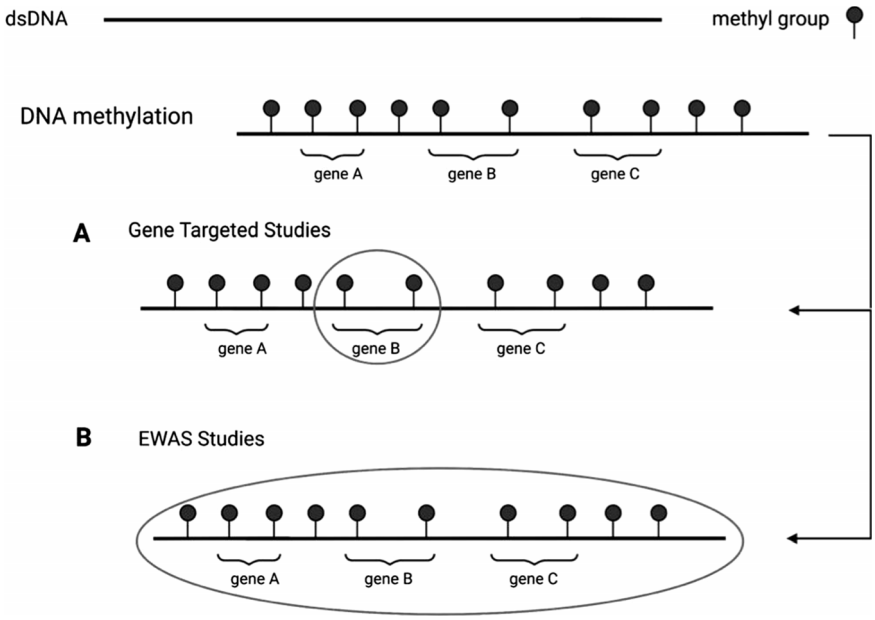

Fig. (1). Different approaches in DNA methylation studies. DNA methylation is a dynamic process consisting of adding a methyl group to the 5' carbonium of cytosine, adenine, or guanine rings. Nowadays, there are different techniques available to study DNA methylation, but most of them can be divided into these two approaches: Gene Targeted Studies (**A**), which focus on a single gene level of methylation, and Epigenome-Wide Association Studies or EWAS (**B**), which allow assessing genome-wide methylation profiles. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

mammalian brain [29]. It is involved in synaptic plasticity, learning, and memory, and alterations in BDNF have been linked to both neurodegenerative and neuropsychiatric disorders [16, 30]. The *BDNF* gene has a complex structure encompassing at least 11 different exons in humans and 9 rodents, with 9 alternative promoters in both species [29, 31]. The coding sequence is located in exon IX in both humans and rodents despite this complexity. All other exons are untranslated regions and have been proposed to represent a spatial code for the regulation of *BDNF* mRNA trafficking in specific neuronal compartments [32].

3.1.1. Clinical Evidence

Several studies consistently reported lower levels of BDNF in both blood serum and post-mortem brains (prefrontal cortex and hippocampus) of people affected by MDD [29, 30, 33, 34]. Accordingly, despite some inconsistencies, the high majority of studies found increased methylation at specific CpG sites within *BDNF* promoters in MDD patients.

The first study analysing methylation at *BDNF* promoters in MDD was published by Fuchikami *et al.* in 2011, who measured CpG islands methylation at *BDNF* promoters I and IV in genomic DNA from peripheral blood of a small sample of MDD patients compared to healthy controls [35]. Although no changes were found in DNA methylation at promoter IV, methylation profiles of CpGs at promoter I showed higher levels in MDD patients compared to healthy controls. This suggested for the first time a putative use of *BDNF* methylation as a diagnostic biomarker of MDD.

This hypothesis was further confirmed by other groups. *BDNF* promoter I methylation was reported to be significantly increased in MDD patients compared to both healthy individuals [36, 37] and patients with bipolar disorder [37,38]. Accordingly, Song and collaborators reported hypermethylation at specific CpG sites within *BDNF* promoter I in DNA from saliva samples of MDD patients compared to healthy controls, while methylation of BDNF exon I was found to be decreased in MDD [39]. Januar *et al.*, analysing DNA obtained from buccal tissue, observed increased DNA methylation at BDNF promoters I and IV in late-life depression (patients ≥ 65 years old) compared to non-depressed individuals of similar age [40], while Roy and colleagues observed significant hypermethylation of BDNF promoter region (no specific analysis for distinct promoters) in MDD patients with suicidal ideation compared to controls [41].

Na and colleagues analysed the correlation between cortical thickness and methylation of *BDNF* promoters as well as serum BDNF levels in MDD patients [42]. Intriguingly, they observed an inverse correlation between cortical thickness and *BDNF* promoter methylation in the MDD group. However, the level of methylation did not correlate with the severity of symptoms [37, 41, 42]. Other authors found significantly higher methylation at CpG site 217 (CpG-217) within *BDNF* promoter IX (not in other CpG sites, where methylation levels were reduced) in MDD patients compared to healthy controls, which was associated with lower plasma BDNF mRNA and protein levels [43].

Changes in *BDNF* methylation were also reported in patients with MDD in comorbidity with other clinical conditions. Kang and collaborators studied *BDNF* gene promoter VII methylation patterns in leukocyte DNA from depressed women 1 week and 1 year after breast cancer surgery [44]. They reported higher levels of average *BDNF* promoter methylation in depressed women than women who did not develop MDD after surgery. Moreover, Kim *et al.* showed that after acute coronary syndrome, patients who developed

MDD showed increased levels of *BDNF* methylation in leukocyte DNA compared to patients that did not develop any psychiatric disorder [45, 46].

Conversely, only a couple of studies reported no increase in *BDNF* promoter methylation in MDD patients. Ferrer and collaborators observed lower methylation at *BDNF* promoter I and promoter IV in the blood of MDD patients compared to healthy controls, which were associated with alterations of cognitive performance [47]. Similarly, Bakusic and colleagues reported reduced *BDNF* promoter I methylation (and no alterations in promoters IV and IX) in MDD patients, which was negatively associated with self-reported anhedonia [48]. However, in both studies, recruited patients were under antidepressant treatment. As described in detail below, recent pieces of evidence found that antidepressants could alter BDNF methylation and suggested that methylation levels at selected loci within the *BDNF* gene might be predictive for an antidepressant response.

For instance, CpG-87 methylation at *BDNF* promoter IV has been consistently associated with a higher probability of depression remission [49, 50]. Indeed, Tadić *et al.* in 2014 observed that non-responders MDD patients after 4 weeks of treatment with fluoxetine or venlafaxine had significantly lower levels of *BDNF* promoter IV methylation at CpG-87 compared to responders, while patients without CpG-87 methylation at baseline had a significantly higher risk for non-response than those with methylation at CpG or non-CpG sites [49]. After this first evidence, the group aimed to replicate the finding in wider sample size [50]. In this second study, although *BDNF* promoter IV methylation was not significantly associated with treatment outcome in the whole population of MDD patients, considering the subgroup with severe MDD, subjects with methylation at *BDNF* promoter IV CpG-87 showed significantly higher remission frequency compared to patients without methylation at CpG-87.

Finally, Hsieh and colleagues reported significantly higher methylation at *BDNF* promoter IX in MDD responders to Selective Serotonin Reuptake Inhibitors (SSRI) antidepressant treatment compared to non-responders [43].

3.1.2. Preclinical Evidence

As for studies on MDD patients, in rodent models of depression, depressive-like behaviour, as well as related alterations of structural plasticity and synaptic function, have been consistently associated with decreased BDNF protein expression, particularly in the hippocampus and prefrontal cortex, while chronic antidepressants were shown to increase the expression of BDNF [51-53]. Moreover, most of the studies on animal models consistently associated depressive-like phenotype and reduced *Bdnf* expression levels with increased methylation at *Bdnf* promoters.

Increased methylation at *Bdnf* I, IV, VI, and IX promoters were found in the hippocampus of prenatally stressed mice at postnatal day (PND) 40 compared to controls, which led to a downregulation of *Bdnf* gene and protein expression [54]. Molecular alterations were associated with depressive-like behaviour in the tail suspension test (TST), forced swimming test (FST), and sucrose preference test (SPT). Ye and collaborators obtained similar results in young adult offspring (PND 40) of dams stressed during the last two weeks of gestation [55]. In prenatally stressed mice, depressive-like behaviour was accompanied by increased methylation at *Bdnf* I, IV, VI, and IX promoters in the hippocampus and decreased BDNF mRNA and protein expression.

A more recent study assessed the cross-generational effect of early life stress (maternal separation) on *Bdnf* promoter IV methylation in rats [56]. In the first generation, maternal separation led to increased *Bdnf* promoter methylation in both sexes and decreased *Bdnf* gene expression in females. Intriguingly, very similar results were also found in the second generation, in which subjects with early life stress lineage expressed higher methylation and lower gene expression levels of *Bdnf*, like their parent generation.

In a different study, chronic administration of the stress hormone corticosterone to adult mice for 21 days induced CpG hypermethylation of *Bdnf* exon IV in the prefrontal cortex, reduced *Bdnf* expression and was associated with depressive-like phenotype in TST, FST, and SPT [57]. Hypermethylation of *Bdnf* IV promoter in the hippocampus was also measured in Flinders Sensitive Line (FSL) rats, a genetic model of depression, and was reversed by sodium butyrate, a histone deacetylase (HDAC) inhibitor with antidepressant and pro-cognitive properties [58]. Similar results were obtained by Francesca Calabrese *et al.* in the genetic rat model of depression obtained by serotonin transporter (SERT) knockout. They found hypermethylation of *Bdnf* exon IV promoter in the hippocampus at PND 7 and more pronounced hypermethylation at PND 21, while in the prefrontal cortex, hypermethylation was higher at PND 7 than at PND 21 [59].

On the contrary, in witnessing the defeat process of a mated partner, a stress model for anxiety-related behaviour, no differences were found between stressed and control mice in the methylation profile of *Bdnf* promoters in the hippocampus [60]. Similarly, although reducing Bdnf exon IV expression in the medial prefrontal cortex, social defeat stress in adolescent mice did not induce any changes in the methylation profile of *Bdnf* IV regions [61].

3.2. Monoaminergic Transmission

Starting from the evidence that drugs increasing the bioavailability of serotonin and noradrenaline exert antidepressant effects, the monoaminergic hypothesis of depression dominated the study of the pathophysiology of MDD for decades [62, 63]. Although the sole role of monoamines cannot give an exhaustive explanation for the development of MDD, the involvement of monoaminergic transmission in both pathogenesis and treatment of depression is definitely supported by convergent clinical and preclinical studies [64]. Consequently, several groups have focused their studies on the evaluation of methylation changes in genes implicated in the modulation of monoaminergic transmission.

The main serotonergic genes explored in clinical studies for changes of methylation profile in depression include the solute carrier family 6 member 4 (*SLC6A4*), serotonin receptors (*HTR1A/1B*), and the brain-specific Tryptophan Hydroxylase 2 (*TPH2*) genes. On the other hand, as detailed below, we were able to find only one preclinical study analysing methylation at serotonergic genes (*HTR1A*, [65]).

3.2.1. Serotonin Transporter (SLC6A4)

The *SLC6A4* gene encodes for SERT, which has a key role in serotonin reuptake and is the main target of SSRI antidepressant drugs. *SLC6A4* has been extensively studied due to its gene polymorphic region in the promoter sequence, an insertion/deletion of 44 nucleotides resulting respectively in either long (L) or short (S) alleles. Several studies have linked the short variant of the allele with reduced *SLC6A4* transcriptional levels and higher risk to MDD development [66, 67]. Intriguingly, this polymorphism was associated with specific alterations of DNA methylation [68].

3.2.1.1. Clinical Evidence

Methylation at the promoter of the *SLC6A4* gene in association with MDD and/or antidepressant response has been studied by several groups, with conflicting results [69].

In a first study, a trend for an association of increased overall *SLC6A4* promoter methylation with a lifetime history of MDD was found in lymphoblast cells [70]. Accordingly, a study conducted on DNA extracted from peripheral blood leucocytes of twin pairs discordant for MDD or posttraumatic stress disorder reported higher methylation levels within the *SLC6A4* promoter region associated with depressive symptoms [71].

Moreover, Iga and collaborators observed higher mean methylation levels of *SLC6A4* promoter in the blood of unmedicated patients with MDD compared to healthy controls [68]. Intriguingly, they also found a positive correlation between *SLC6A4* promoter methylation and the L polymorphism in MDD patients, suggesting that depressed subjects carrying the non-risk allele also had higher promoter methylation.

Similar results were also reported in patients with comorbidities: in 2013, Kim *et al.* reported increased *SLC6A4* gene promoter methylation in the blood of patients that developed MDD after stroke [72].

On the contrary, other groups obtained different findings.

Devlin and colleagues analysed *SLC6A4* promoter methylation in venous blood of pregnant women, with and without depressive symptoms, in the third trimester and neonatal cord blood. Mothers with depressive mood and their infants showed a reduction in *SLC6A4* methylation [73]. Differently, no alterations of *SLC6A4* promoter methylation were reported in buccal cells of a cohort of adolescents suffering from MDD, compared to healthy controls of the same age [74] nor in peripheral lymphocytes of subjects suffering from MDD comorbid with systemic lupus erythematosus (SLE), compared to both SLE without MDD and healthy controls [75].

Some studies also investigated the association between *SLC6A4* promoter methylation and antidepressant response. Kang and collaborators analysed the effect of childhood trauma on *SLC6A4* promoter methylation in leukocytes of patients with MDD [44]. Patients who experienced childhood trauma had higher levels of methylation in the *SLC6A4* promoter. Moreover, increased methylation was associated with a lower response to antidepressant treatment, suggesting that *SLC6A4* methylation could be a biomarker for predicting treatment response [44]. This hypothesis was further investigated by other groups, although with different results.

Domschke *et al.* analysed DNA methylation at nine CpG sites of *SLC6A4* transcriptional control region upstream of exon 1A, in the blood of MDD patients before 6 weeks of antidepressant treatment with escitalopram [76]. Differently from Kang *et al.* [44], lower levels of methylation were associated with impaired treatment response and higher methylation with better treatment response. In line with this evidence, Okada and colleagues observed a positive correlation between *SLC6A4* methylation and antidepressant response [77]. Moreover, they reported no difference in the methylation of *SLC6A4* promoter and the first section of intron 1, between unmedicated MDD patients and healthy controls, neither with clustering analysis nor comparing methylation rate of specific CpG units. Accordingly, Booji and collaborators observed no alterations of *SLC6A4* promoter methylation in the blood of female patients with MDD compared to healthy controls and increased methylation in patients under treatment with SSRI *vs.* patients treated with dual medication and untreated patients [78]. Moreover, Schiele *et al.* observed that average *SLC6A4* blood methylation levels predicted changes in HAM-D score in MDD patients treated for 6 weeks with monoaminergic drugs: average hypomethylation of *SLC6A4* was associated with lower remission of symptoms [79].

On the other hand, Bakusic and colleagues, although observing increased average *SLC6A4* promoter methylation in the blood of patients with MDD under antidepressant treatment compared to healthy individuals, did not find any correlation between *SLC6A4* promoter methylation and antidepressant response [80]. This result could be, however, affected by the fact that in this study, patients were already under treatment before taking part in the trial.

3.2.1.2. Preclinical Evidence

Despite the good number of studies assessing *SLC6A4* methylation in peripheral cells of MDD patients, with and without antidepressant treatment, to the best of our knowledge, surprisingly, there are no papers in the literature assessing *Slc6a4* methylation in brain areas of animal models of depression.

3.2.2. Serotonin Receptors

3.2.2.1. Clinical Evidence

There are just a couple of studies analysing DNA methylation at serotonin receptors genes in humans and reporting inconsistent results.

Gassò and collaborators found a negative correlation between the average methylation level of CpGs in the *HTR1B* promoter region and clinical improvement after fluoxetine treatment in children and adolescents affected by either MDD, obsessive-compulsive disorder, or generalized anxiety disorder [81]. The main limit of this study is that some patients were co-medicated with antipsychotics, benzodiazepines, or mood stabilizers.

Conversely, Wang and collaborators studied DNA methylation at 96 CpG sites within *HTR1A,* and *HTR1B* promoter regions in blood DNA from MDD patients treated with escitalopram and observed that remitters showed significantly higher methylation at CpG-668 of *HTR1A* and CpG-1401 of *HTR1B* compared to non-remitters [82]. Although average

methylation was not correlated with escitalopram response, four CpG sites of *HTR1B* (CpG-336, CpG-105, CpG-107, and CpG-1443) showed higher levels of methylation after treatment compared to baseline levels.

Given that these are the only two studies focusing on *HTR1A*/*HTR1B* methylation in MDD, more data are required to better understand the putative role of *HTR1A*/*HTR1B* methylation in predicting antidepressant response.

3.2.2.2. Preclinical Evidence

Only one paper analysed changes in *Htr1a* methylation in brain areas of male BALB/c mice exposed to Unpredictable Chronic Mild Stress (UCMS, one of the most widely used models of depression, [83]) and effects of chronic treatment with the antidepressant imipramine [65]. Chronic stress increased methylation at the CpG-681 repressing promoter site (which is conserved between rodents and humans), leading to reduced binding of the repressive transcription factor Sp4 and increased 5-HT1A receptor expression in both midbrain and prefrontal cortex. Intriguingly, chronic imipramine treatment fully reversed the UCMS-induced increase of methylation (and *Htr1a* expression) in the prefrontal cortex but not in the midbrain. The authors argued that the incomplete reversal by imipramine of stress-induced changes in *Htr1a* methylation and expression might indicate the persistence of stress vulnerability.

3.2.3. Other Monoaminergic Genes

3.2.3.1. Clinical Evidence

TPH2 protein is the rate-limiting enzyme accountable for the synthesis of serotonin in the brain. *TPH2* promoter does not contain any CpG islands, but it has sparse CpG sites [84]. Only one clinical study explored the relationship between *TPH2* promoter methylation in blood cells, life stress, and antidepressant treatment response in MDD patients [85]. Although some sex-specific and site-specific changes in the methylation profile of *TPH2* promoter were associated with MDD and antidepressant treatment outcome, none of the CpG sites resisted multiple testing corrections, suggesting that further studies are needed to validate the relationship between methylation/childhood stress and antidepressant therapeutic response.

SLC6A2 encodes for norepinephrine transporter, responsible for its reuptake from the synapsis. Based on the idea that hypermethylation at CpG islands of *SLC6A2* promoter was linked to disfunction in the norepinephrine transporter in panic disorder [86], Bayles and collaborators compared methylation levels of *SLC6A2* promoter in genomic DNA from the blood of MDD, panic disorder, and healthy controls, without finding any significant change [87]. However, in a small sample of MDD patients and panic disorder patients, the treatment with SSRIs increased *SLC6A2* promoter methylation levels compared to baseline [87].

Monoamine oxidase A (MAO-A) is an enzyme responsible for the degradation of monoamines such as norepinephrine, serotonin, and dopamine in the brain. There are contrasting results regarding *MAO-A* exon promoter methylation. Melas and collaborators analysed differences in DNA methylation levels of CpG sites spanning the first exon/intron junction of *MAO-A* in saliva samples from patients with either MDD, mixed anxiety depression, or dysthymia compared to healthy individuals [88, 89]. Intriguingly, they observed an average hypomethylation of 10 CpG sites only in female patients with MDD compared to sex-matched healthy controls [88, 89]. In a more recent study, CpG methylation at sites spanning the first exon and part of the first intronic region of *MAO-A* promoter was assessed in saliva samples from female psychiatric patients that experienced sexual and/or physical abuse compared to healthy women [90]. Patients shared a history of abuse that could be sexual, physical, or both and were diagnosed with one of the following disorders: alcohol abuse, drug abuse, depressive disorder, anxiety disorder, or conduct disorder. In this study, a significant correlation was found between *MAO-A* hypermethylation and sexual or physical abuse, while, specifically in sexually abused patients, increased average methylation of exon 1 was linked to current depression. Moreover, sexual abuse and hypermethylation of the *MAO-A* region of interest were independently associated with lifetime depression.

On the other hand, Domschke and colleagues studied the correlation between DNA methylation at promoter 1, exon 1, and intron 1 of *MAO-A* and response to 6 weeks of treatment with escitalopram in peripheral blood from MDD patients, without finding any significant change [76]. However, lower methylation at two specific CpG sites in the MAO-A promoter area was nominally related with impaired response to antidepressant treatment in females alone, which however did not resist to multiple testing.

Although more evidence is required, these studies concur to suggest that *MAO-A* methylation should be more in-depth investigated as a putative MDD predictive and/or diagnostic biomarker, particularly in women.

Catechol-O-methyltransferase (COMT) is an enzyme responsible for catecholamine degradation, specifically involved in maintaining levels of dopamine in the prefrontal cortex. In 2006, Dempster and collaborators analysed mRNA and methylation levels of *COMT* in post-mortem cerebellum tissue of patients who suffered from MDD, compared to healthy individuals, without finding any significant change [91]. Conversely, Na and colleagues observed significantly lower levels of *COMT* promoter methylation in peripheral blood from MDD patients compared to healthy controls, while antidepressant treatment did not exert any effect on *COMT* methylation [92]. Moreover, in the same study, they observed a significant effect of methylation on white matter connectivity in MDD patients: the group used diffusion tensor imaging to calculate the fractional anisotropy (FA) and radial diffusivity (RD) of the white matter tracts related to the prefrontal cortex and observed that in MDD patients FA values were lower and RD values were higher than controls.

3.3. P11

P11 is a calcium-binding protein, a member of the S100 EF-hand protein family, and is widely expressed throughout the brain [93]. P11 has an established function in the intracellular trafficking of transmembrane proteins to the cell surface, modulation of ion channels and receptors, and regulation of neuronal function. Compelling evidence consistently implicated P11 in the pathophysiology of depression as well as in response to antidepressants, which were shown to increase brain levels of p11 [93].

3.3.1. Clinical Evidence

P11 methylation has been studied in MDD patients in relation to antidepressant response.

Neyazi *et al.* found that *P11* promoter methylation predicts the antidepressant effect of electroconvulsive therapy in pharmacoresistant MDD [94]. Indeed, *P11* promoter methylation measured before each electroconvulsive therapy session was significantly higher in responders than in non-responders.

Other studies were published in the context of the "EMC Trial - (early medication change trial)", which was aimed at investigating whether non-improver after 14 days of the antidepressant escitalopram were more likely to remit after 8 weeks of treatment with an early medication change, compared to patients treated according to current guideline recommendations (ClinicalTrials.gov identifier n◦: NCT00974155 [50]). In this study, *P11* methylation was not correlated with MDD, antidepressant treatment response, or memory dysfunctions [50, 95].

3.3.2. Preclinical Evidence

The first paper studying changes in *p11* promoter methylation profile associated with depressive-like behaviour in animal models reported hypermethylation in the prefrontal cortex of FSL rats (and reduced p11 protein expression), which was fully reversed by chronic treatment with escitalopram [96]. More recently, increased methylation at *p11* exon 1, accompanied by reduced gene expression, was found in the prefrontal cortex and blood of rats exposed to 3 weeks of chronic mild stress [97]. Intriguingly, changes in *p11* methylation were associated with stress vulnerability, suggesting a key role in maladaptive processes leading to psychopathology.

3.4. Genes Involved in the Stress Response

The association between affective disorders and behavioural stress comes from compelling clinical and preclinical evidence (reviewed in [13-16, 18, 98]). Stress is one of the main risk factor for psychiatric disorders, and MDD patients often present dysfunctional hypothalamus-pituitary-adrenal (HPA) axis and elevated basal levels of cortisol [99-101]. Under stress conditions, neurons in the paraventricular nucleus (PVN) of the hypothalamus release into blood vessels arginine vasopressin (AVP), oxytocin (OXT), and corticotropin-releasing hormone (CRH). AVP and OXT regulate blood pressure, blood volume, and hydration. Moreover, AVP activates the locus coeruleus-norepinephrine system to support the "fight-or-flight" response mediated by epinephrine and norepinephrine through the sympathetic nervous system. On the other hand, CRH stimulates in the pituitary the production from the prohormone proopiomelanocortin (POMC) of the adrenocorticotropic hormone (ACTH), which in adrenal glands induces the release of glucocorticoids hormones (cortisol in humans and corticosterone in rodents). These hormones coordinate the physiological, behavioural response to stress. In turn, glucocorticoids hormones modulate the HPA system by negative-feedback loops, thus inhibiting the hypothalamus-pituitary activity. However, chronic exposure to stress leads to glucocorticoid insensitivity and decreased negative feedback [102, 103]. Glucocorticoids activate two types of receptors, mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), both of which are widely expressed in the brain [104]. Since hormone affinity to GR is higher than for MR, GR plays an important role in the stress response when cortisol concentration is high. GR sensitivity is regulated by a complex of the chaperone proteins FKBP5 (FKBP Prolyl Isomerase 5), HSP70 (Heat Shock Protein 70), and HSP90 (Heat Shock Protein 90). FKBP5 gene expression is induced *via* GR activation but binding to GR. and decreases affinity for cortisol, thus acting as a mechanism for GR desensitization. Consequently, FKBP5 availability and activity are critical for HPA axis modulation.

Accumulating studies in both MDD patients and animal models of depression analysed alterations in the methylation profile of genes associated with stress response, including *FKBP5*, *CRH*, CRH binding protein (*CRHBP*), CRH receptors (*CRHR*), glucocorticoid receptor (*NR3C1*), *AVP*, *OXT,* and *POMC*.

3.4.1. FKBP5

3.4.1.1. Clinical Evidence

Roy and colleagues reported increased *FKBP5* methylation in the blood of MDD patients compared to controls [41]. They also observed no significant difference between patients with or without suicidal ideation, although *FKBP5* gene expression was reduced exclusively in MDD patients with suicidal ideation.

Han and collaborators studied the interaction between the risk allele of *FKBP5* rs1360780 polymorphism (TT allele, which leads to overexpression of *FKBP5* following glucocorticoid receptor activation and dysregulated negative feedback on the stress-hormone system, [105]) and methylation of *FKBP5* intron 7 in MDD patients (intron 7 was selected because it contains one methylation site within a glucocorticoid response element, [18, 105]). Although not observing any significant diagnosis-by-genotype interaction for DNA-methylation percentage in MDD patients, the TT allele was associated with reduced *FKBP5* methylation only in the MDD group. This result is in line with previous evidence suggesting that the reduction of specific methylation at the *FKBP5* risk allele is associated with the development of psychiatric disorders [105, 106]. Accordingly, Tozzi and colleagues observed reduced *FKBP5* intron 7 methylation associated with reduced grey matter concentration in the inferior frontal orbital gyrus, which inversely correlated with depression severity and resulted in less activation in MDD patients than controls [102]. Moreover, lower methylation at *FKBP5* intron 7 was significantly associated with higher exposure to childhood adversity in MDD patients with the TT allele. This result was replicated by Klinger-König *et al.*, who observed decreased methylation at *FKBP5* intron 7 in patients with MDD and TT allele, compared to both patients with the C allele (no-risk allele) and healthy individuals [103]. Differently, HÖhne and collaborators compared subjects carrying the TT allele and observed increased methylation in MDD patients compared to healthy controls [107].

Conversely, Bustamante and collaborators failed to demonstrate any involvement of *FKBP5* methylation in the relationship between early life stress and MDD, as well as any significant change of *FKBP5* methylation in MDD patients compared to healthy controls [108]. Nevertheless, in the same study gene expression of *FKBP5* was found to be increased in the blood of patients with MDD. Similarly, Farrell and collaborators reported no statistically significant

difference of *FKBP5* intron 7 average or individual CpG methylation between patients with MDD and healthy controls [109].

Overall, despite some inconsistency, most studies showed that the TT allele of FKBP5 rs1360780 polymorphism was associated with decreased methylation in FKBP5 intron 7 in subjects exposed to childhood trauma and MDD patients [18, 102, 103, 110]. Therefore, hypomethylation at *FKBP5* intron 7 has been proposed by some authors as a predictor for the development of MDD, particularly when associated with the presence of the TT allele [105, 106].

3.4.1.2. Preclinical Evidence

Only a few studies from the same group analysed changes in *Fkbp5* methylation in an animal model of depression [111, 112]. In line with most of the clinical evidence, reduced methylation of *Fkbp5* was reported in the blood, whole hippocampus, and hypothalamus of mice exposed to chronic treatment with corticosterone, which persisted for several weeks after cessation of hormone application [111, 112].

3.4.2. CRH, CRH Binding Protein, and CRH Receptors

3.4.2.1. Clinical Evidence

In the previously cited work of Roy and collaborators, besides changes in *FKBP5* methylation, the authors found significant hypermethylation of *CRHBP* exclusively in the MDD group with suicide ideation compared to healthy controls (no differences between MDD with or without suicide ideation, nor between MDD without suicide ideation and controls) [41].

Moreover, Humphreys and colleagues investigated if methylation within six key HPA-axis genes (*NR3C1*, *NR3C2*, *CRH*, *CRHR1*, *CRHR2*, *FKBP5*) was a predictor of MDD development in a sample of healthy adolescent girls at high or low familiar risk of developing depression when participants had no current or past MDD diagnosis [113]. DNA methylation levels within CpG sites in *CRH*, *CRHR1*, *CRHR2* (and *NR3C1*) were associated with risk for MDD across adolescence and young adulthood, suggesting a predictive validity of methylation at HPA-axis genes.

3.4.2.2. Preclinical Evidence

Sterrenburg *et al.* studied changes of *Crh* DNA methylation in the PVN, bed nucleus of the stria terminalis (BST), and central amygdala (CeA) of male and female rats exposed to chronic variable mild stress (CVMS) [114]. Total *Crh* methylation was higher in the PVN of stressed rats compared to control females while reduced by stress in the BST of males and the CeA of females. These results suggest that chronic stress exerts brain area- and sex-specific changes in *Crh* methylation.

Another study showed that prenatal dexamethasone administration in late pregnancy decreased the methylation of Crhr promoter in the hippocampus of first-generation (F1) offspring, inducing depressive-like behaviour and increasing hippocampal CRH and CRHR protein expression [115].

3.4.3. Glucocorticoid Receptor (NR3C1)

3.4.3.1. Clinical Evidence

NR3C1 hypermethylation, specifically at exon 1 F, was consistently associated with MDD in several clinical studies [41, 80, 109,116]. Nantharat *et al.* measured hypermethylation of *NR3C1* in female MDD subjects [116]. Roy and collaborators observed that NR3C1 hypermethylation in DNA isolated from blood mononuclear cells of MDD patients was independent of suicide ideation [41]. In the study of Farrell *et al.*, hypermethylation of *NR3C1* in the blood of patients suffering from MDD was found to be not correlated with early life adversities [109]. Moreover, Bakusic and collaborators observed that increased average methylation at *NR3C1* was associated with lower cortisol response in MDD patients compared to healthy controls and negatively correlated with symptom improvement after chronic antidepressant treatment [80].

Increased promoter *NR3C1* methylation was proposed as a predictive marker for MDD risk by Van der Knaap and coworkers, who reported that *NR3C1* methylation in adolescence was positively associated with risk of lifetime internalizing disorders, and with depressive and anxiety symptom scores at 3-year follow-up [117]. On the other hand, Melas and collaborators found an association between early parental death and hypermethylation of one CpG site at 1F region of *NR3C1*, but no differences in MDD patients compared to healthy controls [88]. Similarly, Alt and collaborators did not identify any significant difference in the methylation pattern of *NR3C1* promoters in post-mortem brains of individuals with MDD compared to healthy controls [118].

Conversely, Tyrka and collaborators reported that childhood adversity and a history of past substance-use disorder and current or past depressive or anxiety disorders were associated with lower levels of *NR3C1* promoter methylation, which was linked to altered cortisol responses to the dexamethasone/CRH test [119]. Finally, Humphreys *et al.* reported a significant association between hypomethylation at *NR3C1* promoter in healthy adolescent girls and the development of MDD in adulthood [113].

3.4.3.2. Preclinical Evidence

Early-life stress in mice was found to induce site-specific hypermethylation at the CGI shore of *Nr3c1* in hypothalamic neurons that produce CHR, thus preventing CRH upregulation under conditions of chronic stress [120]. This interesting observation supports the idea that early-life stress can buffer against subsequent lifetime stressors [120-122]. On the other hand, the combination of adverse maternal environment and high-fat diet impaired learning and memory in male adult offspring mice, concurrently with decreasing DNA methylation at *Nr3c1* promoter and increasing hippocampal expression of GR [123]. Daniels *et al.* found no significant difference in *Nr3c1* promoter methylation in the hippocampus of adult rats exposed to maternal separation in early life [124].

Overall, these results suggest that the effects of stress in early life on *Nr3c1* methylation might vary depending on the species, brain areas, as well as stress type/intensity/duration.

3.4.4. Vasopressin and Oxytocin

3.4.4.1. Clinical Evidence

Mothers with perinatal depression showed increased levels of methylation in 16 out of 22 CpG sites of OXT receptor and in 2 out of 6 CpG sites on AVP gene compared to healthy mothers [125]. Interestingly, in the same study, chil-

dren not exposed to postnatal depression had lower levels of OXT receptor methylation.

Conversely, Sanwald *et al.* reported a significant hypomethylation of *OXT* promoter in peripheral blood of MDD patients and healthy controls [126]. Moreover, they highlighted that in 7 out of the 9 CpG sites studied, methylation was significantly higher in female patients compared to males.

3.4.4.2. Preclinical Evidence

Only one study analysed changes in *Avp* gene methylation in animal models of depression [127]. Early-life stress in mice was found to induce long-lasting alterations (up to one year) of HPA axis function, including elevated CRH, AVP, and glucocorticoid. This was accompanied by *Avp* gene hypomethylation in multiple CpG residues, suggesting that early-life stress can dynamically control DNA methylation in postmitotic neurons to generate stable changes in AVP expression that trigger neuroendocrine and behavioural alterations that are frequent features in depression [127].

3.4.5. Proopiomelanocortin

3.4.5.1. Clinical Evidence

There is only one study that investigates the correlation between MDD and methylation of *POMC*. *POMC* methylation level in blood DNA was higher in both male and female adolescent MDD patients than healthy controls [128].

3.4.5.2. Preclinical Evidence

Wu and co-workers reported that early-life stress reduced *Pomc* methylation in the serum of male mice, which was associated with an increase of *Pomc* mRNA [129]. Although in conflict with the results obtained in the above-mentioned clinical study in depressed adolescents [128], reduced serum *Pomc* methylation and increased mRNA expression persisted in mice subjected to early-life stress for up to one year [129].

More studies are needed to unveil the involvement of *Pomc* methylation in depressive behaviour.

3.5. Glutamatergic Transmission

Glutamate is the main excitatory neurotransmitter in the central nervous system, where it exerts diverse and complex effects by binding to a large range of receptors with distinct structural and functional properties [130]. Glutamate receptors are broadly divided into two groups: the cation-permeable ionotropic glutamate receptors (iGluR) and the G protein-coupled metabotropic glutamate receptors (mGluR). The iGluRs are subdivided into N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-proprionate (AMPA), and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) receptors, while mGluRs are subdivided into three groups (groups I–III) and eight subtypes (mGluR1-8).

Accumulating clinical and preclinical evidence highlighted the key involvement of glutamatergic transmission in the aetiopathogenesis of depression and antidepressant effect [131, 132]. Studies of the neurobiological basis of morpho-functional alterations in cortico-limbic areas implicated in depression have revealed main glutamatergic alterations, and the evidence of the rapid antidepressant effect of glutamatergic agents (namely ketamine) further supports the glutamatergic hypothesis of depression [133-135].

3.5.1. Clinical Evidence

Despite the well-acknowledged involvement of glutamate transmission in depression and antidepressant effect, we retrieved from literature no articles directly addressed the study of the association of methylation in glutamatergic genes with MDD or antidepressant treatment. However, two epigenome-wide association studies (EWASs) on MDD obtained interesting results regarding methylation levels of genes encoding NMDA subunits 1 (*GRIN1*) and 2A (*GRIN2A*) [110, 136].

3.5.2. Preclinical Evidence

The study of epigenetic changes on the *Grm1* gene (encoding mGluR1) induced in the hippocampus by maternal care revealed a long-term increase of *Grm1* exon 2 methylation in the hippocampal tissue of offspring of mothers with low licking/grooming behaviour, compared to adult mice which received high levels of maternal care [137]. This was associated with decreased levels of histone 3 lysine 9 acetylation and histone 3 lysine 4 trimethylation of *Grm1*, decreased mGluR1 mRNA and protein expression, and depressive-like behaviour.

In a different study, the percentage of average methylation at the promoter of the glutamic acid decarboxylase 1 (*Gad1*, an enzyme catabolizing glutamate in GABA) was reported to be significantly higher in the hippocampus of adult offspring with low-maternal care, which also displayed decreased GAD1 protein expression [138]. Overall, these findings suggest that variations in maternal care can influence hippocampal function and cognitive performance through the epigenetic regulation of genes implicated in glutamatergic synaptic signalling.

Moreover, Labontè *et al.*, together with finding that growth arrest and DNA damage beta (*Gadd45*) plays a key role in mechanisms of susceptibility to chronic social defeat stress, observed that viral *Gadd45* downregulation in the nucleus accumbens rescued the susceptibility phenotype and increased *Gad1* promoter methylation [139]. This suggests that changes in *Gad1* promoter methylation could be involved in *Gadd45*-dependent mechanisms regulating susceptibility to chronic social defeat stress.

3.6. Inflammation and Oxidative Stress

A growing body of evidence indicates that inflammation accompanied by increased oxidative and nitrosative stress may play a crucial role in MDD pathogenesis [140, 141]. Indeed, increased peroxidation of lipids, elevated production of reactive oxygen species (ROS), mitochondrial dysfunction, as well as elevated levels of proinflammatory cytokines (including IL-1β, IL-6, IL-8, IL-12. IL-18, interferon-gamma), were reported both peripherally and centrally in depressed patients [142]. Accordingly, antidepressant agents were found to induce anti-inflammatory effects, while some anti-inflammatory agents have properties of antidepressants [143].

Only a few papers focused on the study of alterations in the methylation pattern of genes associated with inflammatory and oxidative pathways in MDD patients and animal models of depression. We found only two clinical reports on

interleukin genes and three papers on oxidative genes in rodent chronic stress models.

3.6.1. Interleukins (Clinical Evidence)

Methylation levels at the *IL-11* promoter were proposed to predict antidepressant response. Particularly, lower levels of DNA methylation were associated with a better response to the antidepressants escitalopram and nortriptyline [144]. Moreover, in the same study, the interaction of *IL-11* promoter with rs1126757 polymorphism was reported in predicting antidepressant response. Individuals homozygous for the G-allele (GG) and higher IL-11 methylation responded better to antidepressant treatment than subjects homozygous for the A allele (AA).

Ryan and colleagues reported that IL-6 promoter methylation in DNA from buccal swabs was lower in old MDD patients (aged ≥65 years) compared to healthy controls [145]. Interestingly, antidepressant use was independently associated with higher IL-6 methylation, thus strengthening the hypothesis of a putative use of IL-6 levels as a biomarker of MDD and antidepressant response.

3.6.2. Oxidative Stress Genes (Preclinical Evidence)

Wigner and co-workers analyzed changes in the methylation of the oxidative genes superoxide dismutase 1 (*Sod1*), superoxide dismutase 2 (*Sod2*), glutathione peroxidase 1 (*Gpx1*), glutathione peroxidase 4 (*Gpx4*), catalase (Cat), nitric oxide synthase 1 (*Nos1*) and nitric oxide synthase 2 (*Nos2*), in brains areas and blood mononuclear cells of rats exposed to CMS and treated with agomelatine or venlafaxine [146,147]. The results obtained in this study indicate that CMS and antidepressant administration differentially affect the methylation of promoters of genes involved in oxidative and nitrosative stress in a tissue-specific manner. In particular, some of the genes were less methylated (and more expressed) in CMS animals, while others were hypermethylated (and less expressed) after antidepressant treatment. Moreover, the changes were different in peripheral and central tissues.

3.7. MORC Family CW-Type Zinc Finger 1

Recent studies have highlighted a number of DNA methylation loci associated with early life stress [17]. MORC Family CW-Type Zinc Finger 1 (*MORC1*) was identified in a cross-species EWAS, where its promoter was hypomethylated in rats, rhesus macaques, and human newborns that had experienced different forms of early life stress [148] (4.2). Starting from this first evidence, the following studies focused on the role of *MORC1* methylation in MDD. *MORC1* encodes for MORC family CW-type zinc finger 1, a highly conserved protein that is probably responsible for epigenetic regulation [149].

3.7.1. Clinical Evidence

Mundorf and collaborators studied the putative association between birth complications and methylation at 13 CpG sites of *MORC1* promoter in buccal cells of healthy individuals [150]. They observed that both mean and total methylation were positively correlated with the presence of birth complications and a higher score on the Beck Depression Inventory scale. On the contrary, Thomas and collaborators studied *MORC1* promoter methylation in the whole blood of patients suffering from MDD who had been exposed to childhood trauma and did not find an association between *MORC1* promoter methylation and childhood trauma [149]. However, they observed a significant association between *MORC1* promoter methylation and depressive score, suggesting that DNA methylation of *MORC1* cannot be used as a biomarker of childhood maltreatment in adults but could be associated with depressive symptoms. *MORC1* promoter methylation was also linked to macrostructural and microstructural alterations in the hippocampus and medial prefrontal cortex [151]. Although these data reinforce the hypothesis of an involvement of *MORC1* in MDD, further investigation is required to strengthen this correlation.

3.7.2. Preclinical Evidence

None.

3.8. Alteration of Methylation in Other Selected Genes

For the sake of completeness, we found in literature other studies specifically addressed the association of methylation at one selected gene of interest with MDD or depressive-like behaviour in animal models. Individual reports, divided into clinical and preclinical, are collected in Table (**1**).

Since only one single paper is available for that specific gene, it is difficult to conclude the real involvement of DNA methylation at the gene in depression, and more studies are required.

4. GLOBAL DNA METHYLATION PROFILING IN DEPRESSION AND ANTIDEPRESSANT TREATMENT

In recent years, thanks to the development of fluorescence-based sequencing methods, an alternative approach to the analysis of DNA methylation changes at specific genes of interest is represented by the introduction of EWASs, which allow assessing genome-wide methylation profiles (Fig. **1B**).

Global DNA methylation profiling has important advantages compared to targeted DNA methylation studies, the main of which relies on the possibility to not merely focus on single pieces of the puzzle but to take a more comprehensive view of the picture of alterations associated with the pathology. Therefore, this approach allows the discovery of new putative pathways involved in MDD and the aetiology of psychiatric illness in general. On the other hand, high-throughput methods provide a huge amount of data, which is considerably challenging.

This section will present studies applying an epigenome-wide approach method to highlight alterations of DNA methylation profiling associated with MDD or depressive-like behaviour in animal models. As detailed below, most of the evidence comes from clinical studies, and to the best of our knowledge, none of them evaluated the effect of antidepressant treatments on DNA methylation. EWAS studies details are reported in Table (**2**).

4.1. Clinical Evidence

Clinical EWASs assessing methylome in MDD patients can be divided into two main groups.

Table 1. Studies reporting changes in DNA methylation at selected genes in MDD patients or animal models of depression.

Clinical Studies								
Gene	**Protein**	**Genomic Region**	**Subjects**	**Tissue**	**MDD Diagnosis**	**N of Subjects**	**Main DNA Methylation Findings**	**Refs.**
GLUT1	Glucose Transporter 1	promoter	MDD *vs.* HC	blood serum	MADRS	52 MDD, 18 HC	↑ in MDD	[152]
PAI-1	Plasminogen Activator Inhibitor-1	promoter	TRD treated with ECT	whole blood and peripheral blood mononuclear cells	HAM-D or MADRS	59 (cross sectional cohort) 28 (longitudinal cohort)	= after ECT	[153]
TPA	Tissue-type Plasminogen Activator	promoter/ exon I	TRD treated with ECT	whole blood and peripheral blood mononuclear cells	HAM-D or MADRS	59 (cross sectional cohort) 28 (longitudinal cohort)	Selected differences between remitters and non-remitters to ECT	[153]
DLG4	PSD-95 (Postsynaptic Density Protein 95)	gene	MDD *vs.* HC	post-mortem brain	DSM-III criteria	6 MDD, 6 HC	= between MDD and HC	[154]
GJA-1	Connexin 43	gene	MDD *vs.* HC	post-mortem brain	DSM-III	6 MDD, 6 HC	= between MDD and HC	[154]
SHATI/ NAT8L	N-acetylaspartate Synthetase	promoter	MDD (>12 y.o.) medicated and non-medicated	peripheral blood	DSM-IV	60 MDD, 69 HC	↑ in unmedicated patients *vs* HC = between medicated patients and HC	[155]
FADS1	Acyl-CoA (8-3)-desaturase	promoter	MDD *vs.* HC	peripheral blood	SCID-I	61 MDD, 59 HC	= between MDD and HC	[156]
FADS2	Acyl-CoA 6-desaturase	promoter	MDD *vs.* HC	peripheral blood	SCID-I	61 MDD, 59 HC	trend towards ↓ in MDD vs HC	[156]
ELOVL5	Elongation of very long chain fatty acids protein 5	promoter	MDD *vs.* HC	peripheral blood	SCID-I	61 MDD, 59 HC	↑ in MDD vs HC	[156]
KLK8	Kallikrein-8	promoter	MDD *vs.* HC	peripheral blood	SCAN	80 MDD, 80 HC	trend towards ↑ in MDD vs HC	[157]
QKI	Quaking	promoter/ exon I	MDD *vs.* HC	post-mortem brain	SCID-I	16 MDD, 13 HC	= between MDD and HC	[158]
HCRT	Orexin A	promoter	MDD *vs.* HC	peripheral blood	ICD-10 DSM-IV	29 MDD, 18 HC	= between MDD and HC	[159]
ACE	Angiotensin Converting Enzyme	promoter/ exon I	MDD *vs.* HC	peripheral leukocytes post-mortem brain	SCID-I	81 MDD, 81 HC	↑ in peripheral leukocytes in MDD vs HC = between MDD and HC in post-mortem brains	[160]
Preclinical Studies								
Gene	**Protein**	**Genomic Region**	**Animal/Strain**	**Animal Model**	**Tissue**		**Main DNA Methylation Findings**	**Refs.**
Arc	Activity Regulated Cytoskeleton Associated Protein	promoter	Sprague–Dawley rats	ECS *vs.* CNT	hippocampus		↑ in ECS	[161]

(Table 1) contd….

Preclinical Studies							
Gene	**Protein**	**Genomic Region**	**Animal/Strain**	**Animal Model**	**Tissue**	**Main DNA Methylation Findings**	**Refs.**
Crmp2	Collapsin Response Mediator Protein 2	promoter	Sprague–Dawley rats	CUMS	hippocampus prefrontal cortex	hippocampus: ↑ in CUMS prefrontal cortex:=	[162]
Dlx5	Distal-Less Homeobox 5	promoter	C57BL/6 J mice	CSDS	nucleus accumbens	↑ in CSDS	[163]
Gdnf	Dlial-Derived Neurotrophic Factor	promoter	BALB Mice	CUMS	ventral striatum, hippocampus	↑ in CUMS DNA methylation restored after imipramine	[164]
Nrtn	Neurturin	promoter	C57BL/6 J mice	CSDS	nucleus accumbens	↑ in CSDS	[163]
Ntrk2	Neurotrophic Receptor Tyrosine Kinase 2	promoter	C57BL/6 J mice	CSDS	nucleus accumbens	↓ in CSDS	[163]
Ntsr1	Neurotensin 1	promoter	Sprague–Dawley rats	MS	amygdala	↑ in MS	[165]
Mkp-1	Mitogen-Activated Protein Kinase Phosphatase-1	promoter	Sprague–Dawley rats	CUS	hippocampus	= in CUS ± antidepressants	[166]
Reln	Reelin	distal promoter	Sprague-Dawley rats	PNS	whole cerebral cortex	↑ in PNS	[167]
Tacr2	Tachykinin Receptor 2	promoter	Sprague–Dawley rats	CUMS	hypothalamus	↓ in CUMS	[168]
Ucn1	Urocortin 1	promoter	Wistar rats	ELS in 5-HTT knockout rats	Edinger–Westphal nucleus midbrain	↓ in ELS GpC-specific alterations in homozygous and heterozygous 5-HTT knockout rats	[169]

Abbreviations: 5-HTT: serotonin transporter; CNT: Control; CoA: Co-enzyme A; CSDS: Chronic Social Defeat Stress; CUMS: Chronic Unpredictable Mild Stress; CUS: Chronic Unpredictable Stress; DLG4: discs large homolog 4; DMS-IV: Diagnostic and Statistical Manual of Mental Disorders IV; DSM-III: Diagnostic and Statistical Manual of Mental Disorders III; ECS: Electro-Convulsive Stimulation; ECT: Electro-Convulsive Therapy; ELS: Early Life Stress; GJA-1: Gap junction alpha-1 protein; HAM-D: Hamilton Depression Rating Scale; HC: Healthy Controls; ICD: International Classification of Diseases; MADRS: Montgomery Asberg Depression Rating Scale; MDD: Major Depressive Disorder; MS: Maternal Separation; PNS: Prenatal Restraint Stress; SCID: Structured Clinical Interview; SHATI/NAT8L: N-Acetyltransferase 8 Like; TRD: Treatment-Resistant Depression.
Up arrow (↑): increased DNA methylation; down arrow (↓): decreased DNA methylation; equal (=): no difference in DNA methylation.

Some studies were aimed at studying aetiopathogenetic mechanisms of depression and were conducted on post-mortem brain tissues. In this case, DNA methylation changes in selected brain areas involved in depression pathophysiology were evaluated as putative pathological determinants. Interestingly, some of these studies revealed significant differences in DNA methylation at genes involved in biological processes already associated with depression, while others allowed for the identification of new putative genes of interest. In most of the studies, the authors applied DNA methylation profiling to DNA extracted from peripheral tissues, aiming at identifying peripheral biomarkers of depression as innovative diagnostic tools.

4.1.1. EWAS on Post-Mortem Brain Tissues

Sabunciyan and colleagues in 2012 presented one of the first genome-wide DNA methylation scans in MDD [170]. Authors compared samples of post-mortem frontal cortex of MDD patients to healthy controls and identified 224 candidate regions with differences in DNA methylation higher than 10%. These regions were highly enriched for neuronal growth and development genes. The greatest difference was detected in the Proline-Rich Membrane Anchor 1 (*PRIMA1*) gene, responsible for anchoring acetylcholinesterase in neuronal membranes, the methylation of which was increased in MDD. However, this result was not confirmed in the replication data.

A work published in 2014 investigated DNA methylation signatures in the prefrontal cortex of suicides with MDD diagnosis, and nonpsychiatric controls, aged 16 to 89 years [171]. The authors found that DNA methylation increases throughout the lifespan and that suicides showed an 8-fold greater number of methylated CpG sites relative to controls, with greater DNA methylation changes over and above the increased methylation observed in normal aging.

These findings suggest that some of the molecular and cellular factors associated with aging may also contribute to the neuropathology of mood disorders, and increased risk of suicide.

Table 2. EWAS in MDD patients or animal models of depression.

Clinical Studies					
Subjects	**MDD Diagnosis**	**Tissue**	**N of Subjects**	**Method**	**Refs.**
MDD *vs.* HC	DSM – IV criteria	frontal cortex	39 MDD, 26 HC	Pyrosequencing of bisulphite-converted DNA	[170]
MDD *vs.* HC	DSM – IV criteria	prefrontal cortex	25 MDD, 28 HC	BeadChip array of bisulphite-converted DNA	[171]
MDD *vs.* HC	DSM – III R criteria	hippocampus, prefrontal cortex	6 MDD, 6 HC	BeadChip array of bisulphite-converted DNA	[136]
MDD *vs.* HC	DSM – IV criteria	prefrontal cortex	76 MDD, 45 HC	MBD-sequencing	[172]
MDD *vs.* non-psychiatric sudden-death controls	SCID - I	cortex (BA11 and BA25	20 MDD, 20 HC	BeadChip array of bisulphite-converted DNA	[173]
MDD *vs.* HC	DSM – IV criteria	brain, blood	113 MDD, 93 HC 812 MDD, 320 HC	MeDIP-sequencing	[174]
MDD *vs.* HC	DSM – III R criteria	dorsolateral prefrontal cortex	31 MDD, 577 HC	BeadChip array of bisulphite-converted DNA	[175]
MDD *vs.* HC twin pairs	DSM - IV criteria	white blood cells	24 MDD vs 24 HC	BeadChip array of bisulphite-converted DNA	[176]
MDD *vs.* HC twin pairs	DSM - IV criteria	whole blood	50 MDD vs 50 HC	MeDIP-sequencing	[177]
MDD *vs.* HC twin pairs	DSM - IV criteria	white blood cells, brain, sperm	103 MDD, 97 HC	Pyrosequencing of bisulphite-converted DNA	[178]
MDD *vs.* HC twin pairs	SCID-I	whole blood	14 MDD, 20 HC	BeadChip array of bisulphite-converted DNA	[179]
MDD *vs.* HC twin pairs	BDI-II	peripheral monocytes	79 MDD, 79 HC	BeadChip array of bisulphite-converted DNA	[180]
MDD *vs.* HC twin pairs	GDS-30	whole blood	58 MDD, 58 HC	RRBS	[181]
MDD *vs.* HC	PHQ-9	whole blood	33 MDD, 67 HC	Microarray Chip of bisulphite-converted DNA	[182]
Subjects with depressive symptoms *vs.* HC	BDI and BDI-II	whole blood	83 subjects with depressive symptoms, 83 HC	BeadChip array of bisulphite-converted DNA	[183]
MDD *vs.* HC	SCID-I	whole blood	30 MDD, 32 HC	ELISA	[184]
MDD *vs.* HC	DSM-IV criteria HAM-D	peripheral leukocytes	20 MDD, 19 HC	BeadChip array of bisulphite-converted DNA	[185]
MDD *vs.* HC	Self reported history	whole blood	100 MDD, 100 HC	BeadChip array of bisulphite-converted DNA	[186]
MDD *vs.* HC	DSM-IV criteria BDI-21	whole blood	8 MDD, 9 HC	BeadChip array of bisulphite-converted DNA	[187]
Preclinical Studies					
Animal/Strain	**Animal Model**	**Tissue**		**Method**	**Refs.**
Rhesus monkeys Sprague-Dawley rats	ELS	CD3+ T cells prefrontal cortex		MeDIP-sequencing	[148]
Wistar-Kyoto rats	MS	hippocampus, PVN, amygdala, septum, medial prefrontal cortex, BNST		ELISA MethylCap- sequencing	[188]
Sprague-Dawley rats	Rats bred for low behavioural response to novelty	amygdala		ELISA MethylCap- sequencing	[189]
Wistar rats	LH	hipocampus prefrontal cortex		EIA	[190]
DBA2/J mice	ELS	striatum, nucleus accumbens, hippocampus, amygdala		ELISA	[184]

Abbreviations: BA: Broadmann Area; BDI: Beck Depression Inventory scale BNST: Bed Nucleus of Stria Terminalis; EIA: Enzyme Immuno Assay; ELISA: Enzyme Linked ImmunoSorbent Assay; ELS: Early Life Stress; GDS-30: Geriatric Depression Scale; HAM-D: Hamilton Depression Rating Scale; HC: Healthy Controls; MDD: Major Depressive Disorder; LH: Learned Helplessness; MeDIP: Methylated DNA immunoprecipitation; MBD: Methyl-CpG Binding Domain; MS: Maternal Separation; PHQ-9: nine-item depression scale of the Patient Health; PVN: paraventricular nucleus; RRBS: Reduced-representation bisulfite sequencing; SCID: Structured Clinical Interview.

The pilot study conducted by Kaut and co-workers in 2015 on 6 MDD patients and 6 healthy control subjects, revealed differential methylation profiles of 11 genes in the hippocampus and 20 genes in the prefrontal cortex [136]. 5 of these methylation loci were selected for replication and, among these, the glutamate receptor ionotropic NMDA 2A (*GRIN2A*) gene was found to be hypermethylated in both the prefrontal cortex and hippocampus of MDD patients.

Another interesting study published in 2015 was focused on the analysis of genome-wide DNA methylation changes in genes associated with astrocytic markers in the prefrontal cortex of depressed subjects compared to healthy controls [172]. The results revealed differentially methylated regions, the majority of which displayed reduced methylation levels in MDD. Reduced methylation at Glutamate Ionotropic Receptor Kainate Type Subunit 2 (*GRIK2*) and Brain Enriched Guanylate Kinase Associated (*BEGAIN*) were the most significant and significantly correlated with gene expression.

Murphy and colleagues in 2017 performed a methylome profiling of cortex samples from depressed suicide completers and sudden-death controls [173]. The upstream Psoriasis Susceptibility 1 Candidate 3 (*PSORS1C3*) gene region ranked first after the application of different strategies to increase the power of the study. The *PSORS1C3*-associated different methylated region has been validated and replicated in a second set of suicide samples and was consistently found to be hypomethylated across all CpG sites in MDD suicide cases compared with controls in both Broadmann Area 11 and Broadmann Area 25. The function of the PSORS1C3 gene product is still unknown, but it is believed to play a potential role in immune system modulation.

In a comprehensive DNA methylation study published in 2019, Chan and colleagues used epigenomic deconvolution to perform cell type-specific methylome-wide association studies in 3 collections of human post-mortem brains and 1 collection of blood samples of MDD cases and controls [174]. The main aim of the work was to apply a cell type-specific methylome-wide association approach to identify multiple findings in neurons/glia that were detected across brain collections and reproducible in physically sorted nuclei from the blood (granulocytes, T cells, B cells, monocytes). Sorted neurons/glia from a fourth post-mortem brain collection were used for validation purposes. Pathway enriched analyses implicated neurotrophin receptor/nerve growth factor signalling and innate immune toll-like receptor signalling, strongly suggesting mechanistic involvement of these processes in the aetiopathogenesis of MDD.

Finally, a study conducted by Huls *et al.* in 2020 aimed to identify epigenome-wide significant associations between brain tissue DNA methylation and late-life MDD in the dorsolateral prefrontal cortex [175]. The most significant CpG sites were found in a region covering an exon of YOD1 Deubiquitinase (*YOD1*) and an intron of 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 2 (*PFKFB2*). *YOD* is a highly conserved deubiquitinase influencing endoplasmic-reticulum-associated degradation and inflammatory responses. Interestingly, brain tissue-based methylation levels were more strongly associated with late-life MDD in men than in women.

4.1.2. EWAS on Peripheral Tissues

4.1.2.1. Studies on Monozygotic Twin Pairs

We found in literature 6 studies assessing changes in DNA methylation profile in peripheral tissues from monozygotic twin pairs discordant for MDD, with the final aim to detect specific epigenetic variants potentially related to depression, avoiding any genetic interference.

The first study from Byrne *et al.* recruited 12 twin pairs discordant for MDD and 12 twin pairs concordant for no MDD and low neuroticism [176]. Although the sample size was insufficient to identify methylation differences that could be attributed to MDD, the study revealed that cases show a highly significantly increased variation in methylation throughout the genome compared with their control co-twins.

The following study published in 2014 involved 50 monozygotic twin pairs and identified 17 differently methylated regions of genome-wide significance, among which hypermethylation of the Zinc Finger and BTB Domain Containing 20 (*ZBTB20*) gene was significantly associated with depressive symptoms and replicated both in the experimental set and in an independent cohort [177]. *ZBTB20* has been implicated in the regulation of hippocampal function.

In Oh *et al.*, peripheral blood DNA from 100 discordant monozygotic twins and prefrontal cortex DNA from 71 MDD patients were analysed for methylome alterations [178]. Although locus-by-locus analysis yielded modest differences, none of which survived correction for multiple testing, differentially modified regions exhibited many overlaps in the tested tissues. Moreover, for most of the twin samples, the combination of small DNA methylation differences was sufficient to accurately classify the phenotypes using machine-learning algorithms.

The study conducted by Cordova-Palomera and co-workers in 2015 involved 17 monozygotic twin pairs [179]. Two analytical strategies were used to identify differentially methylated probes and variably methylated probes, both of which were located in genes within enriched biological pathways and previously associated with depression.

The work of Zhu and colleagues published in 2019 analysed the genome-wide DNA methylation from peripheral monocytes of 79 discordant monozygotic twins [180]. Among the 39 different methylated regions, network analysis revealed distinct gene modules enriched in signalling pathways related to stress responses, neuron apoptosis, insulin receptor signalling, mechanistic target of rapamycin (mTOR) signalling, and nerve growth factor receptor signalling.

The last paper on twin samples was recently published by Wang *et al.* [181]. They conducted an epigenome-wide association study in a sample of 58 depression score-discordant monozygotic twin pairs and showed an association of DNA methylation levels at 66 CpG sites with depression scores. The top CpG sites were located at 34 genes, especially Receptor-type tyrosine-protein phosphatase N2 (*PTPRN2*), Transcription factor HES-5 (*HES5*), Endothelial transcription factor GATA-2 (*GATA2*), Probable histone-lysine N-methyltransferase 7 (*PRDM7*), and Kv channel-interacting protein 1 (*KCNIP1*). No validation analysis was done, while

many ontology enrichments were highlighted, including the Notch signalling pathway, Huntington disease, p53 pathway by glucose deprivation, hedgehog signalling pathway, DNA binding, and nucleic acid metabolic process.

4.1.2.2. Studies on Depressed Patients

To the best of our knowledge, the first study assessing genome-wide methylation profile in MDD patients was published in 2011 by Uddin and colleagues, who analysed whole blood DNA from patients with a lifetime history of depression and healthy controls [182]. The authors found genes involved in inflammatory-related pathways and processes previously implicated in depression in differentially methylated regions. Moreover, an inverse correlation between methylation of *IL-6* CpG and circulating IL-6 and C-reactive protein CRP levels was found in MDD patients.

In 2014, the study of DNA methylation profile in peripheral blood from men who were separated from their parents during childhood compared to controls, although highlighting no differences in DNA methylation associated with early life, found changes of DNA methylation signature in association with the development of at least mild depressive symptoms over the subsequent 5–10 years [183]. In particular, hypomethylation was identified in a number of genes with roles in brain development and/or function in association with depressive symptoms. This was further validated in a pilot clinical study published by Catale and colleagues [184].

In 2015, Numata and co-workers assessed genome-wide methylation profiles from peripheral leukocytes in medication-free patients with depression compared to healthy controls [185]. The authors found lower DNA methylation at 363 CpG sites, most of which were located at CpG in promoter regions, among which 17 CpG sites were validated as discriminating of a pathological condition in an independent set of samples.

Crawford and colleagues measured genome-wide patterns of DNA methylation in whole blood-derived DNA obtained from individuals with a self-reported history of depression and individuals without a history of depression, identifying six significant depression-associated differentially methylated regions, including immune-related genes [186].

Ämmälä and colleagues aimed at testing the hypothesis that sleep and depression have independent epigenomic effects on brain development and plasticity in adolescents and recruited 8 medication-free adolescent boys with depression and sleep symptoms and 9 healthy controls [187]. Although no genome-wide significant differences in methylation between cases and controls have been observed, pathway analysis identified the enrichment of differentially methylated positions on genes related to synaptic Long-Term Depression.

4.2. Preclinical Evidence

Only a few preclinical studies applied genome-wide methods to the analysis of changes in DNA methylation profile in animal models of depression, essentially focusing on the effects of early life stress.

The analysis of changes in DNA methylation profile induced by early life stress, as well as conserved alterations in peripheral tissue and the brain, published by Nieratschker *et al.*, validated hypomethylation of Morc1 as associated with both depressive-like behaviour and depression [148].

McCoy and colleagues, in 2016, quantified global genome methylation levels in limbic brain regions of adult rats exposed to maternal separation in early life compared to controls [188]. Methylome profiling revealed reduced methylation (which was confirmed by quantitative real-time PCR in the hippocampus) at intragenic sites within two key nodes of insulin signalling pathways: insulin receptor (*Insr*) and one of its major downstream targets, mitogen-activated protein kinase kinase kinase 5 (*Map3k5*). The same group in 2017 published another work in which they used rats bred for low response to novelty (Low Responders, LRs), which display anxiety/depression-like behaviour, compared to High Novelty Responder (HR) rats [189]. The analysis of the methylome revealed 793 differentially methylated genomic sites between the groups, with most of the differentially methylated sites hypermethylated in HR versus LR, suggesting that increased DNA methylation is associated with anxiety/depression-like phenotype in this model.

In an elegant study from 2018, Sales and Joca investigated the effects induced by acute and repeated antidepressant treatment on DNA methylation and DNMT expression (1, 3a, and 3b isoforms) in different brain regions of learned helplessness rats (a validated stress model of depression) [190]. Stress increased DNA methylation, DNMT3a, and DNMT3b expression in the dorsal hippocampus and prefrontal cortex. Chronic, but not acute, imipramine administration attenuated stress effects only in the cortex, thus suggesting that the regulation of DNA methylation in the cortex may be an important mechanism for antidepressant-like effects.

More recently, Catale *et al.* asked whether different early life stress experiences in mice resulted in differential impacts on global DNA methylation levels in the brain and blood samples [184]. Exposure to social isolation-induced remarkable global DNA methylation remodelling in the brain, which was found to be stronger than that caused by exposure to a socially threatening environment. The direction and magnitude of these effects depend on the tissue (brain/blood), the brain structure, and the cellular population.

CONCLUSION

The body of evidence collected here shows that both depression/depressive-like behaviour and antidepressant effect are accompanied by remodelling of DNA methylation signatures. Nevertheless, the characterization of specific alterations and the discussion about the putative use of DNA methylation as a diagnostic and/or prognostic biomarker of depression are still at their initial stage. Indeed, the number of studies is still limited, and consistent results are scarce. Table (**3**) resumes the main findings of the involvement of DNA methylation at specific genes in depressive behaviour and antidepressant response, as assessed in at least two different studies. As regards MDD patients, the highest degree of concordance can be observed for four genes: BDNF, SLC6A4, FKBP5, and NR3C1. In more detail, most studies reported increased DNA methylation at BDNF, SLC6A4, and NR3C1 and decreased DNA methylation at FKBP5 in association with depression. More importantly, transcriptional and protein

Table 3. Overview of the most consistent findings about DNA methylation signature at selected genes reviewed in the present manuscript. The table resumes the alterations of DNA methylation associated with depression/depressive behaviour and antidepressant response in clinical and preclinical studies at genes for which at least two papers are available in literature. See the text for more details.

	Depression/ Depressive Behaviour		Antidepressant Response	
GENE	**Clinical Evidence**	**Preclinical Evidence**	**Clinical Evidence**	**Preclinical Evidence**
BDNF	↑ PBMC, leukocytes, whole blood, saliva (↓ whole blood)	↑ hippocampus, prefrontal cortex (= hippocampus, prefrontal cortex)	↑ leukocytes, whole blood	↓ hippocampus, prefrontal cortex
SLC6A4	↑ whole blood, lymphocytes (↓ whole blood) (= buccal cells, lymphocytes)	-	↑ leukocytes, whole blood ↓ whole blood	-
HTR1A	-	↑ prefrontal cortex, midbrain	↑ whole blood	↓ prefrontal cortex = midbrain
HTR1B	-	-	↑/↓ whole blood	-
MAO-A	↑/↓ saliva	-	-	-
COMT	= cerebellum (↓ whole blood)	-	-	-
P11	= plasma	↑ prefrontal cortex, whole blood	↑ whole blood = plasma	↓ prefrontal cortex, whole blood
FKBP5	↓ whole blood (=/↑ whole blood)	↓ whole blood, hippocampus, hypothalamus	-	-
CRF	↑ saliva	↑ BST, central amygdala ↓ hippocampus	-	-
NR3C1	↑ whole blood, PBMC (↓ whole blood, saliva) (= leukocytes, post-mortem brains)	↑ hypothalamus ↓/= hippocampus	-	-
OXT	↑ saliva ↓ whole blood	-	-	-
AVP	↑ saliva	↓ PVN	-	-
POMC	↑ whole blood	↓ serum	-	-
MORC1	↑ whole blood, buccal cells	-	-	-

Abbreviations: BST: Bed nucleus of Stria Terminalis; PBMC: peripheral blood mononuclear cell; PVN: paraventricular nucleus.
Up arrow (↑): increased DNA methylation; down arrow (↓): decreased DNA methylation; equal (=): no difference in DNA methylation. In brackets inconsistent findings.

expression studies largely confirmed the functional role of these epigenetic changes.

Conversely, although the number of studies associating differences in DNA methylation with the antidepressant response is still very small, there is some evidence of higher remission frequency in patients showing increased methylation levels at selected sites within the BDNF gene (CpG-87 methylation at promoter IV), which however needs to be further confirmed in a higher number of patients. Similarly, the predictive validity of methylation levels at other genes in antidepressant response has been tested, although further research is needed.

On the other hand, EWAS approaches, which have increased substantially in recent years, hold promise for the detection of new regulatory mechanisms and candidate pathways relevant for psychiatric disorders. In the majority of genome-wide studies revised here, DNA methylation at genes involved in inflammation, immune and neurotrophic pathways showed the most significant and robust association with MDD. However, since specific limits of EWASs include the use of various techniques to assess genome-wide DNA methylation (ranging from microarrays to next-generation sequencing methods) and of different statistical approaches (with different degrees of or less stringency), the consistent identification of specific DNA methylation pat-

terns applicable as reliable biomarkers of depression would require bigger sample sizes, meta-analyses, and collaborations between research groups.

Moreover, some main concerns should be considered before drawing any general conclusion. First, MDD heterogeneity, sex- and age-dependent effects, and different diagnostic criteria could exert significant confounding effects in the search for common and specific DNA methylation changes related to depression and antidepressant treatment. Second, different DNA methylation sites within the same gene could be differently modulated in MDD, as already reported by some papers [35,48]. Therefore, for the identification of a diagnostic/prognostic biomarker, studies should converge on a limited number of specific methylation sites, as for example already proposed for CpG-87 at BDNF promoter IV [49, 50], FKBP5 intron 7 [102, 103, 105], or NR3C1 exon 1 F [41, 80, 109, 116].

Third, the use of peripheral DNA for methylation analysis may represent a significant issue for the identification of specific alterations associated with depression and/or antidepressant response. Indeed, peripheral alterations could not be related to pathological processes undergoing in the brain of subjects with a psychiatric disorder, thus increasing the risk of occurring confounding factors and making it difficult to obtain reproducible results. Importantly, the attempt to find conserved DNA methylation alterations among central and peripheral tissues in MDD failed to get encouraging results [178]. This could impact both the study of peripheral biomarkers and the aetiopathological mechanisms of MDD.

In this context, our bibliography search effort revealed that most of the studies had been performed in MDD patients, while preclinical evidence in animal models of the pathology is still scarce and mainly focused on a small number of genes (Table **3**). This could represent the main issue, especially in the identification of DNA methylation signature implicated in pathogenetic mechanisms of depression. Indeed, despite the limits of animal models, which can only reproduce selected endophenotypes [190], they offer the possibility to analyse the mechanistic link between brain area- and cell type-specific changes in DNA methylation and behaviour. Last (but not least), the use of animal models may help characterize epigenetic mechanisms associated with antidepressant response, thus fostering the identification of new putative molecular targets for the development of innovative therapeutic strategies.

Overall, the study of DNA methylation involvement in depression has attracted considerable attention in recent years, and convergent clinical evidence suggests that selected peripheral DNA methylation markers could be used as tools to discriminate between patients with MDD and non-psychiatric subjects. Nevertheless, much remains to be done to unveil the functional role of DNA methylation in aetiopathogenetic processes as well as mechanisms of antidepressant response (if any). Animal models, allowing the study of the role of brain epigenetic alterations in shaping behavioural tracts, might aid in the understanding of processes that could be targeted by novel biological therapeutic approaches.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

Funding for this work was supported by Fondazione Cariplo (Prog. 2019-3357).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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