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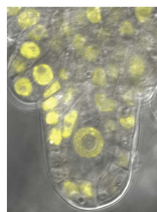
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RESEARCH PAPER



Longitudinal DNA methylation changes at *MET* may alter HGF/c-MET signalling in adolescents at risk for depression

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ABSTRACT

Unrecognized depression during adolescence can result in adult suicidal behaviour. The aim of this study was to identify, replicate and characterize DNA methylation (DNAm) shifts in depression aetiology, using a longitudinal, multi-tissue (blood and brain) and multi-layered (genetics, epigenetics, transcriptomics) approach. We measured genome-wide blood DNAm data at baseline and one-year follow-up, and imputed genetic variants, in 59 healthy adolescents comprising the discovery cohort. Depression and suicidal symptoms were determined using the Development and Well-Being Assessment (DAWBA) depression band, Montgomery-Åsberg Depression Rating Scale-Self (MADRS-S) and Suicide Assessment Scale (SUAS). DNAm levels at follow-up were regressed against depression scores, adjusting for sex, age and the DNAm residuals at baseline. Higher methylation levels of 5% and 13% at cg24627299 within the *MET* gene were associated with higher depression scores ($p_{\text{raw}} < 1e-4$) and susceptibility for suicidal symptoms ($p_{\text{adj}} < 0.005$). The nearby rs39748 was discovered to be a methylation and expression quantitative trait locus in blood cells. mRNA levels of hepatocyte growth factor (*HGF*) expression, known to strongly interact with *MET*, were inversely associated with methylation levels at cg24627299, in an independent cohort of 1180 CD14+ samples. In an open-access dataset of brain tissue, lower methylation at cg24627299 was found in 45 adults diagnosed with major depressive disorder compared with matched controls ($p_{\text{adj}} < 0.05$). Furthermore, lower *MET* expression was identified in the hippocampus of depressed individuals compared with controls in a fourth, independent cohort. Our findings reveal methylation changes at *MET* in the pathology of depression, possibly involved in downregulation of HGF/c-MET signalling the hippocampal region.

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Introduction

Depression in adolescents is a prediction marker for suicidal behaviour in adulthood [1,2]. Approximately one million people die by committing suicide every year worldwide [3] and over 90% of them have a diagnosable mood disorder including depression [4]. Moreover, adolescent-onset recurrent major depression disorder (MDD) is linked to more severe psychosocial impairment during adulthood compared with adult-onset recurrent MDD [5]. The development of depression is the result of an interaction between environmental and genetic risk factors [6].

Emerging evidence suggests that psychosocial stressors affect epigenetic patterns in blood and brain tissues [7–9], which can lead to long-term

gene expression changes [10]. The best-characterized epigenetic mark, DNA methylation (DNAm), reflects the interplay between environmental stress and genetic contribution to depression over the lifespan [11,12]. DNAm involve the addition of a methyl group to DNA, typically at a cytosine nucleotide adjacent to a guanine (CpG). Studies have investigated DNAm of candidate genes in depression aetiology, such as *BDNF*, *SLC6A4* and *NR3C1* [13,14]. Importantly, DNAm patterns are notoriously tissue-specific [15], with moderate correlations between DNAm variation in blood and post-mortem brain tissue [16–18]. To date, epigenome-wide association studies (EWAS) of depression mainly focused on the adult popula-

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 Supplemental data for this article can be accessed [here](#).

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tion, using whole blood [19–23], brain [24,25] or saliva samples [26]. These studies identified differentially methylated CpGs within genes such as *ZBTB20*, *WDR26* and *GHSR* and differentially methylated regions in the *GRIK2*, *BEGAIN* and *PRIMA1* genes. Differences in tissue sources, ethnicity, co-morbidity diagnoses and medication history, may partly explain why these studies failed to find common CpGs robustly associated with depression. Therefore, using both whole blood and brain tissues would allow gaining more insight into the regulatory mechanisms of depression.

Adolescent neurodevelopment is modulated by environmental signals [27], yet without being affected by a large variety of abundant psychiatric comorbidities and medication usage. Moreover, analysing DNA signatures at early stages reduces the noise created by stochastic DNAm changes that happen with advancing age [28]. Only two studies reported methylome-wide findings associated with depression in adolescents [29,30]. One study that identified inverse association between methylation at *MIR4646* and gene expression was underpowered ($n = 11$ adults). Another study reported increased DNAm at *STK32C* in blood of 18 pairs of monozygotic adolescent twins discordant for depression, and replicated the findings in cerebellar tissue, but did not investigate the downstream effects on RNA expression [30]. Moreover, both studies were cross-sectional, while a longitudinal approach tracks changes in depressive symptoms over time and identifies DNA methylation shifts in a more controlled environment [31].

Shifts in epigenetic patterns may be an early mechanism at play in depression and thus, this work aims to uncover longitudinal epigenetic patterns in depression development. We used a longitudinal epigenome-wide approach to identify blood DNAm changes associated with changes in depression in otherwise healthy adolescents. We then tested whether these associations could be correlated with nearby SNP presence by conducting a mQTL analysis. In addition, we tested for replication of the associations between DNAm and depression in an independent dataset of brain tissue. We further investigated the functional relevance of these depression-associated DNAm signatures by integrating them with gene expression in a third dataset.

Importantly, we showed in a fourth cohort that genes annotated to these DNAm signatures were differentially expressed in the hippocampus of depressed individuals. Using four independent cohorts consisting of blood and brain samples and a statistical framework for analysing longitudinal epigenetic data, we uncovered a broad epigenetic-genetic network in the pathology of depression. Our results are summarized in Figure 1.

Results

Demographic and clinical characteristics of discovery and replication cohorts

The discovery cohort consisted of 59 Caucasian adolescents who had available imputed genetic data, recorded psychiatric phenotype and blood DNAm levels at both baseline and 1-year follow-up. Depression scores were assessed by the standardized Development and Well-Being Assessment (DAWBA) depression band at baseline and follow-up, and by the Montgomery-Åsberg Depression Rating Scale-Self report (MADRS-S) at follow-up only. Adolescents defined as cases and controls based on the DAWBA and MADRS-S scores at follow-up did not differ in age, sex distribution or body mass index (BMI). While the majority of controls had low depression scores at both baseline and follow-up, 19 adolescents showed an increased risk for depression from baseline to follow-up (Table 1).

The replication cohort comprised of 45 male and female Caucasians (Table 2), with DNAm profiling in brain tissue. Individuals diagnosed with MDD and controls did not differ in age or sex distribution.

Differential methylation associated with depression risk, suicidal behaviour and mRNA expression in the blood

In the discovery cohort, we aimed to identify epigenome-wide associations between changes in depression risk and changes in methylation levels, focusing on CpGs located in promoter regions (from baseline to 1-year follow-up).

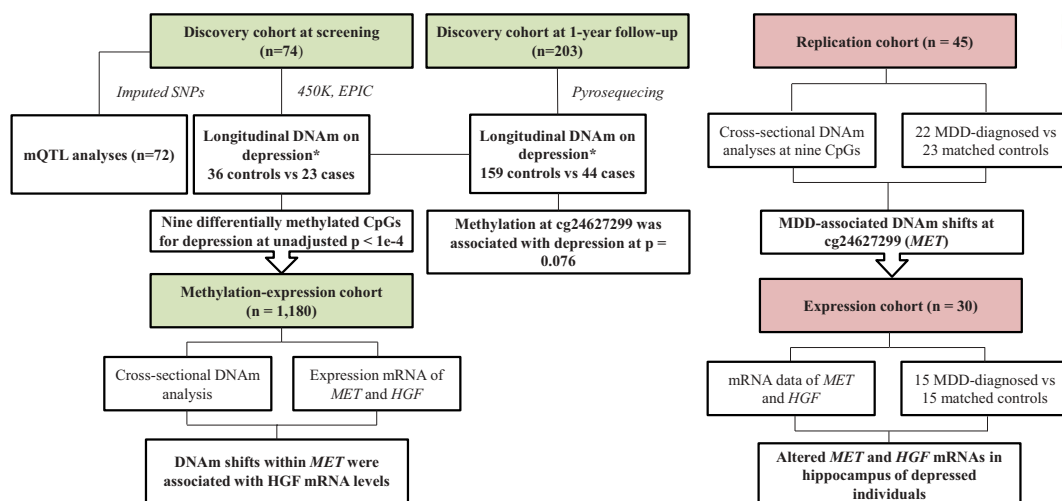


Figure 1. Study design for methylation and expression association with depression using four cohorts. Blood associations between DNA methylation and depression risk, genotype and mRNA expression were investigated using two independent cohorts, i.e. the discovery cohort and methylation-expression cohort. In brain, methylation and mRNA levels were separately analysed in relation to MDD in two independent cohorts. * Individuals with depression DAWBA band risk scores below 15% and MADRS-5 scores <9 were defined as ‘Controls’, while individuals with depression DAWBA level bands 3 ($\approx 15\%$), 4 ($\approx 50\%$) or 5 ($>70\%$) and MADRS-5 scores ≥ 9 were assigned to the ‘Cases’ category. SNPs: single nucleotide polymorphisms; MDD: major depression disease.

Depression risk at follow-up (i.e. control/case) was used as the outcome variable and corrected for methylation residuals at baseline, sex and age. A total of 5,785 CpG sites were nominally ($p_{\text{raw}} < 0.05$) associated with changes in depression risk from baseline to follow-up, 53% of which were hypomethylated in cases. However, none of these sites remained significant after adjustment for multiple testing (Figure 2), so a stricter nominal threshold of $p = 10^{-4}$ [19,20] was chosen. Nine CpG sites were associated with depression risk at $p_{\text{raw}} < 10^{-4}$ (Table 3). The calculated inflation factor of our epigenome-wide analysis was $\lambda = 0.89$, suggesting that there was no overestimation of the level of statistical significance. The largest methylation difference was detected at cg24627299 within the hepatocyte growth factor receptor (MET) gene, DNAm being 5% higher in cases (0.54 ± 0.03) than in controls (0.49 ± 0.04) (Figure 3). Methylation levels at this site were measured by pyrosequencing in a larger sample of 203 individuals at both baseline and follow-up. The normally distributed methylation values at follow-up were regressed against the dichotomous depressive phenotype variable, including the methylation residuals at baseline, age, sex and technical covariates. A clear tendency (two-sided $p = 0.076$) of increased methylation at cg24627299

in ‘cases’ compared to methylation levels of their healthy counterparts was observed.

We also investigated a possible association between the continuous scores for suicidal symptoms in SUAS and the previously identified nine CpGs. Four of the nine methylation sites associated with depression risk were also associated with suicide (Table 3). Similar to the observation made in relation to depression scores, methylation levels were higher for increased suicide symptoms at three out of four CpG sites. The largest effect size of methylation shifts was seen at the aforementioned cg24627299 within the MET gene.

The HGF gene is located in the same chromosomal region (7q21-11) as its receptor the c-MET tyrosine kinase (7q.31) encoded by the MET gene. Given the interaction of c-MET and HGF and their close chromosomal localization, methylation levels at the MET gene were investigated in relation to both MET and HGF expression levels using a methylation-expression cohort (Figure 1). A suggestive inverse association between blood methylation levels at cg24627299 and mRNA expression of HGF (transcript ID: ILMN_1801586, $p_{\text{raw}} = 0.046$, estimate = -0.050) was identified. No association at this CpG site with expression levels of MET gene was identified.

Table 1. Characteristics of the adolescents from the discovery cohort at follow-up.

	Controls (n = 36)	Cases (n = 23)	p-value**
Male: Female	7 (19.4):29 (80.6)	3 (13.1):20 (86.9)	0.77
Age (years)	18.6 ± 0.72	18.6 ± 0.87	0.46
Body Mass Index (kg/m ²)	22.68 ± 3.08	23.72 ± 4.13	0.30
SUAS	9.38 ± 4.49	25.7 ± 9.26	1.20e-08
DAWBA level bands*			
General band	4 (11.1)	100 (100)	3.68e-9
Panic disorder	0 (0)	1 (4.3)	0.0037
Posttraumatic disorder	0 (0)	5 (21.7)	0.0084
Separation anxiety disorder	2 (5.6)	2 (8.7)	0.035
Social phobia	0 (0)	8 (34.8)	0.0017
Obsessive-compulsive disorder	0 (0)	2 (8.7)	0.00045
Conduct disorder	0 (0)	3 (13.0)	0.22
Specific phobia	1 (2.8)	5 (21.7)	0.11
DAWBA level bands at baseline*			
Depression	3 (8.3)	4 (17.4)	0.19
General band	7 (19.4)	13 (56.5)	0.028
Panic disorder	0 (0)	2 (8.7)	0.25
Posttraumatic disorder	1 (2.7)	0 (0)	0.32
Separation anxiety disorder	1 (2.7)	0 (0)	0.064
Social phobia	1 (2.7)	5 (21.7)	0.0087
Obsessive-compulsive disorder	1 (2.7)	0 (0)	0.37
Conduct disorder	1 (2.7)	1 (4.3)	0.65
Specific phobia	0 (0)	4 (17.4)	0.071

Continuous variables are shown as mean ± standard deviation or number (percentage)

*All listed DAWBA bands refer to 'cases' (defined by DAWBA bands = 3, 4 or 5) of e.g. general band, depression, panic disorder.

**Two-tailed Student's t-test for continuous variables and Chi-square test for categorical variables. (Likelihood ratio)

Individuals with depression DAWBA band risk scores <15% and MADRS-S scores <9 were defined as controls; individuals with depression DAWBA level bands 3 (≈ 15%), 4 (≈ 50%) or 5 (>70%) and MADRS-S scores ≥9, were defined as cases.

Abbreviations: DAWBA, Development and Well-Being Assessment; SUAS, Suicide Assessment Scale; MADRS-S, Montgomery-Åsberg Depression Rating Scale-Self report

Lower methylation at cg24627299 and mRNA levels of MET and HGF are associated with MDD in the brain

Methylation levels at the nine CpG sites ($p_{\text{raw}} < 10^{-4}$) associated with depression in the blood discovery cohort were investigated in a replication cohort consisting of post-mortem cortical tissues of MDD-diagnosed and matched-controls (n = 45). Methylation data were available for both neuronal and glial cells. Methylation at cg24627299 (*MET*) adjusted for age and sex, was associated with depression in neurons (FDR-adjusted $p = 0.0437$, absolute difference = 2.6%) (Figure 4), yet the significance did not survive after FDR adjustment in the glial cells (Table 4). In contrast

Table 2. Characteristics of individuals in the replication cohort.

	MDD (n = 22)	Controls (n = 23)	p-value*
Male: Female	12 (54.5):10 (45.5)	12 (52.2):11(47.8)	1
Age (years)	33.6 ± 16.4	33.4 ± 16.2	0.95

Continuous variables are shown as mean ± standard deviation or number. (percentage)

*Two-tailed analysis tests the difference between the 'MDD' and 'Controls' groups using the Student's t-test for continuous variables and the Chi-square test for categorical variables. (Likelihood ratio)

MDD: major depression disorder.

to the findings in blood, methylation levels were lower in the brain from individuals diagnosed with MDD compared to controls.

To investigate HGF-MET signalling dysregulation in depression, we contrasted *MET* and *HGF* mRNA expression levels in hippocampus region of depressed and healthy adults in a fourth independent cohort (see Methods). By alternative splicing, the genes give rise to several transcript sequences. The HEEBO array covers five oligo sequences of *MET* and *HGF*, associated with two accession numbers, i.e. NM_000245 and NM_001010932. Binomial tests were run separately for the dentate gyrus and CA1 sub-regions of hippocampus. More than 50% of the average expression values were higher in the matched controls compared to the MDD-diagnosed individuals ($p < 0.05$), indicating lower expression levels of *MET* and *HGF* in the adult-depressed hippocampus (Supplementary Table 1).

Methylation levels at cg24627299 showed functional relevance in the brain tissue but not whole blood

Using the GTEx online database, *MET* gene was found highly expressed in the cortex, frontal cortex and hippocampus, contrasting with low expression in whole blood (Figure 6(a)). The *HGF* gene was highly expressed in whole blood, frontal cortex, cortex and BA24 region (Figure 6(a)). Moreover, exploration of chromatin states of this locus revealed that cg24627299 is located within active chromatin regions (i.e. TSS or enhancer) in all brain tissues except the anterior caudate and substantia nigra. In contrast, cg24627299 was located in a region repressed by PolyComb proteins in PBMCs (Figure 6(b)). Furthermore, based on

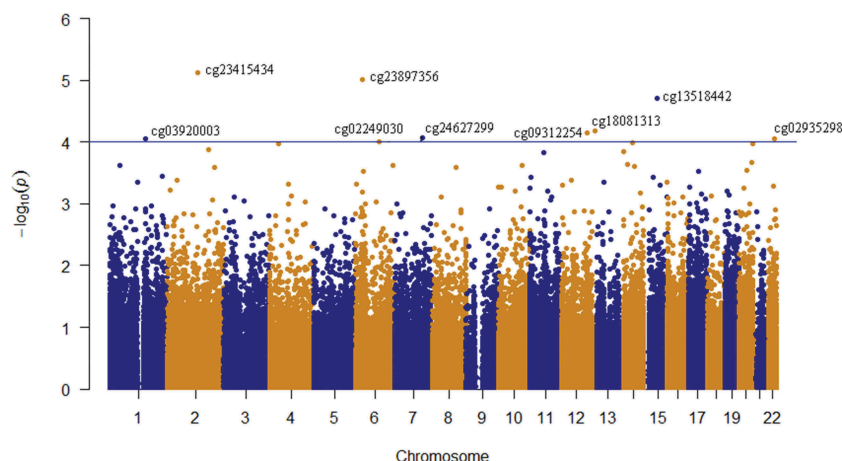


Figure 2. Manhattan plot of the longitudinal epigenome-wide association of depressive-transformed variable in whole-blood samples of 59 adolescents. The depressive-transformed variable was calculated based on depression DAWBA band risk scores and MADRS-S scores. The blue line represents the significance level at $p = 10^{-4}$.

Table 3. List of differentially methylated CpG sites in longitudinal analyses.

Probe	Chr	Position ^a	Depression phenotype				Suicide symptoms			
			Distance to closest TSS [bp]	Closest gene	Raw p-value	Estimate	Methylation Difference %	Raw p-value	FDR-adjusted p-values	Estimate
cg23415434	2	131,515,339	1768	<i>ARHGEF4</i>	7.61e-06	-0.27	3.5	0.048	0.1	-0.0077
cg23897356	6	30,795,992	-143	<i>TUBB</i>	9.72e-06	0.17	<1	0.00051	0.0034	0.0065
cg13518442	15	56,146,171	-758	<i>ALDH1A2</i>	1.93e-05	-0.14	2	0.061	0.1	-
cg18081313	12	130,944,145	-1086	<i>ULK1</i>	6.55e-05	0.18	<1	0.094	0.12	-
cg09312254	12	110,935,362	10	<i>AX747754</i>	7.03e-05	0.11	<1	0.083	0.12	-
cg24627299	7	116,098,732	-962	<i>MET</i>	8.40e-05	0.27	5	0.00077	0.0034	0.012
cg03920003	1	152,848,631	-1283	<i>ADAR</i>	8.75e-05	0.50	3	0.11	0.13	-
cg02935298	22	41,366,678	-127	<i>ATP5L2</i>	8.93e-05	-0.16	<1	0.13	0.13	-
cg02249030	6	100,068,675	1298	<i>USP45</i>	9.75e-05	0.13	<1	0.0038	0.011	0.0049

Position was according to Human Reference Genome build 37 (hg19).

P-values < 0.05 are written in bold.

The depression phenotype was calculated based on depression DAWBA band risk scores and MADRS-S scores. Participants with depression DAWBA band values ≥ 3 and MADRS-S scores ≥ 9 were categorized as 'cases', while participants with depression band values <3 and MADRS scores <9 were categorized as 'controls'. Susceptibility for suicidal symptoms was measured with the SUAS questionnaire.

Chr.; chromosome; TSS: transcription start site; bp: base pair; DAWBA: standardized Development and Well-Being Assessment; MADRS-S: Montgomery-Åsberg Depression Rating Scale-Self; SUAS: Suicide Assessment Scale.

the online BECon tool resource of comparing methylation levels in whole blood and three brain regions, blood methylation levels at cg24627299 inversely associated with BA10, BA20 and BA7 methylation levels, supporting an opposite pattern of methylation observed originally in whole blood and brain.

Genotypes at rs39748 are associated with differential methylation at cg24627299 in whole blood

Taking into account the influence of genetic variants on both methylation [32,33] and gene

expression [34], we performed mQTL and eQTL analyses of SNPs within 100 kbp (up- and downstream) of cg24627299 using 74 individuals from the discovery cohort that had both imputed SNPs and DNA methylation data at follow-up. Using the hg19 version of the UCSC genome browser, we identified 39 genetic variants within 100 kbp of cg24627299. Twenty independent genetic variants were not in LD ($r^2 < 0.8$) (Figure 5) and had MAF > 0.3 in the discovery cohort. Based on an additive model, we identified one genetic variant (rs39748), located in intron 1 of *MET* and ~16 kbp downstream of cg24627299, that was nominally associated with

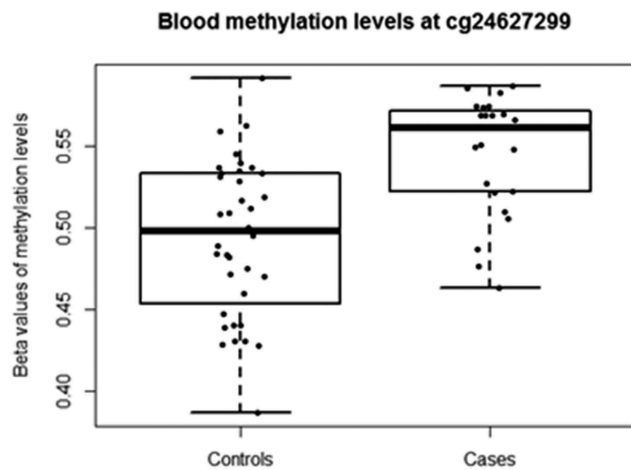


Figure 3. Blood DNAm levels (β -values) at cg24627299 within the *MET* gene for adolescents defined as ‘controls’ and ‘cases’. Individuals with depression DAWBA band risk scores below 15% and MADRS-S scores <9 were defined as ‘Controls’, while individuals with depression DAWBA level bands 3 ($\approx 15\%$), 4 ($\approx 50\%$) or 5 ($>70\%$) and MADRS-S scores ≥ 9 were assigned to the ‘Cases’ category.

methylation levels at cg24627299 ($p = 0.045$, effect size (CC: CG) = 1% (CC: GG) = -2.8%), after adjusting for age and sex. Methylation levels at cg24627299 at rs39748

genotypes were 0.51 ± 0.05 at CC, 0.52 ± 0.04 at CG and 0.48 ± 0.04 at GG. In the discovery cohort, the MAF at rs39748 was 0.36, similar to the whole-population frequency of 0.34. Notably, this variant was also associated with *MET* mRNA expression, and the highest effect sizes were seen in brain regions (Table 5).

Discussion

Here, we conducted a comprehensive analysis of epigenetic, transcriptomic and genetic information in four independent cohorts and two tissues, including longitudinal EWAS, to uncover epigenetic shifts associated with high risk for depression. Higher methylation at cg24627299 within the *MET* gene was associated with a high risk for depression and susceptibility for suicidal behaviour in blood, while lower methylation at cg24627299 was observed in cortical tissue of depressed adults. Importantly, methylation shifts at this site were inversely associated with expression levels of the *MET* ligand *HGF* in blood, indicating a possible functional relationship between

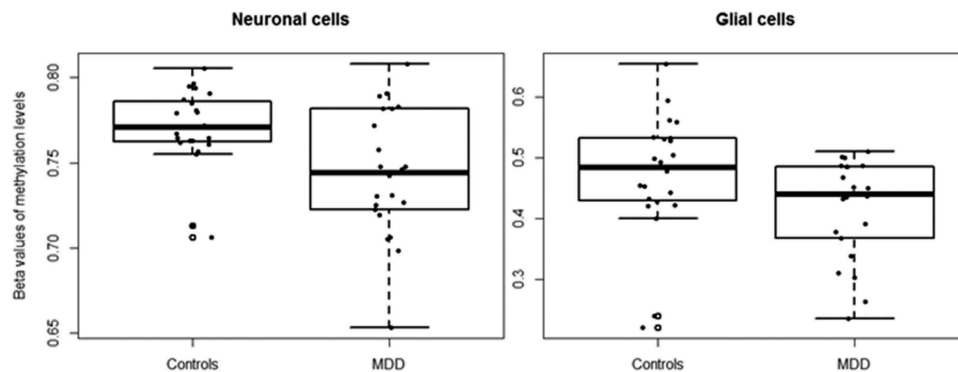


Figure 4. Methylation levels at cg24627299 (*MET*) in brain samples of MDD-diagnosed individuals and controls.

Table 4. Differentially methylated CpG sites in the brain.

Probe	Raw p-value [neuri]	FDR adjusted p-value [neuri]	Methylation Difference % [neuri]	Raw p-value [glia]	FDR adjusted p-value [glia]	Methylation Difference % [glia]
cg02249030	0.71	0.80	0.51	0.78	0.78	0.11
cg02935298	0.64	0.80	0.32	0.17	0.38	0.91
cg03920003	0.80	0.80	0.38	0.09	0.38	1.7
cg09312254	0.32	0.80	0.23	0.70	0.78	0.081
cg13518442	0.76	0.80	0.16	0.77	0.78	0.36
cg18081313	0.54	0.80	0.72	0.42	0.63	1.3
cg23415434	0.66	0.80	0.33	0.12	0.38	2.8
cg23897356	0.062	0.28	0.35	0.23	0.41	0.21
cg24627299	0.0041	0.037	2.6	0.021	0.19	5.5

P-values were obtained using linear regression models, contrasting DNAm levels at the nine CpGs and the yes/no depression diagnosis, together with covariates age and sex.

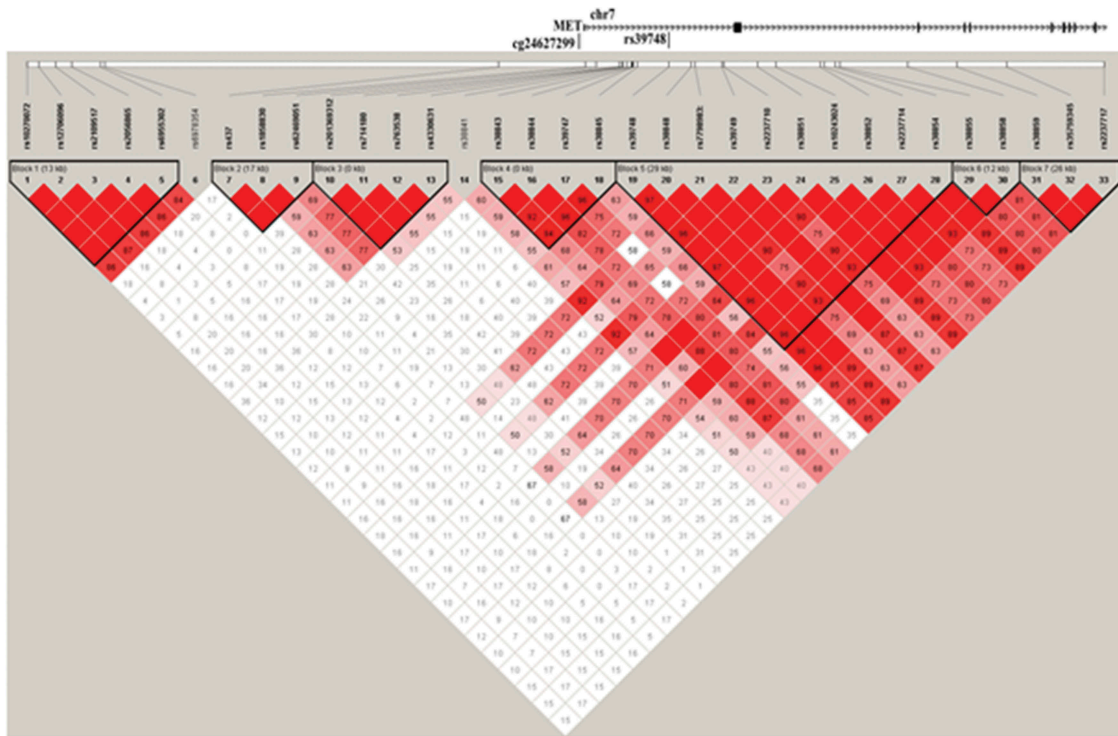


Figure 5. LD matrix of the 39 investigated genetic variants within the ± 100 kbp of the differentially methylated CpG site (cg24627299, *MET* gene).

methylation and expression. Interestingly, both *MET* and *HGF* mRNA expressions were altered in hippocampus of depressed individuals. These findings highlight the interplay between methylation and expression of *HGF*, as well as on HGF/c-MET signalling in the development of depression.

Our discovery of the changes in blood methylation levels in adolescents at risk for depression, supported by the confirmatory approach in adult brain samples, sheds light on depression aetiology. *MET* encodes a tyrosine kinase receptor called c-MET that has high affinity for HGF. Although c-MET was shown to be regulated by a number of transcription factors including p53 [35] and Daxx [36], less is known about the role of DNA methylation in regulating c-MET expression. Loss of *MET* promoter methylation is involved in HGF-dependent increase in tumorigenicity and metastatic potential of hepatocellular carcinoma via upregulation of *c-Met* and *HGF* expression [37]. Importantly, both *MET* and *HGF* are expressed in the developing nervous system, especially in the cerebral cortex [38,39], strengthening the importance of our replication in cortical tissue samples. In the current study, altered gene expression of the

MET ligand *HGF* could be caused by methylation changes at *MET* in the brain. This finding is supported by the locus harbouring the identified differentially methylated CpG exerts a regulatory role on transcription in the brain regions (Figure 6(b)), supporting a functional link with *MET* and *HGF* expression in the hippocampus. Our results suggest that the interplay of these two molecules is regulated at the gene expression level. Therefore, altered activity of HGF/c-MET signalling due to

Table 5. Distribution of the rs39748 eQTL contribution across the tissues.

p-Value*	NES	Tissue
4.6e-18	-0.70	Brain – Caudate (basal ganglia)
5.9e-16	-0.71	Brain – Putamen (basal ganglia)
1.2e-10	-0.64	Brain – Hypothalamus
2.1e-10	0.24	Artery-Tibial
2.6e-10	-0.48	Brain – Nucleus accumbens (basal ganglia)
1.8e-9	0.34	Artery-Aorta
4.3e-9	-0.65	Brain – Substantia nigra
1.0e-8	-0.45	Brain – Cerebellum
1.8e-6	-0.58	Brain – Spinal cord (cervical c-1)
2.9e-6	-0.42	Brain – Cerebellar Hemisphere
1.9e-5	0.12	Lung
6.0e-5	0.16	Thyroid

*P-values NES and tissue regions were identified using quantitative trait loci (eQTL) mapping, using the GTEx Consortium Abbreviations: NES: normalized effect size.

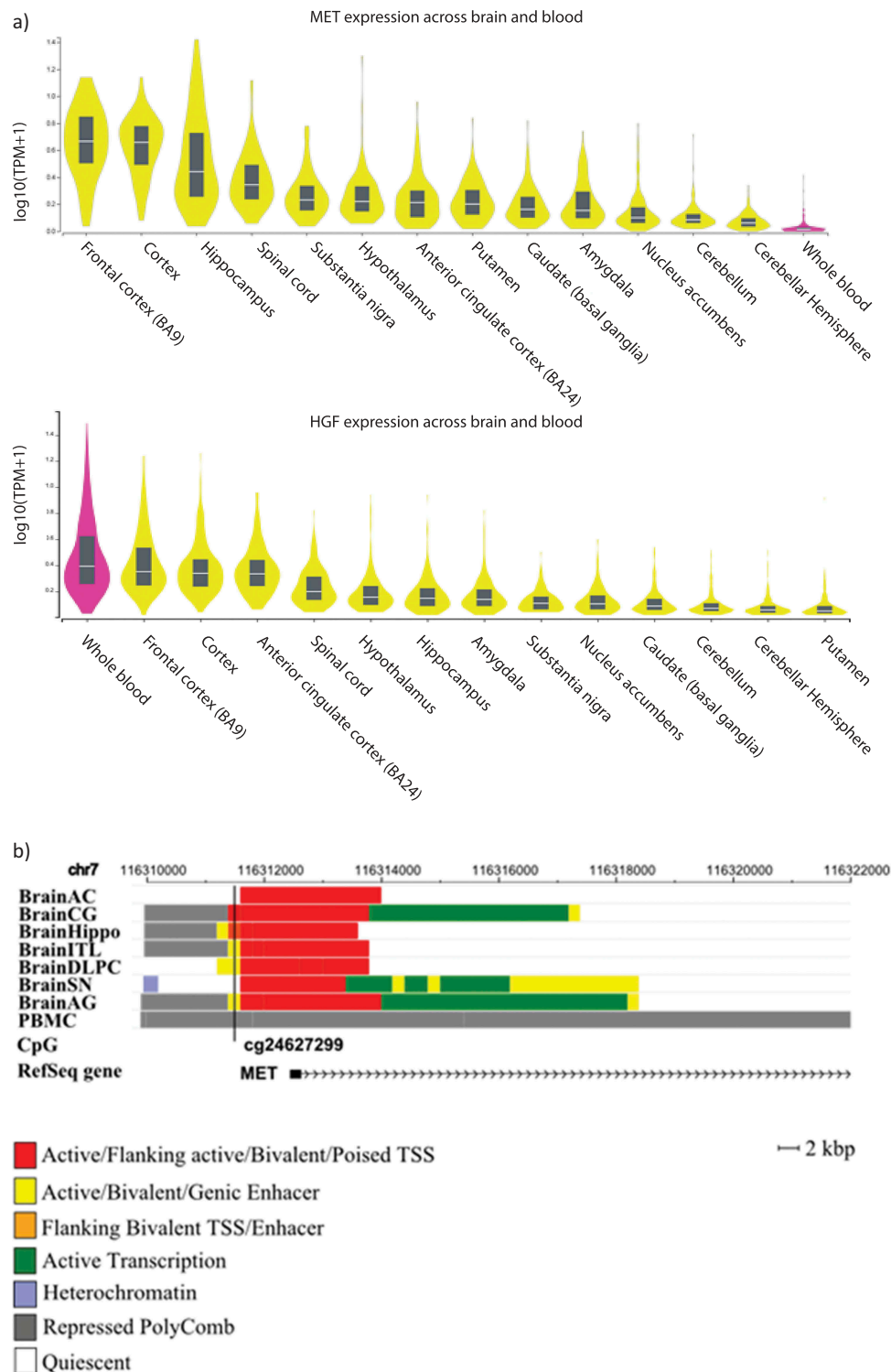


Figure 6. a) *MET* and *HGF* expression levels among different brain regions and whole blood. b) Genomic context of the most significant CpG site associated with the depressive phenotype in the longitudinal analyses. Genomic positions of RefSeq genes are displayed in the bottom part and indicated by arrows. The position of the significant CpG site is highlighted by black lines. Since analyses were performed based on data obtained in blood, chromatin marks overlapping in brain and blood cells were investigated. Chromatin states of eight tissues downloaded from the 37/hg19 WashU Epigenome Browser are illustrated. Each functional role of a segment is indicated by a particular colour. BrainAC: brain anterior caudate; BrainCG: brain cingulate gyrus; BrainHIPPO: brain hippocampus; BrainITL: brain inferior temporal lobe; BrainDPC: brain dorsolateral prefrontal cortex; BrainSN: brain substantia nigra; BrainAG: brain angular gyrus; PBMC: peripheral blood mononuclear primary cells.

methylation shifts may interfere with HGF-mediated action on interneuron migration, disrupting neuronal growth in the cortex [40].

DNAm levels at the identified CpG site within *MET* gene in depression were associated with suicidal thoughts assessed by the SUAS questionnaire. Previous studies investigating DNAm in relation to both depression and suicidality found that decreased anterior prefrontal *GABRA1* levels in major depressive suicide victims may be due to increased anterior prefrontal *GABRA1* gene promoter methylation [41]. An EWAS in MDD suicide cases compared with non-psychiatric, sudden death controls, found hypomethylation at *PSORS1C3* across both cortical brain region, i.e. BA11 and BA25 [42]. DNAm levels at the *GRIK2* and *BEGAIN* genes involved in synaptic communication were associated with astrocyte dysfunction in depression and suicide in the dorso-lateral prefrontal cortex [24]. Lastly, among MDD patients, altered DNAm levels at *ELOVL5* were found in suicide attempters when compared with age- and sex-matched healthy volunteers [43].

Although we could not test the regulatory role of genetic variants on *MET* and *HGF* mRNA expression levels, we found that the genetic variant rs39748, located in intron 1 of the *MET* gene, was associated with differential methylation at cg24627299 at follow-up. During this time window (18–19 years), the within-individual adjustment to environmental factors and its plasticity may be under genetic control. Intriguingly, reduced protein levels of c-MET have been associated with the C allele at the *MET* promoter variant rs1858830 in post-mortem autistic brain samples [44,45]. These findings indicate that changes in DNA methylation may compensate and/or mediate the inter-individual genetic variation [46] and its effect on gene expression.

Intriguingly, we found decreased levels of *MET* and *HGF* in hippocampus of depressed individuals compared with control hippocampi. A number of studies have already examined depressive symptoms in relation to dysregulation of the hippocampus [47]. Negative psychosocial factors induce changes in hippocampal neural activity and synaptic plasticity [48] as well as in the prefrontal cortical region [49]. Moreover, the disturbed activity of the hippocampus can also be caused by a weakening of excitatory synapses in, e.g. CA1, which plays a critical role in the determination of affect

and reward [50]. Importantly, the hippocampus is a prominent site for the expression of *HGF* and *MET* in the developing and adult brain [51] (Figure 6(a)). It has been demonstrated that HGF and MET are clustered at the postsynaptic density of excitatory synapses in CA1 and increased HGF levels improve expression and clustering of excitatory synaptic proteins at sites along dendrites [52], highlighting their importance for neuronal activity. Importantly, our results showed lower mRNA levels of both *HGF* and *MET* in the hippocampus of depressed adults. Consistently, a decreased HGF protein level was reported in individuals with obsessive-compulsive disorder, bipolar disorder and depression [53–55]. Downregulation of HGF/c-MET signalling reduces the axonal growth of hippocampal neurons through deterioration of the autocrine feedback loops [56], resulting in a reduced arborization at excitatory synapses [57]. Processing of the input stimuli is thus altered and an inadequate response is formulated [58], leading to depressive symptoms (Figure 7). In addition, alteration of HGF/c-MET at the excitatory synapses in the CA1 region may disturb the connection between the axons in the entorhinal cortex with the distal dendrites of pyramidal cells, a pathway required for long-term consolidation memory [59]. Interestingly, as serotonin potentiates these synapses, treatment of depression by conventional agents such as selective serotonin reuptake inhibitors results in restoration of the CA1 pyramidal spine density and excitability [60–62].

Our findings on methylation changes at the *MET* gene in whole blood were validated in an independent cohort of prefrontal cortex samples, and further extensively investigated in the brain tissue. This is the first study which identifies differential DNAm at the *MET* gene in depression. This may be the result of the fact that our findings derive from a longitudinal study, taking into account the association of DNAm levels and depression scores at two-time points, compared to earlier EWASs that only used a cross-sectional set-up. Whole blood is commonly used as surrogate for brain in DNAm studies due to accessibility and its potential connection with neuropsychiatric diseases through hormonal and immune regulation [63]. However, given the tissue specificity of DNAm, a cross-tissue analysis approach, using

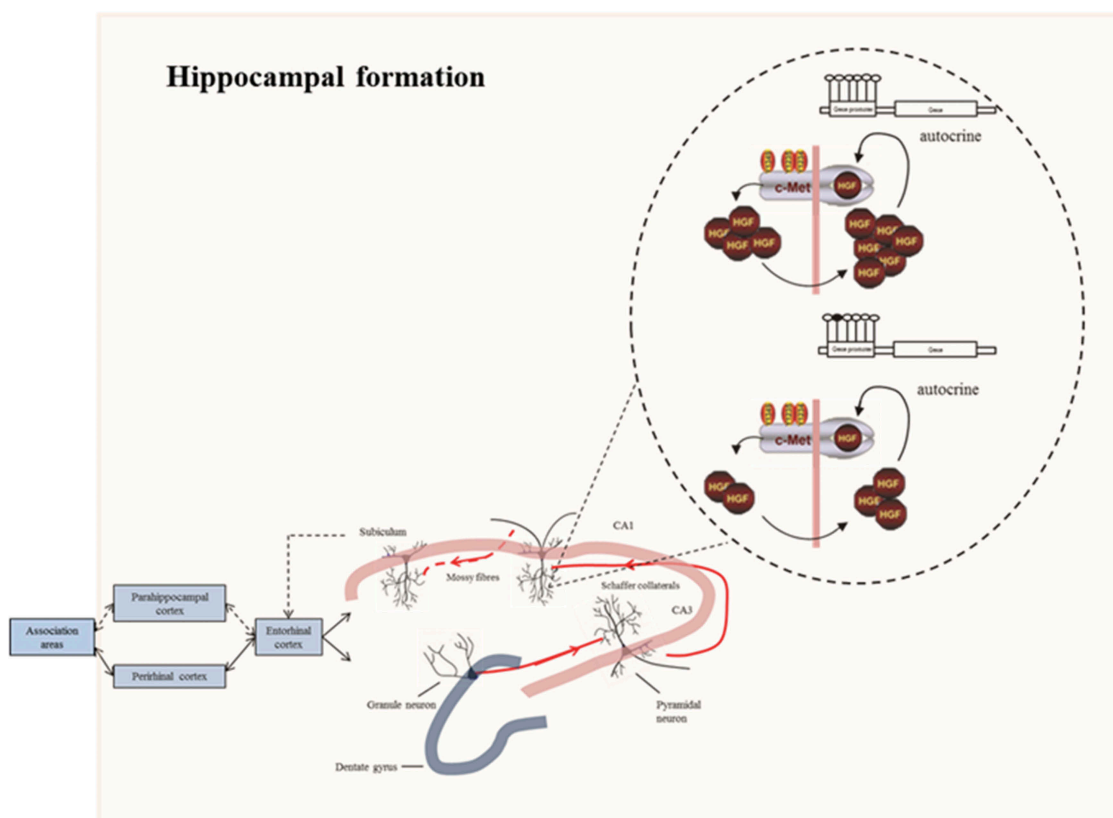


Figure 7. Connections in the hippocampal formation. In the hippocampus, sensory information arrives from the cerebral association neocortex via the entorhinal cortex, parahippocampal gyrus and/or perirhinal cortex. The hippocampal subfield CA1 sends axons to both subcortical areas and the deep layers of the entorhinal cortex, either directly or through the subiculum. Processed information is sent back from the hippocampal CA1 area to the cerebral association cortex through the same pathway. Higher methylation levels at the promoter of *MET* may alter the autocrine mechanism of HGF/c-MET signalling necessary for promoting axonal growth at the excitatory synapses of the CA1 hippocampal pyramidal neurons. The response to the environmental stimuli may therefore be altered, leading to depressive symptoms.

both brain and blood, may help in identifying more relevant DNAm patterns in psychiatric populations. Additionally, the EPIC array used in our discovery analysis showed higher proportion of correlated CpGs between blood and brain than the previously used 450K array [64]. Using separate cohorts, we discovered that higher methylation levels were associated with lower expression levels at the *HGF* gene in whole blood in adolescents at risk for depression and lower *HGF* expression levels in adult depressed hippocampus, indicating that the association between DNAm and expression levels may be translated to the hippocampus. However, in the prefrontal brain samples of depressed adults, we observed an opposite direction of methylation patterns from blood, i.e. lower DNAm at the CpG site. The finding is not surprising, as DNAm dynamics are not only tissue-specific, but may also significantly differ

across different regions of the brain [65]. Whole blood can be assumed to reflect a partial summation of methylation changes at this CpG site occurring in the brain. The observed opposite direction of methylation patterns in blood and prefrontal brain samples found in our study was also supported by an inverse correlation of methylation levels at the identified CpG site between blood and BA10 medial frontal gyrus region using the BECon omics platform.

Our findings have to be evaluated in light of their limitations. Firstly, due to the relatively small sample size and effect sizes detected, no probe reached the multiple-testing levels of significance. As expected in longitudinal studies, methylation differences are moderate (~5%), and, while the directionality could be replicated using pyrosequencing in an extended cohort, significance could not be reached, likely due to the 5%

sensitivity limit of such a locus-specific method [66]. In addition, our discovery and replication cohorts were different in age (adolescents vs. adults). This limitation was taken into consideration by using statistical models adjusting for this factor. A strength of our study is our longitudinal, multi-tissue (blood and brain) and multi-layered (genetics, epigenetics, transcriptomics) approach affirming our findings from whole blood in the prefrontal brain samples, indicating that the association may be of relevance for regulatory pathways in the brain. Importantly, adolescents were free of medication with psychotropic effects, which may affect methylation signatures. Lastly, our psychiatric phenotype was assessed using two questionnaires, i.e. the MADRS-S and DAWBA depression band, increasing the sensitivity of analysis.

Conclusions

We present evidence that stable blood-based epigenetic changes are associated with risk for depression and susceptibility for suicidal behaviour in adolescence. The complementary epigenetic and genetic analyses may provide mechanistic support for downregulation of the *HGF* and *MET* genes in the hippocampus of depressed individuals. Our findings suggest an important pathogenic role of HGF/c-MET signalling in the pathophysiology of depression, partly characterized by impaired neuronal growth.

Methods

Study participants

Discovery dataset

The discovery cohort was a sample of a cohort of 786 unrelated Swedish adolescents aged 17–19, recruited between 2012 and 2017 in Uppsala, Sweden. This longitudinal study aims to investigate potential psychiatric risk factors in youth and followed up the subjects approximately 1 year after study enrolment. Exclusion criteria at recruitment included any severe cognitive impairment or medication for psychiatric disorders. Height, age and medication intake were self-reported. We measured body weight at the clinic and calculated for

body mass index (BMI). Methylation analysis at baseline was available for 221 non-related adolescents, analysed in two batches [29,32]. Out of 130 adolescents analysed in the first batch, a subset of 61 came at the follow-up and therefore had extracted DNA available. From the second batch of 91 adolescents, 11 were selected out of the 34 adolescents who answered positively to the follow-up based on their very low/high psychiatric-related scores at the DAWBA general band. In total, longitudinal DNA methylation analyses were applied on a sample of 74 adolescents. The study was approved by the Regional Ethics Committee in Uppsala, corresponding to the Drnr 2011/446. All participants volunteered to participate in the study, and written informed consent has been obtained.

Psychiatric phenotype of depression and disease outcome

Participants were given a questionnaire at baseline and at follow-up to assess both their baseline psychiatric phenotype and any potential change in psychiatric health over the one-year period. The standardized DAWBA questionnaire assesses the probability that an adolescent (aged 5–17 years) displays anxiety disorders, depression, post-traumatic stress disorder, autism, separation anxiety disorder and obsessive compulsive disorder [67]. The generated *in silico* scores include six band scores for risk, i.e. 0 (<0.1%), 1 (\approx 0.5%), 2 (\approx 3%), 3 (\approx 15%), 4 (\approx 50%) and 5 (>70%), based on Diagnostic and Statistical Manual of mental disorders (DSM-IV) and International Statistical Classification of Diseases and Related Health Problems (ICD-10) [68]. The DAWBA depression band consists of questions relating to the occurrence of depression symptoms in the last 4 months, such as e.g. the level of sadness, irritability, sleep activity, wish to die and to what extent general mood is affected. Individuals with a score ≥ 3 were considered at high risk for depression [69].

At follow-up, participants were given two additional questionnaires, in order to provide more support to the psychiatric-related phenotype. One of the most commonly used instruments for the assessment of depressive symptoms is the MADRS self-report

questionnaire [70]. The MADRS-S comprises nine questions assessing individuals' mood, feelings of unease, sleep, appetite, ability to concentrate, initiative, emotional involvement, pessimism and zest for life. Items are rated on a 7-point Likert scale (from 0 to 6) leading to a total score ranging between 0 and 54. Study participants were instructed to rate symptom severity over the last three days. Individuals with a score ≥ 9 were broadly considered depressive [71]. The MADRS-S questionnaire was available at follow-up only.

We further evaluated individual's susceptibility for suicide or suicidal attempts with the Suicide Assessment Scale (SUAS). This questionnaire assesses suicidal symptoms such as despair, hostility, seclusion, loss of control, somatization, death wishes and suicidal thoughts and behaviour. The overall score ranges from 0 to 80.

Our study includes a population-based cohort of adolescents; therefore, the prevalence of psychiatric phenotypes is not that high, yet normal for a population-based study [72,73]. In order to increase the analysis power and sensitivity, both the DAWBA depression band and MADRS-S scores were transformed into a binary depressed/not-depressed variable and used in further analyses. Participants with depression band values ≥ 3 and MADRS-S scores ≥ 9 were categorized as 'cases', participants with depression band values < 3 and MADRS-S scores < 9 were categorized as 'controls'. Subjects showing depression band values of ≥ 3 and MADRS-S scores < 9 were assigned as 'missing' and omitted from the analysis. A total of 59 individuals were categorized as cases or controls, defining the 'discovery dataset'.

Genome-wide DNA methylation profiling and processing

Blood was collected at two-time points (baseline and one-year follow-up). Baseline DNAm profiles were assessed with the Illumina 450K methylation Beadchip. Data preprocessing was performed as described in [32,69]. It should be noted that baseline methylomes were profiled in two different time batches within a time

range of 1 year [32]. This time difference in DNAm analyses at baseline will further be referred to as 'batch' and taken into consideration in the methylation analyses at baseline.

At follow-up, genomic DNA was extracted from EDTA blood and 250 ng of DNA was bisulphite-converted using the EZ DNA Methylation Kit (Zymo Research Product, Germany). DNA was eluted in 15 μ l buffer according to the manufacturer's protocol, evaporated to a volume of $< 4 \mu$ l, and methylomes were assessed with the Illumina Methylation EPIC array. The results were analysed with GenomeStudio 2011.1 from Illumina Inc. A total of 74 samples had methylation data available for quality control procedures. The signal intensities from the methylated and unmethylated channels were normalized with the *noob* procedure in R [74] and the DNA methylation level, called beta value, was calculated by the ratio of the methylated to the sum of methylated and unmethylated intensities. All samples successfully passed quality control (they had $\geq 75\%$ probes with a detection p-value $\leq 5 \times 10^{-5}$). Probes were filtered out based on the following criteria: 1) probes with $\leq 75\%$ samples showing detection p-values ≤ 0.01 ; 2) probes located on the sex chromosomes; 3) probes not targeting CpG sites; 4) probes with common polymorphisms (MAF $> 5\%$ in European population). After filtering, 765,396 probes remained. Batch effects were corrected using the ComBat function [75]. Relative proportions of white blood cell types, i. e. CD4+ and CD8 + T cells, monocytes (Mono), granulocytes (Gran), B cells (Bcell) and natural killer (NK) cells, were calculated using the Houseman algorithm [76]. These proportions were included in linear regression models prior analyses. As analyses were based on both baseline and follow-up, only CpG-sites present on both chips were analysed. The expanded annotation table by Price *et al.* was used for CpG site annotation [77], defining for each CpG site the associated gene and distance to the closest transcriptional start site (TSS). With the exception of miRNA genes, only probes that were within 2,000 base pairs down/upstream of the TSS of genes were taken into consideration, based on the assumption that DNA methylation and gene

expression are closely related within this region [78]. The two methylation data sets, i.e. at baseline and follow-up, were merged, resulting in 148,788 CpG sites to be analysed.

Genotyping and imputation

All study participants at baseline ($n = 786$) were genotyped using the Infinium Global Screening Array at the SNP&SEQ SciLife Platform at Uppsala University, interrogating a total of 700,078 genetic variants. Prior imputation, quality control steps were performed using Plink 1.9 [79] as following: samples were excluded based on genotyping call rate ($<95\%$), high heterozygosity >2 standard deviations (SD) and missingness rate >0.05 ($n = 21$ samples); probes were removed based on the minor allele frequency (MAF) ($<1\%$), Hardy-Weinberg equilibrium (HWE) ($<1e-6$) and missing rate ($>5\%$) ($n = 203,774$ probes). A total of 496,304 genotypes of 765 individuals were included in the pre-phasing approach implemented in SHAPEIT version 2.7 (r904) [80] and then imputed using IMPUTE2 software (version 2.3.2) [81], taking the 1000 Genome Project phase 3 integrated variant set as a reference (release October 2014). Data were further filtered using HWE cut-off of 0.01, imputation info score >0.3 and MAF >0.01 . A *cis* analysis was performed for the 74 participants at follow-up who had measured both methylation and genetic data. Common single nucleotide polymorphisms (SNPs) within $\pm 100\text{kbp}$ of the differentially methylated CpG site position in Human Reference Genome build 37 (hg19) were investigated with the genome browser [82].

Replication dataset

We interrogated an already pre-processed, publicly available dataset of 58 post-mortem cortical tissues from MDD-diagnosed individuals ($n = 29$) and 29 matched controls [83]. In these samples, neuronal and glia cell proportions were determined by fluorescence-activated cell sorting (FACS). Methylation levels were separately measured in both cell types using Illumina 450K methylation Beadchip determining 480,492 methylation sites. The two methylation datasets

are available under the GEO accession number GSE41826. To avoid confounding of methylation signal by ethnicity, only data from Caucasian individuals were included in further analyses ($n = 45$).

Methylation-expression dataset

To integrate the methylome with the transcriptome, a third independent open-access dataset (E-GEOD-56,047) was used, containing both transcriptome and methylome data from CD14+ samples derived from 1,202 participants (44–83 years-old) [84]. The current cohort is part of a larger study that investigated cardiovascular diseases in a cohort of Caucasians, African Americans and Hispanics [85]. The deposited data contained information about age, race/ethnicity, study site, sex, methylation/expression chip, B-cells, T-cells, neutrophils and natural killer cell proportions. For the purpose of our analyses, methylation data were filtered for outliers based on a graphical boxplot ($n = 22$ outliers removed). Methylomes were profiled with the Illumina 450K methylation Beadchip and transcriptomes were measured with the Illumina HumanHT-12 V4.0 expression Beadchip. Details about methylation data pre-processing and transcriptomic quality control can be found elsewhere [84].

Expression cohort

Gene expression profiles in the dentate gyrus and CA1 subregions of the hippocampus were analysed in 15 patients diagnosed with MDD and 15 age-, sex- and race-matched controls using data from a publically available case/control study (ArrayExpress accession E-GEOD-24,095). Data were analysed using the cDNA hybridization to 48K human HEEBO whole-genome microarrays. Average expression values for all hybridizations were available for each individual [86]. Data were normalized using linear-Log normalization to stabilize the variance of low expressing genes, and then spatial plus intensity based LOWESS to remove spatial and intensity related biases, using the MAANOVA library [86].

Evaluation of genomic sites in brain and blood

The functional relevance of the differentially methylated DNA sites was further investigated in blood and brain, using chromatin state analysis [87] and Spearman correlations [63]. Based on the availability of chromatin state data as predicted with Hidden Markov Models (HMMs), seven brain tissues were analysed: brain angular gyrus (BrainAG), brain anterior caudate (BrainAC), brain cingulate gyrus (BrainCG), brain hippocampus (BrainHIPPO), brain inferior temporal lobe (BrainITL), brain substantia nigra (BrainSN), brain dorsolateral prefrontal cortex (BrainDLPC) and peripheral blood mononuclear primary cells (PBMC). Data were loaded from Roadmap Epigenomics Project of 37/hg19 version of human genome in WashU Epigenome Browser <http://epigenomegateway.wustl.edu/legacy/>. For easier visualization, the 18-state model for the production of the segmentations according to the gene regulatory role was reduced to five regions, i.e. active/flanking active/bivalent/poised transcription start site (TSS), active/bivalent/genic enhancer, flanking bivalent TSS/enhancer, active transcription and repressed polyComb state. We used Spearman correlations to assess DNA methylation correspondence between blood and three brain regions, i.e. Brodmann area (BA) 10 (prefrontal cortex), BA20 (temporal cortex) and BA7 (parietal cortex), using the online BECon tool <https://redgar598.shinyapps.io/BECon/> [63]. The cohort consisted of methylation data of 16 individuals. More details about the preprocessing of this DNA methylation dataset are available in [63]. The transcription levels in different brain tissues and whole blood of the associated genes to the differentially methylated CpGs were investigated using GTEx <https://gtexportal.org/home/> [88]. Moreover, using a population sample in the GTEx Consortium, genotypes at genetic loci were included in linear regression models and the significance to the gene expression was measured [88]. Thus, the identified genetic variants in the mQTL analysis were classified as eQTLs if they showed contribution to the gene expression using the quantitative trait loci (eQTL) mapping.

Statistical analysis

We investigated genome-wide methylation differences with depression risk in the discovery cohort

of $n = 59$ adolescents, using a longitudinal approach. All statistical analyses were performed in R version 3.4.1. In order to reduce the unwanted variability in DNAm caused by intrinsic (age, sex) and environmental (baseline depression, batch effect) factors, DNAm profiles were first regressed against baseline depression DAWBA scores, age, sex and batch. The resulting residuals were then included as covariates in the following linear regression model: $\text{methylation}_{\text{follow-up}} \sim \text{residuals}_{\text{baseline}} + \text{depression-transformed variable}_{\text{follow-up}} + \text{age}_{\text{follow-up}} + \text{sex}$. The inflation factor lambda was calculated for the linear models at follow-up, by dividing the median of chi-squared test statistics with the expected median of chi-squared distribution. In the next step, methylation M-values at the most significant DNA loci were also regressed against the SUAS scores in linear regression models, adjusting for sex and age at follow-up. Unadjusted (raw) p-values $< 10^{-4}$ were considered significant.

In replication analyses using data from brain tissue, methylation levels of significant CpG sites from the discovery analysis were regressed against the yes/no depression diagnosis, adjusting for age and sex. Analyses were run separately for neurons and glia cells and FDR adjustment was applied to the raw p-values. Associations between methylation and RNA expression levels at the identified genes were tested in an independent cohort. Methylation data were firstly regressed against age, race, study site, gender, methylation chip and the sample contamination with non-targeted cells, i.e. non-monocytes. The resulting residuals were included in linear regression models as an independent variable, together with expression values as dependent variables. Covariates were age, race, study site, gender, expression chip and the sample contamination with non-targeted cells. Given that expression data was obtained as normalized log2 ratio representing MDD/control mRNA expression, quantitative analysis could not be performed. Expression data were analysed using a two-sided binomial test, assuming a 0.5 probability threshold.

Given the relatively small sample size ($n = 72$) having both genotype and methylation data in the discovery cohort, only SNPs in no linkage disequilibrium ($r^2 < 0.8$) and with MAF > 0.3 were

analysed. Linkage disequilibrium was calculated using LDlink web-tool [89] and Haploview [90]. The genotypes were coded as '0' (wild-type individuals), '1' (heterozygous of minor allele) and '2' (homozygous of minor allele). The modulatory effect of genetic sequence variant was shown on the surrounding methylation levels [32]. Methylation quantitative trait locus analysis (mQTL) was performed using likelihood ratio tests, adjusted for age and sex. The p-value of the SNP effect was calculated from the Chi-square distribution with 1 degree of freedom.

Pyrosequencing

Methylation levels at cg24627299 the CpG sites showing the highest DNA methylation difference were experimentally measured using pyrosequencing at baseline and follow-up. Out of the 786 adolescents recruited at the baseline, a subset of 203 was followed-up and had available extracted DNA. A total of 44 individuals were defined as 'cases' based on the DAWBA depression band ≥ 3 and MADRS-S scores ≥ 9 and 159 as 'controls'. Primers were designed using PyroMark Design software (Qiagen) (Fwd: ATGATTGAATAATTGGTATGAGAGT, 5'-biotinylated Rev: AATTAAACCCTAAAAAACTTCCATTT, sequencing: TTAATATAATTATTTT AATAGAGT). Genomic DNA (500 ng) was bisulphite (BS)-converted using DNA methylation-Gold Bisulphite Kit (Zymo Research) and eluted in 25 μ l of elution buffer. BS-DNA (~15 ng) was applied as a template in the PCR performed with the PyroMarks PCR kit (Qiagen) following manufacturer's recommendations. To verify the efficiency and sensitivity of the PCR-pyrosequencing, we used Epitect unmethylated and methylated human BS-DNA samples (Qiagen) as controls. The entire PCR product, 4 pmol of the sequencing primer, and streptavidin sepharose high-performance beads (GR Healthcare) were used for pyrosequencing on the PSQ 96 system and PyroMark Gold 96 reagent kit (Qiagen). The PyroMark CpG software 1.0.11 (Qiagen) served for generating methylation levels. Technical covariates, i.e. bisulphite conversion batch, pyrosequencing plate and date of the pyrosequencing analysis were tested against methylation values, separately at baseline and follow-up. Methylation levels at baseline were firstly regressed against age, sex and technical covariates, and

the resulting residuals were included in linear regression models at the follow-up. The following model was applied for the measured methylation levels at follow-up: methylation follow-up ~ residuals baseline + depression-transformed variable follow-up (0/1) + age follow-up + sex + technical covariates.

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Authors' contributions

DMC and HBS conceived and designed the study. DMC analyzed and interpreted the data and wrote the manuscript. JM interpreted the data and helped writing the manuscript. SV helped in analyzing and interpreting the results and writing the manuscript. LK and MJ performed the pyrosequencing analyses and helped writing the manuscript. NW recruited participants, gathered data and help writing the manuscript. JJ helped at interpretation of the results. HBS helped at the data interpretation.

Disclosure statement

No potential conflict of interest was reported by the authors.

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