Epigenome-Wide Association Studies in Cases and Controls With High and Low Genetic Risk for Schizophrenia

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Abstract

Schizophrenia (SCZ) is a severe psychiatric disorder caused by a complex interplay of genetic and environmental factors. Genome-wide association studies (GWASs) have identified numerous genetic variants associated with SCZ, yet genetic risk does not fully predict the disorder. Some individuals with a high genetic risk do not develop SCZ, whereas others with lower genetic risk do. This discrepancy suggests that non-genetic factors, potentially mediated by epigenetic modifications, play a role in SCZ etiology. Our study aimed to identify DNA methylation differences between SCZ patients and healthy controls within high and low genetic risk groups for SCZ, thereby reducing genetic risk heterogeneity and focusing on non-genetic influences. In our cohort of 491 SCZ patients and 765 controls, we selected those in the highest and lowest 30% of the SCZ polygenic risk score (SCZ-PRS) distribution. We then conducted targeted epigenome-wide association studies (EWASs) in these specific high and low genetic risk groups. This analysis identified two differentially methylated regions (DMRs) in the high genetic risk group, annotated to *PF4* and *ZNF727*, and two DMRs in the low genetic risk group, mapped to *EMILIN1* and *HLA-DPB2* (comb-p seed $p = 0.001$, Šidák-corrected $p < 0.05$). Additionally, our findings suggest that a general case-control EWAS, adjusted for SCZ-PRS, might miss epigenetic markers unique to either the high or low genetic risk groups. In conclusion, performing EWASs on PRS-stratified groups can identify novel DNA methylation signatures associated with disease status.

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Introduction

Schizophrenia (SCZ) is a severe psychiatric disorder that is caused by a complex interplay between genetic and environmental risk factors[1]. Numerous genetic variants associated with SCZ have been identified through genome-wide association studies (GWASs)[2,3], with the largest GWAS identifying 342 significant single nucleotide polymorphisms (SNPs)[3].

Polygenic risk scores (PRS) help quantify the cumulative effect of these SNPs on SCZ risk, estimating an individual's genetic liability towards the disease. To calculate the PRS for SCZ (SCZ-PRS), an individual's sum of SCZ-associated alleles is weighted by their effect sizes from GWAS summary statistics[4]. Although SCZ-PRSs are not reliable predictors of a SCZ diagnosis, they can identify individuals with a higher risk: those in the highest decile of the SCZ-PRS distribution in the general U.S. healthcare system had up to 4.6-fold higher odds for SCZ compared to those in the lowest decile[5]. However, some individuals with a high genetic risk for SCZ (i.e. high SCZ-PRS) do not develop the disorder, while others with a low SCZ-PRS do develop it. This suggests that factors beyond genetics, such as environmental or developmental factors, play a role in SCZ etiology.

SCZ risk factors may affect disease-relevant genes through epigenetic modifications, including DNA methylation. This modification involves the reversible addition of a methyl group to cytosine, typically at cytosine-phosphate-guanine dinucleotides (CpGs). Epigenome-wide association studies (EWASs), which investigate the association between DNA methylation patters and a phenotype of interest in a genome-wide fashion, have identified CpGs where DNA methylation is associated with SCZ[6]. Since EWASs can capture DNA methylation signals influenced by non-genetic factors, they can serve as an effective method to identify risk and protective loci beyond genetics.

In our study, we aimed to identify DNA methylation differences between SCZ patients and healthy controls with high and low genetic risk for SCZ, employing a novel two-step approach in our EWAS. We first stratified individuals into groups with high (≥ 70th percentile) and low (≤ 30th percentile)

SCZ-PRS, then conducted case-control EWASs within these high and low genetic risk groups. By performing our EWASs in groups homogenous in their genetic risk for SCZ, we sought to identify DNA methylation signatures reflecting non-genetic influences. This targeted approach is based on the hypothesis that different mechanisms, arising from the complex interplay of environmental and genetic factors, could underlie SCZ in these distinct genetic risk groups. Our results were compared with a general EWAS adjusted for SCZ-PRS, aiming at identifying SCZ-associated methylation signals that are not influenced by genetic risk in the wider population. We hypothesized that this broader approach might dilute methylation signals specific to the high and low genetic risk groups.

Materials and Methods

Sample

Our study included SCZ patients and healthy controls from the *Thematically Organised Psychosis (TOP)*[7] and *Early Treatment and Intervention in Psychosis 2 (TIPS-2)*[8] clinical cohorts. Eligible SCZ patients were recruited from in- and outpatient psychiatric units in the Oslo and Stavanger regions in Norway. They were diagnosed with a schizophrenia spectrum disorder by trained clinicians using the *Structural Clinical Interview for DSM-IV Axis I Disorders (SCID-I)*[9]. Healthy controls were randomly recruited from the same geographic regions using the *Statistics Norway Register*. General inclusion criteria were: 1) absence of head trauma or physical illness potentially impacting central nervous system function, 2) IQ above 70, 3) aged 18-65, and 4) reported European ancestry. Dontrols with a personal or familial (first-degree relative) history of severe mental disorders were excluded. Participants provided written informed consent, and the data collection and analysis were approved by the Regional Committees for Medical Research Ethics South East Norway (#2009/2485), East Norway (#1.2007.2177), and West Norway (#S-0801b).

Genotyping

The DNA extraction and genotyping were performed as previously described[10]. DNA was extracted from blood and genotyped using the Human Omni Express-24 v.1.1 BeachChip array (Illumina Inc., San Diego, CA, USA). Quality control followed standard pipeline as described in Werner et al[10], with genotype imputation performed using the MaCH[11] software with European reference haplotypes from the *1000 Genome Project*[12].

Polygenic risk score calculation

SCZ-PRSs for each individual were calculated using the PRSice2[13] tool, with the latest SCZ GWAS meta-analysis by the Psychiatric Genomics Consortium (PGC) Schizophrenia Working Group (wave 3)[3] as training dataset, and our *TOP* and *TIPS-2* cohorts as test dataset. TOP cohort data was

excluded from the PGC GWAS to avoid overlap, and the *TIPS-2* cohort was not initially included in the GWAS. The training dataset consisted of 50 965 SCZ cases and 68 049 controls. The SCZ-PRSs were computed using the clumping and p thresholding method as described by Werner et al[10]. In brief, SNPs in the SCZ PGC wave 3 GWAS underwent quality control for minor allele frequency (MAF), imputation quality, and presence in at least half of the cohorts used in the meta-analysis. Variants in the major histocompatibility complex (MHC) region were omitted. The remaining SNPs were then clumped into independent regions. SCZ-PRSs in the test dataset were calculated using allelic dosage coefficients of independent variants with an association $p \leq 0.05$ in the training dataset, which explained the most variance in liability in the SCZ PGC wave 3 meta-analysis[3]. Individuals with SCZ-PRS beyond three standard deviations from the mean were excluded (1 patient and 2 controls).

Adjustments for biological and technical factors were made using a linear regression model, creating residualized PRSs accounting for sex, age, genotyping batch, and the first two genetic principal (PCs; **Supplementary Table S1**). The normal distribution of the SCZ-PRSs was assessed by a Shapiro-Wilk Test.

Stratification by genetic risk for SCZ

We adopted a balanced approach between specificity and statistical power when defining individuals with low and high genetic risk, i.e. low and high SCZ-PRS, respectively. Prioritizing specificity by minimizing genetic risk variance within these groups led to decreased power and increased case-control imbalance (**Supplementary Table S2**). Thus, individuals with a SCZ-PRS ≥ 70th percentile were classified as having a high genetic risk to SCZ, while those with a SCZ-PRS ≤ 30th percentile were defined as having a low genetic risk.

Quantification of DNA methylation

DNA isolated from blood was bisulfite-converted, and DNA methylation was measured on the Illumina Infinium MethylationEPIC array (Illumina Inc., San Diego, CA, USA) at *Life and Brain* (Bonn,

Germany). The DNA methylation data were generated in three batches (1000 samples in batch 1, 283 samples in batch 2, and 1082 samples in batch 3).

DNA methylation pre-processing and quality control

Quality control and pre-processing were performed for each batch separately using the following Bioconductor packages in R (version 4.0.0): *minfi*[14], *wateRmelon*[15], *sva*[16], and *ChAMP*[17]. Probes were removed based on the following criteria: 1) detection p value >0.01 in more than 1% of the samples, 2) bead count <3 in 5% of the samples, 3) mapped to the sex chromosomes, 4) known for cross-hybridization or SNPS close to the target CpGs as reported by Zhou et al.[18]. The following samples were removed: 1) >1% of probes with a detection p value > 0.01, 2) mismatch between reported and predicted sex based on DNA methylation data, 3) mismatch between the 59 SNP genotypes obtained from the EPIC array and the genotypes obtained from SNP data for the same sample, and 4) a mismatch between reported and predicted ethnicity. Ethnicity was predicted from genetic data using a Random Forest classifier trained on populations of the 1000 genome project[12]. 5) Samples from individuals with a probability <0.9 of being European were excluded as well.

DNA methylation was normalized using functional normalization[19]. Twenty PCs generated from DNA methylation data were used to normalize the first two batches and 25 PCs for the third batch. The ComBat[20] method was used to correct for batch effects, accounting for array position, array ID, and scanner ID. A second ComBat[20] correction was applied to correct for batch effects between the three batches after merging them. To ensure the quality of the merged data, we confirmed that technical replicates among the typing batched (10 samples from each batch 1 and batch 2 were repeated in batch 3) clustered together in a principal component analysis (PCA).

For this study, we extracted a subset of samples of 490 SCZ patients and 763 healthy controls from the merged quality-controlled DNA methylation data (batch 1: 551 samples; batch 2: 222 samples; batch 3: 400 samples). To visually control for any potential technical artifacts after selecting the subset

of samples, PCA using the *stats*[21] package were performed separately for the high and low SCZ-PRS groups, and the first five PCs colored for possible technical artifacts such as typing batch were plotted (**Supplementary Figures S1 & S2**). We did not observe an influence of technical artifacts on our selection.

Estimation of cell-type proportions and smoking score

The *Houseman* algorithm[22] implemented by the *estimateCellCounts2* function from the *FlowSorted.Blood.Epic*[23] package was used to estimate the cell proportions of six specific cell types ($CD4^+T$, $CD8^+T$, natural killer cells, B-cells, monocytes, and neutrophils) from DNA methylation data. Smoking scores were also calculated from DNA methylation based on an algorithm by Elliott et al.[24], using weights from an EWAS on tobacco smoking by Zeilinger et al.[25]

After QC for methylation and PRS selection, our study sample comprised 216 patients with schizophrenia, 22 with schizophreniform disorder, 56 with schizoaffective disorder, and 458 healthy controls. A total of 728 individuals belonged to the *TOP* cohort, while 24 individuals were part of the *TIPS-2* cohort.

Data analysis

All analyses were performed with R (version 4.1.2)[21], unless otherwise stated. Plots were generated using the *ggplot2*[26] R package. To assess potential differences in age and sex between SCZ cases and controls within the high and low genetic risk groups, we performed Student's t-tests and chi-squared tests, respectively.

Differentially methylated positions (DMPs)

DMPs associated with SCZ in both the high and low genetic risk groups were assessed using the *limma*[27] package. By using linear regression models, beta values for each probe were regressed against case-control status. Age, sex, smoking score, estimated blood cell type proportions, and the first two genetic PCs were included as covariates. Quantile-quantile (Q-Q) plots were generated using

171 the *qqman*[28] package. DMPs were annotated using the
172 *IlluminaHumanMethylationEPICanno.ilm10b4.hg19*[29] package (version 0.6.0). P values were corrected
173 for multiple testing within each genetic risk group using false-discovery rate (FDR) correction[30]. FDR-
174 corrected p values < 0.05 were considered significant.

175 **Differentially methylated regions (DMRs)**

176 We used the *comb-p*[31] pipeline (Python version 3.8.8) to investigate DMRs associated with SCZ in
177 both the high and low genetic risk groups using a seed p value of 0.001 and a window size of 750
178 base pairs. Regions were considered associated if they contained at least four probes and had a
179 Šidák[32]-corrected $p < 0.05$. DMRs were annotated using the
180 *IlluminaHumanMethylationEPICanno.ilm10b4.hg19* package[29]. The location of the DMRs was
181 manually checked in the UCSC Genome Browser (https://genome.ucsc.edu/), genome assembly
182 hg19. For each DMR, the mean difference in DNA methylation between SCZ cases and controls was
183 calculated as ΔDMR methylation = $M_{SCZ} - M_{Controls}$, where M is the average methylation across CpG
184 sites for the SCZ cases and controls, respectively.

185 The correlation of DNA methylation of the CpGs in the DMRs between whole blood and brain was
186 assessed using the *Blood–Brain Epigenetic Concordance (BECon)*[33] tool and the *Blood Brain DNA*
187 *Methylation Comparison Tool*[34]. To compare the expression of genes annotated to the identified
188 DMRs between whole blood and the brain, available data on the GTExPortal[35] was accessed on May
189 30th, 2023.

190 **SCZ-PRS-adjusted EWAS**

191 A case-control EWAS adjusting for SCZ-PRS was performed on a subset of 1209 individuals from our
192 study. This subset represented nearly the entire cohort of our study and was selected due to its
193 overlap with the sample employed in the SCZ EWAS conducted by Tesfaye et al[6]. The control group

consisted of 763 individuals, with a mean age of 33.17 years (SD: 8.68) and 44.7% female. The SCZ group included 446 individuals with a mean age of 31.67 years (SD: 10.26) and 43.0% female.

Initially, adding only SCZ-PRS into the DMP model led to inflated p values. To address this, we included additional covariates in our adjustments, aligning with those used in Tesfaye et al.'s SCZ EWAS[6]. The final model for our analysis was: beta values ~ case-control status + SCZ-PRS + sex + age + smoking score + cell type proportions + 3 PCs from DNA beta values + 5 control probe PCs + 10 genotyping PCs + 10 surrogate variables (SVs). A Q-Q plot was generated, and DMPs and DMRs were identified as previously described.

Association between SCZ-PRS and DNA methylation in the DMRs

In the same individual subset from the SCZ-PRS adjusted EWAS, we tested the association between SCZ-PRS and DNAm at CpGs in DMRs using a modified linear model: beta values ~ SCZ-PRS, sex, age, smoking score, cell type proportions, 3 PCs, 5 control probe PCs, 10 genotyping PCs, and 10 SVs. P values were adjusted for multiple testing across DMRs using FDR correction[30].

Results

Selecting cases and controls with a high and low genetic risk for SCZ

SCZ-PRS values were normally distributed (p = 0.178, **Figure 1**) and, SCZ cases had significantly higher SCZ-PRS values (mean = 0.325, SD = 0.906) than the controls (mean = -0.208, SD = 0.902; p < 0.001), confirming that SCZ patients have a higher genetic risk for developing SCZ when compared to healthy individuals, as expected.

Thirty percent of the individuals with the highest and lowest SCZ-PRS were assigned to the low (PRS ≤ -0.4998076) and high (PRS ≥ 0.4421547) genetic risk groups (**Table 1**). The difference in case/control ratios between the high (ratio = 1.28) and low (ratio = 0.28) genetic risk groups reflects the difference in SCZ-PRS distribution between cases and controls. A significant age difference between cases and controls was observed in the low genetic risk group, which we adjusted for in subsequent analyses.

Table 1: Sample description of the selected individuals with a high and low genetic risk for schizophrenia. CTRL: control, SCZ: schizophrenia, SD: standard deviation.

	high genetic risk			*low genetic risk*		
	CTRL	SCZ	p value	CTRL	SCZ	p value
n	165	211		293	83	
mean age (SD)	32.96 (8.63)	32.22 (9.55)	0.437	33.50 (8.94)	29.61 (10.78)	0.003
%females	44.8%	42.2%	0.679	44.4%	49.4%	0.492

Differentially-methylated positions (DMPs)

The Q-Q plots of our SCZ case-control EWASs showed no elevation (high genetic risk group: λ = 0.95; **Figure 2A**) and slight elevation (low genetic risk group: λ = 1.15; **Figure 2C**). No single CpG site was significantly associated with SCZ in either the high or low genetic risk groups after correction for

multiple testing (**Figure 2, Supplementary Tables S3 & S4**). The top hit in the high genetic risk group was cg17587981 (p=9.02e-08, FDR-corrected p = 0.069), located in exon 1 of *PSMC2* on chromosome 7, while the top hit in the low genetic risk group was cg22373683 (p = 4.29e-07, FDR-corrected p = 0.326) in the *SDHAP4* pseudogene on chromosome 3.

Differentially-methylated regions (DMRs)

We performed DMR analyses separately for the high and low genetic risk groups using comb-p[31] and identified two DMRs in each of the groups (**Figure 2B and 2D, Table 2**). The DMRs spanned 6 to 15 probes and did not overlap between the high and low genetic risk groups, as expected. All DMRs were hypermethylated in the SCZ patients compared to the healthy controls, with a maximal observed difference in average beta values of 3.2% between the two groups (**Table 2**).

Table 2: Significant differentially methylated regions (DMRs) associated with SCZ status. The DMR analysis was performed separately for the high and low genetic risk groups using comb-p[31], and p values were corrected using the Šidák[32] method. avg: average, bp: base pairs, CTRL: control, DNAm: DNA methylation, min: minimum, SCZ: schizophrenia

genomic location	min p	n probes	length (bp)	Šidák p	Δ avg. DMR beta values (SCZ-CTRL)	Genes	in CpG island
high genetic risk group							
chr4:74847710-74848016	6.90e-12	7	306	1.23e-13	0.032	*PF4*	yes
chr7:63505638-63505871	2.00e-05	6	233	1.12e-06	0.016	*ZNF727*	no
low genetic risk group							
chr6:33084554-33085063	1.27e-07	14	509	2.20e-09	0.010	*HLA-DPB2*	yes
chr2:27301369-27301597	2.48e-05	8	228	7.12e-04	0.008	*EMILIN1*	no

In the high genetic risk group, we identified a DMR spanning the 5'untranslated region (5'UTR)/exon 1 region of the *Platelet Factor 4* (*PF4*) gene on chromosome 4 (Šidák-corrected p = 1.23e-13) and a DMR mapped to the 5'UTR/exon 1 region of the *Zinc Finger Protein 727* (*ZNF727*) gene on chromosome 7 (Šidák-corrected p = 1.12e-06).

In the low genetic risk group, we identified one DMR mapped to the intron1/exon2/intron2 region of the pseudogene *Major Histocompatibility Complex, Class II, DP Beta 2* (*HLA-DPB2*) on chromosome 6 (Šidák-corrected p = 2.20e-09) and one DMR in the 5'UTR/exon 1 region of the *Elastin Microfibril Interfacer 1* (*EMILIN1*) gene on chromosome 2 (Šidák-corrected p =7.12e-04). For more details, see **Table 2**.

To explore if the DNA methylation signatures we identified might reflect those in the brain, we examined the correlation of the DNA methylation of the CpGs in the DMRs between blood and brain using two different tools: *Blood–Brain Epigenetic Concordance (BECon)*[33] and the *Blood Brain DNA Methylation Comparison Tool*[34] (**Supplementary Table S5**). Data for all CpGs in the DMRs mapped to *PF4*, *ZNF727*, and *HLA-DPB2* were available, with the DMR mapped to *ZNF727* missing just one CpG in the *BECon* tool. For the DMR mapped to *EMILIN1*, only data for one out of four CpGs was available. We observed the highest overall correlation between blood and brain, averaged over all CpGs and across all measured brain regions, in the DMR mapped to *PF4* (*BECon* mean r_s = 0.562, *DNA Methylation Comparison Tool* mean r =0.642). Among the different brain regions, the correlation was highest in the prefrontal cortex, both in the *BECon* tool (mean r_s = 0.692) and the *DNA Methylation Comparison Tool* (mean r = 0.662). The DMR mapped to *ZNF727* showed a low to moderate average blood-brain correlation (*BECon* mean r_s = 0.203, *DNA Methylation Comparison Tool* mean r = 0.569), similar to the DMR mapped to *HLA-DPB2* (*BECon* mean r_s = 0.304, *DNA Methylation Comparison Tool* r = 0.587). Finally, the only CpG measured in the *EMILIN1* DMR exhibited low average correlation values of r_s = -0.149 in the *BECon* tool and r = 0.116 in the *DNA*

Methylation Comparison Tool. For details on the individual CpG sites and measured brain regions, see **Supplementary Table S5**.

To gain deeper insights into the potential role of the identified genes in the brain, we assessed their expression in whole blood and 13 brain regions, using available data from the *GTExPortal*[35] (**Supplementary Figure S3**). In short, *ZNF727* and *EMILIN1* showed higher expression in all brain regions than in the whole blood, while the opposite pattern was observed for *PF4*. *HLA-DPB2* displayed more similar expression levels in blood and the brain compared to the other three genes, with particularly high gene expression in the cerebellum.

We examined the association between SCZ-PRS and DNA methylation in all four DMRs, including *PF4* and *HLA-DPB2*, which were previously associated with SCZ-PRS in post-mortem brain tissue[36]. In our analysis, only two CpGs in *PF4* exhibited nominal significance, and several others approached significance, but none remained significant after adjusting for multiple tests. No associations were identified in the other DMRs (**Supplementary Table S6**).

SCZ-PRS-adjusted EWAS

Besides using a PRS-stratified approach, an alternative approach to focus on DNA methylation differences between SCZ cases and healthy controls that are not driven by common SCZ risk variants is to adjust the case-control EWAS by the SCZ-PRS. To test if adjusting for the SCZ-PRS results in the same findings as the PRS-stratified approach, we performed this analysis in 446 SCZ cases and 763 healthy controls.

The Q-Q plot showed no inflation (lambda = 1.02, **Supplementary Figure S4**), and our analysis identified a single significant DMR associated with the *Programmed Cell Death 1 (PDCD1)* gene (chr2:242802009-242802192). This DMR, comprising 5 CpG sites, exhibited a Šidák-corrected p value of 2.871e-05. Importantly, this DMR did not overlap with any DMRs found in the PRS-stratified analysis, either in the high or low PRS groups. Additionally, even with a more lenient seed p value

291 threshold of 0.05 in the comb-p analysis (**Supplementary Table S7**), we were unable to detect the

292 same DMRs.

Discussion

In our study, we adopted a novel approach by conducting SCZ case-control EWASs within groups stratified by their genetic risk for SCZ. Our goal was to identify DNA methylation differences between cases and controls in both high and low genetic risk groups. Reducing genetic risk heterogeneity within the groups allowed us to focus on DNA methylation differences potentially driven by non-genetic factors. This strategy led to the identification of two DMRs in the high genetic risk group and two additional DMRs in the low genetic risk group. These findings are particularly noteworthy as these DMRs have not been previously reported in SCZ case-control EWASs, offering new insights into the epigenetic mechanisms involved in SCZ[6,37]. The DMRs did not overlap between the high and low genetic risk groups, suggesting distinct mechanisms in these two groups.

In the high genetic risk group, one DMR annotated to the *Platelet Factor 4* (*PF4*) gene, encoding a chemokine involved in immune processes and platelet aggregation, was hypermethylated in SCZ. This DMR, showing the greatest methylation difference (average beta value difference of 3.2%) among the four identified DMRs, is located in a CpG island within *PF4*'s 5'UTR/exon 1 region. Hypermethylation of CpG islands in promoters is typically associated with gene silencing[38], aligning with previous findings of *PF4* downregulation in the blood of SCZ patients[39].

DNA methylation in the *PF4* DMR was highly correlated between blood and brain[33,34], suggesting peripheral DNA methylation changes might mirror central epigenetic regulation. This is supported by Viana et al.[36], who found a DMR in *PF4*, largely overlapping ours, associated with SCZ-PRS in post-mortem brain tissues, with this association not attributed to direct genetic factors (discussed further below)[36]. Interestingly, the most recent SCZ GWAS did not find genetic variants near *PF4* to be associated with SCZ[3,40], indicating that DNA methylation differences in this region could indeed stem from non-genetic influences.

316 In mouse models, PF4 administration has been shown to reduce T cell exhaustion,
317 neuroinflammation, and improve cognitive function[41,42], indicating its role in cognitive processes and
318 potential involvement in SCZ-related cognitive impairment. While previous studies have associated
319 DNA methylation in *PF4* with SCZ-PRS and differential *PF4* expression in SCZ[36,39], our study is the first
320 to report *PF4* in a SCZ case-control EWAS. This finding contributes to the growing evidence of *PF4*'s
321 link to SCZ, possibly through DNA methylation as an epigenetic regulatory mechanism.

322 The second DMR in the high genetic risk group is in the *Zinc Finger Protein 727* (*ZNF727*) gene, which
323 encodes a transcription factor not been previously associated with SCZ. No genetic variants near
324 *ZNF727* was close to reaching genome-wide significance in the latest SCZ GWAS[3,40], supporting the
325 involvement of non-genetic factors in the DMR. While *ZNF727* is more highly expressed in the brain
326 than in blood[35], the CpGs in its DMR exhibit only low to medium correlation between blood and
327 brain DNA methylation[33,34], suggesting that these CpGs might not mirror those in the brain. Hence,
328 the role of this DMR should be further investigated.

329 In the low SCZ-PRS group, we identified a DMR mapped to the non-coding *HLA-DPB2* pseudogene,
330 whose exact role is yet to be fully understood. This DMR overlaps considerably with another DMR
331 associated with SCZ-PRS in the prefrontal cortex[36], indicating a potential role in the brain despite
332 only low to moderate correlation of DNA methylation between blood and brain. However, in our
333 study, we observed no association between DNA methylation in the *HLA-DPB2* DMR and SCZ-PRS.
334 While methylation changes in *PF4* and *HLA-DPB2* have been associated with SCZ-PRS in post-
335 mortem brain tissue[36], our EWAS is the first to identify these genes after correction for multiple
336 testing. A previous study with 353 SCZ cases and 322 controls also found an association of a CpG in
337 *HLA-DPB2* with SCZ, though only at a discovery threshold of $p < 5e\text{-}5$[43], giving further support to
338 the connection between *HLA-DPB2* and SCZ.

Importantly, Viana et al.'s study on post-mortem brain DNA methylation and SCZ-PRS concluded that methylomic variation associated with SCZ-PRS is not a direct result of genetic influences, as indicated by the lack of enrichment in GWAS regions and independence from the genetic variants used in SCZ-PRS calculation[36]. This unexpected finding supports our hypothesis that the DNA methylation alterations in SCZ that we identified involve non-genetic factors. Considering ours and Viana et al.'s[36] identification of associations between *PF4* with SCZ-PRS, and the gene's link to case-control status in the high genetic risk group in our study, there could be a potential interaction effect. This interaction might not be driven by single SNPs, given their small effect sizes, but could be the result of an environmental impact amplified by a high genetic load. This observation warrants further investigation to better understand the complex relationship between DNA methylation, SCZ-PRS, and environmental factors.

The second DMR identified in the low genetic risk group is in the *Elastin Microfibril Interfacer 1* (*EMILIN1*) gene, which encodes a structural extracellular matrix glycoprotein. *EMILIN1* is more highly expressed in the brain than in blood. However, the only CpG site in *EMILIN1*'s DMR with available blood-brain DNA methylation correlation data showed a low correlation. This limited correlation is inadequate for drawing conclusions about the implications of the blood-identified DMR for potential brain-related functions.

The DNA methylation differences between SCZ cases and healthy controls in the high and low genetic risk groups could be driven by environmental factors. Known environmental risk factors for SCZ, such as cannabis use, childhood trauma, and birth complications, have been associated with altered DNA methylation[44,45,46]. We speculate that in the low genetic risk group, differential methylation might indicate the influence of environmental risk factors[47] on SCZ patients, surpassing the effect of protective genetic variants. In contrast, in differential methylation in the high genetic risk group may reflect protective environmental factors like a nurturing family environment[47],

363 counterbalancing the effects of a heightened genetic risk for SCZ in healthy controls. It could also
364 represent an interaction of adverse environmental factors with genetic risk variants in SCZ, together
365 amplifying the disease risk[48,49]. Similar gene-environment interactions mediated by DNA methylation
366 changes have been observed in the context of post-traumatic stress disorder and child abuse[50]. While
367 environmental factors likely contribute to the observed DNA methylation differences, developmental
368 factors[51,52] and other factors, such as undetected rare SCZ risk variants or the potential for high
369 genetic risk healthy individuals to develop SCZ later, should also be considered.

370 With a similar goal of identifying disease-associated DNA methylation signatures specific for distinct
371 genetic risk backgrounds, other studies on bipolar disorder and suicidal behavior compared DNA
372 methylation between individuals with high and low PRSs[53,54]. Our novel approach, however, compares
373 cases and controls within the same PRS group, more effectively capturing the influences of non-
374 genetic factors. Another strategy to reduce genetic risk variation involves studying families with
375 known risk alleles, examining DNA methylation differences between affected and unaffected
376 members carrying the risk allele. This method was used in a study on a family, in which multiple
377 members were diagnosed with bipolar disorder and major depressive disorder[55] Similarly, genetic
378 risk variation was limited in another study by comparing DNA methylation between monozygotic
379 twins discordant for schizophrenia and bipolar disorder[56]. However, such family- and twin-based
380 studies often struggle with small sample sizes, limiting their ability to achieve epigenome-wide
381 significance[55,56].

382 The SCZ-PRS-adjusted case-control EWAS failed to identify the DMRs that our SCZ-PRS-stratified
383 approach found, even when using a less strict seed p-value threshold. While a SCZ-PRS-adjusted
384 model can detect DNA methylation differences in a broader population, thus improving
385 generalizability, it may dilute specific signals in groups with high or low genetic risk. This is especially
386 true when distinct mechanisms are involved in these groups. Interestingly, although the SCZ-PRS-

adjusted model had a larger sample size and more overall statistical power, our SCZ-PRS-stratified approach identified more DMRs. This indicates that a targeted approach, focusing on individuals with similar genetic risk, is more effective in identifying DNA methylation markers that are particularly relevant to these distinct risk contexts.

The findings of this study must be interpreted in the light of some limitations. First, the small sample size could have reduced our power to detect significant DMPs, though it was adequate to detect DMRs. Second, despite selecting individuals with the highest and lowest SCZ-PRSs, some variation in genetic risk liability persisted within the groups. However, we reduced the potential impact of this genetic variation on DNA methylation by adjusting for the first two genetic PCs. Third, even though we adjusted for age, we cannot guarantee that this correction eliminated its impact, especially in the low genetic risk group. Fourth, in case-control EWASs, there is potential for reversed causality, i.e., DNA methylation differences could either be causal or a result of the disease. Influences like pharmacotherapy and higher smoking rates in SCZ patients might impact DNA methylation. To address this, we adjusted our analyses using a DNA methylation-derived smoking score. Fifth, while SCZ is a psychiatric disorder, the impracticality of large-scale studies on live brain tissue let us use peripheral blood in our study. However, DNA methylation is cell-specific and epigenetic variation identified in the blood may not entirely reflect SCZ-relevant processes in the brain. Thus, we assessed the blood-brain correlation of each CpG in our DMRs individually. Finally, as our study was limited to participants of European ancestry due to limited numbers from other ethnic groups, our findings may not extend to other ethnicities.

Our findings require validation in a larger, independent cohort, potentially identifying additional SCZ-associated DNA methylation signatures. Experiments in cellular models could clarify how the DMRs impact gene transcription. Delving deeper into the DNA methylation differences we observed, it would be interesting to test whether environmental factors correlate with DNA methylation in the

identified genes. Further research should extend to post-mortem brain studies, non-European populations, and other complex polygenic disorders, using a similar methodology.

In conclusion, we identified differential DNA methylation associated with SCZ in individuals with high and low genetic risk for SCZ. By limiting genetic risk variability within these groups, we likely captured differences reflecting non-genetic influences. Our novel approach of performing EWASs within PRS-stratified subgroups holds the potential to uncover disease-associated mechanisms not only for SCZ but also for other complex disorders. Such insights could deepen our understanding of the complex interplay between genetics and epigenetics in these conditions.

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Conflict of Interest

OAA is a consultant to cortechs.ai and received a speaker's honorarium from Otsuka, Janssen, Sunovion and Lundbeck. The remaining authors declare no conflicts of interest.

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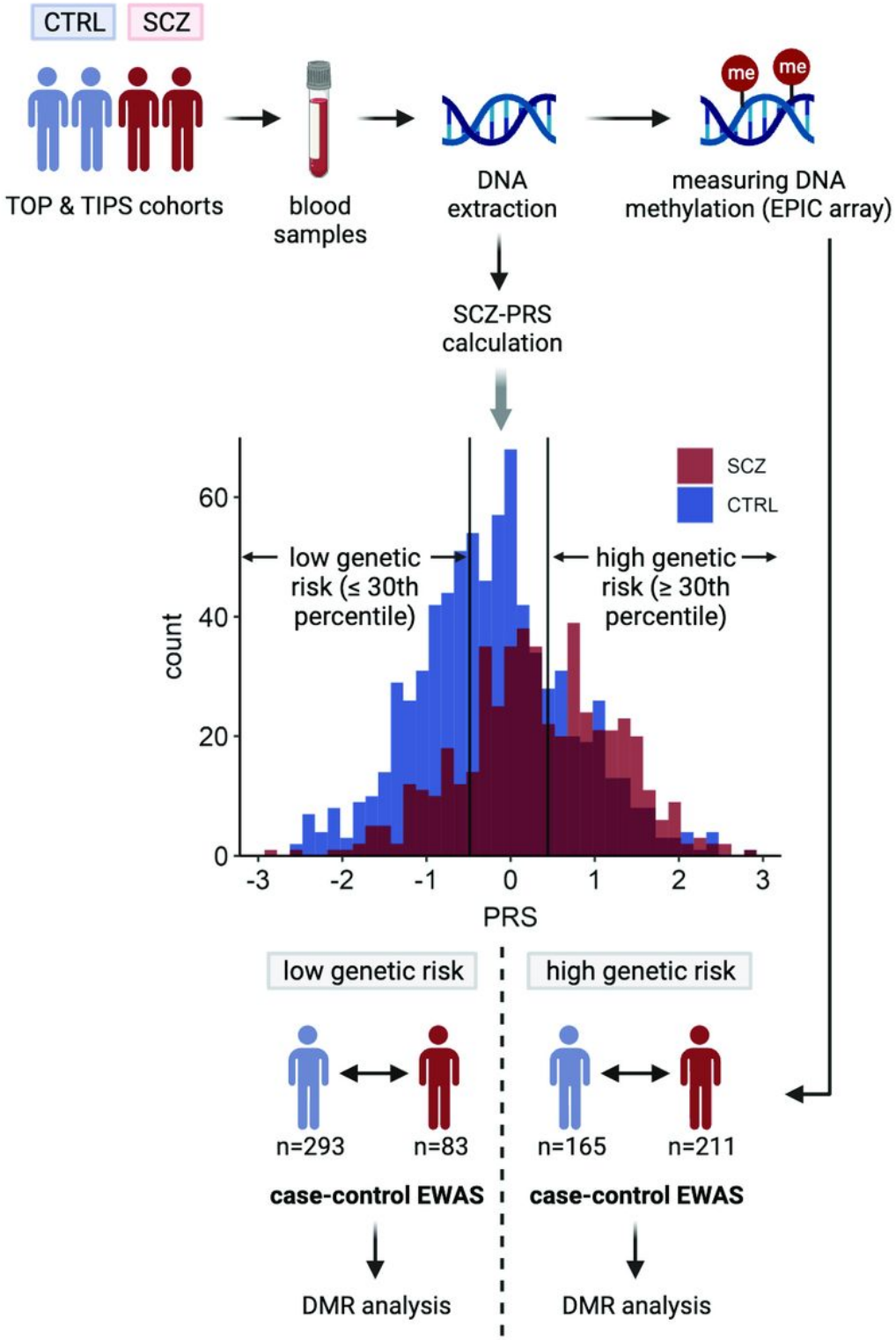
Figure Legends

Figure 1: Overview of the study design and distribution of polygenic risk scores (PRSs) for schizophrenia (SCZ). The high and low genetic risk groups were defined as 30% of individuals with the highest and lowest SCZ-PRS, respectively. CTRL: control, EWAS: Epigenome-wide association study; DMR: differentially methylated region.

Figure 2: Q-Q-plots (A,C) and Manhattan plots (B,D) for SCZ case-control EWASs in individuals with a high (A-B) and low (C-D) genetic risk for SCZ. -log10(p) values of each CpG site analyzed in case-control EWASs are reported on the y axis of the Manhattan plots, and the chromosomes are displayed on the x-axis. Differentially methylated regions are highlighted in blue.

Figures



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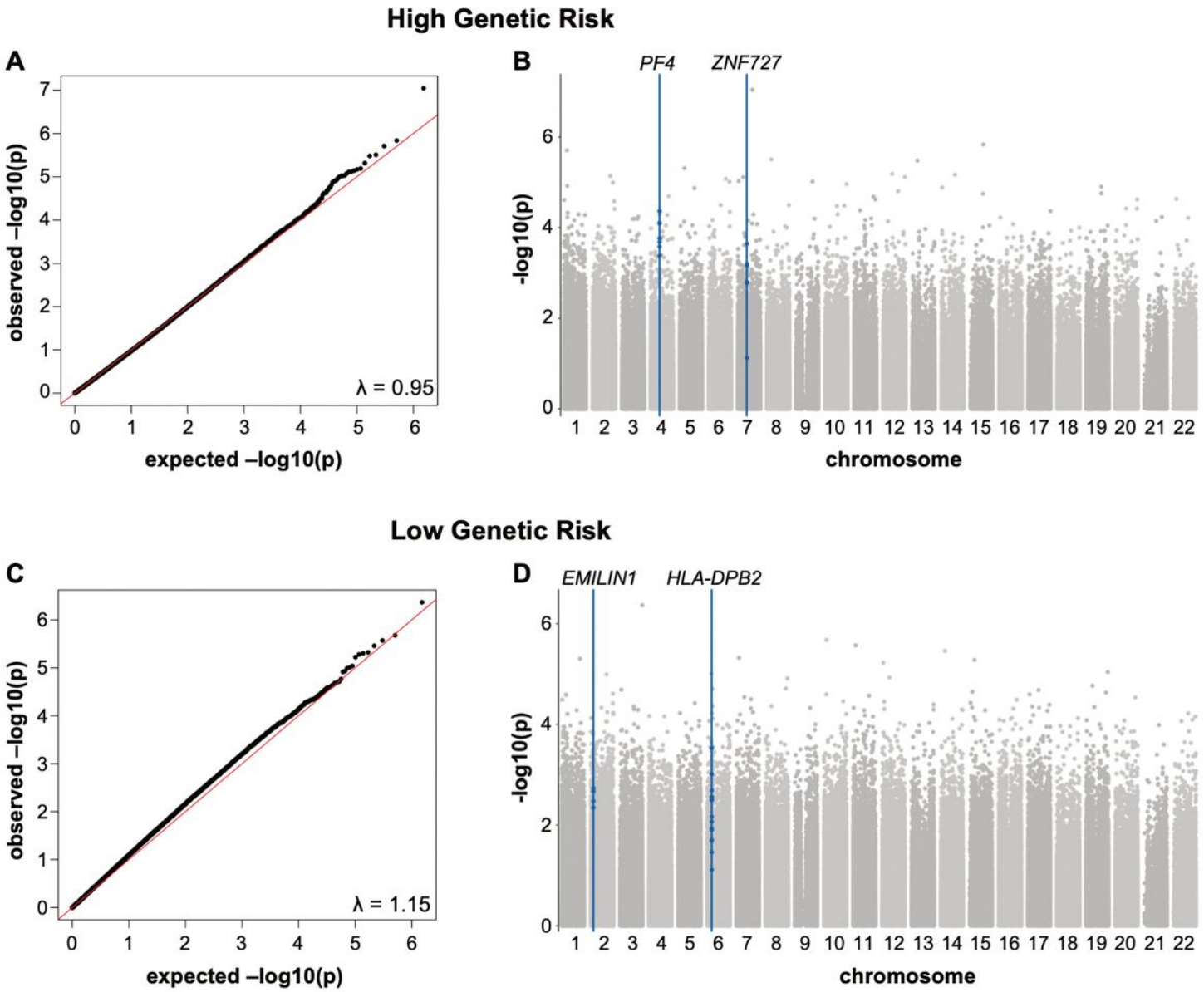

Figure 2

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