

## ORIGINAL ARTICLE

## Methylomic changes during conversion to psychosis

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The onset of psychosis is the consequence of complex interactions between genetic vulnerability to psychosis and response to environmental and/or maturational changes. Epigenetics is hypothesized to mediate the interplay between genes and environment leading to the onset of psychosis. We believe we performed the first longitudinal prospective study of genomic DNA methylation during psychotic transition in help-seeking young individuals referred to a specialized outpatient unit for early detection of psychosis and enrolled in a 1-year follow-up. We used Infinium HumanMethylation450 BeadChip array after bisulfite conversion and analyzed longitudinal variations in methylation at 411 947 cytosine–phosphate–guanine (CpG) sites. Conversion to psychosis was associated with specific methylation changes. Changes in DNA methylation were significantly different between converters and non-converters in two regions: one located in 1q21.1 and a cluster of six CpG located in GSTM5 gene promoter. Methylation data were confirmed by pyrosequencing in the same population. The 100 top CpGs associated with conversion to psychosis were subjected to exploratory analyses regarding the related gene networks and their capacity to distinguish between converters and non-converters. Cluster analysis showed that the top CpG sites correctly distinguished between converters and non-converters. In this first study of methylation during conversion to psychosis, we found that alterations preferentially occurred in gene promoters and pathways relevant for psychosis, including oxidative stress regulation, axon guidance and inflammatory pathways. Although independent replications are warranted to reach definitive conclusions, these results already support that longitudinal variations in DNA methylation may reflect the biological mechanisms that precipitate some prodromal individuals into full-blown psychosis, under the influence of environmental factors and maturational processes at adolescence.

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## INTRODUCTION

The identification of clinical ultra-high-risk state for psychosis (hereinafter UHR; also known as the ‘at-risk mental state’) has been a relatively recent development in the field of psychiatry, and it has provided a means to capture the prepsychotic phase and to describe individuals with prodromal symptoms that may transition into psychosis and schizophrenia.<sup>1</sup> Operational criteria for UHR have been proposed,<sup>2</sup> based on specific comprehensive interviews. In individuals reaching these criteria, the conversion rate of individuals at UHR to full-blown psychosis is 30–40% in the following 24 to 36 months.<sup>3</sup> Nevertheless, the populations reaching UHR criteria remain heterogeneous with the possibility of several outcomes, including symptomatic regression or development of non-psychotic disorders, rather than psychosis, which underscores the need for more predictive markers. Deciphering the biological mechanisms underlying the onset of psychosis requires longitudinal measures in help-seeking patients that includes characterization of their outcomes.

Understanding the different pathophysiological pathways leading to conversion to psychosis is a major issue of the field. The literature about conversion to psychosis, however, is still maturing and molecular findings remain limited. From the molecular point

of view, conversion to psychosis is viewed as the complex interaction between biological vulnerability and exposure to many potentially harmful environmental risk factors.<sup>4</sup> This is in line with the overall gene × environment interaction hypothesis in schizophrenia, whereby the influence of the environment is thought to induce epigenetic changes.<sup>5</sup> Until now, however, the epigenetic signature of conversion to psychosis has not been studied. Epigenetic regulation involves dynamic processes that have a role in controlling gene expression levels, among which histone posttranscriptional modifications and methylation of genes on cytosine–phosphate–guanine (CpG) dinucleotides have been the main focus of the research. New methylomic technologies enable investigation of CpG methylation sites at the genomic scale. A large methylome-wide-association study recently compared patients with established schizophrenia to controls and found 139 differentially methylated CpGs, including *FAM63B* and *RELN*.<sup>6</sup>

To our knowledge, no previous study has considered pangenomic methylation longitudinal changes accompanying conversion to psychosis. In this study, we explored blood methylation biomarkers associated with conversion to psychosis in a methylomic association study involving young help-seeking individuals who were enrolled in a longitudinal follow-up

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program. We were able to detect significant differentially methylated regions (DMRs). We conducted an original exploration of multiple CpG sites followed by pathway and cluster analyses of the top methylation changes. Then we confirmed the top findings using pyrosequencing.

## MATERIALS AND METHODS

### Population

Our study was approved by the institutional ethics committee 'Comité de protection des personnes, Ile-de-France III, Paris, France', and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. Help-seeking individuals (16–30 years) consecutively referred to the Adolescent and Young Adults Assessment Centre (Service Hospitalo-Universitaire, Hôpital Sainte-Anne, Paris, France) between 2009 and 2013 were enrolled in the ICAAR collaborative study promoted by Sainte-Anne Hospital as already described.<sup>7</sup> Inclusion criteria were alterations in global functioning (Social and Occupational Functioning Assessment Scale score < 70) during the past year that were associated with psychiatric symptoms and/or subjective cognitive complaints. Exclusion criteria included manifest symptoms of psychosis, pervasive developmental or bipolar disorders and individuals with other established diagnoses, such as obsessive-compulsive disorder (fulfilling Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria). Other exclusion criteria were: current antipsychotic treatment (> 100 mg Chlorpromazine equivalent) for > 12 weeks, psychoactive substance dependence or abuse during the previous year and/or > 5 years, serious or non-stabilized somatic and neurological disorders head injury and intelligent quotient < 70. All subjects were examined with the Comprehensive Assessment for at-risk mental state (CAARMS,<sup>8</sup> in its translated version<sup>9</sup>) by specifically trained psychiatrists followed by a consensus meeting for best-estimate diagnoses. Individuals fulfilling the criteria for at-risk mental state were characterized as UHR; conversion to psychosis was characterized using the CAARMS-defined psychosis onset threshold (that is, supra-threshold psychotic symptoms—thought content, perceptual abnormalities and/or disorganized speech—present for > 1 week) (see Supplementary Table S1) was used. All subjects excepted those above the psychosis threshold at baseline (M0) were included in the longitudinal follow-up, whether or not they were UHR. Subjects who reach the psychosis threshold during follow-up were classified as converters. The clinical assessment and blood sample collection were repeated after 6 and 12 months or after psychosis onset. In this study, 39 individuals were included and enrolled in the longitudinal follow-up, among whom 14 subsequently developed full-blown psychosis (converters), whereas 25 did not (non-converters). There were no significant differences between these two groups at baseline in sex ratio, age, follow-up duration, body mass index, substance abuse or psychotropic treatment introduction (Table 1). Of the 25 non-converters individuals, 13 were UHR and 12 were non-UHR at baseline. Non-UHR individuals had variable subthreshold symptoms (anxiety and depressive symptoms) without reaching criteria of a fully characterized disorder.

### Genome-wide analysis of DNA methylation

**Preparation.** For each individual, genomic DNA (500 ng) was extracted from whole blood and treated with sodium bisulfite using the EZ-96DNA Methylation KIT (Catalog No D5004, Zymo Research, Irvine, CA, USA) following the manufacturer's standard protocol. Methylation was measured at M0 and after the longitudinal follow-up (MF) by the same technique at the same time for all samples. Genome-wide DNA methylation was assessed using Illumina Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA), which interrogates the DNA methylation profile of > 485 000 CpG loci across the genome at single-nucleotide resolution.

**Data preprocessing and clean up.** Illumina GenomeStudio software (Illumina) was used to extract signal intensities for each probe. All computations and statistical analyses were performed within the R statistical analysis environment (<http://www.r-project.org>), and all scripts are available on request from the authors. R packages methylumi and watermelon were used for data quality check and normalization. Steps used for data clean-up procedure and normalization comprised gender check between phenotype file and methylation data set and evaluation of single-nucleotide polymorphism genotypes concordance between the two samples from the same individuals. Subsequent clean-up steps comprised flagging and removing individuals with no result or gender discrepancies

**Table 1.** Clinical description of population

	Converters	Non-converters	Significance
	n = 14	n = 25	
<i>Clinical variables mean (s.d.)</i>			
Sex ratio (M/F)	9/5	13/12	$P = 0.52^a$
Age	21.9 (3.6)	23.8 (4.1)	$P = 0.17^b$
Body mass index	20.9 (3.5)	21.9 (4.7)	$P = 0.66^b$
<i>Follow-up</i>			
Biological interval in months	10.1 (7.2)	11.4 (5.8)	$P = 0.62^b$
Clinical follow-up in months	10.7 (7)	12.7 (5.7)	$P = 0.22^b$
<i>Substance use (user/non-user)</i>			
Lifetime cannabis use	7/7	5/20	$P = 0.07^a$
Alcohol use (once a week during 6 months)	6/8	13/12	$P = 0.74^a$
Daily/regular tobacco use	7/7	10/15	$P = 0.39^a$
<i>Psychotropic treatment during follow-up</i>			
Antipsychotic or valproate introduction	6/8	4/21	$P = 0.12^a$
Other psychotropic medication introduction	4/10	2/23	$P = 0.16^a$

Abbreviations: F, female; M, male. Biological interval represents time between the two blood samples. Clinical follow-up represents time between inclusion and final status assessments. <sup>a</sup> $P$  is given by Fisher's test. <sup>b</sup> $P$  is given by non-parametric Mann-Whitney test.

or discordant genotypes, samples with  $\geq 1\%$  of sites with a detection  $P$ -value  $\geq 0.05$ , probes with beadcount < 3 in  $\geq 5\%$  of samples, probes with  $\geq 1\%$  of samples with a detection  $P$ -value  $\geq 0.05$ . Additionally, probes on chromosomes X and Y, single-nucleotide polymorphism probes, probes with a single-nucleotide polymorphism at the CpG site and non-specific probes that map to more than one location in the genome were removed.<sup>10</sup> The initial methylation data file includes 485 577 probes, and after normalization and data clean up, 411 947 probes were kept for the final analysis (Supplementary Table S2). R Minfi package was used for supplementary quality control (Supplementary Figures S1 and S2), and no sample was removed. No batch effect was detected (according to Combat R Package using SVA function). Cellular populations were estimated by EstimateCellCounts function (Minfi package).

**Association analysis.** Global methylation change was investigated by computing the difference between mean methylation changes for all probes in each individual and comparing converters to non-converters. DMRs were investigated using Minfi package in R (script available upon request). In summary, time and group were used as factors in a linear model adjusted by cellular populations with a paired design. DMR analysis was performed using bumpHunter function (bootstrap with 1000 permutations and a methylation differential cutoff of 10). Significance was established for *fwer* correction < 0.1.

**Multi-CpG analysis.** Because methylation changes could occur in different CpG from various genes converging to the same pathways, we developed a new pipeline to examine methylation longitudinal changes at each CpG site. Linear model with moderated  $t$ -statistic was used.<sup>11</sup> Statistical analysis was performed with R script (using R Limma Package) testing the following model: Difference in methylation (MF – M0) = psychosis (converters vs non-converters) + gender. The multiple-testing-adjusted significance threshold for probe-wise analysis was established at  $P = 1.2 \times 10^{-7}$  (0.05/411 947 analyzed probes). QQ-plot is displayed in Supplementary Figure S3. Top results of this analysis were pasted in ConsensusPathDB<sup>12</sup> and Enrichr<sup>13</sup> and an overrepresentation analysis based on Reactome, wikipathways and KEGG database was used with a hypergeometric correction to explore whether our top findings are linked to specific biological pathways more than expected by chance. Clustering analysis of the same top results was performed using the MultiExperimentViewer software (version 4.9.0).<sup>14</sup> Methylation differential rates were inputted and normalized by genes.

**Table 2.** DMRs identified by Minfi (absolute  $\beta$  value >10; number of CpG >1)

Chromosome	Start	End	$\beta$ value	Number of CpG	P-value	fwer
chr1	146549909	146550467	0.19	4	0.00007	0.026
chr1	110254662	110254835	0.14	6	0.00068	0.092
chr5	1594282	1594733	0.13	7	0.00076	0.116
chr3	195489306	195489782	0.15	3	0.00182	0.225
chr5	1856713	1857477	0.13	4	0.00372	0.355
chr11	325915	325964	0.15	2	0.00452	0.475
chr19	17599784	17600122	0.14	2	0.00705	0.572
chr2	121496875	121497334	-0.13	2	0.01013	0.647
chr15	101093834	101093900	0.13	2	0.01055	0.655
chr5	176797999	176798049	-0.13	2	0.01303	0.705
chr19	13875014	13875111	-0.13	2	0.01517	0.725
chr17	724273	724374	0.12	2	0.03272	0.812
chr8	143751796	143751801	-0.12	2	0.03554	0.819
chr22	24348549	24348715	-0.11	3	0.03692	0.677
chr1	110254919	110255096	0.12	2	0.04309	0.835
chr9	128776861	128777132	-0.12	2	0.05500	0.862
chr19	55013946	55013954	-0.11	2	0.10390	0.92
chr22	50981121	50981406	0.11	2	0.11146	0.927
chr17	4081325	4081428	0.10	2	0.12249	0.931

Abbreviations: CpG, cytosine-phosphate-guanine; DMR, differentially methylated region.

**Table 3.** Significant pathways in overrepresentation analysis of the multi-CpG pipeline

Biological pathway	Genes	Corrected Q-value
Axon guidance	NRP1: neuropilin 1; CHL1: cell adhesion molecule L1-like; EFNA3: ephrin-A3; COL9A2: collagen, type IX, alpha 2; AP2A2: adaptor-related protein complex 2	0.012
IL-17 signaling pathway	IL17RE: interleukin-17 receptor E; AKT1: v-akt murine thymoma viral oncogene homolog 1; TRAF3IP2: TRAF3 interacting protein 2	$1.7 \times 10^{-4}$

Abbreviation: CpG, cytosine-phosphate-guanine.

For hierarchical clustering, we used 'K-Nearest Neighbors imputation engine' (number of neighbors = 10) and 'Average linkage clustering' using Pearson correlation and asked to construct a gene/sample tree.

### Confirmation by pyrosequencing

Infinium HumanMethylation450 BeadChip is a current and reliable array to detect CpG methylation.<sup>15</sup> However, we propose to compare some of our findings using a technical reference based on pyrosequencing.<sup>16</sup> After bisulfite conversion by EpiTect Plus Bisulfite Kits (Qiagen, Hilden, Germany) and DNA purification on column, non-methylation specific PCR were achieved using Platinum Taq DNA polymerase kit (Invitrogen—Life Technologies, Carlsbad, CA, USA). MAEL promoter was used as positive control for the bisulfite treatment<sup>17</sup> in bisulfited and non-bisulfited samples (Supplementary Figures S4 and S5). Two findings were assessed: we chosen to confirm one specific CpG selected from the top results of multi-CpGs analysis (CpG located in CHL1 gene) and one significant DMR in GSTM5 (the only one significant DMR including a promoter) identified by the Minfi package. Primers were designed by the PyroMark Assay design Software 2.0 (Qiagen), and technical conditions for PCR are shown in Supplementary Table S3; examples of results are shown in Supplementary Figure S6. Biotinylated primers were used to keep the single DNA strand for pyrosequencing. Pyrosequencing was performed using PyroMark Q24 (Qiagen) according the manufacturer's instructions, and data about methylation in each CpG were extracted and analyzed using the PyroMark Q24 2.0.6.20 software (Qiagen).

## RESULTS

### Longitudinal global methylation change in converters vs non-converters

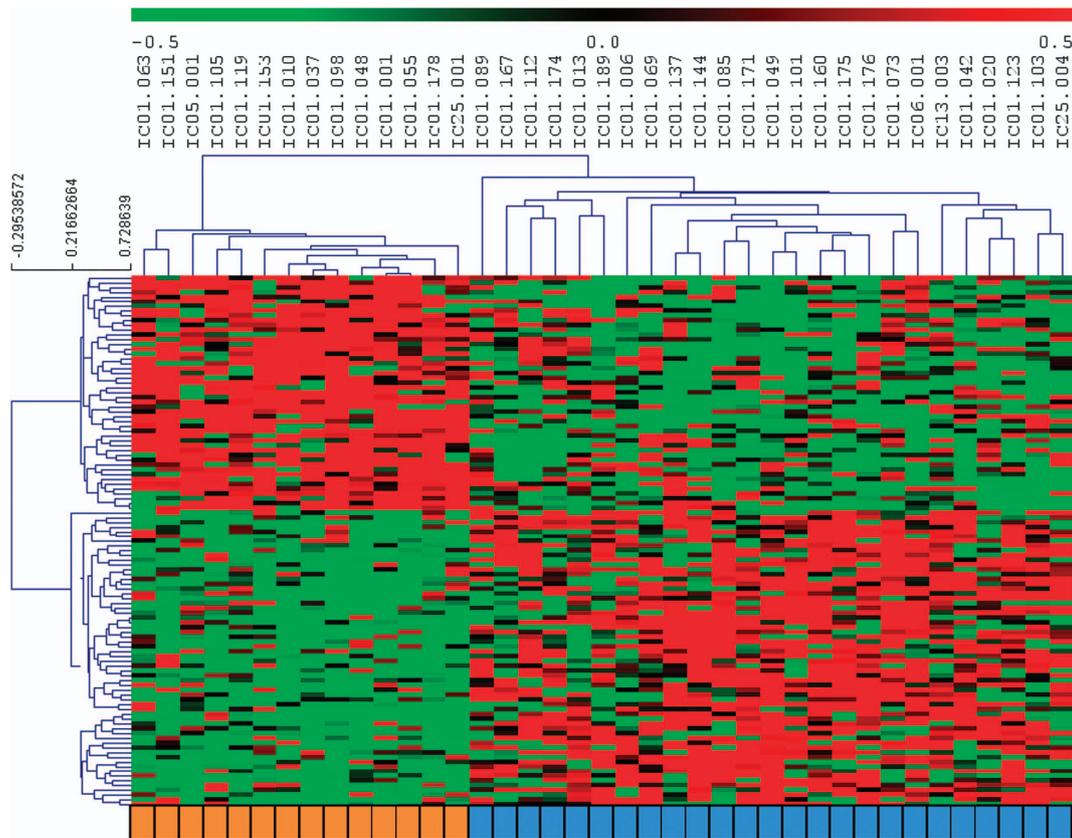
No significant changes in global methylation were associated with the occurrence of conversion to psychosis ( $P = 0.41$ ).

### Longitudinal methylation changes at specific regions in converters vs non-converters

After paired analysis, we identified two significant DMRs ( $fwer < 0.1$ ), including at least two CpGs. The region including HLA-DQ and HLA-DRB (chromosome 6 [32523136; 32633163]) was excluded because of frequent recurrence of this finding by the Minfi package, suggesting spurious results due to the algorithm (according to its authors). The two DMRs were identified in chromosome 1: first region located in [146549909;146550467] corresponding to 1q21.1 and second region in [110254662; 110254835] including the GSTM5 gene promoter. Significant and suggestive results are shown in Table 2. These DMRs are quite stable across time, which could suggest that differences in methylation pattern in these regions could predate conversion to psychosis. We conducted a transversal exploratory analysis comparing subjects at M0 and subjects at MF (Supplementary Table S4). Fifteen DMRs were concordant before and after conversion but three appeared different across groups after transition only. Interestingly, two of these three DMRs were in 22q11 region and are located near GSTT1 and GSTP1, two genes from the same family as GSTM5.

### Longitudinal methylation changes in CpG sites between converters and non-converters

We tested whether changes of methylation in different CpG sites located in distinct genes were associated with psychotic transition. Longitudinal methylation changes at specific CpGs associated with conversion to psychosis are shown in a Manhattan plot (Supplementary Figure S7, see also the top 100 CpGs in Supplementary Table S5). None of the individual CpG changes alone reached significance at a genome-wide level. The best



**Figure 1.** Multi-CpGs clustered and classified as converters (in orange) and non-converters (in blue). CpG, cytosine–phosphate–guanine.

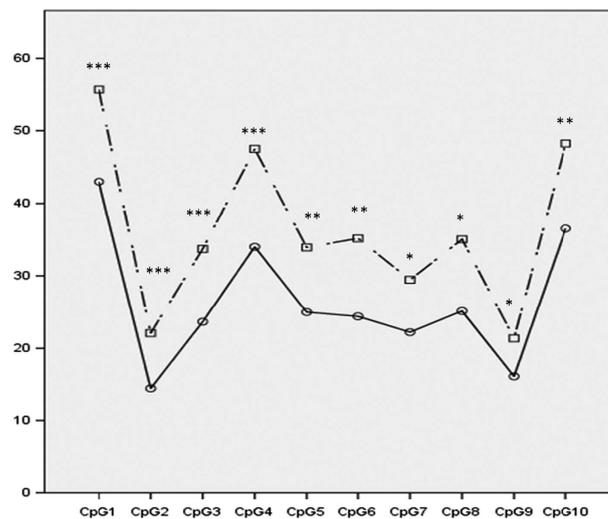
associated CpG sites with conversion to psychosis (top 100 CpGs) were kept for biological pathways analysis and revealed two networks implicating eight genes: an axon guidance pathway and the interleukin (IL)-17 signaling pathway (Table 3).

We performed a cluster analysis of individual data from the top 100 CpGs. Hierarchical clustering of the methylation changes of these top 100 CpGs successfully discriminated between the converters and non-converters (Figure 1). We examined whether the prescription of psychotropic treatment in 10 individuals (6 converters and 4 non-converters) during follow-up could account for the observed methylomic changes. We tested whether the same top CpGs sites display significant methylation modifications in patients in whom medication (antipsychotics or valproate) is initiated compared with those who have no treatment changes. Only two DNA methylation profiles showed a significant difference in methylation change in relation to medication initiation (cg 09270366 located in the inositol-polyphosphate 5-phosphatase gene (nominal  $P=0.0019$ ) and cg 05768558 located in the Lin28 homolog A gene (nominal  $P=0.03$ )).

#### Confirmation of significant results by pyrosequencing

We performed pyrosequencing of CHL1 gene (ch3: 240139) in the 78 samples. Pyrosequencing results were significantly correlated with Meth450K beadchip results ( $P=0.005$ ; Spearman's  $\rho=0.32$ ).

We also performed pyrosequencing of GSTM5. It shows large and significant differences between converters and non-converters regardless of the time of assessment (Figure 2; Supplementary Figure S8), with converters showing hypermethylation of GSTM5 promoter. Whereas bio-informatical analyses identified a cluster of six differentially methylated CpG, pyrosequencing further revealed that four additional CpGs located in the promoter, not targeted by the Meth450 beadchip, showed significant methylation change.



**Figure 2.** Mean of methylation in each CpG (cytosine–phosphate–guanine) located in the GSTM5 promoter. Full line = non-converters; dash line = converters. Mann-Whitney test: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

#### DISCUSSION

To the best of our knowledge, this study represents the first genome-wide analysis of DNA methylation profiles in individuals during conversion to psychosis and one of its strengths is the longitudinal design. We observed that conversion to psychosis was not associated with a global change in methylation and there was no individual CpG significantly associated with psychotic

transition, in line with previous findings showing that one individual CpG is rarely associated with one disease. By contrast, we found that conversion to psychosis was associated with specific methylation changes in genes involved in axon guidance, as well as genes of the IL-17 pathway and the glutathione-S-transferase family.

Both genome-wide and confirmatory experiments suggested that methylation changes, especially in the 1q21.1 region and in the promoter of the GSTM5 gene, were associated with psychosis onset. Deletion of 1q21.1 region has previously been associated with schizophrenia.<sup>18</sup> This deletion classically encompasses several genes, including HYDIN2 associated with macrocephaly and autism, suggesting an alteration of neurodevelopment.<sup>19</sup> GSTM5 is a member of glutathione-S-transferase family and is implicated in the synthesis of glutathione and protection against oxidative stress, which seems to be part of the pathophysiology of schizophrenia.<sup>20</sup> Oxidative stress has recurrently been suggested to be related to different stages of schizophrenic illness.<sup>21</sup> GSTM5 is selectively expressed in the brain<sup>22</sup> and is the most commonly expressed member of its gene family in this tissue.<sup>23</sup> Its involvement in dopamine metabolism has also been suggested.<sup>24</sup> Moreover, its expression has been shown to be decreased in the prefrontal cortex of patients with schizophrenia.<sup>25</sup> Furthermore, GSTM5 levels displayed an inverse correlation with promoter DNA methylation in brain tissue, supporting the idea that GSTM5 CpG methylation status controls gene expression.<sup>26</sup> Interestingly, our exploratory approach provided evidence that two other genes of GST family might be differentially methylated after conversion to psychosis: the GSTT1 and GSTP1 regions were hypomethylated and hypermethylated in converters, respectively, without differences between the groups at baseline. These findings suggest the possibility that conversion to psychosis may depend on the specific control of oxidative metabolism and balance between these genes.

Cluster analysis showed that a subset of top CpGs with the most significant changes in methylation during psychotic conversion correctly classified converters and non-converters, with no influence of medication initiation. Pathway analysis revealed that these top epigenetic changes were overrepresented in certain biological pathways, including an axon guidance pathway and the IL-17 pathway. The axon guidance pathway included the neural cell adhesion protein CHL1 gene (cell adhesion molecule L1-like), which codes for the L1CAM2 protein. The L1 family encompasses immunoglobulin-class recognition proteins that promote axon growth and migration in developing neurons.<sup>27</sup> In preclinical models, a deficit of CHL1 in adult mice impairs working memory,<sup>28</sup> social behavior and synaptic transmission.<sup>29</sup> Genetic variants in the CHL1 gene have been found to be associated with schizophrenia.<sup>30–32</sup> Neuropilin1 (NRP1), also included in this pathway, acts as a receptor that mediates axonal inhibition or repulsion. Neuropilin1 colocalizes with L1CAM2 in the thalamic axons<sup>33</sup> and in immature neurons,<sup>34</sup> they interact together in growth cone collapse, a process important for developing axons.<sup>33</sup> EFNA3, the third gene found in our analysis, is highly expressed in mature neurons, suggesting that an imbalance in expression exists during cerebral maturation between CHL1, NRP1 and EFNA3. EFNA3 encodes ephrin-A3, which is a critical protein for the regulation of synaptic function and plasticity in astrocytes.<sup>35</sup> The second signaling pathway, namely the IL-17 pathway, is involved in the regulation of inflammatory factors and in the immune response to bacterial pathogens. Variations in genes involved in immune response is a recurrent finding in association studies of schizophrenia.<sup>36,37</sup> A recent proteomic study identified ILs as potential diagnostic biomarkers in the onset of psychosis.<sup>38</sup> Differences in the level of several inflammatory cytokine were found in individuals with schizophrenia compared with healthy controls, with a positive correlation between the levels of cytokines in the IL-17 pathway and scores on the Positive and Negative Symptoms Scale.<sup>39</sup> This pathway

includes AKT1, a serine–threonine kinase and a critical mediator of growth-factor-induced neuronal survival in the developing nervous system.<sup>40</sup> Decreased AKT1 protein levels and phosphorylation activity were documented in the lymphocytes and brains of individuals with schizophrenia.<sup>41,42</sup> In addition, it was reported that AKT1 genetic variants were associated with schizophrenia, in relation to cannabis use.<sup>43</sup>

The genome-wide approach, without predefined candidate regions, was crucial for identifying new relevant regions that undergo differential methylation or demethylation changes in converters and non-converters across the baseline and follow-up intervals. Illumina Infinium HumanMethylation450 BeadChip interrogates about 485 000 CpG sites after bisulfite conversion of unmethylated cytosines. This design is valuable as it does not require the selection of a small number of 'candidate' genes or methylation sites. Further, we compared these results with a reference method based on pyrosequencing; the correlation was significant between the beadchip and pyrosequencing as reported in the literature.<sup>15</sup> Pyrosequencing identified additional differentially methylated neighboring CpGs in GSTM5 promoter (four additional CpGs not initially interrogated by the Meth450K array), further strengthening the methylomic results.

The genes identified in our study had not been previously reported in methylomic studies of schizophrenia.<sup>6,44,45</sup> These differences may be due to the fact that, in addition to methodological issues (notably differences in methylome coverage), we employed an original methodology based on longitudinal variation in methylation levels, which cannot easily be compared with methylation measurements from single time point studies in subjects with established schizophrenia. Moreover, in two of the three published studies, patients were aged 30 years older, on average, than our participants and methylation changes with age.<sup>46</sup>

The present work was conducted in adolescents and young adults consecutively referred to a clinic specialized for early detection of psychosis and enrolled in a longitudinal follow-up program. We did not find any differences in environmental exposure between those who converted to psychosis and those who did not, and the methylation changes associated with conversion to psychosis were not related to the initiation of medication. The observed modifications in methylation are thus more likely to be linked to psychosis conversion than to medication initiation or other environmental changes. Even if the sample sizes were sufficient to identify some significant DMRs, larger samples are needed to identify other DMRs. Another issue to identify DMRs is the molecular and clinical heterogeneity between individuals, a well-known issue in the genetics of psychosis.<sup>47</sup> The sample size could not allow us to overpass this heterogeneity.

The amplitude of methylation changes in DMR was similar to that found in previous studies in peripheral tissues comparing individuals with psychiatric disorders and healthy controls<sup>44,48</sup> and seems to be biologically relevant (>10%). However, our observations suggest that individual methylation levels are relatively stable. The extent to which these findings (which were based on peripheral markers) reflect methylation processes in the brain cannot be definitively concluded. Mounting evidence favors a relative concordance between methylation profiles in the brain and blood peripheral cells,<sup>49,50</sup> although the amplitude of peripheral methylation levels might be lower for the equivalent loci in central tissues.<sup>45</sup> Blood and brain convergence has been investigated by the beadchip suggesting that subset of peripheral data may proxy methylation status of brain tissue.<sup>51</sup> Within-subjects design, as we performed here, are recommended.

Our study has several strengths: We conducted a long-term prospective follow-up in both individuals at UHR for psychosis and non-UHR subjects. We used rapidly frozen samples enabling the study of a larger number of methylation sites (even more labile ones). We report longitudinal variations in methylation, which are more suitable for reflecting dynamic epigenetic processes

compared with single time point analyses. We used a genome-wide strategy rather than limited candidate genes strategy. We used newly developed pathway and clustering analyses to investigate the functional relevance of top CpG methylation sites. Several issues need to be addressed in future studies, however, including the problem of clinical heterogeneity and the possible influence of a larger number of environmental factors (for example, early stressful events). It will also be important to make direct measures of maturational changes (for example, using brain imaging) and to examine interactions between CpG methylation and other mechanisms of epigenetic regulation. Finally, inter-individual heterogeneity raises a yet-to-be-investigated hypothesis that private epimutations might be involved in the conversion to psychosis.

In conclusion, we found that the conversion to psychosis in young help seekers is accompanied by epigenetic changes in genes involved in relevant genes and pathways. We also identified possible candidate mechanisms, including alterations in oxidative stress regulation, axon guidance and in inflammatory pathways. These candidate genes could represent multiple theaters for the disruption in homeostasis that accompanies the emergence of full-blown psychosis. At this point, it is unknown whether the observed methylation changes have a causal role in the processes leading to psychosis or whether they are simply reflective of psychosis onset. These new observations shed light on the biological processes underlying the interactions between early vulnerability, late environmental response and maturational processes at adolescence that can precipitate some UHR individuals into full-blown psychosis. This exploratory study is a first step toward the identification of epigenetic changes accompanying the onset of psychosis and opens new perspectives for early intervention and prevention in psychosis. Replications in larger and/or independent samples are warranted to reach definitive conclusions. Future developments should also investigate the functional impact of these methylation changes.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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