

Overexpression of complement component C4 in the dorsolateral prefrontal cortex, parietal cortex, superior temporal gyrus and associative striatum of patients with schizophrenia

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1	OVEREXPRESSION OF COMPLEMENT COMPONENT C4 IN THE DORSOLATERAL PREFRONTAL								
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43 **ABSTRACT** (259 words)

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Background: In schizophrenia, abnormal synaptic pruning during adolescence may be due to altered expression of the Complement component 4 (*C4*). Overexpression of *C4* genes has been identified in the total cerebral cortex and in 6 different brain regions of schizophrenic patients compared to controls. These alterations should be replicated and extended to other brain regions relevant to schizophrenia. Moreover, it remains unknown whether cerebral and peripheral *C4* expression levels are related.

51 *Methods:* We explored *C4* genes expression both at the cerebral and peripheral levels. Using 52 *shiny*GEO application we analyzed *C4* expression from eight Gene Expression Omnibus 53 datasets obtained from 196 schizophrenic patients and 182 control subjects. First, we 54 compared *C4* expression between schizophrenic patients and controls in postmortem 55 cerebral samples from 7 different brain regions. Then, we compared *C4* expression between 56 schizophrenic patients and controls in 4 peripheral tissues.

57 *Results:* At the cerebral level, we provide further evidence of *C4* overexpression in 58 schizophrenic patients. Consistently with a previous report, we found *C4* overexpression in 59 the dorsolateral prefrontal cortex and in the parietal cortex of schizophrenic patients. The 60 observation of *C4* overexpression was further extended to the superior temporal cortex and 61 the associative striatum of schizophrenic patients. Conversely, no significant alteration of *C4* 62 expression was observed in peripheral tissues.

63 *Conclusions:* Our results support the hypothesis of an excessive Complement activity in 64 various brain regions of schizophrenic patients which may disrupt the synaptic pruning 65 process occurring during adolescence. *C4* overexpression may be specific to the cerebral

66 tissue while other alterations of the Complement system may be detected at the systemic

67 level.

- 68
- 69 Keywords:
- 70 Schizophrenia; Complement component 4; Neuronal plasticity; Brain; Leucocytes; Gene
- 71 expression
- 72
- 73

74 **1. Introduction**

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76 Schizophrenia (SZ) affects approximately 1% of the worldwide population (Laidi et al., 2018) and is responsible for a dramatic increase in mortality due to somatic diseases (especially 77 78 those related to inflammatory processes i.e. metabolic, cardiovascular pathology and 79 cancers) (Laursen et al., 2014). Thus, SZ can be considered as a systemic disorder in which 80 pathological processes take place not only at the cerebral level but also in peripheral tissues 81 (Al-Diwani et al., 2017). Although SZ pathogenesis is partly understood, there is strong 82 evidence that SZ is a complex trait resulting from genetic and environmental influences (Sullivan et al., 2003). 83

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85 Among the several susceptibility loci for SZ identified by Genome Wide Association Studies, 86 the strongest genetic association involves variation in the major histocompatibility complex 87 (MHC) locus (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). 88 This latter hosts the human leukocyte antigens (HLA) loci that encode cell surface proteins essential for the immunological recognition of self and non-self in vertebrates. The 89 90 MHC/HLA cluster is physically divided into three regions corresponding to the HLA-class I, II 91 and III clusters. Class III genes encode components of the Complement system which 92 consists of at least 30 circulating and cell-surface proteins that interact with each other 93 leading to the elimination of pathogens while protecting the self from subsequent damage 94 (Presumey et al., 2017). At the cerebral level, components of the classical Complement 95 cascade also participate to non-immunological processes such as the elimination of synapses 96 (Schafer et al., 2012; Stevens et al., 2007); this so-called "pruning" occurs during normal 97 postnatal development. Among the various proteins involved in the classical pathway, the

98 Complement component 4 (*C4*) is a critical factor (Stevens et al., 2007) previously implicated 99 in the pruning process of mouse models (Schizophrenia Working Group of the Psychiatric 100 Genomics Consortium et al., 2016).

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102 In SZ, accumulating evidence support the hypothesis of a disruption of the synaptic pruning 103 in the brain during adolescence (Johnson and Stevens, 2018; Rocco et al., 2017). 104 Consistently, recent results have established a link between the C4 locus (C4A and C4B loci) 105 and abnormal synaptic pruning in SZ patients (Gandal et al., 2018b, 2018a; Schizophrenia 106 Working Group of the Psychiatric Genomics Consortium et al., 2016). In six different brain 107 regions of SZ patients and healthy controls (HC), it has been shown that C4A and C4B 108 expressions are under the genetic control of various C4 molecular structures (Gandal et al., 109 2018b; Schizophrenia Working Group of the Psychiatric Genomics Consortium et al., 2016). 110 Notably, the increase of C4A expression parallels that of the C4A gene copy numbers on the 111 one hand and the insertion of a human endogenous retroviral (HERV) element on the other. 112 Moreover, each C4 allele is associated with schizophrenia risk in proportion to its tendency 113 to produce greater expression of C4A in the brain (Fromer et al., 2016; Gandal et al., 2018b; 114 Schizophrenia Working Group of the Psychiatric Genomics Consortium et al., 2016). 115 Consistently, an overexpression of C4A has been identified in the total cerebral cortex 116 (Gandal et al., 2018a), as well as in 6 different brain regions (namely, the prefrontal, anterior 117 cingulate, orbital frontal, parietal cortices, the cerebellum and the corpus callosum) of SZ 118 patients vs. HC (Gandal et al., 2018b; Schizophrenia Working Group of the Psychiatric 119 Genomics Consortium et al., 2016). Furthermore, a C4B overexpression has been reported in 120 the prefrontal cortex (PFC) of SZ patients vs. HC (Gandal et al., 2018b) suggesting a potential

involvement of *C4B* in SZ pathogenesis. Altogether these data implicate both *C4A* and *C4B*overexpression in SZ pathogenesis (Woo et al., 2020).

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124 To assess the clinical and biological significance of the present knowledge in SZ patients, 125 alterations previously reported should be replicated and extended to other brain regions 126 relevant to SZ. In this regard, C4 expression measured by microarrays, which reflects the 127 combined expression of C4A and C4B genes, constitutes a valuable resource. Moreover, it 128 remains unknown whether cerebral and peripheral C4 expression levels are related. A single 129 study compared C4A expression in the peripheral blood mononuclear cells (PBMCs) of SZ 130 patients vs. HC and no difference was found (Melbourne et al., 2018). Additional studies in 131 peripheral tissues are needed since they are considered as promising and readily accessible 132 surrogate systems to study the molecular mechanisms implicated in SZ (Cai et al., 2010; 133 Kálmán et al., 2016; Lavoie et al., 2017).

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Using the bio-informatic application *shiny*GEO, we aimed to identify altered *C4* expression (reflecting the combined expression of *C4A* and *C4B* genes) both at the cerebral and peripheral level in SZ patients. We compared *C4* expression between SZ patients and HC (i) in postmortem cerebral samples from 7 different brain regions and (ii) in 4 peripheral tissues from independent samples. Finally, in order to evaluate the neurobiological relevance of the changes identified in peripheral tissues, we compared them to those observed in brain regions relevant to SZ.

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143 **2. Material and Methods**

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145 **2.1. Search and inclusion criteria of primary datasets**

146 Gene Expression Omnibus (GEO) database provides microarray expression results from 147 postmortem brain or peripheral tissues samples from SZ patients and HC, along with 148 demographic, clinical and quality data (Barrett et al., 2013). With the aim to identify altered 149 expression of C4 genes both at the central and peripheral level in SZ patients, we explored 150 GEO database for datasets providing microarray expression results from SZ patients and HC. 151 Relevant microarray datasets were searched using the following search terms: 152 (("schizophrenia"[MeSH Terms] OR schizophrenia[All Fields]) AND "Homo sapiens"[porgn]) 153 AND "Homo sapiens" [porgn] AND ("gse" [Filter] AND "Expression profiling by array" [Filter]). 154 We included datasets from original studies (i) involving brain or peripheral tissues samples 155 from SZ patients and HC and (ii) using the Affymetrix Human Genome U133 plus 2.0 156 (HG-U133_Plus_2) or Affymetrix Human Gene 1.0 ST (Human Gene 1.0 ST) or Affymetrix 157 Human Gene 1.1 ST (Human Gene 1.1 ST) arrays. These chips can technically interrogate C4 158 expression. Human Gene 1.0 ST and Human Gene 1.1 ST arrays exhibit comparable detection 159 thresholds and are highly concordant with HG-U133 Plus 2 array (Pradervand et al., 2008; 160 Robinson and Speed, 2007).

In this study, eight independent GEO datasets were included (Barnes et al., 2011; Cattane et al., 2015; Chen et al., 2018, p. 2, 2013; Horiuchi et al., 2016; Lanz et al., 2019; Maycox et al., 2009; van Beveren et al., 2012), obtained from 196 SZ patients and 182 HC. The included GEO datasets provided data from seven different brain regions relevant to SZ (Grot et al., 2017; McCutcheon et al., 2019; Singh et al., 2018; Yeganeh-Doost et al., 2011) and four peripheral tissues (Table 1). All the data used in this project were acquired in previous studies, all of which conformed to Ethical Standards (Barnes et al., 2011; Cattane et al.,

2015; Chen et al., 2018, p. 2, 2013; Horiuchi et al., 2016; Lanz et al., 2019; Maycox et al.,
2009; van Beveren et al., 2012).

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171 **2.2. Demographic characteristics and quality data**

For each included GEO dataset, the quality data of the samples and demographic characteristics of the subjects who provided brain or peripheral tissues are summarized in Table 2 and Table 3, respectively. There was no significant difference in age, sex distribution, postmortem interval (PMI) and RNA integrity number (RIN) between the SZ patient and HC groups. For the brain pH, a significant difference was observed in the anterior PFC BA10 (p=0.019) and in the superior temporal gyrus (STG) (p=0.002).

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179 2.3. Statistical analysis

XLSTAT software (XLSTAT 2017: Data Analysis and Statistical Solution for Microsoft Excel.
Addinsoft, Paris, France, 2017) was used to perform statistical analysis. For each included
GEO dataset, demographic characteristics of subjects and quality data of samples in HC and
SZ patient groups were compared using unpaired t-test (for age, PMI, brain pH and RIN), and
Fisher exact test (for gender).

For each tissue, we used *shiny*GEO application to realize differential expression analysis between SZ patients and HC (Dumas et al., 2016). *shiny*GEO is implemented using R [https://www.r-project.org/] and shiny [http://shiny.rstudio.com/]. *shiny*GEO downloads processed gene expression datasets from GEO using GEOquery package (Davis and Meltzer, 2007). *C4* expression values are then normalized using *HSP90AB1* as the reference housekeeping gene. *HSP90AB1* was selected based on the following criteria: (i) *HSP90AB1* is one of the endogenous genes listed by Casares as potential housekeeping genes for

microarray analysis (Casares, 2016), (ii) *HSP90AB1* and *C4* genes are involved in different cellular functions, (iii) there was no significant difference in *HSP90AB1* expression between the SZ patients and HC groups in any brain region or peripheral tissue. Normalized *C4* expression values are log2-transformed to stabilize the variance.

ANCOVA testing was conducted to evaluate whether normalized *C4* expression was impacted by relevant covariates. In each brain region and peripheral tissue, ANCOVA analysis was conducted using the normalized *C4* gene expression as a dependent variable and with age, PMI, pH and RIN as quantitative independent variables, and sex as categorical independent variable.

Normal distribution was tested using Shapiro-Wilk test. For differential expression, exact pvalues were calculated using unpaired, two-tailed t-tests. When a deviation from normality was detected, exact p-values were calculated using unpaired, two-tailed Mann-Whitney U tests. In case of violation of the equal variance assumption (Supplementary Tables 1-2), exact p-values were calculated using unpaired, two-tailed Welch tests. Gene expression comparisons were considered to be statistically significant for p<0.05.</p>

207

208 **3. Results**

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3.1. Demographic characteristics of the subjects and quality characteristics of the samples

ANCOVA testing of each potential covariate (age, sex, PMI, pH, RIN) revealed no significant influence on normalized *C4* expression in any of the brain regions or peripheral tissues, except in the cerebellum where the pH was significant (pH=0.014).

- **3.2. Differential expression analysis in the brain tissues**
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Compared to HC, we identified a significant overexpression of *C4* in the DLPFC (BA46) (fold change=1.46; p=0.005), STG (BA22) (fold change=1.78; p=0.035), parietal cortex (fold change=1.20; p=0.039) and associative striatum (fold change=1.32; p=0.034) of SZ patients (Fig. 1). In the anterior PFC (BA10) of SZ patients vs. HC, there was a trend to *C4* overexpression (fold change=1.37; p=0.076). No alterations were found in the hippocampus and cerebellum of SZ patients vs. HC.

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3.3. Differential expression analysis in the peripheral tissues

No significant difference in *C4* expression was identified in the olfactory epithelium, skin
fibroblasts, lymphoblastoid cell lines (LCLs) and PBMCs of SZ patients vs. HC (Fig. 2).

226

227 4. Discussion

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229 Compared to HC, we observed a C4 overexpression in the DLPFC (BA46), STG (BA22), 230 parietal cortex and associative striatum of SZ patients. At the peripheral level, no difference 231 in C4 expression was observed in SZ patients vs. HC. All the original studies from which are 232 derived the included datasets used a genome-wide approach. As a principle, the expression 233 of all genes was analyzed, including C4. However, those results were not discussed in the 234 original experiments. The present study re-analyzed the original data using a candidate-gene 235 approach. Since only C4 gene expression was re-analyzed in each dataset, no requirement 236 for multiple tests correction was needed. Such an approach allowed us to extend previous 237 knowledge on schizophrenia-associated C4 overexpression to other brain regions.

At the cerebral level, our results expand previous evidence reporting *C4A* overexpression in the total cerebral, prefrontal, anterior cingulate, orbital frontal, parietal cortices, cerebellum and corpus callosum (Gandal et al., 2018b, 2018a; Schizophrenia Working Group of the Psychiatric Genomics Consortium et al., 2016), *C4B* overexpression in the PFC (Gandal et al., 2018b; Pérez-Santiago et al., 2012) and *C4A/C4B* overexpression in the thalamus (Chu and Liu, 2010) in SZ patients.

245 First, in the PFC of SZ patients vs. HC, we report C4 overexpression in BA46 and a trend to C4 overexpression in BA10. This refines previous results reporting C4A and/or C4B 246 247 overexpression in the PFC (mixed data from BA46, BA9 and BA10) and C4B overexpression in 248 BA9 of SZ patients vs. HC (Gandal et al., 2018b; Pérez-Santiago et al., 2012). As an 249 exploratory analysis with *shiny*GEO, we replicated *C4* overexpression (fold change=2.03; 250 p=0.003) in the BA46 of 29 SZ patients and 30 HC from another independent GEO dataset, 251 GEO: GSE21138. These results suggest that C4 is overexpressed in different sub-regions of 252 the PFC in SZ patients.

Moreover, we identified a *C4* overexpression in the parietal cortex of SZ patients vs. HC. Our findings are consistent with the *C4A* overexpression found in the parietal cortex of SZ patients (Schizophrenia Working Group of the Psychiatric Genomics Consortium et al., 2016) and support the correlation between *C4A* gene copy number and neuropil contraction observed in the parietal regions of young adult-onset SZ patients (Prasad et al., 2018).

Furthermore, we observed for the first time *C4* overexpression in the associative striatum and STG (BA22) of SZ patients vs. HC. In the associative striatum, *C4* overexpression is consistent with glial activation and neuro-inflammation, as well as increased expression of pro-inflammatory transcripts in drug-naïve first episode psychosis (FEP) patients (Plitman et al., 2016; Sivaraman et al., 2018) and SZ patients (Lanz et al., 2019), respectively. In the STG,

263 *C4* overexpression is in line with the involvement of immune related genes in synaptic
264 events in the BA22 region of chronic SZ patients (Schmitt et al., 2011).

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266 Notably, the C4 expression changes identified in the present study were of modest 267 size (fold change (FC) from 1.3 to 1.8). Consistently, Sekar et al. reported that the median 268 C4A expression in brain tissues was 1.4-fold greater in SZ patients compared to HC (Schizophrenia Working Group of the Psychiatric Genomics Consortium et al., 2016). 269 270 Moreover, altered expression changes identified in SZ patients compared to HC are usually 271 modest. Indeed, in the postmortem DLPFC, hippocampus and associative striatum of 272 SZ patients versus HC, after false discovery rate correction, 99% of the differentially 273 expressed genes exhibit a FC<|2| (Lanz et al., 2019). Mixed results are available regarding 274 the functional consequences of such modest gene expression changes. On the one hand, in 275 SZ patients, small gene expression changes have been associated with abnormal brain level 276 of the encoded protein (Gibbons et al., 2020; Narayan et al., 2008; Pandey et al., 2018), and 277 cognitive deficits (Cai et al., 2020; Horiuchi et al., 2016). In animal models of SZ as well, association between modest brain expression changes and behavioral abnormalities have 278 279 been reported (Hsueh et al., 2018). In contrast, in SZ patients, other experiments failed to 280 report that small gene expression changes have functional consequences on the encoded 281 protein level (Boerrigter et al., 2017; Sinclair et al., 2013; Uhrig et al., 2016) or clinical scales (Ansoleaga et al., 2015; Gouvea et al., 2016). In this regard, while consistent with previous 282 283 evidence involving C4 overexpression in SZ pathogenesis (Schizophrenia Working Group of 284 the Psychiatric Genomics Consortium et al., 2016), our results require additional studies to 285 demonstrate that C4 overexpression in the brain of SZ patients is associated with abnormal 286 C4 protein levels, disease severity and functional impairment.

Altogether our results are consistent with the hypothesis of an excessive 288 Complement activity in schizophrenic disorders (Schizophrenia Working Group of the 289 290 Psychiatric Genomics Consortium et al., 2016). Previously, Sekar et al. showed that each of 291 the four common C4 structural haplotypes is associated with schizophrenia risk in proportion 292 to its tendency to produce higher C4A brain expression (Schizophrenia Working Group of the 293 Psychiatric Genomics Consortium et al., 2016). In SZ patients, these results suggest a 294 genetically-determined lifelong high expression of C4. Since the average age of the brains 295 investigated in the present study ranged from 42 to 73 years, our findings are consistent 296 with the persistence of C4 overexpression in the brain of SZ patients long after the synaptic 297 pruning period. However, since our samples were obtained long after adolescence, we could 298 not demonstrate that C4 overexpression is already present during adolescence. To date and 299 to our knowledge, no human study has explored C4 brain expression during adolescence.

This may be due to the scarcity of brain collection tissues which are mainly obtained from old subjects. Since our present results suggest that peripheral *C4* expression poorly reflects *C4* brain expression, future research needs to gain access to *C4* expression in the central nervous system during human adolescence. In this regard, measuring *C4* expression in cerebrospinal fluid, although more invasive and expensive as compared to peripheral measurements, may provide meaningful information about *C4* expression during the synaptic pruning period occurring in adolescence.

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Paradoxically, while increasing evidence implicate the hippocampus and the cerebellum in SZ pathogenesis (Yeganeh-Doost et al., 2011; Nakahara et al., 2018; Lieberman et al., 2018; Ding et al., 2019), no significant difference in *C4* expression was found in these

311 regions in SZ patients vs. HC. To date, findings related to C4 genes expression in these 312 regions in SZ patients are mixed. Regarding the hippocampus, while increased predicted C4A 313 expression was associated with poorer episodic memory performance in both SZ patients 314 and HC (Donohoe et al., 2018), C4A gene copy number was not associated with hippocampic 315 neuropil contraction in SZ patients (Prasad et al., 2018). Additionally, no change was 316 observed in the hippocampic level of C4 protein in a neurodevelopmental mouse model of 317 SZ (Mattei et al., 2017). Regarding the cerebellum, our results are in line with those of 318 Prasad et al. who reported no association between C4A gene copy number and cerebellum 319 neuropil contraction in SZ patients but contrast with those of Sekar et al. who observed C4A 320 overexpression in the cerebellum of SZ patients (Schizophrenia Working Group of the 321 Psychiatric Genomics Consortium et al., 2016).

322

In the olfactory epithelium, skin fibroblasts, LCLs and PBMCs of SZ patients vs. HC, we observed no significant difference in *C4* expression. Although skin fibroblasts and olfactory epithelium are considered as promising surrogate systems for the study of SZ pathogenesis (Kálmán et al., 2016; Lavoie et al., 2017), our results suggest that they may not be affected by transcriptional alterations of *C4*.

The absence of difference in *C4* expression in the lymphoblasts of SZ patients vs. HC is consistent with a previous RNA sequencing study on LCLs (Sanders et al., 2013). In this latter study, no difference in *C4* expression was observed although an overexpression of four other Complement components was reported. In SZ disorders, these results suggest that *C4* overexpression may be specific to the cerebral tissue while other alterations of the Complement system may be detected at the systemic level possibly in relation with other Complement-mediated processes.

335 In PBMCs, the absence of significant difference in C4 expression is consistent with the 336 single previous study exploring C4A mRNA expression in the PBMCs of SZ patients vs. HC 337 (Melbourne et al., 2018). When considering C4 protein levels in the peripheral blood of SZ 338 patients, discrepant results reporting increase (Cooper et al., 2017; Hakobyan et al., 2005; 339 Mayilyan et al., 2006; Walss-Bass et al., 2019), decrease (Mayilyan et al., 2008) or no 340 difference (Ali et al., 2017; Idonije et al., 2012; Kopczynska et al., 2019; Santos Sória et al., 341 2012) have been observed. Specifically, C4 protein level has been found increased in 342 newborn babies who later develop SZ (Cooper et al., 2017), Ultra-High Risk patients (Laskaris 343 et al., 2019), in advanced stages of SZ disorders or in chronic patients (Laskaris et al., 2019) 344 contrasting with normal C4 protein levels in FEP patients (Kopczynska et al., 2019; Laskaris et 345 al., 2019). However, among FEP patients, recent findings suggest that increased C4 protein 346 levels may be restricted to treatment non-responders (Mondelli et al., 2020). The mismatch 347 between mRNA and protein results might be related to the biological source (i.e. plasma or 348 serum), the specific stages of SZ illness, the techniques involved in the measurement of 349 mRNA and protein levels and the various levels of regulation between transcript and protein product (Maier et al., 2009). Notably, while most studies investigating mRNA-protein 350 351 expression correlation used a genome-wide approach (correlation between mRNA 352 expression and protein expression across all genes), only a limited number of individual gene 353 correlation analyses (correlation between the expression of a mRNA and its protein product 354 across multiple samples or conditions) have been performed (Koussounadis et al., 2015). 355 This lack of individual gene correlation analyses is relevant since higher correlations have 356 been observed for certain classes of genes while poorer correlations have been attributed to 357 post-transcriptional regulation (Koussounadis et al., 2015). To date and to our knowledge, 358 no individual gene correlation analysis has explored both C4 expression and C4 protein level in brain or peripheral tissues of SZ patients vs. HC. It may thus be worthwhile for future
studies to look for a correlation between *C4* mRNA and C4 protein level in a given tissue.

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362 All these findings raise the possibility that direction and distribution of C4 altered 363 expression in brain and peripheral tissues, may depend on the characteristics of SZ patients. 364 On the one hand, the heterogenous clinical features of the included samples of SZ patients, 365 especially the stage of the disorder, the acute or chronic status, the clinical severity, the level 366 of treatment-resistance and the various antipsychotic treatments may constitute 367 confounding factors. Regarding symptomatology, a positive correlation between C4A 368 expression and PANSS positive factor scores was found in the PBMCs of SZ patients 369 (Melbourne et al., 2018) while no results are available concerning such a correlation in the 370 brain tissues. Therefore, we can't exclude that C4 brain overexpression identified in SZ 371 patients may also reflect the severity of the pathological process. Unfortunately, we could 372 not explore this issue since no information about severity of symptomatology was provided 373 in the included GEO datasets. A single study reported no influence of antipsychotic treatments on C4A expression in the PBMCs of SZ patients vs. HC (Melbourne et al., 2018). 374 375 Since no previous study explored the influence of antipsychotics in brain tissues, a potential 376 impact of such treatments on C4 brain expression could not be ruled out.

On the other hand, the biological heterogeneity of SZ patients may impact the observed *C4* expression levels. Indeed, *C4* overexpression may be restricted to a subset of SZ patients characterized by *C4* alleles generating high levels of *C4* mRNA (Schizophrenia Working Group of the Psychiatric Genomics Consortium et al., 2016). This is in accordance with the concept of SZ disorders as a group of heterogeneous disorders underpinned by various mechanistic processes (Clementz et al., 2016; Mothi et al., 2018). More studies combining structural

383 genetic data and gene expression data are warranted in brain and peripheral tissues to 384 evaluate whether *C4* overexpression is only observed in a subset of individuals with SZ 385 disorders. A classification of SZ patients according to underlying biology rather than clinical 386 symptomatology would be a further step to a precision psychiatry approach (Fernandes et 387 al., 2017).

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389 In this study, the transcriptional alterations identified in cerebral tissues of 390 SZ patients were not observed in the peripheral tissues. These discrepancies may be due to 391 the fact that brain and peripheral tissues samples used in the present study were not 392 derived from the same subjects. Alternatively, our results suggest that the olfactory 393 epithelium, skin fibroblasts, LCLs and PBMCs do not reflect the brain expression of C4 in SZ 394 patients. Our results are not consistent with previous studies reporting that blood shares 395 significant gene expression similarities with brain tissues (Sullivan et al., 2006). Regarding C4 396 expression in SZ disorders, our results rather suggest that brain and peripheral tissues may 397 be affected by distinct alterations (Al-Diwani et al., 2017; Cai et al., 2010; Liew et al., 2006; Sullivan et al., 2006) and that C4 overexpression may be restricted to specific brain regions in 398 399 SZ disorders. Since C4 expression level is related to gene copy number and HERV status 400 (Schizophrenia Working Group of the Psychiatric Genomics Consortium et al., 2016) which 401 are identical throughout the whole body, it may be suggested that additional epigenetic 402 factors are involved in the regulation of C4 expression at the cerebral level, a wide concept 403 named tissue-specificity. Consistently with this hypothesis, previous studies using mouse 404 models have shown that early environmental risk factors for SZ disrupt the Complement 405 system (Duchatel et al., 2018) even in the absence of genetic background (Mattei et al., 406 2017).

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408 Finally, the microarrays used to acquire the GEO datasets included in the present 409 study could not technically distinguish between C4A and C4B transcripts. Thus, the relative 410 involvement of C4A and C4B transcripts in the C4 overexpression identified in the DLPFC 411 (BA46), the STG (BA22), the parietal cortex and the associative striatum can't be specified. 412 We could not exclude the possibility that C4 overexpression may reflect C4B overexpression. 413 Although the majority of available results conclude that risk for SZ is associated with C4A 414 overexpression (Gandal et al., 2018b, 2018a; Schizophrenia Working Group of the Psychiatric 415 Genomics Consortium et al., 2016), recent results also implicate C4B overexpression in SZ 416 pathogenesis. Firstly, an increased expression of C4B was reported in the PFC and thalamus 417 of SZ patients vs. HC (Chu and Liu, 2010; Gandal et al., 2018b; Pérez-Santiago et al., 2012). 418 Secondly, in two independent samples of SZ patients, an association between C4B gene copy 419 number and increased neuropil contractions was observed in various brain regions 420 associated with SZ (Prasad et al., 2018). Additionally, although mouse models provided the 421 experimental evidence implicating C4 expression in the pruning processes (Schizophrenia 422 Working Group of the Psychiatric Genomics Consortium et al., 2016) such models are of 423 limited interest to explore the specific role of C4A and C4B in the altered pruning process of 424 SZ since mice lack the two isoforms of C4 found in humans. Altogether these results raise the 425 hypothesis of the potential involvement of C4B overexpression in SZ pathogenesis. To 426 further explore this issue, we performed an exploratory analysis with shinyGEO. We selected 427 the GEO dataset GEO: GSE62191 which was acquired using a microarray specifically 428 exploring C4B expression. In line with our latter hypothesis, we observed a trend to C4B 429 overexpression (fold change = 1.17; p = 0.077) in the frontal cortex of 29 SZ patients vs.

430 30 HC. Additional studies are needed to further investigate *C4B* overexpression in SZ431 disorders.

432

433 **5. Limits**

434

435 Interpretation of the present results is affected by limiting factors. Firstly, brain and 436 peripheral tissues were obtained from 8 different and modest-sized SZ samples. Brain 437 tissues were obtained from rather old men and women (Table 2), whereas PBMCs were 438 collected from younger men (mean age \pm SD: 23.02 \pm 4.03 for the controls and 23.90 \pm 4.08 439 for the SZ patients). Therefore, the gene expression changes identified in the cerebral tissues 440 may reflect the influence of age, while the absence of results in the PBMCs may be gender 441 specific. However, previous studies observed no influence of age or sex on C4 expression in 442 brain tissues (Schizophrenia Working Group of the Psychiatric Genomics Consortium et al., 443 2016) or PBMCs (Melbourne et al., 2018) of SZ patients. Consistently, in the present study, 444 ANCOVA testing did not report a significant influence of age or sex on normalized C4 445 expression in any of the brain regions or peripheral tissues. Thus, it is unlikely that age or sex 446 explain our results. Moreover, it should be noted that the modest size of the samples may 447 have led to type II errors, especially in the anterior PFC (BA10) where we identified a trend 448 to C4 overexpression (FC=1.37, p=0.076). Independent, larger samples are thus needed to replicate our results. 449

450 Secondly, factors inherent in post-mortem brain studies, and beyond the investigator's 451 control, might have influenced our results: (i) A concern is the effect of brain pH on our 452 results since we observed a significant difference in brain pH between HC and SZ patient 453 groups in the PFC (BA10) and STG (BA22). However, ANCOVA testing did not report a

significant influence of pH on normalized *C4* expression in any of the brain regions or peripheral tissues, except in the cerebellum, (ii) Another limitation is the lack of information regarding potential confounding factors such as drug consumption, tobacco smoking or alcohol abuse history even if no significant effect of smoking on *C4* transcript levels was observed in the PBMCs of SZ patients (Melbourne et al., 2018). Brain tissue collections associated with high-quality phenotype data are warranted to allow replication of the present results.

461 Thirdly, all the datasets included in the present study were acquired through microarray 462 technology. Our results are thus subject to the methodological limitations that lies within 463 the use of microarrays: (i) background hybridization limit gene expression measurement, 464 especially for low abundance transcripts, (ii) probe performance may have led to specificity 465 issues (through cross-hybridization and non-specific hybridization), (iii) only genes for which 466 probes are designed can be explored. In contrast, RNA-sequencing (RNA-seq) has higher 467 sensitivity and specificity and thus allows the detection of a higher percentage of 468 differentially expressed genes, especially those with low expression. Moreover, RNA-seq can 469 detect novel transcripts, isoforms and reveal splice variants (Zhao et al., 2014). Recently, 470 using RNA-seq in the prefrontal cortex of SZ patients compared to HC, Gandal et al. reported 471 decreased expression levels of two C4A isoforms as well as an overexpressed and a 472 decreased C4B isoforms (Gandal et al., 2018b). Thus, specific splice variants of C4A and C4B may be associated with the altered synaptic pruning in SZ and remain to be explored. 473 474 Additional RNA-seq studies are needed to disentangle the specific role of C4A and C4B splice 475 variants to better understand the mechanisms underlying the dysregulation of pruning in SZ disorders. 476

Fourthly, postmortem tissue samples may have different white/grey matter ratios leading to different relative amounts of neuronal or glial transcripts in the extracted mRNA. Further research using single-cell RNA-seq techniques is needed to take into account this issue. Indeed, in comparison to traditional profiling methods that assess bulk cellular populations, single cell RNA-sequencing has the ability to study specific cell populations, allowing the study of white and grey matter-derived transcripts.

In the light of these different limits, replication studies in larger, independent samples using high-throughput sequencing techniques such as single-cell RNA-seq technology are needed to confirm, refine and extend our results. In this regard, high-quality brain tissue collections are needed to allow optimized cell dissociation and single-cell preparation, thus ensuring meaningful analyses and conclusions.

488 Finally, the gene expression changes identified in the present study were of modest size 489 raising the question of whether or not they are associated with functional consequences. 490 Additional studies in SZ patients are thus needed to demonstrate that C4 brain 491 overexpression is associated with abnormal C4 protein levels, disease severity and functional 492 impairment. Especially, while our study provides additional clues towards an excessive 493 Complement activity in the brain of SZ patients, our results do not allow us to link C4 494 overexpression to abnormal synaptic pruning. Hence, additional studies are needed, at 495 different time points of life, to confirm that C4 cerebral overexpression disrupts the synaptic 496 pruning during adolescence which in turn increases risk for schizophrenic disorders (Johnson 497 and Stevens, 2018).

498

499 **6.** Conclusions

500

501 Using eight independent gene expression datasets, we provide further evidence of 502 C4 overexpression at the cerebral level in SZ patients. Consistently with a previous report, 503 we found C4 overexpression in the DLPFC (BA46) and in the parietal cortex of SZ patients 504 compared to HC. In SZ patients, C4 overexpression was further extended to the STG (BA22) 505 and the associative striatum. Conversely, no significant alteration of C4 expression was 506 observed in peripheral tissues suggesting that C4 overexpression may be specific to the 507 cerebral tissue while other alterations of the Complement system may be detected at the 508 systemic level. Our results support the hypothesis of an excessive Complement activity in 509 various brain regions of SZ patients which may disrupt the synaptic pruning process 510 occurring during adolescence.

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518

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523

524 **Declaration of Competing Interest**

525 The authors report no biomedical financial interests or potential conflicts of interest.

526

527 Data Availability Statement

528 The data that support the findings of this study are openly available in Gene Expression

529 Omnibus (NCBI) at <u>https://www.ncbi.nlm.nih.gov/geo/</u>, reference numbers GEO: GSE53987,

530 GEO: GSE17612, GEO: GSE35977, GEO: GSE35974, GEO: GSE21935, GEO: GSE27383,

531 GEO: GSE73129, GEO: GSE62333, GEO: GSE21138, GEO: GSE62191.

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TABLE LEGENDS

Table 1

Characteristics of the included GEO datasets.

Table 2

Demographic characteristics and quality data of the post-mortem brain samples.

Table 3

Demographic characteristics of the peripheral tissues samples.

FIGURE LEGENDS

Fig. 1. Expression of *C4* in the postmortem brain tissues of SZ patients and HC subjects. Fold change in gene expression was provided by shinyGEO based on the processed gene expression datasets downloaded directly from GEO. *p < 0.05 vs HC subjects.

Fig. 2. Expression of *C4* in the peripheral tissues of SZ patients and HC subjects. Fold change in gene expression was provided by *shiny*GEO based on the processed gene expression datasets downloaded directly from GEO. *p < 0.05 vs HC subjects.



Peripheral tissues



Table 1

Characteristics of the included GEO datasets.

GEO Accession Number	Tissue	Region / Cell Type	Microarray	Schizophrenic patients	Control subjects	Sample collection
GSE53987 (Lanz et al., 2019)	Brain	DLPFC (BA46)	HG-U133_Plus_2	15	19	University of Pittsburgh brain bank
GSE53987 (Lanz et al., 2019)	Brain	Hippocampus	HG-U133_Plus_2	15	18	University of Pittsburgh brain bank
GSE53987 (Lanz et al., 2019)	Brain	Associative striatum	HG-U133_Plus_2	18	18	University of Pittsburgh brain bank
GSE17612 (Maycox et al., 2009)	Brain	Anterior prefrontal cortex (BA10)	HG-U133_Plus_2	28	23	Tissue collection of the Charing Cross Hospital, Imperial College London, UK
GSE21935 (Barnes et al., 2011)	Brain	Superior temporal cortex (BA22)	HG-U133_Plus_2	23	19	Tissue collection of the Charing Cross Hospital, Imperial College London, UK
GSE35977 (Chen et al., 2018, 2013)	Brain	Parietal cortex	HuGene-1_0-st	51	50	Stanley Medical Research Institute's Neuropathology Consortium and Array Collections
GSE35974 (Chen et al., 2013)	Brain	Cerebellum	HuGene-1_0-st	14	14	Stanley Medical Research Institute's Neuropathology Consortium and Array Collections
GSE27383 (van Beveren et al., 2012)	Blood	PBMCs	HG-U133_Plus_2	43	29	Erasmus University Medical Center, Rotterdam, The Netherlands
GSE73129 (Horiuchi et al., 2016)	Olfactory Epithelium	Neural layers	HG-U133_Plus_2	19	19	Johns Hopkins Schizophrenia Center, Baltimore, MD, USA
GSE73129 (Horiuchi et al., 2016)	Blood	Lymphoblasts	HG-U133_Plus_2	36	41	Johns Hopkins Schizophrenia Center, Baltimore, MD, USA
GSE62333 (Cattane et al., 2015)	Skin	Fibroblasts	HuGene-1_1-st	20	20	Samples from Brescia and Verona Italy

DLPFC dorsolateral prefrontal cortex, BA Brodmann area, PBMCs peripheral blood mononuclear cells

Table 2

Demographic characteristics and quality data of the post-mortem brain samples.

Brain region	Subjects (n)	Age (mean ± SD)		Gender (M/F)		Post-mortem interval (mean ± SD)		pH (mean ± SD)		RIN (mean ± SD)						
		Control	Schizophrenia	p-value ¹	Control	Schizophrenia	p-value ¹	Control	Schizophrenia	p-value1	Control	Schizophrenia	p-value1	Control	Schizophrenia	p-value1
DLPFC (BA46)	19 HC / 15 SZ	48.1 ± 10.6	46 ± 8.6	0.549	10/9	7/8	1	19.5 ± 5.1	18.9 ± 6.7	0.779	6.6 ± 0.2	6.5 ± 0.4	0.551	7.8 ± 0.6	7.6 ± 0.7	0.06
Hippocampus	18 HC / 15 SZ	48.2 ± 10.9	45.7 ± 8.8	0.493	9/9	9/6	0.728	19.4 ± 5.2	19.4 ± 7.2	0.775	6.6 ± 0.2	6.4 ± 0.3	0.358	7.4 ± 0.6	6.5 ± 0.5	0.51
Associative striatum	18 HC / 18 SZ	48.4 ± 10.8	45 ± 8.8	0.301	10/8	10/8	1	19.8 ± 5.1	19.9 ± 7.1	0.797	6.6 ± 0.2	6.5 ± 0.4	0.337	8.2 ± 0.7	7.9 ± 0.8	0.454
Anterior prefrontal cortex (BA10)	23 HC / 28 SZ	69 ± 21.6	73.3 ± 15.2	0.411	13/10	19/9	0.561	9.9 ± 4.4	8.7 ± 7.0	0.609	6.5 ± 0.3	6.1 ± 0.2	0.019*	-	-	
Cerebellum	50 HC / 44 SZ	45.8 ± 9.3	43.2 ± 9.5	0.182	31/19	32/12	0.283	-	-		6.5 ± 0.3	6.4 ± 0.2	0.776	-	-	
Parietal cortex	50 HC / 51 SZ	45.5 ± 9.0	42.6 ± 9.9	0.132	35/15	37/14	0.828		-		6.5 ± 0.3	6.4 ± 0.3	0.631	-	-	
Superior temporal cortex (BA22)	19 HC / 23 SZ	67.7 ± 22.2	72.2 ± 16.9	0.462	10/9	13/10	1	9.1 ± 4.3	7.1 ± 5.7	0.516	6.5 ± 0.3	6.2 ± 0.2	0.002*	-		

DLPFC dorsolateral prefrontal cortex, BA Brodmann area, RIN RNA integrity number, HC healthy controls, SZ schizophrenic patients

¹Unpaired t-tests and Fisher exact tests were conducted to assess group differences for continuous and discrete variables, respectively. * p<0.05

Table 3

Demographic characteristics of the peripheral-tissues samples.

Peripheral Tissue	Subjects (n)	Age (mean ± SD)			Gender (M/F)		
		Control	Schizophrenia <i>p</i> -value ¹		Control	Schizophrenia	p-value1
PBMCs	29 HC / 43 SZ	23.02 ± 4.03	23.90 ± 4.08	0.378	29/0	43/0	1
Olfactory epithelium	19 HC / 19 SZ	39.7 ± 11.6	38.9 ± 11.4	0.829	13/6	13/6	1
Skin fibroblasts	20 HC / 20 SZ	48.4 ± 12.2	44.6 ± 12.7	0.340	9/11	10/10	0.758
Lymphoblasts	41 HC / 36 SZ	37.1 ± 11.6	37.6 ± 10.9	0.864	29/12	24/12	0.806

PBMCs peripheral blood mononuclear cells, HC healthy controls, SZ schizophrenic patients

¹Unpaired t-tests and Fisher exact tests were conducted to assess group differences for continuous and discrete variables, respectively. *p<0.05