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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**
2 **CORTEX, PARIETAL CORTEX, SUPERIOR TEMPORAL GYRUS AND ASSOCIATIVE STRIATUM**
3 **OF PATIENTS WITH SCHIZOPHRENIA**

4 Running title: C4 overexpressed in brain regions in schizophrenia

5 Original full-length research report

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41

42 **Word count: 5194**

43 **ABSTRACT** (259 words)

44

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

51 *Methods:* We explored *C4* genes expression both at the cerebral and peripheral levels. Using
52 *shinyGEO* 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**

75

76 Schizophrenia (SZ) affects approximately 1% of the worldwide population (Laidi et al., 2018)
77 and is responsible for a dramatic increase in mortality due to somatic diseases (especially
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
83 (Sullivan et al., 2003).

84

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
89 essential for the immunological recognition of self and non-self in vertebrates. The
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).

101

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

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

123

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).

134

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

142

143 **2. Material and Methods**

144

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).

161 In this study, eight independent GEO datasets were included (Barnes et al., 2011; Cattane et
162 al., 2015; Chen et al., 2018, p. 2, 2013; Horiuchi et al., 2016; Lanz et al., 2019; Maycox et al.,
163 2009; van Beveren et al., 2012), obtained from 196 SZ patients and 182 HC. The included
164 GEO datasets provided data from seven different brain regions relevant to SZ (Grot et al.,
165 2017; McCutcheon et al., 2019; Singh et al., 2018; Yeganeh-Doost et al., 2011) and four
166 peripheral tissues (Table 1). All the data used in this project were acquired in previous
167 studies, all of which conformed to Ethical Standards (Barnes et al., 2011; Cattane et al.,

168 2015; Chen et al., 2018, p. 2, 2013; Horiuchi et al., 2016; Lanz et al., 2019; Maycox et al.,
169 2009; van Beveren et al., 2012).

170

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

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

178

179 **2.3. Statistical analysis**

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

185 For each tissue, we used *shinyGEO* application to realize differential expression analysis
186 between SZ patients and HC (Dumas et al., 2016). *shinyGEO* is implemented using R
187 [<https://www.r-project.org/>] and shiny [<http://shiny.rstudio.com/>]. *shinyGEO* downloads
188 processed gene expression datasets from GEO using GEOquery package (Davis and Meltzer,
189 2007). *C4* expression values are then normalized using *HSP90AB1* as the reference
190 housekeeping gene. *HSP90AB1* was selected based on the following criteria: (i) *HSP90AB1* is
191 one of the endogenous genes listed by Casares as potential housekeeping genes for

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

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

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

207

208 **3. Results**

209

210 **3.1. Demographic characteristics of the subjects and quality characteristics of the samples**

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

214

215 **3.2. Differential expression analysis in the brain tissues**

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

222

223 **3.3. Differential expression analysis in the peripheral tissues**

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

226

227 **4. Discussion**

228

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.

238

239 At the cerebral level, our results expand previous evidence reporting *C4A*
240 overexpression in the total cerebral, prefrontal, anterior cingulate, orbital frontal, parietal
241 cortices, cerebellum and corpus callosum (Gandal et al., 2018b, 2018a; Schizophrenia
242 Working Group of the Psychiatric Genomics Consortium et al., 2016), *C4B* overexpression in
243 the PFC (Gandal et al., 2018b; Pérez-Santiago et al., 2012) and *C4A/C4B* overexpression in
244 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*
246 overexpression in BA10. This refines previous results reporting *C4A* and/or *C4B*
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 *shinyGEO*, 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.

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

258 Furthermore, we observed for the first time *C4* overexpression in the associative striatum
259 and STG (BA22) of SZ patients vs. HC. In the associative striatum, *C4* overexpression is
260 consistent with glial activation and neuro-inflammation, as well as increased expression of
261 pro-inflammatory transcripts in drug-naïve first episode psychosis (FEP) patients (Plitman et
262 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).

265

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
269 (Schizophrenia Working Group of the Psychiatric Genomics Consortium et al., 2016).
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,
278 association between modest brain expression changes and behavioral abnormalities have
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
282 (Ansoleaga et al., 2015; Gouvea et al., 2016). In this regard, while consistent with previous
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.

287

288 Altogether our results are consistent with the hypothesis of an excessive
289 Complement activity in schizophrenic disorders (Schizophrenia Working Group of the
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.
300 This may be due to the scarcity of brain collection tissues which are mainly obtained from
301 old subjects. Since our present results suggest that peripheral *C4* expression poorly reflects
302 *C4* brain expression, future research needs to gain access to *C4* expression in the central
303 nervous system during human adolescence. In this regard, measuring *C4* expression in
304 cerebrospinal fluid, although more invasive and expensive as compared to peripheral
305 measurements, may provide meaningful information about *C4* expression during the
306 synaptic pruning period occurring in adolescence.

307

308 Paradoxically, while increasing evidence implicate the hippocampus and the
309 cerebellum in SZ pathogenesis (Yeganeh-Doost et al., 2011; Nakahara et al., 2018; Lieberman
310 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

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

328 The absence of difference in *C4* expression in the lymphoblasts of SZ patients vs. HC
329 is consistent with a previous RNA sequencing study on LCLs (Sanders et al., 2013). In this
330 latter study, no difference in *C4* expression was observed although an overexpression of four
331 other Complement components was reported. In SZ disorders, these results suggest that *C4*
332 overexpression may be specific to the cerebral tissue while other alterations of the
333 Complement system may be detected at the systemic level possibly in relation with other
334 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 S3ria 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
350 product (Maier et al., 2009). Notably, while most studies investigating mRNA-protein
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

359 in brain or peripheral tissues of SZ patients vs. HC. It may thus be worthwhile for future
360 studies to look for a correlation between *C4* mRNA and *C4* protein level in a given tissue.

361

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
374 treatments on *C4A* expression in the PBMCs of SZ patients vs. HC (Melbourne et al., 2018).
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.

377 On the other hand, the biological heterogeneity of SZ patients may impact the observed
378 *C4* expression levels. Indeed, *C4* overexpression may be restricted to a subset of SZ patients
379 characterized by *C4* alleles generating high levels of *C4* mRNA (Schizophrenia Working Group
380 of the Psychiatric Genomics Consortium et al., 2016). This is in accordance with the concept
381 of SZ disorders as a group of heterogeneous disorders underpinned by various mechanistic
382 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).

388

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;
398 Sullivan et al., 2006) and that *C4* overexpression may be restricted to specific brain regions in
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).

407

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 SZ
431 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
449 replicate our results.

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

454 significant influence of pH on normalized *C4* expression in any of the brain regions or
455 peripheral tissues, except in the cerebellum, (ii) Another limitation is the lack of information
456 regarding potential confounding factors such as drug consumption, tobacco smoking or
457 alcohol abuse history even if no significant effect of smoking on *C4* transcript levels was
458 observed in the PBMCs of SZ patients (Melbourne et al., 2018). Brain tissue collections
459 associated with high-quality phenotype data are warranted to allow replication of the
460 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*
473 may be associated with the altered synaptic pruning in SZ and remain to be explored.
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
476 disorders.

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

483 In the light of these different limits, replication studies in larger, independent samples using
484 high-throughput sequencing techniques such as single-cell RNA-seq technology are needed
485 to confirm, refine and extend our results. In this regard, high-quality brain tissue collections
486 are needed to allow optimized cell dissociation and single-cell preparation, thus ensuring
487 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 <https://www.ncbi.nlm.nih.gov/geo/>, 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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839

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

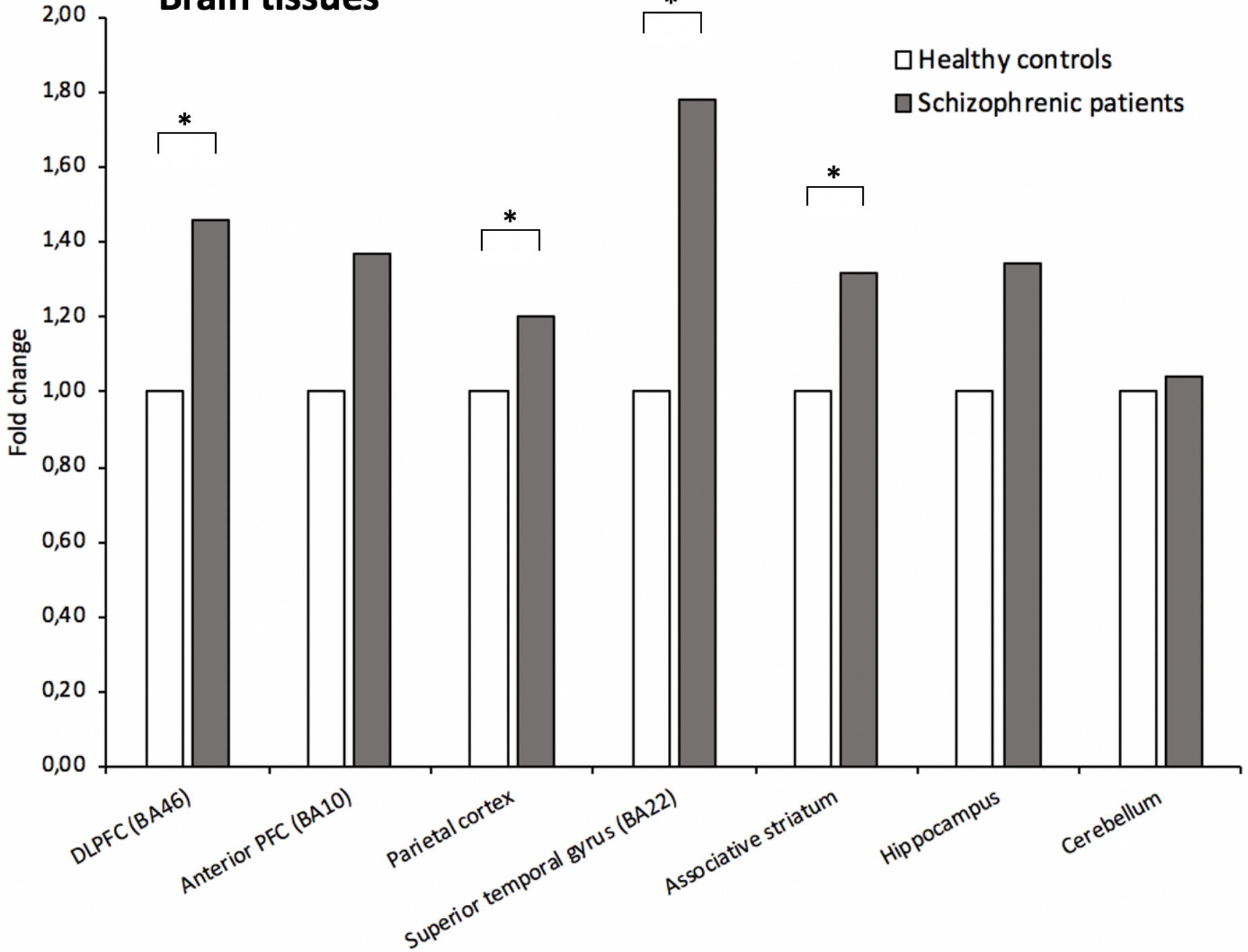
Demographic characteristics of the peripheral tissues samples.

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.

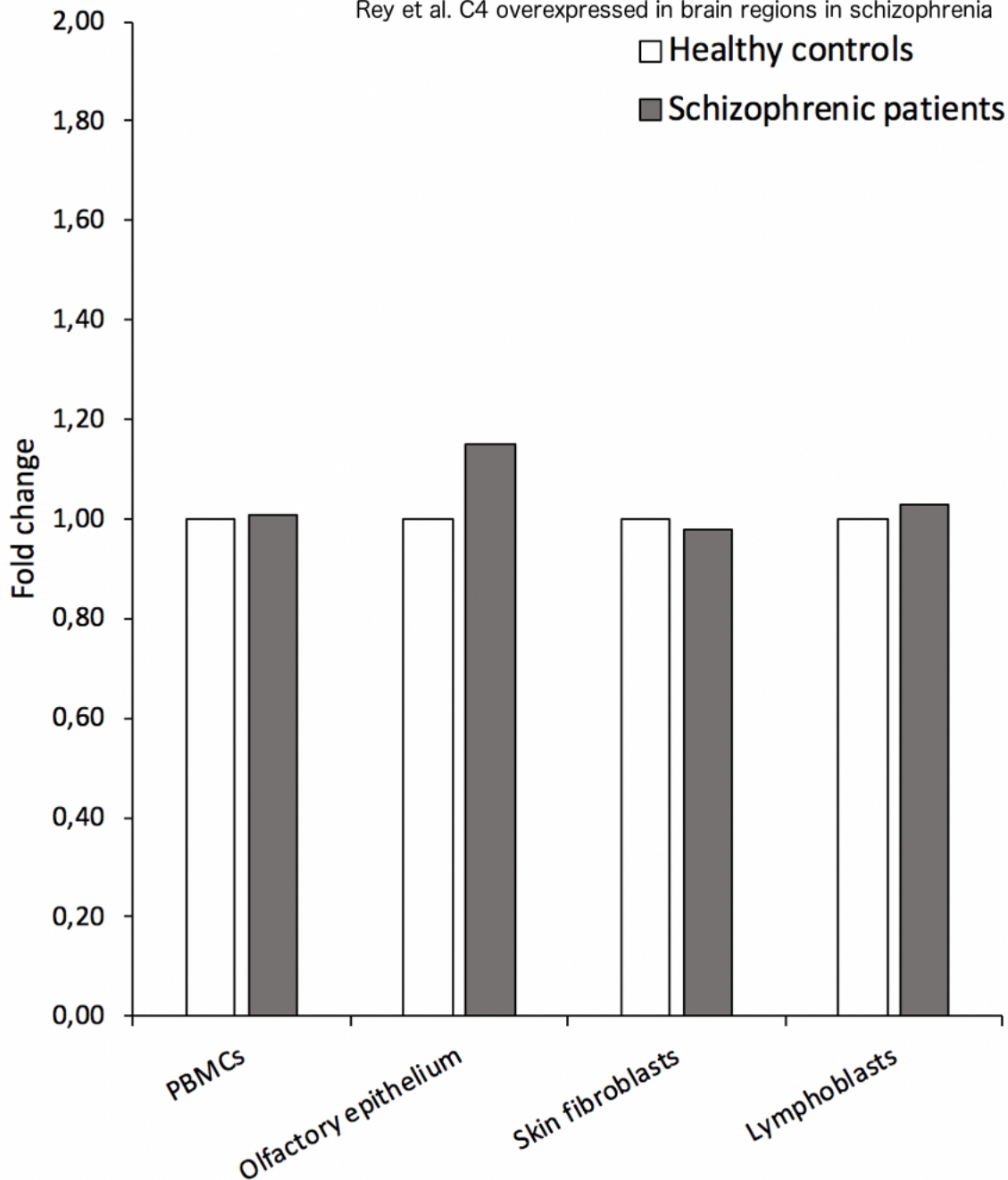
Fig. 2. Expression of *C4* in the peripheral tissues of SZ patients and HC subjects. Fold change in gene expression was provided by *shinyGEO* based on the processed gene expression datasets downloaded directly from GEO. * $p < 0.05$ vs HC subjects.

Brain tissues



Peripheral tissues

Rey et al. C4 overexpressed in brain regions in schizophrenia



Rey et al. *C4* overexpressed in brain regions in schizophrenia

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

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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	<i>p</i> -value ¹	Control	Schizophrenia	<i>p</i> -value ¹	Control	Schizophrenia	<i>p</i> -value ¹	Control	Schizophrenia	<i>p</i> -value ¹	Control	Schizophrenia	<i>p</i> -value ¹
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

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Table 3

Demographic characteristics of the peripheral-tissues samples.

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