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## **Immune signatures of treatment resistant schizophrenia: a FACE-SZ study**

### **Running title: Immune profile of treatment-resistant schizophrenia**

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## Abstract

Treatment resistant schizophrenia (TRS) affects around 30% of patients with schizophrenia resulting in poor functioning, relapses and reduced quality of life. Convergent findings show that inflammation could contribute to resistance. We thus search for immune signatures of patients with TRS/UTRS in a sample of community-dwelling outpatients with schizophrenia. 195 stabilized SZ patients (mean age = 31.2 years, 73% male gender) were consecutively included in the network of the FondaMental Expert Centers for Schizophrenia in France and received a thorough clinical assessment. At inclusion, psychotic symptomatology was evaluated by the Positive and Negative Syndrome scale for Schizophrenia (PANSS). Circulating serum/plasma levels of a large panel of markers reflecting the main inflammatory pathways were evaluated. TRS was defined by current treatment by clozapine and Ultra TRS (UTRS) by current clozapine treatment + PANSS total score  $\geq 70$ . The frequency of TRS and UTRS patients were respectively 20% and 7.7% and were defined using Multivariable analysis elevated by high levels of IL12/IL23p40, IL17A, IL10 and B2M and IL12/IL23p40, IL17A, IL6, IL10, IFN $\gamma$  and B2M respectively.

These observations suggest that resistance and ultra-resistance to clozapine treatment are underpinned by pro-inflammatory molecules mainly belonging to the T helper 17 (Th17) pathway, a finding making sense given the interplay between inflammation and anti-psychotic treatment responses. If confirmed, our findings may allow to consider IL-23/IL 17 pathway as a therapeutic target for patients with resistance to antipsychotics.

Keywords: schizophrenia, immuno-inflammatory, signatures, profile, Treatment-Resistant, clozapine

## Introduction

Treatment resistant schizophrenia (TRS) is usually defined as non-response to at least two first-line of antipsychotic treatments of adequate duration and dose with documented compliance<sup>1</sup>. TRS affects approximately 30% of all individuals with schizophrenia and results in poorer functioning, reduced quality of life and higher rates of unemployment, as compared to responders to treatment<sup>2</sup>, altogether leading to a 3 to 11 fold increase of health care costs in comparison to the overall patients with schizophrenia<sup>3</sup>. The only approved evidence-based treatment for TRS is Clozapine (CLZ). However, due to its adverse effects, CLZ is only licensed as a third-line treatment (NICE, 2014). Howes et al,<sup>4</sup> showed that CLZ is offered years after the onset of the disorder, with an average delay of 3.9 years. Nevertheless, as it has been shown that patients with a short delay before CLZ initiation experience a better prognosis<sup>5</sup>, it would be of the utmost importance to be able to identify early predictors of TRS<sup>6</sup>. Because of the specific role of CLZ in the treatment of TRS, it constitutes an often used proxy of resistance. A step beyond, ultra-treatment resistant schizophrenia (UTRS) designs cases of failure to respond to CLZ, a sub-group representing up to 60% of patients with TRS who do not respond<sup>7</sup>.

Identifying biomarkers of resistance would not only help in its early prediction but would also add to our understanding of underlying causes of TRS and inform development of future treatment. Current hypotheses for the biological basis of TRS focus on differences of dopamine functioning, changes in glutamate or of other pathways<sup>8</sup>. In particular, elevated levels of inflammation at baseline have clearly been suggested to contribute to resistance. Convergent findings show that inflammation plays a role in the pathogenesis of psychotic disorders<sup>9</sup> with increased cytokine levels in peripheral blood and cerebrospinal fluid, both at illness onset and in later stages, particularly in TRS. Neuroinflammation early in life followed by chronic overactivation has been hypothesized to contribute to the etiology of TRS<sup>1,10,11</sup>. We have previously shown that CRP us was associated with ultra-high resistance to SZ, defined by CLZ resistance<sup>12</sup>. However, this finding was not replicated in a sample of first episode schizophrenia who later developed TRS<sup>13</sup>. Strawbridge et al thus suggested that a composite measure of inflammatory markers would better contribute to predicting resistance in depression than single marker of inflammation<sup>14</sup>.

The objective of our study is thus to search for immune signatures of patients with TRS/UTRS in a population of 195 patients with schizophrenia assessed clinically and biologically tested for a large number of immuno-inflammatory markers.

## **Materiel and Method**

### ***Study population***

This is a cross-sectional study based on a national cohort issued from FondaMental Academic Centers of Expertise (FACE), the FACE-SZ cohort<sup>15</sup>. The FACE-SZ cohort is based on a French national network of 10 Expert Centers which is coordinated by the Fondation FondaMental ([www.fondation-fondamental.org](http://www.fondation-fondamental.org)) and funded by the French Ministries of Research and of Health in order to develop, establish and provide precision medicine and adaptive care for patients suffering from schizophrenia spectrum disorders. All individuals included were outpatients receiving stable medication for more than 4 weeks. A subgroup of 195 patients with thorough description of their current and past psychotropic treatments along with clinical assessment and measures of serum inflammatory markers were included in this analysis. The assessment protocol was approved by the relevant ethical review board (CPP-Ile-de-France IX, January 18<sup>th</sup>, 2010). All data were collected anonymously.

### ***Data collection***

Diagnostic interviews were carried out by psychiatrists according to the Structured Clinical Interview for Mental Disorders (SCID 1.0)<sup>16</sup>. Information about socio-demographic data, onset and course of illness, duration of untreated psychosis (DUP), comorbidities, in particular history of suicide attempt, lifetime alcohol and/or cannabis abuse, body mass index (BMI) were recorded. Psychotic and general psychopathology was assessed using the Positive and Negative Syndrome Scale (PANSS)<sup>17</sup>. Current depressive symptoms were evaluated using the Calgary Depression Rating Scale for Schizophrenia (CDRS)<sup>18</sup>. Daily global functioning was evaluated with the Global Assessment of Functioning Scale (GAF)<sup>19</sup>. Ongoing and past psychotropic treatment were recorded and self-reported adherence to pharmacological treatment was evaluated using the Medication Adherence Rating Scale (MARS)<sup>20</sup>.

### ***Biological data***

Circulating serum/plasma levels of a large panel of markers reflecting the main inflammatory pathways, including pro- and anti-inflammatory cytokines, chemokines, growth factors, adhesion molecules and non-specific inflammatory molecules were evaluated. More precisely IL1 $\alpha$ , IL1 $\beta$ , IL2, IL4, IL5, IL6, IL7, IL8, IL10, IL11, IL12-IL23p40, IL12p70, IL13, IL15, IL16, IL17A, IL21, IL22, IL23, IL27, IL31, IFN  $\gamma$ , TNF  $\alpha$ , TNF  $\beta$ , VEGF, MIP 1, MCP 1, GM-CSF, BAFF, BAFF, BDNF, eotaxin, eotaxin 3, ICAM1, IP10, M1, MCP4, MDC,

MIP1 $\alpha$ , MIP1 $\beta$ , MIP3 $\alpha$ , PD1, PDL2, SAA, TARC, and VCAM1 were measured using a MesoScale Discovery (MSD) human V-Plex electrochemoluminescence assay (MSD, Rockville, Maryland, USA) while soluble isoforms of beta 2 microglobulin (B2M), and HLA-E by commercially available ELISA kits (BioVendor, Brno, Czech Republic and Cloud-Clone Corp, Katy, Texas, USA respectively). Both methods were performed according to manufacturer's recommendations. Biological markers with more than 15% of missing data and cytokines with more than 50% of concentrations outside the limit of detection (LLOD) were excluded from the analysis. For the remaining cytokines, concentrations below or upper limit of detection were respectively replaced by LLOD divided by 2 and by maximum value +1.

***Definition of response to treatment status:***

***TRS***

Treatment-Resistant schizophrenia (TRS) was defined by current treatment by clozapine.

***UTRS***

Among TRS individuals, Ultra Treatment-Resistant schizophrenia (UTRS) was defined by failure to respond to CLZ as reflected by a mean PANSS score  $\geq 70$ , which corresponds to “moderately to severely ill” patients.

***Statistical analysis***

Categorical variables are presented using frequency distribution and continuous variables by measure of means (SD) or median (IQR) depending of the variable's distribution. Normal distribution was examined using the Shapiro-Wilk test. Comparison between TRS and non-TRS individuals, i.e. good responders as well as between UTRS and non-UTRS individuals regarding demographic and clinical characteristics were performed using the Chi-square or Fisher exact test for categorical variables and Student *t* tests or Mann-Whitney Wilcoxon test for continuous variables. Immuno-inflammatory makers were  $\log(1+x)$  transformed and separate multivariable models for each immuno-inflammatory marker were performed. Variables significantly associated with TRS/UTRS in univariate analysis were included in the multivariable analysis of covariance model. As more traditional levels (such as 0.05) can fail to identify variables known to be important, sensitivity analysis including variables with *p* value  $<0.20$  in univariate analysis were performed. Multivariable analyses were then adjusted for (i) age, gender, age of SZ onset, DUP, psychotic symptomatology, global functioning, history of suicide attempt, current smoker, lifetime cannabis abuse, first generation



antipsychotic, antidepressant medication, anticholinergic treatment and the number of baseline psychotropic medication for TRS and (ii) age, gender, age of SZ onset, DUP, duration of illness, psychotic symptomatology, global functioning, depressive symptoms, history of suicide attempt, current smoker, lifetime cannabis abuse, body mass index, anticholinergic treatment and the number of baseline psychotropic medication for UTRS. All statistical tests were two-tailed, with  $\alpha$  level set at 0.05. Data were conducted using R software.

## Results

We included a sample of 195 SZ patients with a mean age of 31.2 (sd=8.3) years (y), mean illness duration 10y (sd=7.9) and a majority of men (73%). The mean PANSS total score was 62.9  $\pm$  19.6. The frequency of TRS and UTRS patients were respectively 20% (n=39) and 7.7% (n=15). The mean duration on clozapine treatment was 34 month (sd=45) for TRS individuals and 45 month (sd= 42) for UTRS patients.

Individuals with TRS had a significantly younger age at onset (18.6y  $\pm$ 3.3 vs 22.4y  $\pm$ 6.5; p=0.0004) and a shorter Duration of Untreated Psychosis (DUP) (1.7y  $\pm$ 4.5 vs 2.0y  $\pm$ 4.7, p=0.0450) as compared to good responders. They also were less likely to be smokers (33.3% vs 53.8%, p=0.0254) and to have lifetime cannabis abuse (20.5% vs 34%, p=0.0354). No association with other clinical variables was observed (Table 1). In comparison to good responders, UTRS individuals had significantly earlier age at onset (18.7y  $\pm$ 3.4 vs 22.4y  $\pm$ 6.5; p=0.0236), shorter DUP (0.35y $\pm$ 1.3 vs 2.0y  $\pm$ 4.7, p=0.0052), lower global functioning, higher depressive symptoms and were more likely to have a history of suicide attempt (40.0% vs 15.4% in UTRS and good responders respectively, p=0.0340). As expected, they had persistent psychotic symptomatology for total and all sub-scores of the PANSS scoring scales. Similarly to TRS, they were less likely to be smokers, to have lifetime cannabis abuse and more likely to be treated with anticholinergic medication.

Table 2 showed the results of univariable analysis of potential associations between immunoinflammatory markers and TRS and UTRS subtypes of patients as compared with the good responders subset. We found that both TRS and UTRS individuals had significantly higher levels of IL12/IL23p40, IL17A, IL6, IFN $\gamma$  and B2M as compared to good responders alongside a trend for increased expression of TNF $\alpha$  which however failed to reach statistical significance. Moreover, TRS individuals exhibited higher level of IL10. The multivariable

analysis confirmed the associations of high levels of IL12/IL23p40, IL17A, IL10 and B2M with TRS ( $p=0.0036, 0.0138, 0.1935, 0.0347, 0.0715$  and  $0.0041$  respectively) and of IL12/IL23p40, IL17A, IL6, IL10, IFN $\gamma$  and B2M with UTRS ( $p=0.0144, 0.0026, 0.0498, 0.0435, 0.0012$  and  $0.0019$  respectively), in comparison to good respondents (Table 3), both after adjustment for potential confounders i.e. age, gender, age of SZ onset, DUP, psychotic symptomatology, global functioning, history of suicide attempt, current smoker, lifetime cannabis disorders, first-generation antipsychotic, antidepressant medication, anticholinergic treatment and the number of psychotropic medication. Additional adjustment for depressive symptoms and body mass index were performed for UTRS. Further cross-analysis of potential interrelationships between the herein associated immuno-inflammatory markers and TRS/UTRS, showed that except IL6, which is only associated with IL10, all other inflammatory parameters are significantly related to each other, representing a network deserving to be dissected in future studies (Supplementary Table 1). Finally, concerning the other studied inflammatory markers, we did not observe any significant difference between the three groups.

Overall, it is important to mention that the increase of the majority of pro-inflammatory molecules (IL17A, IL6, IL10, and B2M) along with the severity status of resistance i.e TRS vs UTRS, strongly suggest the major implication of inflammatory processes in the mechanism of resistance to anti-psychotics in SZ (Figure 1).

## **Discussion**

Resistance to treatment (TRS) is a major topic in schizophrenia as it often contributes to clinical deterioration, increased number of hospitalizations, chronicity and poor quality of life<sup>21</sup>. TRS may be driven, at least in part, by deregulated immune processes reflected by fluctuating levels of inflammatory molecules<sup>10,12</sup>. We found that resistance and ultra-resistance to clozapine treatment are associated with high level of cytokines mainly belonging to the T helper 17 (Th17) pathway, a finding suggesting that inflammatory processes might be at work especially given the interplay between inflammation and anti-psychotic treatment responses<sup>22,23</sup>. In terms of immune signatures, it seems that TRS and UTRS are associated with elevated circulating levels of IL12/IL23p40, IL17A, IL10 and B2M and IL12/IL23p40, IL17A, IL6, IL10, IFN $\gamma$  and B2M respectively. In the two situations, the concomitant increased levels of IL17A, IL12/IL23p4, and IL-6 in the UTRS group, evoke the implication of the Th17 immune pathway which make sense both for inflammation and schizophrenia.

Indeed, Th17 subset produces pro-inflammatory cytokines, a property explaining their well-known link with both inflammatory/autoimmune disorders<sup>24</sup> and schizophrenia<sup>22,23</sup>. Th17 cells predominantly produce IL-17 along with IL-22 and TNF- $\alpha$ . Their development and differentiation require coordinated actions of multiple factors in particular of IL-6 and IL-23 as differentiation and maintenance elements respectively<sup>25</sup>. Due to its potent role in the maintenance of pathogenic Th17 cell populations, IL-23 is also recognized as one of the critical cytokine involved in autoimmune/inflammatory processes. IL-23-activated Th17 cells are known to be essential to sustain chronic inflammation in the brain during infection and auto-immunity<sup>26</sup>. IL-17 molecules are expressed on endothelial cells of the blood-brain-barrier (BBB) and can disrupt tight junction thus impairing its integrity<sup>27</sup>.

Dysregulations of the Th17 pathway in psychosis have been reported by several studies although measurement of circulating of IL-17 levels yielded discrepant results. Indeed, while high levels of IL-17, and high proportion of TH17 cells were found in drug naïve SZ patients<sup>28,29</sup> and in subjects at ultra-high risk of schizophrenia<sup>30</sup>, diminished levels have also been observed in first episode SZ patients<sup>31</sup> and in stable SZ patients<sup>32</sup>. Associations between elevated<sup>33</sup> or low IL-17 levels have been found with disease severity<sup>34</sup>. Finally, a recent meta-analysis of IL-17 levels in drug-naive first episode psychosis found no implications of IL-17 in the pathophysiology of SZ<sup>35</sup>. Altogether such controversial findings might be attributable to differences in study designs, methods of cytokine measurement and/or to patient's heterogeneity in terms of time of analysis, disease progression, treatment, and cognitive decline.

Beside increased level of IL-17, we also observed an upregulation of the common IL-12 and IL-23 cytokines' p40 subunit in the two resistant subgroups of individuals. IL-12 and IL-23 are two immunoregulatory cytokines mainly produced by dendritic cells and are both pivotal for anti-infectious immune responses. Both cytokines exist as heterodimers with a common p40 subunit. The p40 subunit mainly functions in conjunction with either p35 (to form IL-12) or p19 (to form IL-23) to support differentiation and maintenance of Th1 or Th17 cells, respectively<sup>36</sup>. While IL-12 promotes Th1 differentiation and IFN- $\gamma$  production, IL-23 contributes to Th17 development and function. Increased levels of these two cytokines were repeatedly reported in various immune settings including autoimmunity. Given the high expression level of IL-17a observed here, it seems plausible that the p40 subunit likely belong to the IL-23 cytokine. It is worthy to mention that increased levels of IL-23 have been found both in first-episode patients, in SZ relapsing patients whatever antipsychotic treatment<sup>37</sup> and in patients under Clozapine. However, the overexpression of INF- $\gamma$  observed only in the

UTRS subgroup cannot exclude a Th1 induction and hence an additional implication of IL-12 cytokine in the resistance to treatment processes. Altogether, these data suggest that activation of IL-17 pathways is possibly present from onset of the disorder, and seem to increase with the progression of the disorder, up to resistance and ultra-resistance. This hypothesis is totally in line with our observation of an increase of the level of pro-inflammatory markers along resistance worsening status i.e. responders, TRS and UTRS. One may thus hypothesize that when inflammatory processes exceed inter-individual homeostatic capacities, resistance to treatment appear and gradually worsen possibly explaining TRS and UTRS. One indirect example in favor of such hypothesis is the *in vitro* demonstrated capacity of Clozapine to mediate protective effects against inflammation-induced damage of Dopaminergic neurons by preventing microglial overactivation in experimental models<sup>38</sup>.

In addition, we found elevated levels of IL-6 in TRS and in UTRS which confirms converging evidence of implications of IL-6 in schizophrenia<sup>39</sup>. As mentioned above, IL-6 is one of the major effector cytokines of Th17 cells. However, it is also possible that the high levels of IL-6 observed in the UTRS subgroup may indicate a skewing toward a chronicity-related Th2 pathway.

We also observed high levels of IL-10 and B2M in TRS and UTRS. The up-regulation of the potent anti-inflammatory IL-10 cytokine may reflect the induction of counteractive homeostatic processes. Whereas that of B2M may reflect either a non-specific hyperactivation of T cells and/or age-dependent negative regulation of cognitive and regenerative functions, processes known to be operating in murine model of SZ<sup>40</sup>.

Beside the discussion around the possible therapeutic strategies to be developed to target and alleviate immune-related mechanisms of resistance, it is also important to take into account the pathophysiology of SZ *per se*. Schizophrenia is best conceived as a neurodevelopmental heterogeneous disorder, induced by environmental triggers, such as prenatal infections, interacting with specific immuno-genetic background<sup>41</sup>. Prenatal infections are known to predispose offspring to increased schizophrenia risk<sup>42</sup> and might lead to the initial differentiation of Th17 cells, via the induction of IL-6 and IL-1b, inducing upregulation of IL-23 expression<sup>43</sup>. In addition, genetic variations in the complement C4 cluster with consequent overexpression of the C4 molecules have been reported to be associated to SZ<sup>44</sup>, an important notion given the capacity of complement proteins to modulate the production of IL-17 and IL-23.

Altogether, as depicted by the above-mentioned cross analysis of inflammatory markers, a global pro-inflammatory pathway seems to underlie both SZ and resistance to treatment but

distinguishing which is the egg or the chicken remains to be elucidated. One may hypothesize that such global pathway induces a vicious circle of inflammation generating a progressively worsened resistance to treatment. Finally, even if the studied patients benefit from a yearly psychiatric and somatic examination, we cannot exclude that an underlying infra-clinic immune-related condition may sustain inflammatory processes contributing hence to the immune profiles observed herein and consequently to resistance to anti-psychotic treatment. Thus, personalized use of anti-inflammatory compounds may disrupt such self-sustaining processes.

Future longitudinal studies are warranted to follow IL-17-related changes according to treatment responses, as well as to stage progression and handicap along cognitive decline aggravation. If confirmed, our findings may allow to consider IL-23/IL 17 pathway as a therapeutic target for patients with resistance against antipsychotics especially as many anti-inflammatory drugs, have been proposed as add-on to treat SZ symptoms such as N-Acetylcystein which seem to reduce the production of IL-17. Future clinical trials targeting this pathway are also warranted to monitor its effects on resistance processes by themselves. Some limitations in our study should be noted: due to the cross-sectional nature of the study, we were unable to draw any firm conclusions concerning the causal nature of the associations observed. Longitudinal studies would be required for this purpose. Furthermore, our study was relatively small and was probably not representative of all patients with schizophrenia, particularly because institutionalized and hospitalized patients were not referred to the participating expert centers.

## **Conclusion**

The IL-23/Th17 axis seem to play a prominent role in the pathophysiology of schizophrenia, as in several chronic inflammatory disorders, including psoriasis<sup>45</sup>, multiple sclerosis and rheumatoid arthritis<sup>46</sup> where upregulation of this pathway induce a pro-inflammatory status. Here we have found that patients with resistant or ultra-resistant schizophrenia show an upregulation of this pathway.

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**Conflict of interest**

None

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Figure 1: Association between pro-inflammatory molecules (IL17A, IL6, IL10, and B2M) along with the severity status of resistance

TRS : Treatment-Resistant Schizophrenia ; UTRS : Ultra Treatment-Resistant Schizophrenia  
\*p value<0.05, \*\* p value <0.005

Supplementary Table 1: Correlation between immune-inflammatory markers

	<b>IL12/ IL23p40</b>	<b>IL17A</b>	<b>IL6</b>	<b>IL10</b>	<b>IFN</b>	<b>B2M</b>
<b>IL12/ IL23p40</b>	1	0.49 <0.0001	0.09 0.21	0.28 <0.0001	0.25 0.0003	0.30 <0.0001
<b>IL17A</b>		1	0.05 0.455	0.18 0.010	0.35 <0.0001	0.30 <0.0001
<b>IL6</b>			1	0.71 <0.0001	0.03 0.643	-0.01 0.867
<b>IL10</b>				1	0.16 0.022	0.12 0.098
<b>IFN</b>					1	0.51 <0.0001
<b>B2M</b>						1