UNIVERSITÀ DEGLI STUDI
DI GENOVA

**Scuola di Scienze Mediche e Farmaceutiche**
**CORSO DI LAUREA IN MEDICINA E CHIRURGIA**

**Tesi di Laurea**

Dipartimento di neuroscienze, riabilitazione, oftalmologia, genetica e scienze materno-infantili - DINOGMI

# "Innate Immunity in Psychotic Disorders"

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*Anno accademico 2022/2023*

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# 1. ABSTRACT

Schizophrenia (SZ) and bipolar disorder (BD) are severe mental illnesses with a multifactorial pathogenesis. Evidence in literature implicates a key role for neuroinflammation and immune dysfunction in the etiology of psychosis. Research has focused on microglia and on peripheral inflammatory markers, such as cytokines and C-reactive protein. Immune cells alterations have recently gained attention as important part of the more complex immune dysregulation in psychotic disorders. The literature on altered levels and functions of innate immune cells in psychosis is limited and inconsistent. In this study, we conducted flow cytometer analysis on NK cells and monocytes from blood samples of SZ and BD subjects (NK analysis: 19 SZ; 28 BD; 26 HC; Monocytes analysis: 8 SZ; 15 BD; 18 HC). NK data were then represented in a space with a reduced dimensionality by FlowSOM and tSNE algorithms. No differences were observed in levels of monocyte subpopulations between patients and controls. FlowSOM and tSNE analysis revealed lower levels of mature NK cells (CD56dim CD16+ CD57+ NKG2C+) and higher levels of less mature NK cells (CD56dim CD16+ CD57+ NKG2C-) in patients compared to controls. Additionally, bipolar patients exhibited elevated levels of the activating receptor NKG2C. This study provides evidence for the presence of abnormal NK cell levels in individuals with psychosis. The elevated levels of the “adaptive” receptor NKG2C suggest a potential role of environmental triggers, such as viral infections, in the development of psychosis.

# 2. SCHIZOPHRENIA AND BIPOLAR DISORDERS

## 2.1 EPIDEMIOLOGY AND DIAGNOSIS

Schizophrenia and bipolar disorders are severe mental illnesses and causes of disability and they have life-changing consequences.

One in a hundred people will develop schizophrenia in lifetime (1), whereas the lifetime prevalence estimated for bipolar disorder is 2,4% (2). A recent meta-analysis demonstrated a higher frequency of schizophrenia in men (3); females and males are affected equally in bipolar disorder type I, instead bipolar disorder type II is more common among females (4). The onset is typically in early adult life, with a peak in the twenties (4,5).

Affected patients show a reduced life expectancy, to which chronic medical conditions (i.e. cardiovascular diseases) and psychiatric comorbidities play a role. Life expectancy is reduced by 13-15 years in schizophrenia, whereas patients with bipolar disorder have twice the risk of death compared to the general population (6,7). In bipolar disorder suicide has a relevant impact; evidence shows that suicide rates among these patients are 20 to 30 times higher than the rates in the general population (8).

Schizophrenia is characterized by two major classes of symptoms: positive symptoms or rather delusions, hallucination and formal thought disorder, and negative symptoms which include lack of volition, reduced speech output and flattening of affect. This dichotomous distinction have been partially overcame by the three-syndrome model which segregates symptoms into three groupings: reality distortion (delusions and hallucinations), disorganization (formal thought disorder, disorganized behavior and inappropriate affect) and negative symptoms or clinical poverty syndrome (9,10). More recently, cognitive impairment has become part of the clinical features of schizophrenia. It is now well known that schizophrenic patients show poor performance on tests of executive function, long-term memory and sustained attention (5).

**Box. *DSM-5* Criteria for Schizophrenia**

Criteria A, B, and C must be fulfilled and other causes of symptoms excluded.

Two or more of the following symptoms must be present for a 1-month period or longer, and at least 1 of them must be item 1, 2, or 3:

1. Delusions
2. Hallucinations
3. Disorganized speech
4. Grossly disorganized or catatonic behavior
5. Negative symptoms, such as diminished emotional expression

Impairment in 1 of the major areas of functioning (work, interpersonal relations, or self care) for a substantial period since the onset of the disturbance.

Some signs of the disorder must last for a continuous period of at least 6 months. This 6-month period must include at least 1 month of symptoms (or less, if treated) that meet criterion A (active-phase symptoms) and may include periods of residual symptoms. During residual periods, only negative symptoms may be present.

Fig. 1: *DSM-5 Diagnostic Criteria for schizophrenia* (11).

Bipolar disorder is defined by the succession of recurring manic or hypomanic episodes with depressive episodes. The presence of manic episodes leads to bipolar type I diagnosis; patients tend to be overconfident, talkative, disinhibit and show decreased need for sleep, highly elevated mood or irritable mood. Delusions and hallucinations often occur in manic episodes. Bipolar II disorder, according to DSM-5, is defined by hypomanic episodes alternating with depression. The DSM-5 introduces the mixed features specifier, characterized by opposite polarity symptoms during a manic or depressive episode (12). Comorbidity with other psychiatric diagnoses and internal medical conditions is a frequent occurrence in bipolar patients. Patients with bipolar disorder have high rates of anxiety (70-90%), substance and alcohol abuse (30-50-%), personality disorder (20-40%) and ADHD (25-45) (13).

**DSM-5 diagnostic criteria**

*Bipolar I disorder*

Criteria met for at least one manic episode, which might have been preceded or followed by a hypomanic episode or major depressive disorder; a depressive episode or psychosis do not have to be present for a diagnosis.

*Bipolar II disorder*

Criteria met for at least one current or past hypomanic episode and a major depressive episode; no manic episodes.

*Cyclothymic disorder*

Hypomanic symptoms that do not meet the criteria for hypomanic episodes and depressive symptoms that do not meet the criteria for major depressive episodes in numerous periods (at least half the time) for at least 2 years (1 year in those aged ≤18 years); criteria for major depressive, manic, or hypomanic episodes have never been met.

*Other specified bipolar disorder*

Bipolar-like phenomena that do not satisfy the criteria for bipolar I disorder, bipolar II disorder, or cyclothymic disorder (ie, short-duration hypomanic episodes and major depressive episodes, hypomanic episodes with insufficient symptoms and major depressive episodes, a hypomanic episode without a previous major depressive episode, and short-duration cyclothymia).

*Unspecified bipolar and related disorder*

Symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning, but do not meet the full criteria for any of the disorders in the bipolar and related disorders diagnostic class.

Fig. 2: *DSM-5 Diagnostic Criteria for bipolar disorder* (13).

## 2.2 ETIOPATHOGENESIS

Studies of the etiopathogenesis of schizophrenia date back more than 50 years ago with the formulation of the dopamine hypothesis according to which alterations in both mesolimbic and mesocortical paths bring to positive and negative symptoms, respectively (14).

Dysregulation in subcortical dopamine is proven by several lines of evidence, but new imaging studies suggest a specific involvement of striatum and mesostriatal dopamine neurons. It has been shown that an increase in striatal dopamine synthesis and release occurs in psychotic patients, but the real question is how these alterations can result in psychotic symptoms (15). A possible answer lies in the physiological role of mesostriatal dopamine neurons in attributing salience to environmental stimuli. In psychosis the dysregulated release of dopamine becomes spontaneous and independent from stimuli and leads to aberrant assignment of salience to both external and internal irrelevant stimuli. In this context, delusional beliefs are supposed to be an attempt to give explanations for these experiences of aberrant salience (16). Moreover, dorsal striatum has a role in signaling threat-related information (17), so this is a possible explanation for why delusions are often

persecutory in nature. Mesostriatal neurons activity is also implicated in the discrepancy between expected and actual rewards, which is called “reward prediction error signal”. The brain uses this discrepancy to update its model of the world. Hallucinations can arise from greater ability of prior expectations to influence perceptions (15).

In addition to dopamine, also glutamate has been implicated as a key neurotransmitter in the pathogenesis of schizophrenia (5).

Findings from brain imaging help explain cognitive impairment and the more recent view of schizophrenia as a neurodegenerative disorder. Patients show a reduction in brain volume, which affects gray matter more than white matter and particularly involves the frontal lobe (18). A reduction of gray matter volume is physiological over adolescence and early adult, due to synaptic elimination or pruning and the reorganization of both structural and functional brain networks. A pathogenetic hypothesis suggests that these processes are disrupted in schizophrenia and leads to cognitive deficits. In support of this theory, post mortem studies have found a lower density of dendritic spines in pyramidal neurons. Eventually, another mechanism potentially implicated is a disbalance between excitatory and inhibitory signals, coming from abnormalities of NMDA receptor and glutamatergic signaling (11).

A reduced cortical thickness of frontal, temporal and parietal cortex has been also found in brain of bipolar patients. In addition, there was evidence of an association with long duration of illness, which causes to think that neuroprogression may have a role in duration and episodes of disease (19).

Bipolar disorder is also associated with hyperactivity of the Hypothalamic-Pituitary-Adrenal (HPA) axis activity, but it is still unclear whether this dysfunction could be a risk factor, a pathophysiological mechanism or a consequence of the disease. Patients show higher levels of basal cortisol and ACTH and increased response to the Dexamethasone/CRH test. These results seem to be more prominent among manic patients, but it is also evident in euthymia (20).

### 2.2.1 GENETICS

It is well known that both pathologies have high genetic predisposition, which make inherited genes prime suspect in the etiology of both diseases.

Results from the largest genome-wide association study indicate that schizophrenia is a polygenic disorder coming from the effects of minimum 108 genetic loci. Genes highlighted in this study include the Dopamine receptor D2D2, are involved in glutamatergic neurotransmission, synaptic plasticity and ion channel signaling, as they have a role in immunity, such as B-lymphocyte lineages and the complement pathway (21).

Some genetic variants involving deletion or duplication of sections of DNA (copy number variants) are associated with an increased risk of Schizophrenia. One of the most known is the deletion at chromosome 22q11.2, which is one of the strongest risk factors for schizophrenia. Indeed, the relative risk in a patient with 22qDS is about 20 to 25 times the lifetime general population risk (22).

Schizophrenia has been also associated with genetic markers across the major histocompatibility complex (MHC) locus, which spans several megabases of chromosome 6. This relationship has not yet been fully explained, but a good amount of studies shown that diverse alleles of the complement component 4 (C4) within the MHC locus may have a role. In particular, C4 expression seems to be elevated in brain from schizophrenic patients. C4 has been also implicated in the pruning of synapses by microglia phagocytosis during adolescence, a process that is disrupted in schizophrenia. Thus, a cooperation between neuron and microglia via an aberrant functioning of complement system may contribute to schizophrenia pathogenesis (23).

Genetic heritability is stronger in bipolar disorder, where accounts for 70-90% of cases. A clear Mendelian pattern of transmission hasn’t been recognized, indicating a multifactorial and more complex pathogenesis. The most recent genome-wide association study in 2019 identified 30 significant loci, even though we are far from conclusions about specific risk genes. Three genes have been investigated: ANK3, which encodes Ankyrin B, a protein involved in axonal myelination, CACNA1C, shared with Schizophrenia, and TRANK1, which encodes an uncharacterized protein that may play a role in maintenance of blood brain barrier. Interestingly, some of these genes are involved in pathways controlling insulin secretion and endocannabinoid signaling (24,25).

2.2.2 ENVIRONMENTAL FACTORS

Genetic findings aren't enough to explain the pathophysiology of psychosis. Literature suggests that genetics and environmental factors act together in increasing the risk of disease development. This is the so-called "Neurodevelopmental hypothesis" by which adverse conditions lead to abnormal brain development during the prenatal and perinatal period, whereas symptoms of the disease appear in early adulthood after the synaptic pruning mentioned above. This hypothesis of early stress experience is consistent with the finding of an alteration in the neuronal network between prefrontal cortex and hippocampus, since the hippocampus has a key role in regulating stress response and providing feedback to the HPA axis (26).

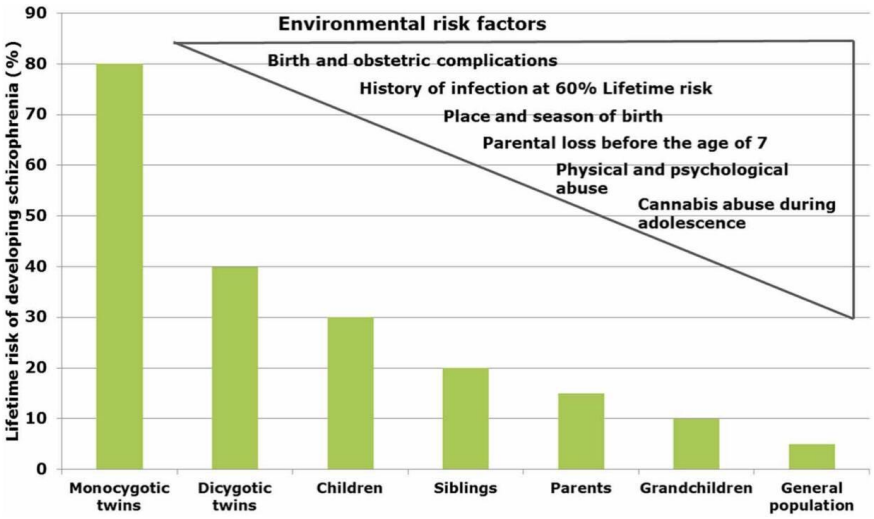



Fig. 3: *Interacting risk genes and environmental factors contribute to increase the risk of schizophrenia* (26)

Adverse childhood experiences, like physical and emotional abuse, have been associated to an increased risk for developing psychosis (27). Serious life events and maternal stress during prenatal period have also been related to schizophrenia, especially during the first and second trimesters. Retrospective studies also show that there is an association between famine and psychiatric disorder (28). This premature stress sensitization probably causes dysregulation of the HPA axis and contributes to dopamine alterations in mesolimbic areas, interestingly supporting the Neurotransmitter hypothesis.

Obstetric complications such as maternal hypertension, bleeding, premature rupture of membranes, polyhydramnios and asphyxia are associated to the development of schizophrenia (29). In particular, schizophrenia is strictly related to perinatal hypoxia (30), whom consequences are neuronal death, impaired myelination and reduced growth of dendrites (31). Apart from white matter and oligodendrocytes, also microglia is activated by hypoxic periods and can contribute to cell damage via synthesis of nitric oxide (32). Fetal growth and development have been related to psychotic disorders. Lower birthweight is a significant risk factor as premature birth and congenital malformations (29).

Maternal infections during pregnancy have gained interest as possible factors increasing the risk of neurodevelopmental injury and psychosis. Influenza, cytomegalovirus, Toxoplasma, herpes simplex virus are known to be potent disrupters of fetal neurodevelopment and for this reason are considered as major candidates in the development of schizophrenia. Several studies showed an increased risk of schizophrenia among individuals who were exposed to documented infections during gestation (33).

Otherwise, more recent findings from meta-analysis suggest caution. In a 2020 Meta-analysis only HSV-2 and infections NOS obtained significance (29). Supporting this view a Swedish Cohort study in 2015 showed that maternal infections during pregnancy were not statistically associated with psychosis, but the synergy between infections and maternal psychiatric disorders could act in psychosis development (34).

Few studies investigated the linkage between infections and bipolar disorder, even though an environmental exposure to infections (especially CMV and Toxoplasma) has been involved. A study of 1207 bipolar patients investigated antibodies to common infections from patient sera. Patients showed an higher CMV-positive and Toxoplasma gondii-negative IgG status compared to controls and this status was particularly associated with bipolar cases type I, non-early onset and history of manic psychosis (35).

To conclude, we may assume that an inflammatory condition secondary to infections can contribute to acute brain injury and then affects brain development. Brain

abnormalities may result from the activation of microglia and astroglia by cytokines levels elevation, in particular maternal levels of IL-8 and TNF-alfa were associated with psychosis in offspring (36).

Thus far all the findings from genetics and environmental factors suggest inflammation as a crucial player in the pathophysiology of schizophrenia and bipolar disorder. There is a flourishing literature investigating Neuroinflammation and alterations in immune system, but results are often conflicting and lack of evidence. We focus on this issue in the belief that can have a relevance in future prevention and treatment.

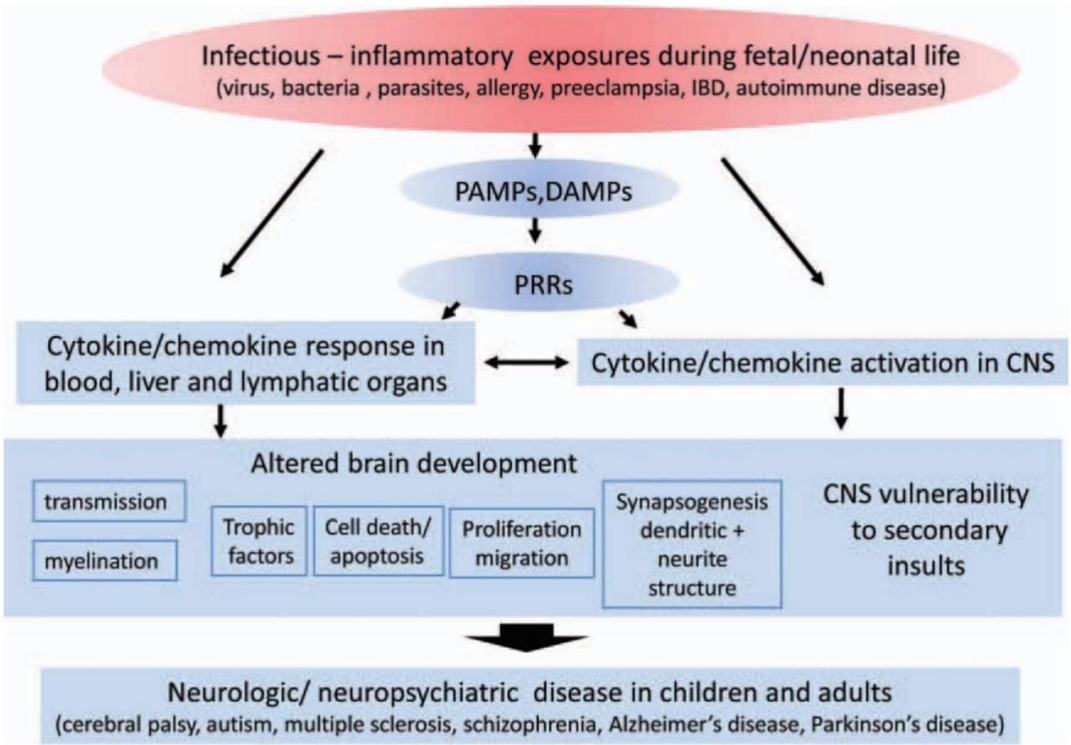



Fig. 4: *Short-term and long-term consequences of perinatal infection/inflammation* (36)

## 3. NEUROINFLAMMATION

The idea of an involvement of inflammation and immunity in the pathogenesis of psychiatric illnesses is well established. Studies about antipsychotic drugs gave the first clue of a possible role for the immune system in the pathophysiology. An immunomodulatory effect of antipsychotic medications had been known since 1950. Firstly phenothiazines and then clozapine were studied for their effects on immunity components, such as granulocytes and cytokines (37). Moreover, Horrobin and Lieb (1981) suggested that immune modulation may be one of Lithium mechanisms of action (38). Over time, several hypothesis about schizophrenia onset have succeeded: general inflammation hypothesis, macrophage-T cell hypothesis, autoantibody hypothesis and microglia hypothesis (39).

Another indicator for immune dysfunction is the high rate of inflammatory comorbidities in Schizophrenia and Bipolar Disorder, suggesting that it can be a common underlying cause. Such inflammatory comorbidities comprehend autoimmune disorders, cardiovascular diseases and metabolic disorders (40,41).

It is, then, clear that a chronic low grade systemic inflammation is a common basis for both schizophrenia and bipolar disorder (40,42).

The CNS has always been considered an immune-privileged site thanks to the blood-brain barrier (BBB) and the immunosuppressive environment created by neurons, glia and microglia (43). In contrast, different studies have shown that a communication between peripheral immune system and the brain parenchyma exist. Brain antigen and cells can reach lymph nodes from the CNS via perivascular spaces communicating with the subarachnoidal space. This state of privilege is not absolute, so it can be undermined since both peripheral and local immune stimulation can induce inflammatory reactions within the CNS. It is well known that common neurodegenerative disorders, like Alzheimer disease or Parkinson disease, are related with Neuroinflammation, with no exception of psychiatric illnesses (36,43).

Moreover, a neurovascular endothelial dysfunction and a hyperpermeability of the blood-brain barrier has been found in patients. Increased permeability can have a role

in facilitating communication between peripheral and brain immune system cells and contribute to the deleterious effects of brain inflammation (44).

Recent discoveries highlighted the importance of meningeal lymphatic vessels in regulating inflammatory processes and immune surveillance of the CNS. They have a role in draining macromolecules and immune cells from the CSF. In particular, T cells from meningeal lymphatic vessels have been demonstrated to reach deep cervical lymph nodes in a CCR7-dependent manner (45).

Several elevated inflammatory markers in the brain supports the presence of Neuroinflammation. Postmortem studies showed an increased expression of pro-inflammatory genes in the brain of schizophrenic patients, including Serpin family A member 3 (SERPINA3), interferon-induced transmembrane protein and the transcripts of TNAalfa, IL1beta, IL6 and IL8 (39).

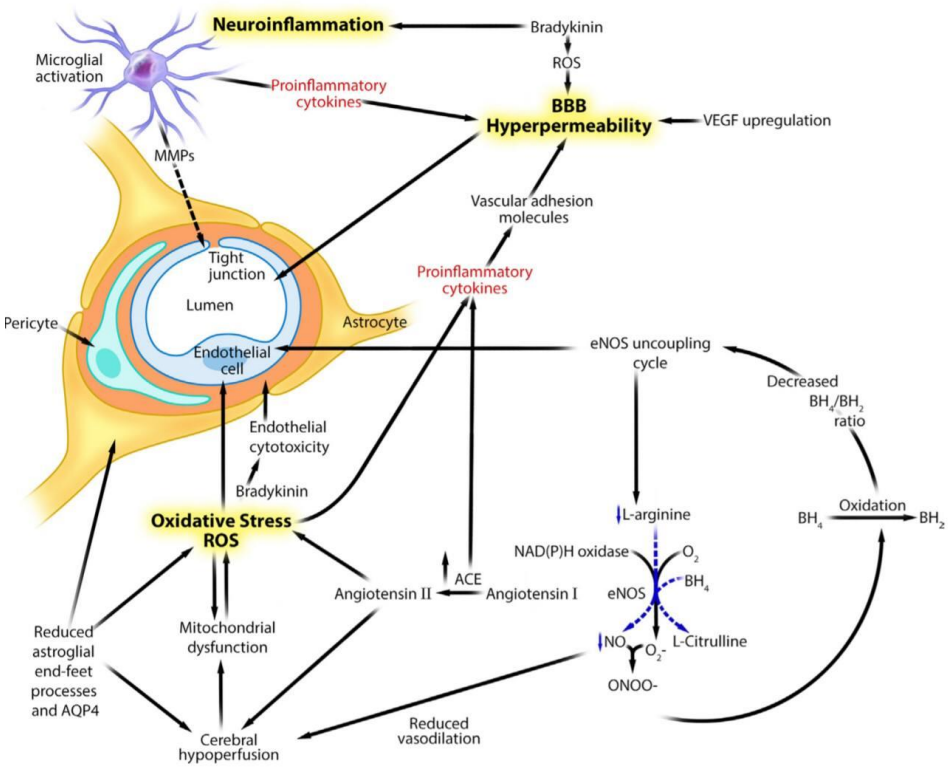



Fig. 5: *Theoretical integration of human and experimental data linking neuroinflammation, oxidative stress, and genetic factors to neurovascular unit dysfunction and blood-brain barrier hyperpermeability in schizophrenia* (44)

## 3.1 MICROGLIA

One of the fundamental aspects of Neuroinflammation is definitely the involvement of microglia. Microglia are the resident macrophages of the central nervous system. These cells present different morphologic phenotypes, ranging from amoeboid to varied degrees of cellular processes ramification. Depending on the location and the stimuli, they undergo morphological adaptions. In resting conditions they are characterized by small soma and elongated processes, while when they meet harmful stimuli they become more mobile.

Microglia have a phagocytic ability and a capacity to produce and release inflammatory mediators such as cytokines, chemokines, reactive oxygen species (ROS), but also growth factors. They exert a crucial modulatory role in formation/maturation of neurons and neural circuits, contributing to brain homeostasis (46,47)

Under physiological conditions, microglia are involved in synaptic pruning in order to get rid of unused neural circuits and to prioritize the most important ones. In case of chronic inflammation, microglia are over-activated and aberrantly prune relevant circuits implicated in the pathogenesis of schizophrenia and bipolar disorder. Microglia release cytokines, which increase inflammation and further microglia recruitment and activation, all resulting in a positive feed-forward loop. Over-reacted microglia also increase the releasement of reactive oxygen species and lead to a local oxidative stress. Oxidative stress is responsible too for brain damage and neurodegeneration and contributes itself to increase inflammation. (40).

Post-mortem and PET studies investigated microglial changes in density and potential signs of microglia activation. Many post mortem studies of brain samples from schizophrenic patients showed a trend in increased density of microglia. A review in 2016 found that microglial markers were increased in 11 studies and unchanged in 8 studies (48). A meta-analysis in 2017 found an increased density of microglia especially in the temporal cortex (49).

Another study found increased expression of CD64 in the dorsal prefrontal cortex of schizophrenic patients, while it did not show any differences for other markers of microglia. CD64 is a high-affinity activating receptor and its increase may imply the

presence of elevated monomeric IgG in the brain, all supporting the theory of inflammation in schizophrenia (50).

Concerning bipolar disorder, to the best of our knowledge there are no studies with solid results. It seems for now that microglia in brain from bipolar patients are not different in numbers and are not immune activated compared to controls (51).

To detect microglia, PET studies used a tracer targeting TSPO, a translocator protein that is expressed in activated microglia. Some studies found increased microglia TSPO expression in schizophrenic patients (52), increased microglia activation in the hippocampus of bipolar patients compared to healthy controls (53), while others did not confirm this finding (54). These conflicting results can be explained by different binding affinity of the radioligands, stage of disease and use of medications. In summary, the available data to our knowledge are not sufficient to correlate disease progression or severity of symptoms with microglia-mediated inflammation (46).

Besides microglia, other immune cells were found in brain tissue of patients, such as perivascular macrophages and increased density of T-lymphocytes and B-lymphocytes (55).

## 3.2 PERIPHERAL INFLAMMATORY MARKERS

Inflammatory proteins have been investigated as possible markers to testify the presence of inflammation in psychotic disorders. C-reactive protein is one of the most important acute-phase proteins and it is produced in response to an inflammatory stimulus, primarily induced by IL-1beta and IL-6.

CRP has been demonstrated to be significantly elevated in both acute and chronic schizophrenic patients (56). The same finding can be found in bipolar patients, where PCR levels were substantially elevated during manic phase and moderately elevated during depression and euthymia. Additionally, CRP concentrations were higher in unmedicated patients (57).

Some studies investigated whether there is a relationship between increased CRP levels and psychotic symptoms. A large cohort study of schizophrenic patients showed that positive and negative symptoms were significantly related to CRP (58).

Concentrations of CRP have been associated to brain pathology and inflammation. Increased levels of CRP were inversely correlated with cortical thickness in frontal,

insula and temporal brain regions of schizophrenic patients (59). Moreover, elevated concentrations of CRP has been related to lower cognitive functioning in both patients with schizophrenia or bipolar disorder. The cognitive assessment was measured with the Repeatable Battery for the Assessment of Neuropsychological Status, RBANS. The relationship of this finding is still unclear (60).

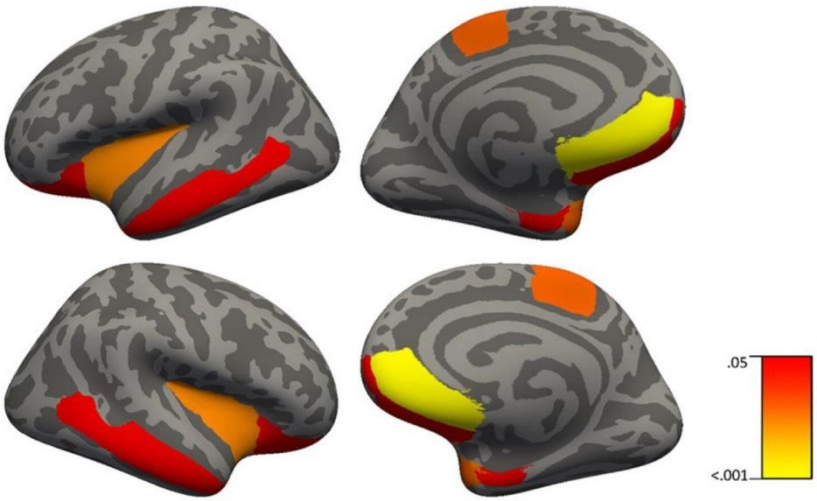



Fig 6*: Brain regions in which C-reactive protein significantly predicts cortical thickness in patients* (59)

The complement system has been investigated since it is known that C4 component has a role in synaptic pruning, as we have seen above. Despite being relevant in the pathogenesis of Schizophrenia, serum levels of C3 and C4 components seem to not differ between schizophrenic patients and controls (61).

### 3.3 CYTOKINES AND CHEMOKINES

Cytokines are the core of inflammation and for their relevance they have been largely investigated in literature. Cytokines are small circulating proteins that allow all immune cells to communicate each other. Since they are key messengers in the cross-talk between CNS and immune cells, they are supposed to mediate the effect of stressors on the development of psychosis. They can have either a pro-inflammatory or anti-inflammatory effect, the same cytokine can act on different cell types

(pleiotropic action) and have overlapping actions (redundant action). Besides fibroblast and endothelial and epithelial cells, the main producers are white blood cells, in particular T helper cells and macrophages (56,62).

Countless studies have reported aberrant levels of peripheral cytokines in both schizophrenic and bipolar patients, but despite the number of studies results are controversial.

A recent meta-analysis show that levels of IL-1beta, IL-1RA, sIL-2R, IL-6, IL-8, IL-10 and TNF-alfa were significantly elevated in both acute and chronic schizophrenic patients, so they can be hypothesized to be trait markers. IL-2 and IFN-gamma were increased in acute schizophrenic patients, instead IL-4, IL-12 and IFN-gamma were decreased in chronic patients, suggesting a potential role as state markers. The suspected state markers are known to have a role in stimulating proliferation of lymphocytes and natural killer cells (56). Abnormalities in cytokines levels imply a underlying and more general immune dysregulation, involving activated T lymphocytes and macrophages, as proposed by Smith and Maes (63).

IL-6 and TNF-alfa concentrations were increased in subjects with bipolar disorder. TNF-alfa was elevated in both depressive and manic episode, potentially representing a state markers, while IL-6 was elevated also in euthymic patients, suggesting a role as trait marker. Unexpectedly, no differences in IL-1beta were found between patients and controls (64). A meta-analysis showed that also IL-4, sIL-2R and IL-10 levels were increased in bipolar patients compared to controls (65). All these findings support the hypothesis of a low-grade inflammation caused by pro-inflammatory cytokines with an anti-inflammatory counterpart.

A systematic review focused on chemokines involvement. Chemokines are cytokines with the capacity to induce chemotaxis to the sites of inflammation or injuries. First-episode psychosis and multiple-episode schizophrenic patients showed elevated levels of monocyte-chemoattractant protein-1 (MCP-1), while only multiple-episode schizophrenic patients showed elevated levels IL-8, eotaxin-1 and macrophage inflammatory protein (MIP-1beta). Bipolar patients showed increased levels of IL-8 and MCP-1 during depressive episodes (66).

Peripherally circulating cytokines can also pass through the blood-brain-barrier and penetrate into the CNS (67). Aberrant levels of cytokines have been found in cerebrospinal fluid (CSF) of schizophrenic and bipolar patients. For example, a meta-analysis in 2018 found that IL-1beta CSF levels were increased in both disease and CSF levels of IL-8 and IL-6 were increased in schizophrenic patients (68).

To support the interconnection between peripheral inflammation and the CNS, a study provided evidence of increased IL-6 and TNF-alfa levels associated with higher white matter free water in schizophrenic patients, which is known to be related to inflammation (69).

Once in the brain, cytokines may alter the structure and function of key brain regions, over-activating microglia and lead to increased oxidative stress. Cytokines can directly and indirectly alter neurotransmitter levels, such as serotonin, dopamine and norepinephrine. Pro-inflammatory cytokines can also up-regulate HPA activity, which is known to have a role in pathophysiology of bipolar disorder (40).

### 3.4 THE KINURENINE PATHWAY

One of the possible candidates to explain the relationship between inflammation and brain alterations is the Kynurenine pathway. It is responsible for the degradation of tryptophan to generate tryptophan catabolites, known as kynurenines, instead of generating serotonin. It is largely regulated by cytokines, for example IL-2 and INF increase the activity of indolamine 2,3-dioxygenase (IDO), the enzyme which converts tryptophan into L-Kynurenine (40,70).

Some of the catabolites have a connections to the NMDA receptor and the glutamatergic neurotransmission, known to be implicated in the pathogenesis of psychosis. In addition, inflammatory cytokines can directly increase glutamate levels acting on NMDA receptors. Kynurenic acid is synthesized by astrocytes and it is an antagonist of the NMDA receptor, which confers neuroprotection. On the contrary, quinolinic acid is synthesized by microglia and it is a potent NMDAR agonist. This pathway has been linked to disruptive effects on neurons through oxidative stress and excitotoxicity.

Pre-clinical and clinical data suggest that alterations in Kynurenines may have a role in the pathophysiology of psychiatric illnesses. Increased levels of quinolinic acid

has been implicated in major depressive disorder, while strangely an increase in kynurenic acid has been associated with schizophrenia and bipolar disorder, despite the supposed role of neuroprotection. An influence of kynurenic acid on positive symptoms via NMDAR antagonism can help explain this finding (71).

As a matter of fact, meta-analysis in literature did not find solid conclusions. Most of the metabolites did not show any significantly differences between schizophrenic patients and controls. There was only a trend for increased kynurenic acid in CSF and lower levels of tryptophan. A reduction of tryptophan and kynurenic acid has been observed in bipolar patients (70,71).

Even though the role of the Kynurenine pathway is far from being understood, we can suggest that a pro-inflammatory state can divert tryptophan metabolism away from serotonin towards kynurenine in some psychiatric illnesses.

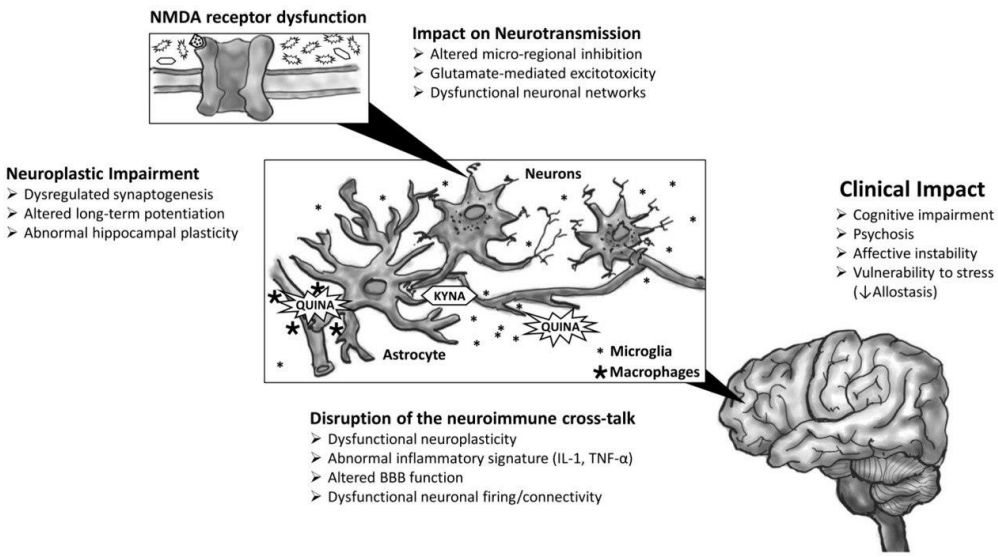



Fig 7: *Summarizes CNS factors that influence tryptophan dynamics* (70)

# 4. AUTOIMMUNITY AND PSYCHOSIS

The first hypothesis of an autoimmune basis of mental diseases dates back to 1930, when Hermann Lehmann-Facius suggested that schizophrenia could be the result of an autoimmune reaction with antibodies against brain tissue. Currently, some support the theory of an autoimmune pathogenesis of psychosis (41).

This idea has firm basis on epidemiological studies. Schizophrenia and bipolar disorder have increased prevalence of autoimmune diseases. A Danish study established that the prevalence of autoimmune diseases in schizophrenic patients is increased by 45% of the occurrence of an autoimmune disease (72).

Non-neurological autoimmune diseases increase the risk of developing psychosis and a meta-analysis found this to be increased by 43%. At the same time, schizophrenic patients have increased risk of subsequent autoimmune diseases and the same meta-analysis estimated it to be around 55% (73).

Schizophrenia and bipolar disorder has been associated with celiac disease, multiple sclerosis, systemic lupus erythematosus, Graves' disease, autoimmune hepatitis, Guillain-Barré syndrome and psoriasis. An association has been seen with autoimmune thyroiditis and type 1 diabetes, but with more heterogeneous results between studies. Rheumatoid arthritis has been negatively associated with psychosis; among more recent studies, some confirm this findings, while others find an increased risk of developing schizophrenia (40,41,73).

It has to be mentioned that some studies did not find evidence of these associations, especially in bipolar disorder. A Danish study found that there is no increase in risk of bipolar disorder associated with autoimmune diseases in general. There is a positive association with ulcerative colitis, psoriasis and rheumatoid arthritis, but only in the period close to the diagnosis. The strongest association was with Guillain-Barré syndrome and autoimmune hepatitis (74).

A possible factor supporting a link between psychosis and autoimmune diseases is the evidence of increased presence of autoantibodies in patients with schizophrenia. In particular, antinuclear antibodies, anti-cardiolipin IgG and IgM, anti-double-

stranded DNA (dsDNA) and anti-single-stranded DNA (ssDNA), anti-gliadin, anti-histone have been found (75).

Schizophrenic patients also showed the presence of antibodies to neuroantigens, in particular the N-methyl-D-aspartate receptor (NMDAR) antibodies have gained specific interest in literature. The NMDA receptors are glutamate-gated ion channel with a tetrameric structure composed by two NR1 subunits and two non-NR1 subunits, such as NR2A, NR2B, NR2C, NR2D, NR3A and NR3B. They have a role in brain plasticity thanks to their capacity to induce long-term changes in synapse structure (76).

NMDAR antibodies are known to be implicated in the pathogenesis of autoimmune encephalitis. Up to two-thirds of patients affected by autoimmune encephalitis initially exhibit psychiatric symptoms. These psychotic and catatonic symptoms seem to be caused by NMDAR antibodies (77). As a matter of fact, once bound, NMDAR antibodies induce NMDAR internalization, a reduced receptors density and eventually a reduced activity. In addition, according to glutamatergic hypothesis of Schizophrenia, what is thought to produce psychotic symptoms is exactly a lower activity of NMDARs (76). All these findings support a key role for anti-NMDAR antibodies in inducing psychosis.

To better understand the possible role of these antibodies in psychiatric diseases, several studies have investigated prevalence and titers of NMDAR antibodies in patients.

A couple of meta-analysis found increased prevalence of anti-NMDAR antibodies in schizophrenic and bipolar patients (78,79). Antibodies titers were found increased in patients with first episode psychosis and acute mania (78). Levels of anti-NMDAR antibodies were also found elevated in both schizophrenic and bipolar patients in a more recent umbrella review (80). At the same time, some studies failed to find any anti-NMDAR antibodies in patients (81).

We have to notice that also major depression and other psychiatry entities have been associated to alterations in glutamatergic neurotransmission, but the relationship with NMDAR antibodies is still unknown. Supporting this field, the current antidepressant treatment comprehends NMDAR inhibitors, such as Esketamine (79).

Other implicated neuroantigens to be thorough are: alfa-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR), dopamine D2 receptor, metabotropic GABA receptors, voltage-gated potassium channels, M1 and M2 cholinergic receptors (39). Some studies found also an increased prevalence of autoantibodies to glutamic acid decarboxylase (GAD) in schizophrenic and bipolar patients (82), just as they has been associated with limbic encephalitis (83).

# 5. ALTERATIONS IN THE IMMUNE SYSTEM

The immune system is our defense against pathogenic microbes, toxic and allergenic substances. The main feature of the immune system is the ability to distinguish essential features of pathogens or toxin from host cells. This provides the immune system the ability not to damage its own tissues. It is divided into two arms: innate (aspecific) system and adaptive or acquired system. The two systems are supposed to act together and amplify their responses one another (84).

The innate system represents the first line of defense and it is required to act rapidly, since it can recognize features expressed broadly on a large number of cells. It has also a pivotal role in repairing aspecific tissue damage (85).
It recognizes pathogens through the pattern recognition receptors (PPRs); PPRs can be soluble, like the mannose-binding lectin (MLB), or they can be membrane bound, such as the more famous Toll-like receptor (TLR).
The main cellular components of innate immunity are: macrophages, monocytes, NK cells, dendritic cells, neutrophils, eosinophils, basophils and mast cells (86).

The adaptive system becomes prominent after some days given that adaptive cells respond with specificity to individual antigens. Adaptive cells are also responsible for creating an immune memory. These characteristics make adaptive immunity more effective in host defensing (84).
Its activity is based on the antigen-specific receptors expressed by T-cells and B-cells, the two main cells populations of adaptive immunity. B-cells express the membrane-anchored immunoglobulin and produce antibodies, the humoral part of adaptive system. Initially, B cells differentiate to mature surface IgM and IgD expressing cells, a process that occurs in absence of antigen. Once activated by Th cells and cytokines, they undergo a isotype switching, which allows B cells to produce antibodies of different isotype, but the same antigenic specificity. At the same time, they undergo a process of affinity maturation, which gives B cells an increased affinity for the antigen and a proliferative advantage in responses (87).

T-cells express the TCR, a protein that binds processed antigens displayed by antigen-presenting cells (APCs). T cells are in turn divided into two major subsets, CD4+ T helper cells, which help to regulate immune responses also through cytokines secretion, and CD8+ T killer cells (88). A portion of the CD4+ T cells are called T regulatory (Treg) cells, due to their important role in down modulating immune responses. After exposure to antigen, CD4+ T cells differentiate into activated subpopulations depending on the cytokines present at the site of activation. Th1 and Th17 cells produce cytokines with pro-inflammatory effects, such as IL-2, IFN-gamma, IL-6 and Il-17, while Th2 cells produce mainly IL-4, IL-5, IL-10 with mixed effects. Th1 cells support cell-mediated immune responses, Th2 cells support humoral and allergic responses, Th17 help to recruit neutrophils.

Immune cells alterations have been studied as a part of immune dysregulation in psychiatric disorders. Abnormal leukocyte count has been historically found in depression, mania and schizophrenia (89,90).
Supporting this, a recent meta-analysis found increased total white blood cells in schizophrenic patients (91).
However, only few studies have investigated specific immune subpopulations disturbances so far and for this reason literature lacks of solid results.
Innate cells alterations are reviewed extensively in the paragraphs below.

## 5.1 INNATE IMMUNITY

### 5.1.1 NK CELLS

NK cells are CD3- and CD56+ lymphocytes with a spontaneous and selective cytotoxicity against transformed and infected cells. They play a crucial role in anti-viral immunity and immune surveillance (92,93).
More recently NK cells are regarded as the founding member of the innate lymphoid cell (ILC) family, even though other ILC family subtypes are characterized by a cytokine secretion profile (94).
NK cells development and maturation primarily lead to CD16-CD56bright and then to CD16+CD56dim subpopulations (95,96). They differ in CD56 expression levels;

moreover, the CD56bright population is more immunomodulatory compared to the more cytotoxic CD56dim population. The CD56dim population is the major component of peripheral blood NK cells, while the CD56bright population is more dominantly present in tissues (97).

NK cells function is strictly regulated by a balance between activating and inhibitory signals, so they recognize and then kill target cells, without prior sensitization. The ability to distinguish healthy "self" cells from abnormal cells comes from the expression of both activating and inhibitory receptors on the NK cell surface (93,97,98).

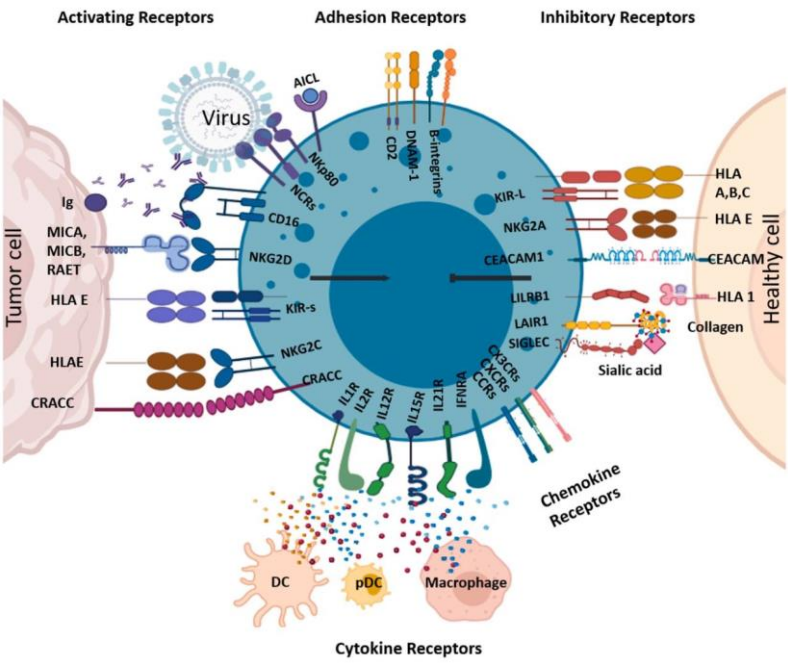



Fig. 8: *Regulation of NK cell via its surface receptors* (97)

MHC-I molecules, expressed by healthy cells, bind to inhibitory receptors, generating negative signals and inducing self-tolerance. On the contrary, target cells have reduced MHC-I molecules expression and show activating receptor ligands, which causes NK cell activation (92,97,99).

There are two major types of MHC-I binding receptors: the polygenic and polymorphic family of killer cell immunoglobulin (Ig)-like receptors (KIRs), both inhibitory and activating, and the non-polymorphic, heterodimeric C-type lectin-like

receptors CD94-NKG2. In the CD94-NKG2 family, the CD94 receptor is combined with either NKG2A (inhibitory) or NKG2C (activating). Whereas CD94–NKG2 receptors are invariant and bind to non-classical MHC-I molecules, such as HLA-E, KIRs bind to classical MHC-I molecules, like HLA-A, -B and –C (93,100,101). Another important family of activating receptors is the superfamily of natural cytotoxicity receptors (NCRs) (93,100).

Once NK cells find a target cell to kill, they release their granules containing perforin (a membrane-disrupting protein) and granzymes (a family of proteolytic enzymes). The granules are responsible for the specific lysis of the target cell (102–104). Activated NK cells also express death-inducing ligands, such as FAS ligand and TNF-related apoptosis-inducing ligand; death receptors on tumour cells bind to these ligands and activate the enzymatic cascade that causes apoptosis (104,105).

Once activated, NK cells are able to secrete cytokines and chemokines. IFNγ is the best known cytokine produced by NK cells, but they can also secrete interleukins (e.g. IL-10), TNF, growth factors and chemokines (93).

Recent discoveries have shed light on an adaptive role of NK cells (106). Adaptive NK cells are increased in cytomegalovirus (CMV) positive patients and persist long after the induction of the anti-CMV immune response (107). In reaction to a CMV infection, adaptive NK cells proliferate and differentiate upregulating HLA-E-specific activating receptor CD94/NKG2C (108). In the peripheral blood of CMV+ individuals, adaptive NK cells are defined as CD56dimCD16bright and display a highly differentiated surface signature (self-KIR+ NKG2A– LILRB1+ CD57+ Siglec7-) (109).

There are only few studies about NK cells and mental diseases. Findings are inconsistent and show little agreement. The results are different between schizophrenic and bipolar patients.

Two studies reported that there are no significant differences in the levels of NK cells between BD patients and controls (110,111). Furlan R. et al. confirmed this finding, but showed higher levels of CD56+ IL17+, CD56+ GMCSF+, and CD56+ INFɣ+, cells. These cytokine-producing NK cells are positively associated with DTI measures of white matter integrity and they play a role in functional connectivity and

response in brain areas with a task in defining emotional experience and mood control (112).

Snijders G. et al., on the contrary, found that higher levels of circulating NK cells in twin BD patients are associated with the liability to develop BD (113).

In schizophrenic patients conclusions are even more heterogeneous. One case-control study showed lower levels of NK cells in SCZ patients with acute episode; in chronically ill patients the NK cells levels normalized over time and treatment (114). Lower levels of NK cells in both medicated and drug-naive patients were also observed by Karpinski P. et al. in 2016 first (110) and then in 2018 (115).

Unlike studies above, the one from Fernandez-Egea E. et al. found that chronic treatment-resistant schizophrenic patients had increased relative numbers of NK cells (116), while in another study there was no evidence of alterations in NK cells levels. The same article investigated also NK cell activity (NKA), following the hypothesis by which an abnormal activity may play a role in the pathogenesis of schizophrenia. According to this study, schizophrenic patients show higher NKA than healthy controls (117).

Inconsistencies in literature may be explained by small size samples and the effect of antipsychotic medications, age, gender, illness duration and smoking. Several studies demonstrated a close correlation between NKA and smoking, especially smokers tend to exhibit reduced NKA (117). Steiner J. et al. found that cotinine levels are related to NK cells counts (118).

There is only one study that analyzed NK cells surface receptors in patients with first episode psychosis (119). CD3- CD56+ NK cells levels didn’t differ between patients and controls, but patients exhibited increased NK cells expressing HLA-DR. NK cells from patients overexpressed the activating NKG2C receptor, a specific marker of CMV and other viral infections. NKG2C expression in patients was surprisingly irrespective of CMV status, but correlated with HLA-DR and CD57 expression, two late activation markers that define the profile of an adaptive NK cells. Concerning NKA, SCZ-derived NK cells showed a suppressed capacity to mount cytotoxic responses with a target, while BD-derived NK cells showed an inability to produce IFN-γ.

5.1.2 MONOCYTES AND MACROPHAGES

Monocytes and macrophages with conventional dendritic cells are part of the "mononuclear phagocyte system". They are able to phagocytize apoptotic cells, cellular debris and microbes marked with immunoglobulin, complement or both (120).

Monocytes are circulating blood cells able to rapidly reach inflamed tissues where they exert both their pro-inflammatory or resolving activities (121). Macrophages are a tissue-resident immune population that have a role in both induction and resolution of inflammation. They have a role in regulating tissue homeostasis and they exhibit unique features depending on the tissue in which they reside (122).

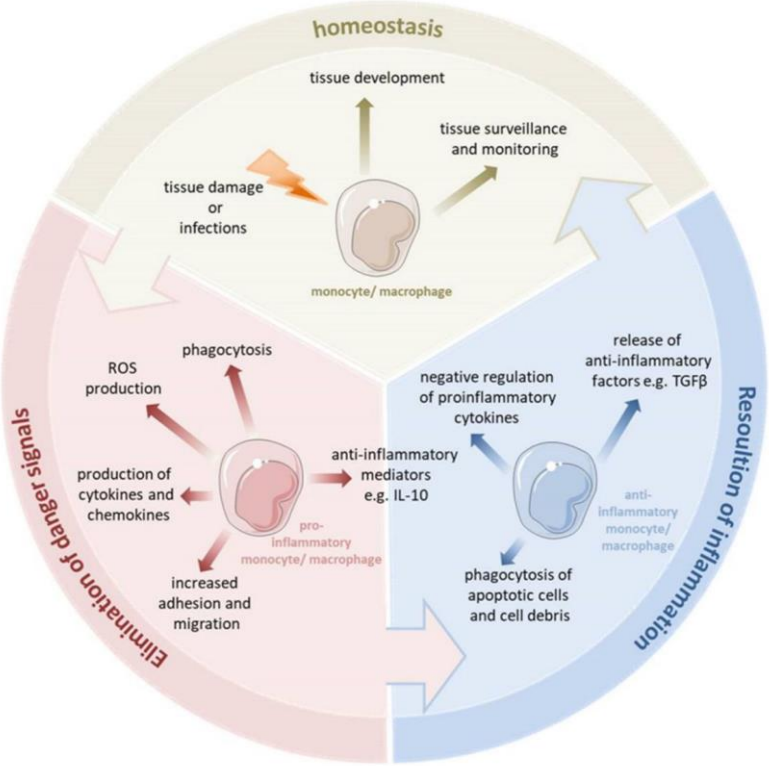



Fig. 9: *Depending on the kind of signal or pathophysiologic condition, monocytes and macrophages can undergo specific phenotypic polarization and thus acquire distinct functional phenotypes* (123)

Historically, monocytes were thought to be immature precursors of macrophages and dendritic cells. Therefore, the traditional view of macrophages origin believed that

tissue-resident macrophages develop from adult monocytes (124). Contrary to this belief, in the last few years, several studies have showed that some tissue macrophages origin from the yolk sac and populate tissues during embryonic development. These macrophages persist during adulthood independently of monocyte recruitment from blood. Examples of these yolk sac derived cells are Kupffer cells, microglia, Largerhans cells and cardiac tissue macrophages (125,126).

Monocytes are traditionally described as homogeneous HLA-DR+ cells with a kidney shaped nucleus. They are divided in three subpopulations depending on the surface expression of CD16 and CD14 markers. CD14+ CD16- monocytes are the so called "classical" monocytes, which represent the majority of the monocytes pool, the CD14low CD16+ monocytes represent the "non-classical" monocytes, while the CD14+ CD16+ monocytes are known as intermediate monocytes (121,127).

Macrophages show consistent plasticity too, as they can switch into different phenotypes with different functions. Classically activated macrophages M1 release pro-inflammatory cytokines such as IFN-gamma, IL-6, IL-12 and TNF, while alternative activated macrophages M2 are induced by IL-4, IL-10 and IL-13 and they exert anti-inflammatory functions via the production of IL-10, IL-1a receptor antagonist and TGF-beta (128).

Under physiological conditions, tissue-resident macrophages exhibit a M2 phenotype, promoting tissue homeostasis. Following infection or injury, M1 macrophages are induced by the engagement between PPRs and PAMPs, such as lipopolysaccharide (LPS). They increase microbicidal activity by producing oxygen and nitrogen radicals, a process which is amplified by T cells IFN-gamma secretion. During the resolution of inflammation, there is a shift to M2 phenotype in order to promote clearance of debris, wound healing and to restore tissue homeostasis (129).

Furthermore, macrophages have a significant role in orchestrating immune responses, as they act as antigen-presenting cells and they are involved in T cells differentiation via cytokines secretion (130).

Eventually, besides being active in acute inflammatory responses, they have a key role in chronic inflammation, which is responsible for the development and progression of autoimmune disease, allergies, granulomatous processes, chronic infections, but also cardiovascular diseases and cancer (123).

As mentioned earlier, the macrophage/T cell theory of depression and schizophrenia has been suggested first in 1992 due to the belief that chronically activated macrophages and T cells can have an impact on brain, predisposing it to develop psychosis.

Supporting this theory, several studies have succeeded showing quantitative and qualitative alterations in monocytes and macrophages.

Almost all the studies in schizophrenic patients showed increased levels of monocytes (131,132). Two different meta-analysis in 2020 confirmed this finding (91,133). Steiner et al. in 2019 showed higher monocyte count in both first episode patients and schizophrenic patients. They followed the same patients for 6 weeks and after this period they found decreased monocyte count in schizophrenic patients, even though the count did not reach controls' levels. Moreover, they associated cells counts with PANSS scores. They found that FEP patients with increased monocyte count showed significantly higher PANSS-P scores at baseline (134). Eventually, the study from Garcia-Rizo only found that monocyte count in patients showed an increased mean value with a trend towards signification ($p = .063$) (135).

Only one study found decreased levels of CD14+ monocytes in schizophrenic and bipolar patients. However, it has to be clarified that the study sample was very small (8 schizophrenic patients and 7 bipolar patients) (136).

Few studies can be found in literature about monocyte count in bipolar patients. The one from Barbosa et al. found higher percentages of monocytes in patients (137), while another one did not find any differences in the percentages of monocytes (138).

For the sake of knowledge, a study also investigated the levels of macrophages in the CSF of Schizophrenic patients. Cytological examination found that patients has a significantly higher proportion of macrophages in the CSF compared to controls (139).

Two different studies have investigated expression of immune genes in monocytes (140,141). In sum, they identified a monocyte inflammatory gene expression signature based on 34 aberrantly expressed genes. In particular, two main subsets of

genes were detected – Cluster 1 and 2. Cluster 1 was composed of pro-inflammatory cytokines and compounds, while Cluster 2 was made of adhesion/motility factors and chemokines. Interestingly, the aberrantly expressed genes were only partially shared between the two disorders. Monocytes from bipolar patients showed an activated gene expression set-point involving both Cluster 1 and 2, whereas the ones from schizophrenic patients showed only up-regulated genes from Cluster 1.

These studies suggest that monocytes from both schizophrenic and bipolar patients can show dysfunctional activation, but with differences between the two groups.

In this regard, Hughes et al. in 2021 aimed to identify any possible differences of functional monocyte responses across schizophrenia and bipolar disorder (142). They found that monocyte-induced macrophages from non-affective psychotic patients produced lower levels of innate cytokine under inflammatory conditions. The affective psychosis group showed a stronger response after inflammatory activation and under alternative activation AFF macrophages produced higher amount of inflammatory cytokines.

Another study focused on macrophages responses after stimulation in bipolar patients. A dysfunctional response was confirmed and a correlation with disease progression has been suggested. Indeed, macrophages from late-stage patients showed a decreased secretion of cytokines compared to early-stage patients (143).

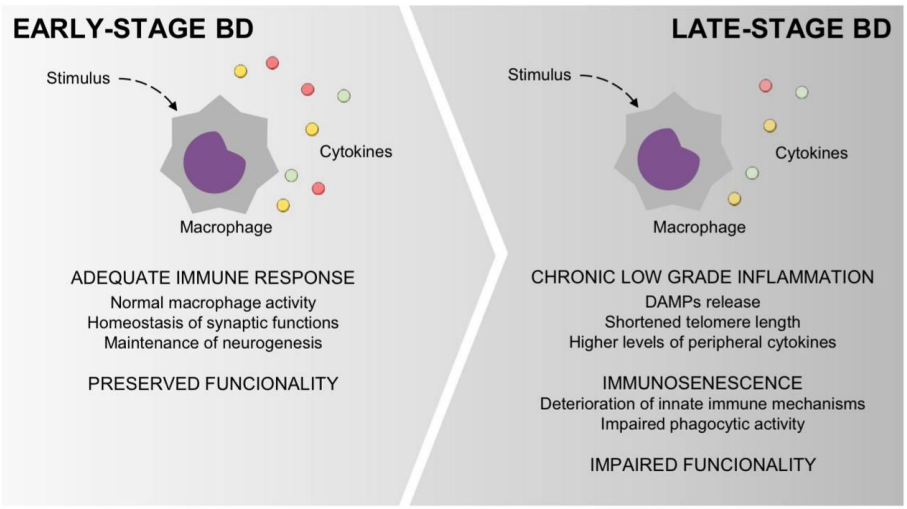



Fig. 10: *Macrophage activity impairment occurs in parallel with the progression of BD* (143)

The monocyte-to-lymphocyte ratio (MLR) is a simple and inexpensive test that has been studied in literature as possible marker of systemic inflammatory response. It has gained interest due to the fact that it may be more predictive in evaluating inflammation than immune cells separately. A meta-analysis from Mazza et al. in 2020 showed increased MLR in non-affective patients compared to controls (144). The same finding has been confirmed in 2022 in bipolar patients (145). For bipolar patients a higher MLR has been related to manic episodes and not to depressive ones (146).

The same studies supposed that increased MLR in patients could represent a peripheral marker of brain inflammation and in particular microglia activation.

### 5.1.3 NEUTROPHILS

Neutrophils are the most abundant components of granulocytic lineage and the predominant white blood cells in mammals. They are the very first cells recruited to inflamed or damaged sites (147).

Neutrophils are characterized by a segmented nuclei (usually 3-5 lobes), which gives them the name of polymorphonuclear cells (PMNs) (147). They develop in the bone marrow from a common myeloid progenitor cell through a process known as granulopoiesis. During their differentiation, they acquire their typical granules, which can be divided into cytoplasmic azurophil granules, specific granules and gelatinase granules. Azurophil granules are defined by the positivity for myeloperoxidase (MPO), but they also contain: cathepsin G, elastase, proteinase 3, bactericidal/permeability-increasing protein and defensins, which can permeabilize and kill a range of pathogens (148). Specific granules contain lysozyme, lactoferrin and lipocalin-2 to sequester iron, transcobalamin II, collagenase, phospholipase A2.

After their release from the bone marrow, they circulate in the bloodstream for less than a day, with a half-life of 19 hours (149). The neutrophil reserve in the bone marrow is 5-6 times larger than the pool of circulating neutrophils and they can be released in case of severe infection (150).

When a site is damaged or inflamed, local macrophages and mast cells secrete TNF-alfa, IL-1beta and other cytokines that activate endothelial cells and induce neutrophil extravasation. Firstly, neutrophils start to interact with endothelial cells via selectin-mediated binding and this interaction contributes to the stability of the rolling neutrophils. The integrin binding to the adhesion molecule ICAM-1 on endothelial cells allows neutrophils to stop and attach to endothelial cells. Eventually, neutrophils go through gaps between pericytes in the vessel wall and migrate into tissues (151).

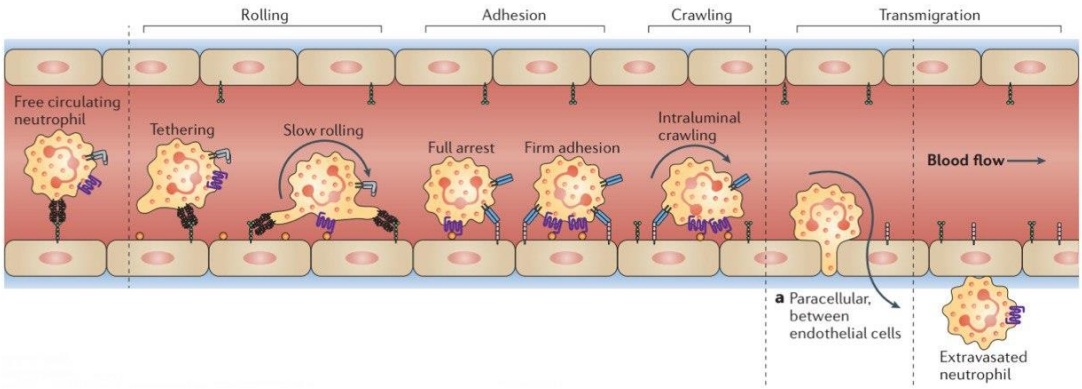



Fig. 11: *The updated classical neutrophil recruitment cascade* (152)

In tissues aged PMNs undergo spontaneous apoptosis within 24 hours and they are then phagocytosed by macrophages (153). In sites of inflammation, neutrophil apoptosis can be delayed in order to enhance clearance of pathogens (147).

Neutrophils can count on many microbicidal mechanisms to kill and remove infectious agents. When neutrophils meet pathogens, they phagocytize them. Once pathogens are in phagosomes, these cells kill them with NADPH oxygenase-dependent mechanism or other antibacterial proteins (152). The antimicrobical protein from granules can be released both in phagosomes and into the extracellular milieu through a process known as degranulation (152).

PMNs, as well as other granulocytes, express the NADPH oxidase, a multiprotein enzyme which synthetizes ROS. In resting state, these enzymes are segregated in specific granules; NADPH oxidase can be activated at the plasma membrane if the stimulus is soluble or it can be activated in the phagosomal membrane if activation follows phagocytosis (154). The final product of NADPH is superoxide anion, which

can be directly toxic to pathogens, but it is usually converted in more powerful ROS. Superoxide anion is converted to hydrogen peroxide by superoxide dismutase (SOD), another enzyme largely present in PMNs. Moreover, PMNs can release cytoplasmic granules including high concentrations of MPO, which catalyzes the production of other potent ROS (155).

Brinkmann and colleagues in 2004 discovered another killing mechanism which was given the name of neutrophil extracellular traps (NETs). They are weblike structures composed of DNA with histones, elastase, myeloperoxidase and cathepsin G that are meant to trap and kill bacteria extracellularly (156). Two main processes of NETs release has been identified. The lytic formation involves the neutrophil membrane rupture after chromatin decondensation, otherwise in the non-lytic release the chromatin is secreted via vesicles, allowing neutrophils to stay alive (157).

Despite being an essential component of innate immune system, neutrophil alterations are less studied in psychotic patients. Hereinbefore, findings in both schizophrenic and bipolar patients have showed increased levels of neutrophils.

A study in 2018 found higher levels of granulocytes in schizophrenic patients (115). The article from Garcia-Rizo et al. showed that non-smoking FEP patients had higher neutrophil count compared to controls (135). The article from Steiner et al. demonstrated that FEP and schizophrenic patients had significantly higher neutrophil count, which decreased after six weeks. Neutrophil count correlated with PANSS-P scores in FEP and schizophrenic patients (134). A meta-analysis has confirmed increased neutrophils in schizophrenic patients (91). Neutrophils has been demonstrated to be higher mainly in manic patients (146).

An interesting study in 2019 have demonstrated that a higher neutrophil count in FEP patients is associated with reduced gray matter volume and increased CSF (158). It can be hypothesized that this brain GM loss may be due to the disruptive effects of peripheral white blood cells on the central nervous system. The same study showed that neutrophil count was also associated with the total PANSS score.

Literature, similar to monocytes, has investigated the neutrophil-to-lymphocyte ratio (NLR). It is a marker of inflammatory state as well and it was originally used for monitoring critically ill patients (159).

In 2020 two different articles and one meta-analysis demonstrated higher NLR in schizophrenic patients compared to controls (134,144,160). Brinn and Stone also showed a positive correlation between mortality and NLR (160). This finding has been confirmed more recently by Bioque et al. in first episode psychotic patients. Moreover, they followed up the same patients for 24 months and mean NLR differences with controls became even higher (161).

Concerning bipolar disorder, a first meta-analysis was conducted in 2018. Bipolar patients showed higher NLR than controls; in more detail, a subgroup analysis revealed that the effect was significant only for studies including patients with manic or any bipolar phase (162). According to the cross-sectional study of Fusar Poli et al., manic patients expressed higher levels of NLR than depressed individuals, which confirms what has been said in the previous meta-analysis (146). On the contrary, Brinn and Stone and Dadouli et al. found significantly higher NLR in bipolar patients with no differences between manic and depressive episodes (145,160).

To sum up, elevated levels of NLR could suggest an imbalance in favor of innate immunity compared to adaptive immunity. Since higher levels are present even in FEP patients, NLR could be a marker of early stages of psychotic diseases.

# 6. AIMS OF THE STUDY

The aims of our study are: i) find quantitative and qualitative alterations in innate immune cells in psychotic patients, ii) identify unique immunologic biomarkers indicative of psychosis.

# 7. MATHERIALS AND METHODS

## 7.1 SUBJECTS

Participants were inpatients of the Psychiatric Clinic of the IRCCS Ospedale Policlinico San Martino (Genoa, Italy) with a diagnosis of schizophrenia and bipolar disorder according to the Diagnostic and Statistical Manual for Mental Disorders-Fifth Edition (DSM-5) criteria. Healthy participants were selected from the general population using advertising in the hospital and underwent a clinical interview to exclude the presence of lifetime or current psychiatric disorders.
Inclusion and exclusion criteria for patients are reported in the following table:

| Inclusion criteria | Exclusion criteria |
|---|---|
| Age > 18 years | Clinical conditions that generate risks for the patient or the stuff during experiments |
| Primary diagnosis of schizophrenia or bipolar disorder | Presence of current substance use history of drug and alcohol abuse |
| Presence of other psychiatric comorbidities | Presence of severe and/or acute medical comorbidities or any conditions that could affect the measured parameters |
| Written informed consent to participate in the study | Inability or refusal to provide a written informed consent to participate in the study |

The current state of the patients was clinically determined by scales: PANSS for schizophrenic patients, Hamilton rating scale for depression and mania rating scale for bipolar patients.
All the patients were under treatment with humor stabilizers, antipsychotics, antidepressants and benzodiazepine.

All the participants were thoroughly informed about study's aims and the procedures. The Ethical Committee of IRCCS Ospedale Policlinico San Martino approved the study, and all participants gave their written informed consent.

### 7.1.1 NK ANALYSIS

A total of 73 participants were enrolled, 19 schizophrenic patients, 28 bipolar patients and 26 healthy controls. Among bipolar patients, 12 were diagnosed with bipolar disorder type 1, 15 were diagnosed with bipolar disorder type 2, 1 patient was diagnosed with NAS mood disorder. Among the schizophrenic group, 6 were diagnosed with schizoaffective disorder and 5 were diagnosed with NAS psychosis. A total of 3 patients were a first episode psychosis patient. Age and duration of illness were heterogeneous within patients. Healthy controls were matched for sex, but not median age to patients.

| | Number | Gender | Age (mean, years) | Disease duration (mean, years) | First episode |
|---|---|---|---|---|---|
| Schizophrenic patients | 19 | 6F/13M | 36,5 | 14,4 | 2 |
| Bipolar patients | 28 | 12F/16M | 44,1 | 15,7 | 1 |
| Controls | 26 | 14F/12M | 30,9 | - | - |

Table 1. *Socio-demographics features of NK patients and controls*

### 7.1.2 MONOCYTES ANALYSIS

A total of 41 participants were enrolled, 8 schizophrenic patients, 15 bipolar patients and 18 healthy controls. Among bipolar patients, 8 were in a depressive phase and 5 were in a manic phase, while 2 were a first episode psychosis patients. Age and

duration of illness were heterogeneous within patients. Healthy controls were matched for median age and sex to patients.

| | Number | Gender | Age (mean, years) | Disease duration (mean, years) | First episode |
|---|---|---|---|---|---|
| Schizophrenic patients | 8 | 5F/3M | 49,25 | 16,85 | - |
| Bipolar patients | 15 | 8F/7M | 44,27 | 16,46 | 2 |

Table 2. *Socio-demographics features of monocytes patients.*

## 7.2 PROCEDURES

We performed a blood test for each participant for a total amount of 4 blood samples. About 20 ml of blood was collected in two heparinized tubes to carry out flow cytometry analysis. In addition, 20 ml of blood was collected in two tubes without anticoagulant in order to obtain serum samples and use them to study inflammatory markers (i.e. cytokines) and autoantibodies specific for target proteins of the brain.

Immunophenotype experiments were conducted on mononuclear cells populations (PBMC), which were isolated from heparinized blood through separation by density gradient.
Cells concentration was brought to 10^7/ml in PBS 1X (Euroclone Dulbecco's Phosphate Buffered Saline w/o Calcium, w/o Magnesium Sterile Filtered).
1x10^6 PBM cells contained in a volume of 100 microL for each tube were stained with specific fluorochrome-conjugated monoclonal antibodies targeting NK cells (Table 3) and monocytes populations (Table 4).
Samples were then processed with flow cytometer Becketon Dickinson (BD) LSR Fortessa X-20 using the software BD FACS DIVA version 8.

| Marker | Fluorochrome |
|---|---|
| CD16 | BV711 |
| CD3 | BV605 |
| HLA-DR | APC-H7 |
| CD159c (NKG2C) | BB700 |
| CD159a (NKG2A) | BV480 |
| CD85j | PE-Cy5 |
| CD337 | PE |
| CD335 (NKP46) | PE-Cy7 |
| CD158e1 (NKB1) | BV421 |
| CD56 | APC |
| CD57 | FITC |

Table 3: *NK markers panel*

| Marker | Fluorochrome |
|---|---|
| CD16 | FITC |
| HLA-DR | APC |
| CD14 | PerCP-cy5.5 |
| CD11c | BV421 |

Table 4: *Monocytes markers panel*

Gating strategy for NK cells (Fig. 12) consisted of the definition of lymphocyte population and the definition of CD3- subpopulation. After that, in CD16 versus CD56 plot, subsets of circulating NK were defined. Three different subsets were identified: CD56bright CD16- or CD16dim; CD56dim CD16+; CD56- CD16+.

Eventually, the cluster of fully mature NK was selected. They are CD56dim or CD56- CD16+ cells with high expression of CD57 and KIRs and the absence of NKG2A.

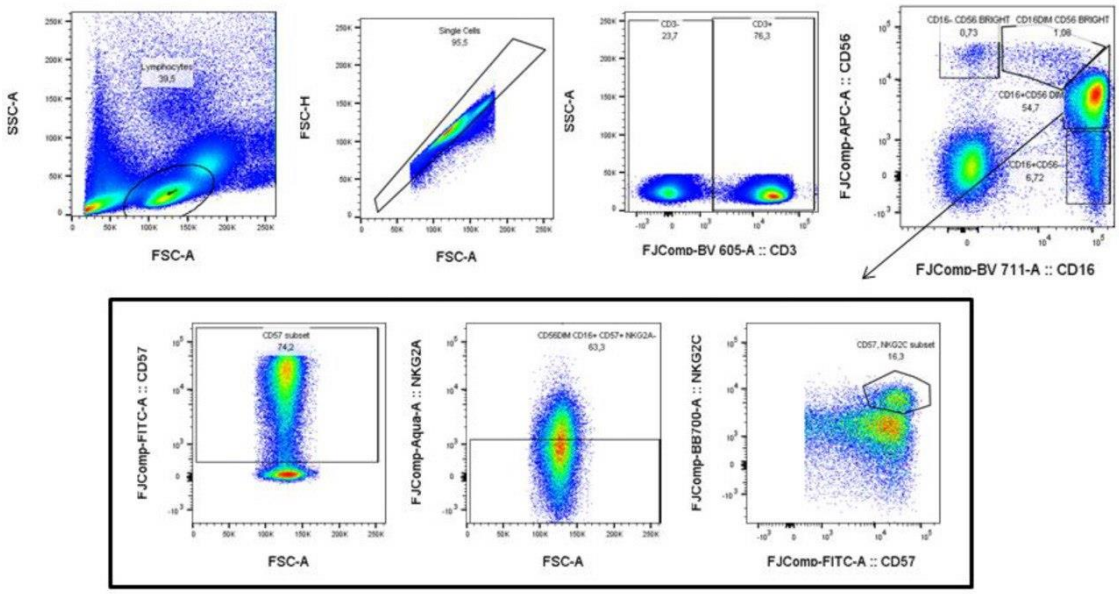



Fig. 12: *Gating strategy for NK cells*

Gating strategy for monocytes consisted of the identification of CD14+ and CD16+ populations in the CD14 versus CD16 plot. Then, monocytes were identified by the expression of HLA-DR and the morphological features in the HLA-DR versus SSC-A plot. The expression of the CD11c marker was used to confirm the selection of monocytes in CD11c versus SSC-A plot. Eventually, three different subpopulations were identified: non-classic monocytes CD14dim CD16bright, intermediate monocytes CD14bright CD16dim and classic monocytes CD14bright CD16-.

The programs FlowJo and FACSDiva were used to analyze and make a multiparametric visualization of NK data.

In order to better analyze NK panel, NK data from the first cohort of patients were first analyzed with bidimensional standard cytometry and then represented in a space with a reduced dimensionality by FlowSOM and tSNE algorithms.

FlowSOM is a clustering algorithm which builds self-organizing maps and provides an overview of markers expression on cells. It can reveal cell subsets and automatically groups cell clusters into higher order metaclusters.

tSNE (t-distribuied stochastic neighbor embedding) is an unsupervised non-linear dimensionality reduction technique which allows to preserve the pairwise similarities between data points in a lower-dimensional space. The output is a bidimensional map with clusters of subpopulations identified through “proximity” features.

## 7.3 STASTICAL ANALYSIS

Statistical analysis were performed by Jamovi softwere version 1.6.

Results relative to identified population were expressed as a percentage value compared to the parental gate. Results about NK markers were expressed as median fluorescence intensity and percentage compared to parental gate. Data were initially described as a graphic using violin plot.

In order to establish significant differences about populations and evaluated parameters within the three groups, the distribution of collected data was subject to a D’Agostino-Pearson normality test. The F test was used to compare variance between samples groups.

In order to establish significant differences about frequency and evaluated parameters within the three groups, a one-way variance analysis was conducted (One-Way ANOVA), followed by the post-hoc Tukey test.

# 8. RESULTS

## 8.1 NK CELLS - FlowSOM AND tSNE ANALYSIS

NK population turned out to be phenotypically complex and showed different stages of differentiation depending on the combined expression of CD56 and CD16 markers. CD56 is a marker of immaturity, while CD16 expression defines maturation and activation stages.

Flow cytometer experiments were conducted separately for each maturation stage; in particular, median fluorescence intensity (MFI) and percentage ( $\geq$ 1) of these markers were evaluated: CD57, CD85J, CD337 (NKP30), HLA-DR, NKB1, NKG2A, NKG2C, NKP46.

Immunophenotype analysis generated high-dimension data that the conventional procedures cannot analyze in the most appropriate way. To better comprehend the complexity of NK cells we used two computational techniques which allowed us to represent original high-dimension data in a bi-dimensional space.

FlowSOM analysis produced a heatmap which consisted of 8 subpopulations identified and gathered together in clusters and meta-clusters (Fig. 13). Each subpopulation is different depending on markers intensity.

The statistical analysis by One-Way ANOVA test showed lower levels of subpopulation number 5 in patients compared to controls ($p = 0,024$). This subpopulation corresponded with mature NK cells which are characterized as CD16+ CD56dim CD57+ NKG2C+ CD337+ CD85Jdim HLA-DR-, NKB1- NKp46- (Table 5).

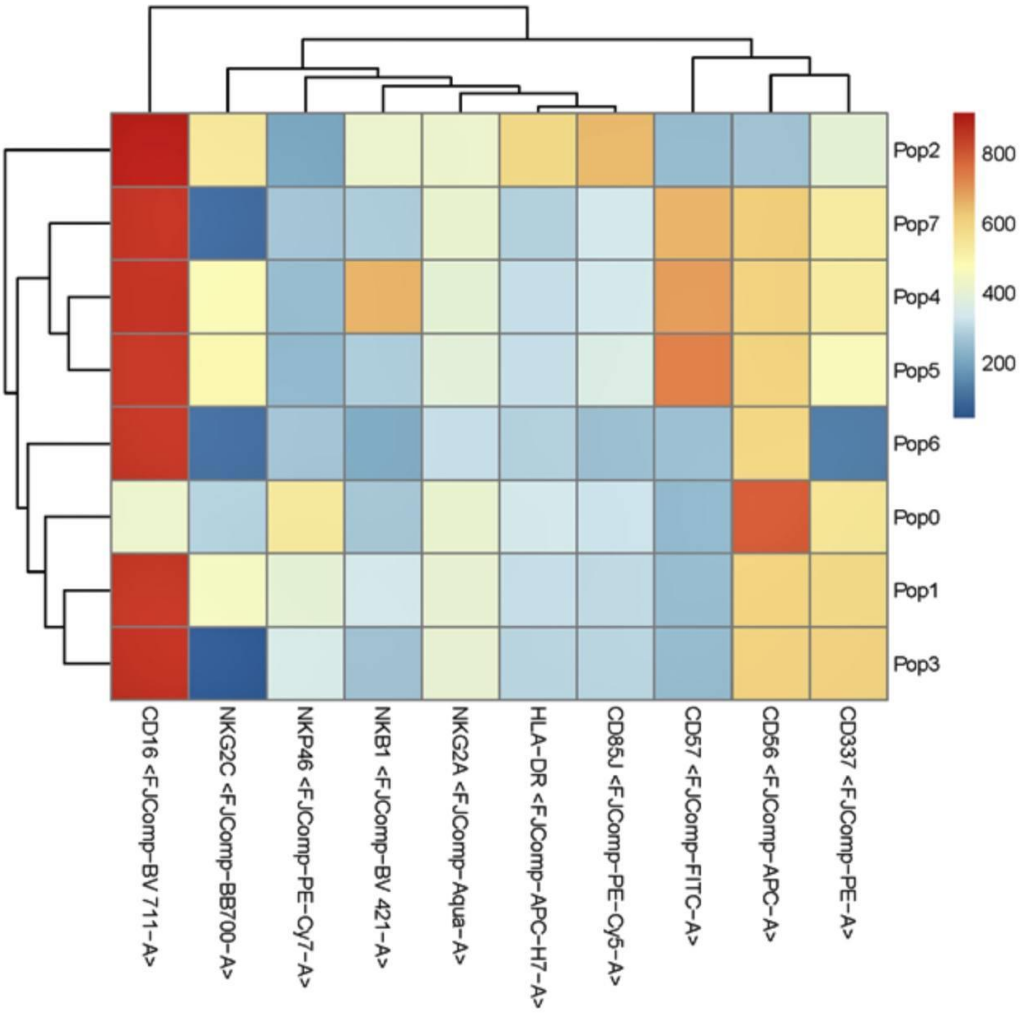



Fig. 13: *FlowSOM heatmap of circulating NK cells*

**One-Way ANOVA (Welch's)**

| | F | df1 | df2 | p |
|---|---|---|---|---|
| POP7 | 2.3428 | 2 | 10.5 | 0.144 |
| POP6 | 1.1904 | 2 | 10.3 | 0.343 |
| POP5 | 5.3230 | 2 | 11.2 | 0.024 |
| POP4 | 0.0135 | 2 | 10.9 | 0.987 |
| POP3 | 1.4142 | 2 | 11.5 | 0.282 |
| POP2 | 0.2410 | 2 | 11.5 | 0.790 |
| POP1 | 0.2345 | 2 | 11.8 | 0.795 |
| POP 0 | 1.9642 | 2 | 10.6 | 0.188 |

Table 5

The same NK populations were then visualized with tSNE plot (Fig. 14). We observed a higher expression of population number 7 in patients rather than controls. This population is characterized as CD16+ CD56dim CD57+ CD337+ CD85Jdim NKG2C-.

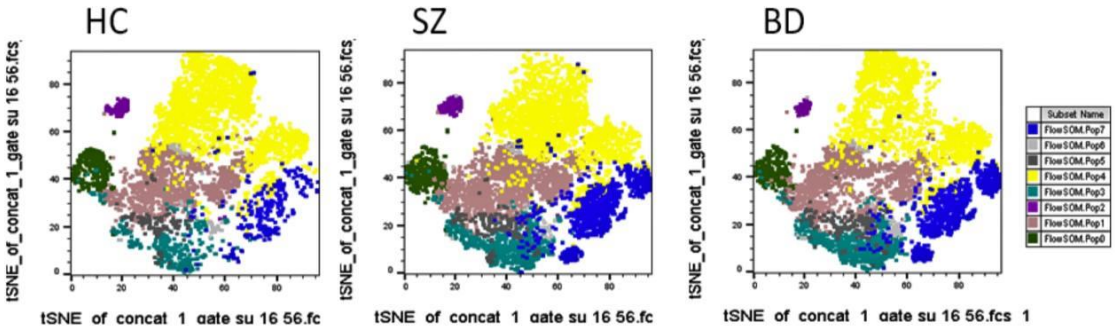



Fig. 14: *FlowSOM subpopulations visualized with the tSNE plot*

The output of tSNE software on NK analysis for each marker is shown in the following figure (Fig. 15). A higher expression of the activating receptor NKG2C has been shown in bipolar patients.

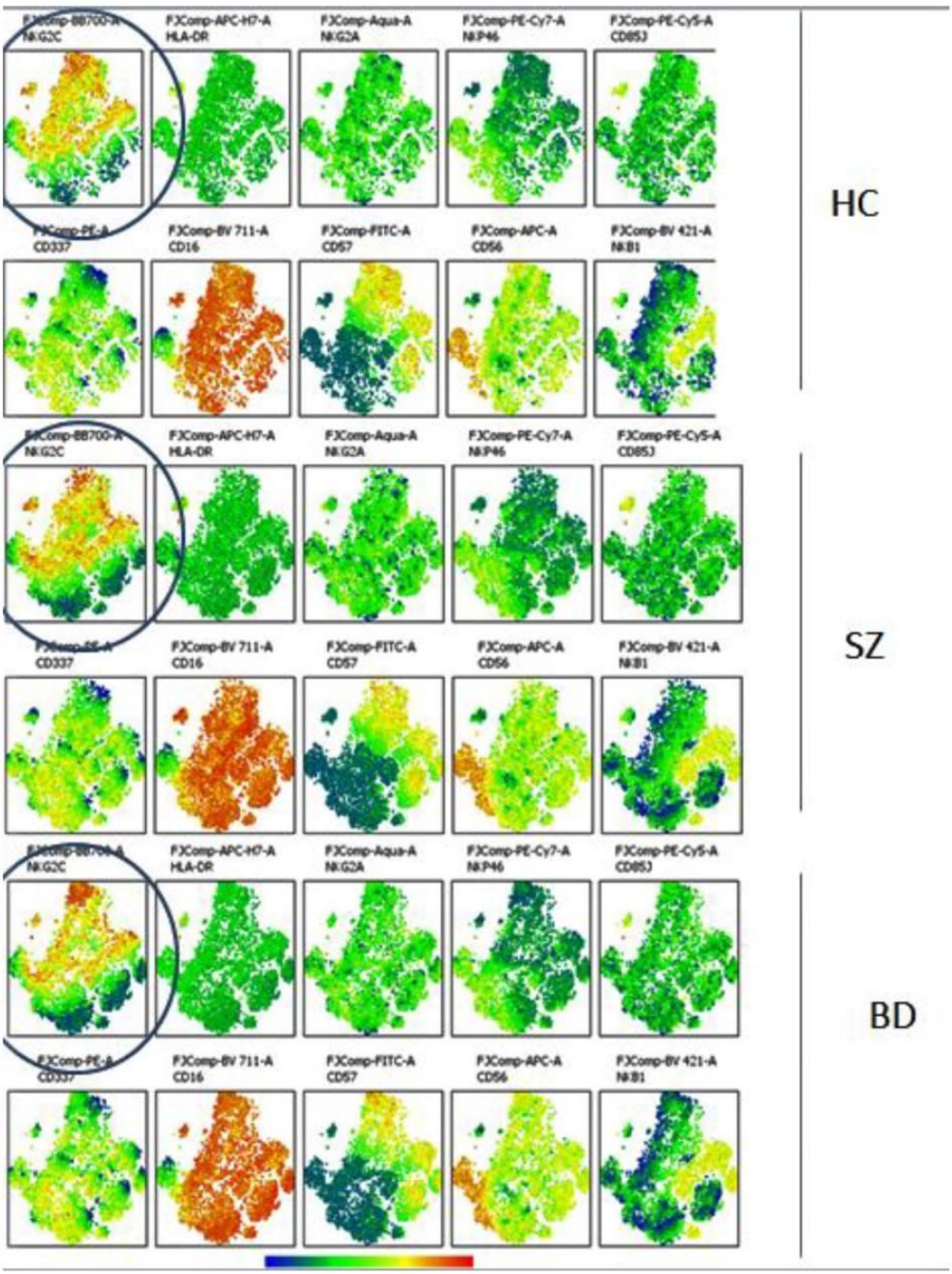
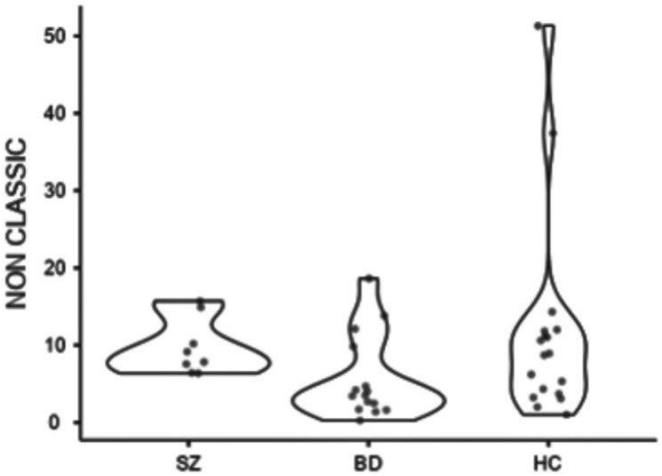



Fig. 15: *Markers graphics from the tSNE output*

## 8.2 MONOCYTES

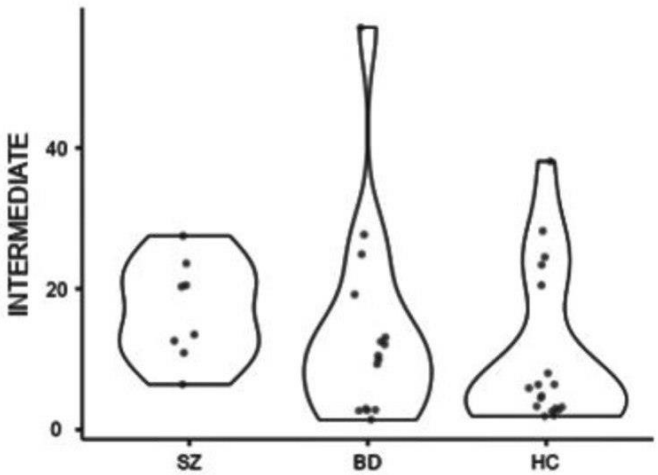
Monocytes levels were studied separately for each subpopulation (CD14dim CD16bright, CD14brigh CD16dim, CD14bright CD16-). There were no differences in levels of each subpopulation between patients and controls (Fig. 16, 17, 18).




Tukey Post-Hoc Test – NON CLASSIC

| | | SZ | BD | HC |
|---|---|---|---|---|
| SZ | Mean difference | — | 4.15 | -1.68 |
| | p-value | — | 0.571 | 0.905 |
| BD | Mean difference | | — | -5.83 |
| | p-value | | — | 0.186 |
| HC | Mean difference | | | — |
| | p-value | | | — |

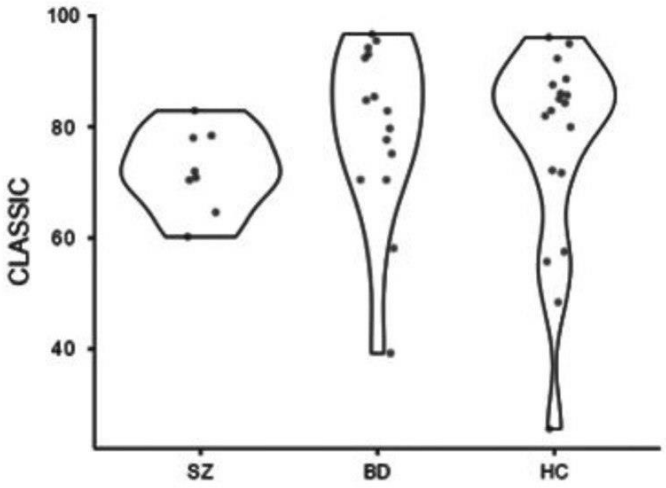
Fig. 16: *Analysis of the median expression of non-classic monocytes subpopulation*




Tukey Post-Hoc Test – INTERMEDIATE

| | | SZ | BD | HC |
|---|---|---|---|---|
| SZ | Mean difference | — | 2.98 | 6.39 |
| | p-value | — | 0.835 | 0.423 |
| BD | Mean difference | | — | 3.41 |
| | p-value | | — | 0.692 |
| HC | Mean difference | | | — |
| | p-value | | | — |

Fig. 17: *Analysis of the median expression of intermediate monocytes subpopulation*




Tukey Post-Hoc Test – CLASSIC

| | | SZ | BD | HC |
|---|---|---|---|---|
| SZ | Mean difference | — | -7.56 | -4.30 |
| | p-value | — | 0.532 | 0.803 |
| BD | Mean difference | | — | 3.26 |
| | p-value | | — | 0.830 |
| HC | Mean difference | | | — |
| | p-value | | | — |

Fig. 18: *Analysis of the median expression of classic monocytes subpopulation*

# 9. DISCUSSION

To the best of our knowledge this is one of the first studies that makes an immunophenotype analysis on innate immune cells of psychotic patients.
Literature lacks of solid results about innate immune cells in psychosis and instead it often focuses on peripheral markers such as cytokines and inflammatory proteins. The majority of studies showed differences only in levels of innate immune cells between patients and controls, but with little agreement within the studies. The heterogeneity of NK cells and monocytes obliges to characterize each subpopulation to better understand their possible role in the pathogenesis of psychosis.

Flow cytometer analysis on monocytes showed no differences in the levels of each subpopulation (non-classic monocytes CD14dim CD16bright, intermediate monocytes CD14bright CD16dim and classic monocytes CD14bright CD16-) among the three groups of participants. The cytometry panel should be completed with function-based markers.
Literature highlights the importance of monocytes in the pathophysiology of psychiatric disorders. It has been supposed that under conditions affecting the CNS, monocytes are recruited from peripheral blood into the brain, where they can cooperate with activated microglia (163). According to this hypothesis, activated monocytes and microglia may represent the systemic activation of the mononuclear phagocyte system and, to a greater extend, a state of systemic inflammation. A better comprehension of morphology and function of patients' monocytes should be aimed since it can give a "glimpse" of brain inflammation or damage in psychotic patients.

Immunophenotype analysis and then the computational analysis on NK cells provided us with significant results.
The first interesting result by FlowSOM is the reduction of the NK subpopulation number 5 in patients compared to controls. This subpopulation is a mature one, with high expression of CD16, CD57 and NKG2C. tSNE plot revealed a higher level of subpopulation number 7 in patients compared to controls. This subpopulation shows high expression of CD16 and CD57, but it is CD56dim and NKG2C negative. The

absence of the activating receptor NKG2C indicates that this subpopulation is less mature and activated compared to the subpopulation number 5. All these findings provide evidence for the presence of abnormal NK cells levels in psychotic patients.
We can speculate that mature NK cells in peripheral blood can be attracted to the inflamed CNS by the release of cytokines and chemokines. They can easily cross the BBB due to a state of BBB hyperpermeability. Once in the brain, NK can have a role in the processes of Neuroinflammation. In summary, the reduction of mature NK subpopulations in periphery can be a sign of NK migration to the brain and a more general sign of Neuroinflammation.
The study from Furlan et al. (112) reported a positive association between cytokine-producing NK cells and DTI measures of white matter integrity, suggesting that cells counts could correlate with integrity of myelin sheaths. Once in the brain, NK cells can interact with other activated immune cells, such as microglia, infiltrating monocytes and lymphocytes, in order to protect the brain from demyelination or dysmelination. This neuroprotective effect of NK cells seems to be related to the increase of IFN-gamma activity.
Higher levels of less mature NK subpopulation number 7 in patients may be explained by a state of systemic low-grade inflammation occurring during psychosis. Indeed, NK cells development in patients can be increased and speeded up by a pro-inflammatory stimulus.

Eventually, the tSNE output showed elevated levels of the activating receptor NKG2C on NK cells from bipolar patients. This receptor has been largely studied during last years as it is a marker of the so-called adaptive NK cells, which are related to previous infections and typically expressed in CMV-seropositive patients.
CMV infection has a deep influence on NK cells development since it induces a persistent redistribution of NK cells receptors and promotes the expansion of NKG2C expressing cells. These cells have memory properties and when exposed to a second viral infection can elicit a more intense response. Indeed, activated NKG2C expressing cells are responsible for an enhanced production of IFN-gamma, the anti-viral cytokine (164).

The greater expression of this receptor in bipolar patients suggests a potential role of infections in the development of psychosis and supports the “Neurodevelopmental theory”. In particular, it may also suggest the specific involvement of CMV infection in the genesis of psychosis.

Another study in 2021 (119) found elevated levels of CD57+ NKG2C+ NK cells in first psychosis patients and gives strength to our finding. Interestingly, the NKG2C overexpression was not related to the CMV status, but it was associated with NK cells functional impairment.

Anyway, this finding requires further investigation and the next step would be to study the positivity of CMV antibodies and the correlation with NKG2C positive cells in patients.

We have to notice that this study has several limitations which suggest caution.

First, the sample was too small and number of participants needs to increased. Moreover, we were not able to access detailed information on sociodemographic features of the majority of participants and our analysis was not adjusted for sex, age or other features. Eventually, all the patients were under treatment and the effects of medications cannot be excluded.

Then, the study did not keep illnesses’ features in consideration and it did not differ between patients with long duration of illness and patients with a first episode. Indeed, it has to be considered that levels of inflammation can modify during the course of illness. Furthermore, we did not divide bipolar patients depending on their mood state, but again we can suppose the presence of differences in inflammation levels between manic phases and depressive phases.

# 10. CONCLUSIONS

This study supports the hypothesis of abnormal immune cells levels in psychotic patients and the role of a disrupted immune system in the pathogenesis of psychosis.

The presence of lower levels of mature NK cells in peripheral blood of patients may be the sign of brain inflammation. The elevated levels of the CMV-related receptor NKG2C on cells from bipolar patients give strength to the hypothesis of an involvement of infections in the pathogenesis of psychosis.

Future direction will be to divide patients into subgroups as the complexity of symptoms suggests. Patients with the same diagnosis have not the same illness's presentation and this may be related to different levels of inflammation. Sign of inflammation can be find only in some subsets of patients and studies reported that approximately 30 to 50% of psychotic patients can present with increased levels of inflammation (165).

Research can be extended to other groups of psychiatric patients and not only to psychotic ones. A disruption in the immune system has been studied also in major depressive disorder, obsessive-compulsive disorder and PTSD (166–168).

The final aim of this study is to identify groups of patients that can benefit from a more targeted therapy. The immune system can be a potential target in the treatment of psychiatric diseases. Therefore, the clinical effects of several anti-inflammatory agents such as NSAIDs, N-acetylcyteine, Pioglitazone, Minocycline has been studied (42,169). The results are encouraging and strongly suggest that immune alterations can be the key for future psychiatric therapies.

## ACKNOWLEDGEMENTS

Ringrazio il mio relatore, il Prof. Andrea Escelsior, per il suo supporto durante la stesura di questa tesi e per avermi dato l'opportunità di lavorare a questo affascinante e stimolante percorso di ricerca.

Ringrazio la clinica psichiatrica dell'Ospedale San Martino, il direttore Gianluca Serafini e il Prof. Mario Amore.

Ringrazio inoltre la Dott.ssa Daniela Fenoglio e Chiara Uras per aver collaborato alla parte immunologica.

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