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Pediatric Acute-onset Neuropsychiatric Syndrome (PANS): new insights

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1. INTRODUCTION

Pediatric Acute-onset Neuropsychiatric Syndrome (PANS) is a clinically heterogeneous disorder described in 2012 as result of the modification of the Paediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections (PANDAS) criteria.

PANDAS was described for the first time in the late 1990s to identify a subset of patients with obsessive-compulsive disorder (OCD) and tics for whom the onset of symptoms appeared related to a Group A β-hemolytic Streptococcus (GABHS) infection (Swedo et al., 1998).

PANS is a recently described syndrome characterized by an abrupt and acute onset (<72h) of obsessive-compulsive symptoms and/or food restriction in association with at least two concomitant cognitive, behavioural, or affective symptoms such as anxiety, obsessive-compulsive, irritability/ depression (Swedo et al., 2012; Chang, Frankovich et al., 2015).

The etiopathogenesis of the disorder is not unique and the hypotheses on the underlying processes are based on the broadening of the study perspective of the PANDAS) (Swedo et al., 1998). PANS can be associated with non-streptococcal triggers, such as Mycoplasma pneumoniae, Epstein Barr virus, influenza or other common viruses, usually located in the upper respiratory tract (Chang et al., 2015). Paediatric autoimmune-mediated inflammatory brain diseases may be triggered by different environmental agents (e.g., stress, substances, virus or bacteria), in subjects with a brain susceptibility to autoimmunity (Van Mater, 2014; Graus et al., 2016).

Neuroinflammation has been postulated to have a pathogenic role in many psychiatric illness (e.g., major depressive, bipolar, schizophrenia, obsessive-compulsive disorders (Najjar et al., 2013).

PANS could therefore be configured as a disorder with an immune-mediated inflammatory genesis characterized by an acute onset and by the presence of a constellation of other symptoms which, associating with OCD and tics, define its peculiarity.

Presently, there are no specific guidelines for PANS treatment. The available suggestions for intervention (Cooperstock et al., 2017; Frankovich et al., 2017) are exclusively based on the clinical experience and the "best practice". They are widely derived from the therapeutic strategies used for other nervous system disorders with inflammatory/ autoimmune pathogenesis, such as autoimmune encephalitis or Sydenham's Korea (Sigra et al., 2018).

PANS, as well as PANDAS, is not yet mentioned in the most accredited diagnostic manual of mental disorders (DSM-5). The controversy concerning the diagnosis and etiology of these syndromes is still ongoing (Chiarello et al., 2017). Regarding the pathophysiology, the research group of Pallanti (2017) claims that unfortunately the levels of antineuronal antibodies, observed in previous studies for PANDAS patients, showed only descriptive information.

Furthermore, it is difficult to sustain a correlation between high levels of antineuronal antibodies in patients with PANDAS and their clinical features with no other biochemical and clinical evidence (Chiarello et al., 2017). The same researchers pose several questions related to how long autoantibodies can be detected in sera after the acute onset, whether antibodies could cross the blood–brain barrier, or whether Immunoglobulin G (IgG) production takes place inside the Central Nervous System (CNS). Demonstrating the existence of a clear immune basis for PANS still remains a challenge for leading research groups, although the focus on cohort studies begins to show correlations between infection and emergence of psychiatric symptoms, especially OCD, in children with PANS.

Concerning the treatment, studies about immunotherapy, antibiotic prophylaxis and tonsillectomy have produced inconsistent results and more studies are needed to confirm and support the use of these modalities for the clinical indication (Wilburn et al., 2019).

At the moment, it would seem unclear whether the diagnostic criteria for PANS delineate a distinct diagnostic entity, although the abrupt onset of psychiatric symptoms is a fundamental criterion for identifying this syndrome in its peculiarity (Wilburn et al., 2019).

Much more needs to be studied and discovered about this syndrome from a diagnostic and pathophysiological point of view, as well as from a therapeutic point of wiew, even thoug a large body of evidences are accumulating in favour of the individuation of this syndrome as a specific clinical entity.

Aim of this work is to regard and describe the PANS feature through three innovative research approaches with the purpose of more accurately defining the clinical and neurobiological characteristics of this controversial clinical entity.

The first approache is based on the study of PANS symptoms and signs by mean the Artificial Neural Networks (ANNs), computational adaptive systems inspired by the functioning processes of the human brain and particularly useful for discovering subtle trends and associations among different variables. Through this method we have tried to grasp the core of the relationship between signs and symptoms in PANS phenotype.

The second approach starts from the evidence that sleep disorders represent one of the most frequent manifestations of PANS, involving around 80% of patients and emerging at the onset of the syndrome.

Through the analysis of the relationships between the sleep polisonnographic characteristic and the symptoms of children diagnosed with PANS, we intended to suggest an interwoven pathophysiological mechanism, putatively involving basal ganglia functional alterations, underling both sleep and PANS symptoms.

The third approache moves from the awareness that the identification of potential new specific biomarkers of PANS is strongly desirable. With this purpose we have used the metabolomics analysis in order to determine whether it is possible to define a specific metabolic pattern of patients affected by PANS compared to healthy subjects.

The whole of the data emerging from these three different research perspecives could provides a useful set of biomarkers very helpful in the identification and the management of PANS patients.

Therefore, we present in this work the three following research studies:

1. Study 1: it explores the associations between the different PANS features in a sample of 39 children diagnosed with PANS, using the Artificial Neural Networks (ANNs) analysis approach, to exploit putative subtle simultaneous connections among the full spectrum of clinical variables and different domains of impairment.

2. Study 2: it describes the clinical and polysomnographic features of sleep in a group of 23 children diagnosed PANS, with neither medications nor nutracetincs for at least 4 weeks before enrolment, and discusses the relashonship between symptoms and sleep variables.

3. Study 3: it analyses the serum metabolomics profile of a sample of children (34 patients and 25 controls) diagnosed PANS, through the Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR) technique, and outlines the separation between PANS patients and controls based on the different serum metabolic profile.

1.1. PANS: DEFINITION, DIAGNOSTIC CRITERIA AND CLINICAL PRESENTATION

As describe above, PANS is a syndrome characterized by an abrupt and acute onset (<72h) of obsessive-compulsive symptoms and/or food restriction in association with at least two concomitant cognitive, behavioural, or affective symptoms such as anxiety, obsessive-compulsive, irritability/ depression, induced by an immune-mediated inflammation of specific brain regions, usually showing a chronic remitting- relapsing pathway, that can be accompanied, in the most severe cases, with a serious functional deterioration and a progressive impoverishment of cognitive and relationship functions (Calaprice et al., 2017). In February 2015, a Consensus Conference launched in 2013, first defined the diagnostic criteria of the disorder and proposed systematic strategies for identifying suspect cases (Chang, Frankovich et al., 2015).

The diagnoses of PANS can be formulated according to PANS working criteria defined by experts convened at the National Institute of Mental Health (NIH) in July 2010 (Swedo et al., 2012), defined as follows:

I. Abrupt, dramatic onset of obsessive-compulsive disorder or severely restricted food intake

II. Concurrent presence of additional neuropsychiatric symptoms (with similarly severe and acute onset), from at least two of the following seven categories:

1. Anxiety

2. Emotional lability and/or depression

3. Irritability, aggression, and/or severely oppositional behaviours

4. Behavioural (developmental) regression

5. Deterioration in school performance (related to attention deficit/hyperactivity disorder [ADHD]-like symptoms, memory deficits, cognitive changes)

6. Sensory or motor abnormalities

7. Somatic signs and symptoms, including sleep disturbances, enuresis, or urinary frequency

III. Symptoms are not better explained by a known neurologic or medical disorder, such as Sydenham chorea (SC).

In particular, when PANS is suspected, the following aspects should be evaluated: Family history; Medical history and physical examination; Psychiatric evaluation; Infectious disease evaluation; Assessment of symptoms and history that points to need forfurther evaluation of immune dysregulation (autoimmunedisease, inflammatory disease, immunodeficiency); Neurological assessment; Assessment of somatic symptoms, including possible sleepevaluation; Genetic evaluation (Chang, Frankovich et al., 2015).

1.1.1. PANS and clinical features

Recently other research groups attempted to describe the PANS clinical profile and the associated symtoms and disorders. Hesselmark and Bejerot (2019), for instance, studied a group of 28 Swedish PANS patients, evaluated with the PANS Scale-R (an unpublished structured interview based on the diagnostic criteria for PANS by Leckman, 2014) and they found out that: the 89% had obsessive–compulsive symptoms and Sleep problems, the 79% Separation anxiety, the 75% Urinary symptoms, the 71% Depression, Emotional lability, Attention deficit and Simple motor tics, the 64% Irritability or aggression, the 63% Loss of academic skills, the 62% General anxiety, the 61% Eating disorder, Contamination (OCD) and Personality change, the 57% Symmetry (OCD), the 46% Causing harm, Phobias, Behavioural regression, Dilated pupils and Motor hyperactivity, the 43% Panic episodes, the 42% Choreiform movements, the 37% Complex motor tics, the 36% Hallucinations, the 25% Hoarding and Sexual or religious (OCD) (see TABLE 1).

Overall the study described a total of 89 patients with psychiatric illness that were classified into three groups: (a) confirmed to have PANS; (b) previously suspected to have PANS but failed to meet the full criteria; (c) never suspected to have PANS.
The same group of patients was also evaluated with the Mini International Neuropsychiatric Interview for Children and Adolescents (MINI-KID) and it was found out that the 71% fulfilled the criteria for the diagnosis of Obsessive–compulsive disorder (current), the 68% for Major depressive episode, the 37% for Tourette syndrome, the 36% for Generalised anxiety disorder (current), the for 33% Oppositional defiant disorder, the 30% for ADHD (inattentive), the 26% for Motor tics disorder, the 22% for ADHD (combined) and Pervasive developmental disorder, the 20% for Specific phobia, the 16% for Dysthymia, the 15% for Vocal tics, the 11% for Suicidality, the 10% for Agoraphobia and Separation anxiety disorder, the 4% for Anorexia nervosa, Hypomania and Psychotic disorders (see TABLE 2).

In particular, sleep disorders represent one of the most frequent manifestations of PANS, involving around 80% of patients, often emerging at the onset of the psychiatric symptoms and subsequently regress after the acute phase or follow the course of the disease (Chang et al., 2015, Gagliano, Galati et al., 2020; Gagliano, Puligheddu et al., 2020 - see Study N.2).

Despite this frequent finding, to date few studies have been conducted analyzing the sleep characteristics of these patients, and only two polysomnographic studies have been published in patients diagnosed with PANS (Gaughan et al., 2016; Santoro et al., 2018). Both these studies report a higher prevalence of sleep disorders in PANS patients than in the general pediatric population.

Among the sleep disorders described in PANS the most frequently mentioned are parasomnias (nightmares, nocturnal *pavor*, sleepwalking or somnambulism); difficulties in falling asleep or maintaining sleep (early or intermediate insomnia); early awakenings (terminal insomnia); REM sleep disorders such as REM Sleep Without Atonia (RSWA); Sleep movement disorders such as Periodic Limb Movement Disorder (PLMD) (Swedo et al., 2012; Santoro et al., 2018).

At the same time, PANS has been matched to other persistent neuroinflammatory disorders, such as multiple sclerosis, Sydenham chorea, Behcet's disease, and asthma, to whom underlining infections and other environmental triggers play a role in provoking an inflammatory brain response, which evolves into a chronic or progressive neuroimmune disorder (Frankovich et al., 2017).

It is clear that a such huge spectrum of symtoms and disorders strongly reduce the specifity of PANS as distinct clinical entity. Moreover, the validity and generalizability of epidemiologic studies about PANDAS and PANS have been criticized for highly biased participant sampling and non-validated causes, effects, and methods (Gilbert et al., 2018).

Currently, PANS is a still controversial clinical entity regarded as a clinical complex constellation of psychiatric symptoms and adventitious movements as well as the expression of different serological variables of an autoimmune/ inflammatory disease (Gagliano et al., 2020).

However, according to the 2013 PANS Consensus Conference (Swedo et al., 2012, Chang et al., 2015), PANS is currently conceptualized as a complex syndrome with a number of aetiologies and disease mechanisms, encompassing psychiatric symptoms, arising from immune abnormalities triggered by a variety of agents (Frankovich et al., 2015; Murphy et al., 2015).

Despite the fact PANS is a diagnostic construct of exclusion without a single defined aetiology or specific clinical symptoms, it has been considered a useful construct for outlining the sudden onset of severe neuropsychiatric symptoms in children with a relapsing-remitting course. Consequentely, the PANS diagnostic construct was proposed as a clinical entity distinct from idiopathic or familial OCD, anxiety or Tourette disorder on the basis of clinical observations.

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1.1.2. Epidemiology

Epidemiological studies relating to PANS are lacking due to the symptomatic heterogeneity and the progressively developing clinical characterization of this condition. Probabily, the still open debate on the introduction of this new nosographic entity is the main reason why large population study on the incidence of PANS or PANDAS have not been conducted yet within the scientific community (Williams and Swedo, 2015).

Basically, it not possible to define how common PANS and PANDAS are. They both are often overlooked by clinicians because they might assume that there is not an underlying medical cause for the neuropsychiatric symptoms (Stanford Medicine, 2020).

Despite this, conservative estimates put the incidence of this syndrome at 1 in 200 children. Thus, PANS/ PANDAS seem to be not a rare disorders ìf we compare its incidence with the incidence of other common medical conditions.

For instance, about 1 in 400 under age 20 is expected to develop a diabetis and 1 in 285 children is expected to have a diagnosis of cancer before age 20 (Greene, 2016). Furthermore, epidemiological studies of pediatric obsessive-compulsive disorder (OCD) and the prevalence of acute-onset cases, report a prevalence of 3-5% of cases meeting the PANS/ PANDAS diagnostic criteria (Jaspers-Fayer et al., 2017). Regarding the distribution by gender, several authors report a marked prevalence of males with an M: F ratio of approximately 3-5: 1 (Santoro et al., 2018; Frankovich et al., 2015).

1.2.   ETIOPATHOGENESIS

As PANDAS, PANS is thought to be sustained by immune-mediated mechanisms, on the model of the so-called "molecular mimicry" that occurs when pathogenic microorganisms possess structures very similar (in terms of sequence or amino acid structure) to "self" or self-antigen structures: the immune response is directed, by cross-reactivity, towards the "self" peptide with consequent activation of self-reactive naive T lymphocytes (Swedo et al., 2015; Spinello et al., 2016).

Under this rationale, the initiation of the symptomatic state results from an event (e.g. Group A Streptococcal infection) causing a localised immune response in the central nervous system (CNS), and the chronic relapsing course is due to the persistence of the immunological imbalance also after the resolution of the acute phase of the infection.

In PANS, different etiological agents, including viruses, Mycoplasma pneumoniae and Haemophilus influenzae, are supposed to act as triggers for the activation of the immune response, in turn responsible for the inflammatory cascade and the consequent release of chemical mediators of inflammation at the CNS level (Hoekstra et al., 2005; Muller et al., 2004; Swedo et al., 2012; Frankovich et al., 2015).

A survey aimed at parents of children with PANS, concerning the disorders present at the time of onset, revealed that Streptococcal and Mycoplasma infections were the most declared (FIGURE 1).

The neuroanatomical correlates of PANDAS, as with tics, OCD and Sydenham's Chorea, are the basal ganglia and dopamine receptors (Swedo et al., 1994). Functional and structural abnormalities of the cortico-basal ganglia circuitry, similar to that seen in those with acute Sydenham's Chorea, have been described in PANDAS. In particular, an enlarged striatal volume and an inflammatory state of the striatum, as measured by positron emission tomography using a marker of microglial activation has been reported in PANDAS (Giedd et al., 2000; Elia et al., 2005; Kumar et al., 2015).

A recent MRI diffusion study identified cerebral microstructural differences in children with PANS in multiple brain structures (eg, Thalamus, Basal Ganglia, Amygdala), putatively related to a neuroinflammatory state (Zheng et al., 2020).

There is evidence that both the basal ganglia and dopamine receptors are targeted by Group A streptococcal infection-associated autoantibodies in this condition (Dale et al., 2005; Dale et al., 2012). Studies have shown children that meet the clinical criteria for PANDAS have higher levels or circulating antibodies targeting the caudate and putamen neuronal surface antigens in the midbrain compared to children with Tourette's syndrome or other tic disorders (Pavone et al., 2004; Church et al., 2003).

Specific antibodies against neuronal surface glycolytic enzymes, lysoganglioside and N-acetyl-β-D-glucosamine, are found to be in higher concentrations and more active in children with tics compared to those children with only Group A streptococcal pharyngitis and without tics (Pavone et al., 2004; Church et al., 2003).

Current findings support the hypothesis that there is an abnormal immunological response in those with the clinical symptoms of PANDAS (Brimberg et al., 2012).

In cohorts of rigorously selected subjects with PANS, evidence of post-infectious autoimmune processes and/ or a condition of neuroinflammation was observed in over 80% of cases (Swedo et al., 2017).

Despite the fact that numerous studies have attempted to replicate and confirm these results identifying specific antibodies and antigens in the serum of individuals with PANS/PANDAS, all these findings have not been consistent across all studies, possibly due to etiological heterogeneity.

1.2.1. Basal Ganglia involment in PANS

The Basal Ganglia (BG), or Basal Nuclei, is a distributed group of subcortical brain structures located within the telencephalon, diencephalon, and mesencephalon that act to control motor, cognitive, and affective functions (Macpherson and Hikida, 2019). They are important stations of the circuits that, mainly, regulate movement, perception, executive functions and emotions (Yaddanapudi et al., 2010).

More precisely, they exert inhibitory influence on upper brain functions (motor & behaviors systems). To achieve goal-directed behaviors, the BG and frontal cortex work together, concerning therefore not only the execution of motor plans, but also: the behaviours that lead to this execution, including emotions and motivation that drive behaviours; the cognition that organizes and plans the general strategy, motor planning (BG as supporting a procedural, or habit, learning system); and again, the execution of that plan (Robbins, 1996; Haber, 2003).

While in the past the neuroscience has mainly focused on how dysfunctions of the BG lead to motor disorders, currently there is much more knowledge about neuropsychiatric disorders (Macpherson and Hikida, 2019). Currently, it is assumed that dysfunction of the Basal Ganglia involves several motor, psychiatric, and cognitive impairments.

Actually, PANS patients, in consideration of their clinical characteristics, might have difficulty in control their thoughts, behaviours and movements, both autonomous and voluntary. They show difficulties in control and fine tune movements, mood and emotion, behaviour, procedural learning, cognition.

Basal Ganglia functioning

A possible explanation of what happens in the brain comes from an interesting study conducted by Topalidou et al. (2018).

They suggested a model that encloses interactions between the cortex, the BG, and the thalamus based on a dual competition. Their hypothesis was that the striatum, the subthalamic nucleus (STN), the internal globus pallidus (GPi), the thalamus, and the cortex were involved in closed feedback loops through the hyper-direct and direct pathways. The ability of BG to make a cognitive decision followed by a motor one, seems to derive from these loops that support a competition process.

A second competition would take place inside the cortex (regarding the lateral cortical interactions), giving the latter the possibility to make a cognitive and a motor decision.

It has shown how the dual competition provides the model with two regimes:

1. The first, driven by reinforcement learning
2. The second, driven by Hebbian learning

Through the combination of these two mechanisms, with a gradual transfer from the former to the latter, the final decision is made.

In particular, the architecture of the model is focused on:

▪ The hyper-direct pathway (cortex → STN → GPi/ Substantia nigra pars reticulata (SNR) → thalamus → cortex)

▪ The direct pathway (cortex → striatum→ GPi/ SNR → thalamus → cortex)

- The cortex where lateral interactions take place (FIGURE 2; FIGURE 3) (Alexander et al., 1990).

The model includes three segregated circuits (cognitive, associative, motor).

The cognitive and motor circuit each comprises a cortical, a striatal, a thalamic, a subthalamic, and a pallidal population while the associative loop only comprises a cortical and a striatal population that communicates with two other circuits via diffused connections to the pallidal regions and from all cortical populations (Topalidou et al., 2018) (FIGURE 3: Arrows, excitatory connections. Dots, inhibitory connections).

Evidences of Basal Ganglia involment in PANS

The hypothesis of the involvement of the Basal Ganglia, in the pathogenesis of PANS, comes from various neuroimaging studies conducted on populations of patients diagnosed with OCD and Tourette's disorder.

It is interesting to note that people with neurological disorders resulting from basal ganglia alterations, such as Sydenham's Chorea and Tourette's disorder (TD) almost invariably present obsessive-compulsive symptoms (Swedo et al., 1989; Williams et al., 2015; De Groot et al., 1994-1995; Kano et al., 2014).

Also tics correlate with a dysfunction of the frontal-subcortical brain circuits involved in movement programming and in children they are associated with a decrease in the volume of the Caudate Nucleus; Peterson, 2003). In both Tourette's and OCD the involvement of Basal Ganglia in the pathogenesis of the disorder is described (Nomura, 2017; Haynes, Clair, et al., 2018), but unlike PANS the onset is not acute and an immune-mediated genesis is not hypothesized.

A PET scanning study (Kumar et al., 2014) aimed to evaluate neuroinflammatory changes in basal ganglia and thalamus in children with clinically diagnosed PANDAS and Tourette syndrome revelead an underlying activated microglia-mediated neuroinflammation in bilateral caudate and bilateral lentiform nucleus in the PANDAS group and in bilateral caudate nuclei only in the Tourette syndrome group, compared to control group.

According to the authors, these differences in the pattern and extent of neuroinflammation also signify a possible difference in pathophysiological etiology between PANDAS and Tourette syndrome patients.

PANS could therefore be configured as a neurodevelopmental disorder with immune-mediated inflammatory genesis of tic and obsessive symptoms, characterized by an acute onset and by the presence of a constellation of other symptoms which, associated with Obsessive Compulsive Disorder (OCD) and tics, define its peculiarity.

Thus, a regional dysfunction related to the onset of a condition of neuro-phlogosis, particularly localized at the level of the BG, becomes a valid hypothesis explaining PANS and PANDAS (Brown et al., 2017). This also because both an injury and an inflammatory condition in the BG can result in release of inhibitory circuits (Frankovich, 2019). As a confirmation of this hypothesis, Giedd et al. (2000) showed volumetric differences of the Basal Ganglia in a group of subjects with PANDAS.

This study analyzed, through the computer-assisted morphometric techniques, the cerebral magnetic resonance images of 34 children with the diagnosis of PANDAS. It has been shown (FIGURE 4) that the average sizes of the caudate, putamen, and globus pallidus, but not of the thalamus or total cerebrum, is significantly greater in the group of children with PANDAS than in the control group.

Moreover, similar differences (FIGURE 5) were found before for subjects with Sydenham's Chorea compared with healthy children (Giedd et al.,1995).

More recently, a neuroimaging study indicates that microstructural differences (i.e. apparent diffusion coefficient, but not regional brain volume nor cerebral blood flow) may be observed in the basal ganglia, thalamus, and amygdala of children and adolescents with PANS (Zheng et al., 2020).

The basic concept is that this group of disorders (PANS/ PANDAS) is based on an atypically directed immune process, which affects or weakens the Blood-Brain Barrier (BBB), especially in some regions such as the BG (Yaddanapudi et al., 2010). The role of antibodies directed against epitopes of the brain tissue, and in particular against the Basal Ganglia, probably similar to the membrane epitopes of some germs, appears of particular interest in the pathogenesis of PANS. Seropositivity for antibodies to Basal Ganglia has been repeatedly documented in these disorders (Kirvan et al., 2003).

A study investigating the presence of these antibodies in patients with ongoing streptococcal infection, without neuropsychiatric symptoms and in patients diagnosed PANS, showed a positivity to these antibodies of 10% in the first group and over 30% in the second group (Pavone et al., 2004).

In conclusion, BG seems to play a crucial role in the pathophysiology of PANS as well as in the related neuropsychiatric disorders.

1.3. CLINICAL MANAGEMENT

In February 2015, a Consensus Conference launched in 2013, preliminarily defined the diagnostic criteria of the disorder and proposed systematic strategies for identifying suspected cases (Chang, Frankovich et al., 2015). Nevertheless, there are still no specific guidelines for therapy and some recent suggestions on intervention modalities (Cooperstock et al., 2017; Frankovich et al., 2017) are based exclusively on the clinical experience of the authors, and on "best practice" and largely borrowed from the therapeutic strategies used for other disorders of the nervous system with inflammatory/ autoimmune pathogenesis, such as autoimmune encephalitis or Sydenham's chorea (Sigra et al., 2018).

Currently, even in the absence of clear biomarkers, a combination of immunomodulatory therapy, antibiotic prophylaxis, associated with targeted treatments on symptoms, is the most used clinical practice (Frankovich et al., 2017).

According to the *General Principles for Treating Pediatric Acute-Onset Neuropsychiatric Syndrome* outlined by Swedo et al. (2017), the management of PANS/ PANDAS concerns:

1. Establish that PANS is the correct "diagnosis of exclusion" by completing a comprehensive diagnostic evaluation (Chang et al., 2015)

2. Provide symptomatic relief with psychiatric medications and behavioural interventions, prioritizing treatment of symptoms causing the greatest distress and interference (Thienemann et al., 2017)

3. Treat underlying infections and consider use of therapeutic or prophylactic antibiotics (Cooperstock et al., 2017)

4. Treat symptoms resulting from neuroinflammation or postinfectious autoimmunity with anti-inflammatory or immunomodulatory therapies, chosen on the basis of symptom severity and disease trajectory (Frankovich et al., 2017)

5. Evaluate effectiveness of the treatment regimen at frequent intervals, making modifications as warranted by improvement or worsening of symptoms

6. Treatment can be tapered downward or stopped when symptoms resolve. However, treatment may be necessary again at some point in the future, given the relapsing–remitting nature of PANS symptoms (quoted in Swedo et at., 2017)

The FIGURE 6 shows the PANS *treatment triangle* (Swedo et al., 2017).

1.3.1. Pharmacological Interventions

In cohorts of PANS patients well-characterized, the evidence of post-infectious autoimmunity and/ or neuroinflammation was found in over 80% of cases (Frankovich et al., 2015; Murphy et al., 2016; Swedo et al., 2012), therefore the choice of the treatment depends on three complementary modalities of intervention:

1. To treat symptoms with psychoactive drugs, psychotherapies (particularly Cognitive Behavioural Therapy) and supportive interventions

2. To remove the source of inflammation with antimicrobial interventions

3. To treat immune system disorders with immunomodulatory and/ or anti-inflammatory therapies (Swedo et al., 2017).

A recent systematic review of the literature, over the last twenty years, has confirmed the tendency of most clinical and research centres dealing with PANS to make use of different therapeutic strategies for the treatment of this disorder, having, as a common rationale, the dysimmune/ inflammatory etiopathogenetic hypothesis (Swedo et al., 2017).

The medical interventions that have been systematically studied for PANS/ PANDAS are:

- ✓ Antibiotics
- ✓ Corticosteroid and non-corticosteroid anti-inflammatory drugs
- ✓ Intravenous immunoglobulins
- ✓ Plasmapheresis
- ✓ Tonsillectomy

Alongside these therapeutic aids, they have also been being used as symptomatic therapy psychoactive medications, mainly selective serotonin reuptake inhibitors and antipsychotics.

Antibiotic and immune-based treatments often have dramatic effects, reducing symptoms to a tolerable level or eliminating them completely (Cooperstock et al., 2017). When antimicrobial therapy is indicated, amoxicillin-clavulanate is generally preferred for acute sinusitis in children with moderate or severe PANS or PANDAS, and for those who have recently been treated with other antibiotics (Cooperstock et al., 2017).

For the treatment of Streptococcal Pharyngitis, Penicillin orally or intramuscularly is indicated as the first choice (Gerber et al., 2009), moreover, in current practice, amoxicillin is oftenused in suspension form for younger children due to its enhanced palatability (American Academy Pediatric, 2015).

Intramuscular therapy is considered the most reliable, although in practice it is only given to children who do not accept oral therapy.

The goal of therapy is to eradicate the infection so as not to incur complications. For children allergic or intolerant to Penicillin, Cefalexin, Clindamycin, Azithromycin or Clarithromycin can be administered (Shulman et al., 2012).

The use of azithromycin as an alternative for the treatment of pharyngeal GAS infection may be less efficacious, due to regional GAS resistance rates as high as 5%–10% or more (Silva-Costa et al., 2015) with an associated potential for the development of sequelae (Logan et al., 2012).

The theoretical advantages of Azithromycin include the fact that it is also active against Mycoplasma Pneumoniae and for its powerful immunomodulation (Obregon et al., 2012).

The use of Corticosteroids for PANS patients should be based on case. Alternatively, the use of nonsteroidal anti-inflammatory drugs in Pediatric Acute-Onset Neuropsychiatric Syndrome might concern patients with mild impairment (Frankovich et al., 2015; Murphy et al., 2016; Swedo et al., 2012).

TABLE 3 shows the general approach to using induction corticosteroids and/ or nonsteroidal anti-inflammatory drug therapies in PANS/ PANDAS (Frankocivh et al., 2017).

IVIG is the Infusion of Immunoglobulins (antibodies) into the vein. This solution is composed of antibodies normally found in adult human blood that provide immunity against disease.

IVIG products are derived from the plasma of a large number of individuals who have formed antibodies to a wide variety of bacteria, viruses, and other proteins (Murphy et al., 2014). Murphy, Gerardi, Leckman (2014) point out that similar to treatment of Sydenham chorea (SC) (Walker et. al., 2012; Garvey et al., 2005) IVIG was found to be superior in a randomized clinical trial for PANDAS (Perlmutter et al., 1999). Furtermore, of interest, IVIG was not effective in a double-blind, placebo-controlled study of adult patients with tics (Hoekstra et al., 2004).

Therapeutic Plasmapheresis is a form of therapeutic apheresis. Therapeutic apheresis is a term used to describe processes, such as decreasing the amount of a patient's blood cells, changing the blood components (plasma, erythrocyte), modifying the blood components, or autologous peripheral stem cell harvesting, performed to achieve clinical benefit (Beşiroğlu et al., 2007). Beşiroğlu (et al., 2007) studied the effect of plasmapheresis treatment in 4 adult cases of obsessive-compulsive disorder and tic disorder triggered by streptococcal infections, showing that all 4 cases plasmapheresis treatment resulted in significant improvement in both obsessive-compulsive symptom and tics.

The presence of mild symptoms in children may not require pharmacological intervention. The most of severe cases might be treated through pharmacologic and behavioural interventions, and because the PANS/ PANDAS symptoms often change during the course of illness, the treatments may require a continuous adjustment (Swedo et al., 2017).

Regarding the role of Adenotonsillectomy, Cooperstock et al., (2017), point out there are no available prospective controlled trials of adenoidectomy and tonsillectomy for PANDAS. They believe that indications for tonsillectomy and/or adenoidectomy should be limited to those recommended for the general population, such as sleep disordered breathing or frequent GAS infections.
(The TABLE 4 shows the general strategies for management of PANS based on disease trajectory; Frankovich et., al 2017).

1.3.2. Non-pharmacological interventions

The main non-pharmacological interventions regarding PANS/ PANDAS include psychoeducational, psychotherapeutic, behavioural, family, and school-based, interventions. These treatments aim to decrease suffering and improve functioning.

Many PANS cases could recover completely and when this happens, behavioural interventions could be interrupted.

At present there are no specific studies on the efficacy of psychotherapeutic treatments on PANS, and new research is needed.

What can certainly be done, is to work on the symptoms in consideration of the syndromic conditions that PANS possesses. That is, symptom-specific psychotherapy should be applied.

Therefore, clinicians should plan to intervene, through non-pharmacological therapies, on:

- ✓ Family Support and Education
- ✓ Obsessive compulsive (OCD) symptoms
- ✓ Restriction of food or fluid intake
- ✓ Tics
- ✓ Irritability and aggression
- ✓ Anxiety
- ✓ ADHD symptoms
- ✓ Sleep disturbances
- ✓ Depression
- ✓ Psychosis
- ✓ Pain (Thienemann et al., 2017).

It would be desirable that Children with PANS always receive a psychotherapy: Cognitive behavior therapy (CBT), specifically the Exposure/ Response Prevention (ERP), and minimizing family accommodation to OCD behaviors are the most effective interventions for pediatric OCD (Lebowitz et al., 2011).

CBT is typically conceptualized as a short-term, skills-focused treatment aimed at altering maladaptive emotional responses by changing the patient's thoughts, behaviors, or both.

Over the years a large number of diverse CBT protocols, have been created (Kaczkurkin and Foa, 2015). Regarding the ERP, the patient is exposed to thoughts, images, objects and situations that generate discomfort, while the part of the Prevention, on the other hand, concerns the choice not to carry out certain behaviours when these manifest their urgency to be implemented (Hezel and Simpson, 2019).

To maximize benefits, treatment with CBT/ ERP should start as soon as possible on an individualized intervention (Thienemann et al., 2017).

An exploratory investigation conducted on 473 patients, which had received some form of psychotherapy with some benefit, revealed that CBT was found to be the treatment with the highest efficacy. Furthermore, interestingly, during this exploratory investigation, it was often claimed that pharmacological therapies allowed CBT to be used more successfully (Calaprice et al., 2018).

The TABLE 5 shows the frequency of use and effectiveness of psychotherapy regarding this study.

2. STUDY N.1. PANS: A DATA MINING APPROACH TO A VERY SPECIFIC CONSTELLATION OF CLINICAL VARIABLES

2.1. RATIONAL AND OBJECTIVES

According to the 2013 PANS Consensus Conference (Swedo et al., 2012; Chang et al., 2015), PANS is currently conceptualized as a complex syndrome with a number of etiologies and disease mechanisms, encompassing psychiatric symptoms, arising from immune abnormalities triggered by a variety of agents (Frankovich et al., 2015; Murphy et al., 2015).

The PANS diagnostic construct was proposed as a clinical entity distinct from idiopathic or familial OCD, anxiety, or Tourette disorder on the basis of clinical observations.

The validity and generalizability of epidemiologic studies about PANDAS and PANS have been criticized for highly biased participant sampling and nonvalidated causes, effects, and methods (Gilbert et al., 2018).

Nevertheless, PANS may be comparable to other persistent neuroinflammatory disorders such as multiple sclerosis, Sydenham chorea (SC), Behcet's disease, and asthma, underlining that infections and other environmental triggers play a role in provoking an inflammatory brain response, which evolves into a chronic or progressive neuroimmune disorder (Frankovich et al., 2015).

Despite the fact that PANS is a clinical diagnosis of exclusion without a single defined etiology or specific clinical symptoms, it could be considered a useful construct for outlining the sudden onset of severe neuropsychiatric symptoms in children with a relapsing-remitting course.

Artificial Neural Networks (ANNs) are computational adaptive systems inspired by the functioning processes of the human brain: they are considered particularly useful to solve nonlinear problems and to discover subtle trends and associations among variables. Based on their learning through an adaptive way (i.e., extracting from the available data the information needed to achieve a specific aim and to generalize the acquired knowledge), the ANNs appear to be a powerful tool for data analysis in the presence of relatively small samples (Buscema et al., 2015).

In the last decade, a fourth generation ANN called Auto-Contractive Map has been increasingly used in medicine (Street et al., 2008; Gironi et al., 2013; Buscema et al., 2015; Narzisi et al., 2015; Toscano et al., 2017; Grossi et al., 2017, 2018).

Overall, literature findings suggest that this method may be a strategic approach to grasp the core of the relationship between signs and symptoms of PANS.

This study aimed to explore the associations between the different PANS features and laboratory and clinical variables in a sample of 39 children diagnosed with PANS. We used the ANN approach to exploit putative subtle simultaneous connections among the full spectrum of clinical variables and different domains of impairment.

2.2.   METHODS

2.2.1. Participants

Consecutive patients referred for obsessive-compulsive or anxiety symptoms and tic disorder, between December 2017 to December 2018 to the outpatient clinics of Child & Adolescent Neuropsychiatric Unit, "G. Brotzu" Hospital Trust, Cagliari and the Child and Adolescent Psychiatry Unit, Policlinico "G. Martino", Messina, were analysed for possible PANS, according to PANS working criteria defined by experts convened at the National Institute of Mental Health (NIH) in July 2010 (Swedo et al., 2012) (see Introduction).

The diagnosis of PANS was confirmed by two child psychiatrists (AG and CG).

Exclusion criteria were the following:
- ✓ Occurrence of immunologic diseases or cancer
- ✓ Presence of other medical or neurological/psychiatric diseases
- ✓ Active treatment with anti-inflammatory or corticosteroid agents
- ✓ Lack of consent form for participating to the study
- ✓ Finally, patients with missing data on clinical records were excluded.

2.2.2. Variables

Patients were assessed according to the Consensus Statement clinical recommendations (Chang et al., 2015).

An extensive physical, neurological and psychiatric examination was performed. Parents were interviewed about family history and child medical history with a focus on the neurodevelopmental course, immune profile (autoimmune diseases, inflammatory diseases, immunodeficiency) and psychiatric conditions; family medical history included information about two generations (grandparents,parents, uncles, aunts, siblings, and cousins).

A blood sample was collected from each subject after the diagnosis and before starting any treatment.

The battery of clinical laboratory test included: complete blood count, renal and liver function test, mineral panel, thyroid function indices, antithyroid antibodies (anti-thyroid peroxidase [anti-TPO], anti-thyroglobulin antibodies, TSH receptor antibodies [anti-TRAb], thyroid stimulating hormone receptor antibody [anti-TSH receptor]) and inflammatory blood markers (C-reactive protein [CRP], erythrocyte sedimentation rate [ESR], and procalcitonin [PCT]).

Serum level of Immunoglobulin G (IgG) and Immunoglobulin M (IgM) Antibodies against Mycoplasma pneumoniae, Chlamydia pneumoniae, Haemophilus influenzae, Epstein Barr virus, and Herpes Simplex Virus –HSV- Type 1, was also measured.

At enrolment, all parents completed a checklist in Italian screening the symptoms and assessed their severity.

The checklist was defined according to PANS criteria (Swedo et al., 2012). It encompasses 10 items and systematically describes both main and additional PANS neuropsychiatric symptoms with multiple choice responses on a 4 point Likert scale 0 = absent; 1 = mild; 2 = severe; 3 = very severe). A narrative description of the meaning and implications of each item was provided to the parents (see TABLE 6) and a clinician helped the parents to fill out the checklist.

In particular, parents were informed that the tool allows rating of how much the symptoms impact their child's life from 0 to 3, where 3 indicates the highest impact.

2.3. STATISTICAL ANALYSIS

All patient's clinical variables (family history, child symptom severity, medical history, and clinical laboratory test results) were collected on a specific database.

The complete list of the variables is shown in TABLES 6, 7 and 8.

The proportion of patients who had a specific symptom, a positive family history and maternal disease, and abnormal laboratory test results was calculated for each variable.

Furthermore, the clinical variables were analysed in the following three steps: Linear correlation analysis, Artificial Neural Networks (ANNs) analysis, and Benchmarking analysis.

2.3.1. Linear correlation analysis

Spearman correlation analysis was performed on the 33 clinical and laboratory test variables expressed in binary format. A p-value <0.05 was considered to be statistically significant. Statistical analysis was performed with XLSTAT package 2018.

2.3.2. Benchmarking Analysis

To handle a benchmarking analysis, the Principal Component Analysis (PCA) and Hierarchical Agglomerative Clustering (AHC) was carried out.
Results of this benchmarking analysis allow to compare findings from the Auto-CM approach with findings from the traditional statistical approach.

2.3.3. Principal component analysis

PCA is mathematically defined as an orthogonal linear transformation of the data to a new coordinate system such that the highest variance by any projection of the data comes to lie on the first coordinate (called the first principal component), the second highest variance on the second coordinate, and so on. PCA is theoretically the optimum transform for given data in least square terms.

2.3.4. Hierarchical agglomerative clustering

AHC is one of the most popular clustering methods which seeks to build a hierarchy of clusters with a "bottom-up" approach: each observation starts in its own cluster, and pairs of clusters are merged as one moves up the hierarchy.
This method works from the dissimilarities between the objects to be grouped together, producing a so called "dendrogram" which shows the progressive grouping of the data.

It is then possible to gain an idea of a suitable number of classes into which the data can be grouped.

PCA and AHC have been carried out with XLSTAT package 2018.

2.3.5. The Artificial Neural Networks (ANNs) Analysis

2.3.5.1.   General features

The Auto Contractive map (Auto-CM) system is a fourth generation unsupervised ANNs, which has already been demonstrated to outperform several other unsupervised algorithms in a heterogeneous class of tasks (Buscema and Sacco, 2017).

Auto-CM is a mapping method able to compute the multidimensional strength association of each variable with all other variables in a dataset, using a mathematical approach based on ANNs.

Auto-CM is especially effective in highlighting any kind of consistent patterns, systematic relationships, hidden trends, and associations among variables. Indeed, this method is able to compute and graph a semantic connectivity map which:

(1) preserves nonlinear associations among variables,

(2) captures elusive connection schemes among clusters,

(3) highlights complex similarities among variables.

The 3-layer architecture and the mathematical models of Auto-CM have been described elsewhere (Buscema and Grossi 2008). In nontechnical terms, this model has both a training phase and a learning phase. After the former, Auto-CM determines the socalled ''weights'' of the vector matrix, which

(1) represent the warped landscape of the dataset and

(2) permit a direct interpretation.

Indeed, these weights are proportional to the strength of many to-many associations across all variables and can be easily visualized by transforming them into physical distances: variables whose connection weights are higher get relatively nearer and vice versa.

By applying a mathematical filter (i.e., minimum spanning tree, MST) (Kruskal, 1956; Fredman and Willard, 1990) to the matrix of distances, a graph named ''semantic connectivity map'' is generated.

This representation allows a visual mapping of the complex web of connection schemes among variables, simplifying the detection of the variables that play a key role in the graph. The adaptive learning algorithms of inference, based on the principle of a functional estimation like ANNs, overcome the problem of dimensionality.

For this reason, we did not apply Bonferroni adjustment (applied in the case of significance tests carried out with dependent variables) preferring to use an exploratory analysis looking for important associations among many independent variables.

2.3.5.2.   Minimum spanning tree

The MST (FIGURE 9 and 10) shows among the full spectrum of possible ways to connect the variables in a tree, the shortest combination. Based on the MST theory, the Auto-CM reveals the connections among variables providing a graph in which the distances among variables reflect their bonding strength (weights) (Buscema and Grossi, 2008, 2017; Buscema et al., 2008). In practical terms, MST shows the best way to connect the variables in a tree and the shortest possible combination allowing to present the data in a simplified graph.

In classical mechanics, Maupertuis's principle (Cheng, 2012) states that the path followed by a physical system is the one of least length. It is a special case of the more generally stated principle of least action.

Using the calculus of variations, it results in an integral equation formulation of the equations of motion for the system.

The kinetic paths from least action principle quantify the transition processes among normal state and pathological state in biological systems.

Also in case of variable interconnection, our assumption is that their system must naturally tend to minimum energy state, well described by the graph generated by MST. This approach provides the map of relevant connections between and among variables and the principal hubs of the system. Hubs can be defined as variables with the maximum amount of connections in the map. The Auto-CM does not pose randomly the initial weights.

Conversely, the Auto-CM starts with the same value. Thus, the resulting graph is reproducible along many runs. In other words, the Auto-CM visualizes in the space' the correlation among the variables (''closeness''), and the graph identifies only the relevant associations organizing them into a coherent picture. The ''central node'' is the inner node that remains after bottom-up recursively pruning away the ''leaves'' nodes.

The MST represents what could be called the 'nervous system' of any dataset. In fact, summing up all of the connection strengths among all the variables, it gets the total energy of the system. The MST selects only the connections that minimize this energy, that is, only the ones that are really necessary to keep the system coherent.

Consequently, all the links included in the MST are fundamental, but, on the contrary, not every ''fundamental'' link of the dataset needs to be in the MST. Such limit is intrinsic to the nature of MST itself: every link that gives rise to a cycle into the graph (viz., that destroys the graph's ''treeness'') is eliminated, whatever its strength and meaningfulness. To fix this shortcoming and to better capture the intrinsic complexity of a dataset, it is necessary to add more links to the MST, according to two criteria:

(1) the new links have to be relevant from a quantitative point of view;
(2) the new links have to be able to generate new cyclic regular microstructures, from a qualitative point of view.

The additional links superimposed to MST graph generate a maximally regular graph (MRG).

2.3.5.3. *Maximally regular graph*

To understand how MRG works we must start remembering the nature of MST. MRG is the graph whose hubness function attains the highest value among all the graphs generated by adding back to the original MST, one by one, the connections previously skipped during the computation of the MST itself. Starting from the MST, the MRG presents the highest number of regular microstructures and highlights the most important connections of the dataset. In other words, to build the MRG the sorted list of the connections skipped during the derivation of the MST must be considered.

Each time we add a new connection to the MST basic structure, to monitor the variation of the complexity of the new graph at every step, with a specific parameter of complexity, called H Function. We call MRG the graph whose H Function attains the highest value among all the graphs generated by adding back to the original MST, one by one, the missing connections previously skipped during the computation of the MST itself. By this way, we draw a "diamond" expressing the complexity core of the system.
The frequency of a given symptom/variable influences the likelihood to become part of the central group of variables (core).

Nevertheless, a high occurrence does not necessarily bring to the inclusion of a given variable in the core domain. In fact, even if the program is influenced by variable frequency, it picks up other kind of inherent information, independent from the simple frequency.

2.4. ETHICAL APPROVAL

The independent Ethics Committee of Cagliari University Hospital approved the study. All the parents were given a full explanation of the study methods and purposes and gave their written consent.

2.5. RESULTS

2.5.1. Clinical variables

From a total of 312 consecutive outpatients referred for obsessive-compulsive or anxiety symptoms and tic disorder, 42 were diagnosed with PANS.

One child was excluded because parents' refusal to take part in research. Two children were excluded due to active treatment with anti-inflammatory agents.

Thus, according with the inclusion and exclusion criteria of the study, 39 patients (13 female and 26 male) were enrolled into this study, corresponding to the 13% of the outpatients with obsessive-compulsive or anxiety symptoms and tic disorder referred to the two units.

Mean age at recruitment was 8.6 yrs. (SD 3.1). For most of these patients (28/39 subjects; 72%), the symptom onset was close to the time of the first clinic assessment (between 1 and 12 weeks).

In particular, 23/ 39 patients (59%) reported symptoms of infections (fever, coughing, ear pain or diarrhea) within 4 weeks from the symptom onset. Instead, for the remaining 11/39 subjects (28%) the symptom onset lied between 3 month and 3 years before the first clinic visit, with relapsing/remitting course or with a single previous episode (spontaneously recovered) before the first observation.

Independently from their onset, all patients showed acute psychiatric symptoms at the time of the enrolment.

As reported in TABLE 6, "anxiety" and "irritability/oppositional defiant disorder" were the most frequent, interesting the 89% of subjects.

The "obsessive-compulsive symptoms" have been reported in the 87% of the sample. The percentage of subjects showing the other symptoms was never below 60%. The less frequent symptoms were"enuresis/urinary frequency" and restricted food intake (respectively, 61% and 66%).

As reported in TABLE 7, a positive family history of Autoimmune Diseases was the most frequent condition associated to PANS (80%). Increased rates of psychiatric disorders (72%) in the family were also reported.

The pregnancy-related/intrapartum complications were reported by 64% of the mothers of the enrolled patients, active infections in 15%, active preexisting or new onset autoimmune diseases during pregnancy in 59%, and Hashimoto's disease during pregnancy in 33%.

As shown in TABLE 8, recurrent maternal infections were frequent (32/39; 82%); allergy and atopic diseases were reported almost in half of the sample (18/39; 46%), atopic dermatitis being the most frequent atopic disease (23/39; 59%).

2.5.2. Laboratory variables

Only 15 patients (39%) showed a high antistreptolysin O antibody (ASO) level (i.e., > 250 IU, laboratory upper reference limit).

Nasopharyngeal culture was positive in 23/39 subjects (59%) and it revealed the presence of Group A beta-hemolytic streptococcus (GABHS) in 8 out of 23 patients; other bacteria isolated by the nasopharyngeal culture were Staphylococcus aureus (11/23), Streptococcus pneumoniae (pneumococcus) (1/23), Haemophilus influenzae (2/23), and Pseudomonas aeruginosa (1/23).

In a large portion of the sample (49%) a high antibody titer against different germs was observed: the most common were antibodies against Mycoplasma and Chlamydia. We found IgG and/or IgM, depending on the length of the interval between infection and neuropsychiatric symptom onset, since the IgM normally disappears after 2–3 weeks of their production.

A serological diagnosis of Epstein–Barr virus infection (IgM antibodies) was made in one case. Lyme disease was suspected for a young girl with IgG antibodies against Borrelia burgdorferi, but further laboratory tests did not confirm the diagnosis.

Antithyroid antibodies were found above the upper reference limit in the 36% of our sample, although no patients with thyroid antibodies positivity showed reduced echogenicity on thyroid sonogram, nor clinical Hashimoto's thyroiditis features, nor overt hypothyroidism. They all showed normal levels of thyroidstimulating hormone (TSH) and free thyroxine (FT4).

The antinuclear antibodies (ANAs) were also found above the upper reference limit (titers > of 1: 120) in 13/39 subjects (33%). The lupus workup was completed for all the children with elevated ANA title: none of them resulted positive for lupus-specific antibodies.

Inflammatory markers like CRP, ESR, and PCT levels above the upper reference limit were observed in only 7/39 subjects (18%).

A reduction of the Natural Killer (NK) absolute cell counts in the peripheral blood lymphocyte was observed in almost all the enrolled children. Among the Lymphocyte subsets (T, B, NK cells), the NK cell (CD3+/CD56+) % value was considered to define two groups of patients (NK ‡3% or NK <3%), based on the lower reference limit (3%). In 72% of our children the percentage of NK cells was less for the lower reference limit (5 patients had 1% or 0% of NK cells).

In 22 patients (56% of the sample) the electroencephalography (EEG) overnight recording showed intermittent or persistent focal or generalized slowing (mainly localized in temporal-frontal regions).

Some patients presented also a persistent and unvarying focal or generalized slow-wave activity in the vigilant state, but none of them have had epileptic seizures.

Minor structural brain magnetic resonance imaging (MRI) abnormalities (T-2 weighted images or contrast enhancement) were observed in 8/39 subjects of the sample (21%). These abnormalities were however considered "incidental": 2 slight ventricular asymmetries, 2 developmental venous anomalies, 1 isolated cerebellar vermis hypoplasia, 1 arachnoid cysts, 1 Arnold Chiari malformation, and 1 mild white matter hyperintensity in the periventricular area. No specific volumetric and/or inflammatory changes in the basal ganglia or cortical areas were found.

2.5.3. Linear correlation analysis

The Spearman linear correlation matrix of variables included in the study showed, as expected, a strong correlation between family history of autoimmune disease and maternal autoimmune disease in pregnancy and allergic and atopic disease ($R = 0.38$ and 0.40, respectively).

Remarkably, family history of autoimmune disease was correlated also to family psychiatric history ($R = 0.39$).

Furthermore, family psychiatric history was related to EEG alteration and ASO positive test ($R = 0.38$ and 0.45, respectively).

Allergic and atopic diseases correlate to the increase of antibodies against other germs (Anti- Mycoplasma pneumoniae, Chlamydia pneumoniae, Epstein Barr virus, Borrelia Burgdorferi, Herpes Simplex Virus –HSV- Type 1) ($R = 0.33$), and against thyroid ($R = 0.38$).

High levels of ANA correlate with family history of autoimmune diseases ($R = 0.44$) and with positive other germs antibodies ($R = 0.51$). With regard to symptoms, ASO positive test is related to restricted food intake ($R = 0.40$) and the ANA positive test is related to enuresis/urinary frequency ($R = 0.37$).

Emotional lability/depression correlates with the presence of positive other germs antibodies (R = -0.32) and with low natural killer cells (R = 0.33).

2.5.4. Benchmarking Analysis

The Principal Component Analysis (PCA) contains all possibly correlated variables distributed along vectors of different sizes (various numerical values), identifying the linearly uncorrelated variables (principal components) along which the variation in the data is maximal.

Here PCA Map provided the identification of two clusters, shown in FIGURE 7.

The first cluster is organized around the obsessive compulsive dimension and encompasses almost all PANS symptoms in the following hierarchy: enuresis/urinary frequency, school performance deterioration, sensory motor abnormalities, sleep disturbance, irritability/ODD, behavioural regression, anxiety and emotional lability/depression.

Among the variables of this cluster we also found the variables ASO test >150 and NK<3. The opposite variables (ASO test <150 and NK≥3) in our PCA, were uncorrelated variables.

The second cluster encompasses most of the familial and personal risk factors and conditions. Among them, autoimmune diseases during pregnancy, ANA > 1:110, Antithyroid antibodies, familial autoimmunity, allergic, atopic disorders and other germs (Anti- Mycoplasma pneumoniae, Chlamydia pneumoniae, Epstein Barr virus, Borrelia Burgdorferi, Herpes Simplex Virus –HSV- Type 1) antibodies are represented by vectors of largest size.

The Agglomerative Hierarchical Clustering (AHC) analysis resulted in a dendrogram which shows the progressive grouping of the variables (FIGURE 8).

The dendrogram shows the clustering of the variables according to a particular kind of mathematics inherent to HAC technique. As stated in the figure legend there are different clusters marked with different colors.

Our dendrogram shows three clusters identified by different colors.

The green cluster encompasses almost all PANS symptoms, with some variables more closely related to each other, as obsessive-compulsive symptoms and school performance deterioration, enuresis/urinary frequency and behavioral regression, sleep disturbance, and irritability/ODD.

Furthermore, emotional lability/depression is linked to NK <3. The red cluster shows a predictable relationship between ''pregnancy/ delivery complications'' and ''infection during pregnancy.'' Noteworthy, both these conditions appear linked with ''ASO test >150'' and are more frequent in female children. The blue cluster encompasses all other laboratory and clinical variables.

2.5.5. Semantic connectivity map

The semantic connectivity map (Auto-CM method) - Minimum spanning tree (MST) graph (FIGURE 9) shows the strength of association across the clinical and laboratory variables visualized by the concept of "closeness": the variables whose connection weights are higher get relatively nearer and vice versa. The links strength values were all above 0.8.

The connection strengths do not influence the solution of MST. They have a different meaning from the weights generated by Auto-CM and have only a descriptive function.

It is important to remember that the frequency of each variable influences the likelihood to become part of the central group of variables (core), but it does not necessarily bring to the inclusion of the variable in the core domain.

For example, family history of autoimmune disease is the most frequent variable, but it is not included in the core domain.

As showed in the FIGURE 10, the "Maximally Regular Graph" superimposed to MST indicates the putative internal structure of the syndrome.

The map shows that PANS symptoms are strictly linked to each other in a central "diamond," originated by the specific mathematical function called MRG (see Method section). It encompasses anxiety, irritability/ODD symptoms, obsessive compulsive symptoms, behavioral regression, sensory motor abnormalities, school performance deterioration, sleep disturbances, and emotional lability/depression.

Two PANS symptoms (enuresis/urinary frequency and restricted food intake) are beyond the "diamond," indicating a less frequent link with the other symptoms. Nevertheless, their high link strength value (≥0.8) with the other symptoms indicates a strong probability of co-occurrence with the other symptoms. In our opinion, the resulting "diamond" well expresses the complexity core of the PANS.The robustness of Auto-CM system analyzing the stability ofconnections in the MST graph is described in Supplementary Data.

2.6. DISCUSSION

Even though the data collected describe the already known characteristics of PANS, our study offers a statistic model for a specific symptoms' constellation (syndrome) clinically distinct from other neurodevelopmental disorders.

2.6.1. PANS described by ANN analysis

The present study is the first one to have adopted Complex network mathematics approach like Auto-CM to face the complexity of PANS phenotype.

The results of the study show that PANS symptoms, as defined by the PANS Collaborative Consortium (Swedo et al., 2012; Chang et al., 2015), are strictly connected one to another, shaping a central ''diamond'' encompassing anxiety, irritability/ODD symptoms, obsessive compulsive symptoms, behavioral regression, sensory motor abnormalities, school performance deterioration, sleep disturbances, emotional lability/depression, and laboratory measures.

The Auto-Contractive Map method also allowed to grasp the core of the relationship between symptomatology, history, and laboratory results of our subjects with PANS. Exploiting all not obvious connections among the full spectrum of clinical variables revealed the simultaneous connections among symptoms and clinical signs, highly consistent with the PANS Collaborative Consortium's description of the syndrome (Chang et al., 2015).

Noteworthy, the classical statistical analysis (Spearman linear correlation) resulted as far less explicative of the real relationships between the variables.

It failed in showing the whole symptomatic dimensions even if it displayed the relationships among them. The traditional statistical analysis approach suffered from some criticisms.

Indeed, due to the heterogeneity in clinical expression of the PANS syndrome (i.e., different degrees of symptom severity along with various pathogenesis), a traditional statistical analysis approach using a ''single symptom approach analysis'' may not provide comprehensive information on the putative nature of the PANS.

The semantic connectivity map (FIGURE 9 and 10) describes the hidden internal construct of PANS and exemplifies the consistency of the PANS working criteria, as they were defined at the NIH in July 2010 (Swedo et al., 2012).

In fact, the PANS symptoms are strictly linked to one another shaping a central "diamond" encompassing anxiety, irritability/ODD symptoms, obsessive compulsive symptoms, behavioral regression, sensory motor abnormalities, school performance deterioration, sleep disturbances, and emotional lability/depression.

In the present semantic connectivity map, the variable "obsessive-compulsive symptoms" node acts as a hub (variable with three or more links) receiving convergence from the other symptom nodes (anxiety, irritability/ODD symptoms, behavioral regression, and sensory and motor abnormalities) and from clinical variable (nasopharyngeal culture and pregnancy/delivery complications).

Therefore, the obsessive-compulsive dimension seems to represent the core symptom of the syndrome. At the same time, it is one of the most frequent symptoms, being reported in 87% of the sample.

Enuresis/urinary frequency is located beyond the "diamond," even though it is strictly related to the other symptoms. This symptom, reported in the 61% of patients, could be underestimated because parents may not be aware of it at the early stage.

The only other symptom located outside the "diamond" is the restricted food intake, even though it is situated along one of the branches directly arising from the main hub (obsessive compulsive symptoms).

Of note, even if this symptom was very common among the subjects of the present sample (66% of the subjects), most of the children had a relatively mild food intake restriction.

It is possible that cultural reasons may explain this finding, since parents (mostly living in the south of Italy) are used to strongly encourage their children to eat even when they tend to refuse the food. This peculiar approach to feeding can also explain the relatively lower frequency of food intake restriction compared to the other symptoms.

However, in the semantic map, this symptom is allocated right beyond the sensory/motor abnormalities. Interestingly, according to our clinical experience, some of the children with PANS that refused to eat also showed atypical sensory interests concerning the food (e.g., odor, consistency, or color of aliments) or compulsions and rituals related to the feeding act (e.g., crumbling the food, taking little bites, long chewing, moving the bite in the mouth, refusing to swallow, or swallowing only in specific positions, and so on).

This suggests that, at least in part, the food intake restriction could be linked to the sensory and compulsive dimension of PANS clinical presentation rather than to simple loss of appetite.

The semantic map also describes the association between obsessive-compulsive symptoms, school performance deterioration, and anxiety symptoms and between emotional lability/depression and behavioral regression, supporting the PANS phenotype description.

Actually, the coherence of these symptoms was already evident in the benchmarking analysis. Both the PCA and the AHC provide clusters of symptoms closely and reciprocally linked.

From the PCA, the first cluster is organized around the obsessive-compulsive dimension and encompasses almost all PANS symptoms in the following hierarchy: enuresis/urinary frequency, school performance deterioration, sensory motor abnormalities, sleep disturbance, irritability/ODD, behavioral regression, anxiety, and emotional lability/depression.

A second cluster encompasses the most of laboratory and clinical variables. This cluster underlines a meaningful relationship between most of the autoimmunity markers studied in our sample ("other germs antibodies," "antithyroid antibodies," "allergic and atopic disorder," "familial autoimmunity," "ANA >1:110," "autoimmune diseases in pregnancy," and "Hashimoto's disease in pregnancy").

It is known that most of the patients with PANS have both autoimmune/ inflammatory diseases (e.g., autoimmune thyroiditis, postinfectious, enthesitis-related, psoriatic, or spondyloarthritis) and higher ANA and antithyroid antibodies than expected in the general population (Frankovich et al., 2015).

In particular, the AHC analysis resulted in a dendrogram, which shows the progressive grouping of the variables. It gains the idea of a suitable number of classes into which the variables can be grouped. One of the clusters of variables in our sample includes almost all PANS symptoms. Among them, it is possible to recognize variables closely related to each other, as obsessive-compulsive symptoms and school performance deterioration, enuresis/urinary frequency and behavioral regression, sleep disturbance, and irritability/ODD.

Furthermore, in our semantic map, both familial and maternal disease factors (familial autoimmunity, family history of psychiatric diseases, Hashimoto's disease in pregnancy, infections in pregnancy, and autoimmune diseases in pregnancy) and children clinical and laboratory variables (allergic and atopic disorders, atopic dermatitis, nasopharyngeal culture, EEG alterations, NK cells % value <3, inflammatory markers, ASO test, other germ antibodies, ANA, and antithyroid antibodies) are closely related to symptoms.

These suggest that it is important to take notice of the familial autoimmune/inflammatory profile and to consider the possible pathophysiologic role of immunological events during pregnancy. Even though factors having less than three links can be considered from a mathematical point of view, of relatively lower importance, they may represent the potential mechanisms, including direct influences of infections in the central nervous system (CNS), immune activation, and inflammatory mediators in PANS syndrome. Furthermore, emotional lability/depression is strongly related with NK cells % value <3.

2.6.2. PANS and familial, clinical, and laboratory variables

A second level of analysis with regard the familial, clinical, and laboratory data was described in our sample. As shown in TABLE 7, a high percentage of our patients had relatives affected by autoimmune disorders (80%) and psychiatric disorders (72%).

Both conditions have been associated with PANS (Chang et al., 2015), even if most of the studies are focused on OCD and tic disorders among first-degree relatives of PANDAS probands (e.g., Lougee et al., 2000).

A recent survey, carried out by PANDAS network (Pohlman, 2018) and based on parent's reports, described 47,34% of 1221 patients with PANS/PANDAS having autoimmune signals in the maternal and paternal lineage, 58,39% having anxiety disorders, and 27,52% having OCD in the maternal and paternal lineage.

In our sample, both autoimmune and psychiatric diseases in the family members ranked higher than the survey data (around 70%).

This difference may be attributed to the different method used to collect the information, because in the present study data were collected from a clinic interview by expert clinicians rather than a questionnaire.

On the whole, a familiar probably genetic (Wang et al., 2015) susceptibility for both autoimmune and psychiatric disorders appears to play an important variable increasing the likelihood of developing PANS. Results of the present study also showed a high prevalence rate of the pregnancy/delivery complications in general (64%) and active infections and autoimmune diseases during pregnancy in particular (15% and 59%, respectively) in our sample (TABLE 7). Prenatal exposure to infection is a risk factor for a wide range of neurodevelopmental and psychiatric disorders according to gene–environment interaction etiological model (Zhou, 2012; Blomstrom et al., 2015).

More recent studies have considered the interaction between maternal immune activation (MIA) and genetic risk factors for alterations in structural/functional neuronal network impairments leading to psychiatric conditions as autism spectrum disorder (ASD) and schizophrenia (Bergdolt and Dunaevsky, 2018).

The link between maternal infection and neurodevelopmental disorders could also be regarded as a MIA potentially inducing prolonged immune alterations in the offspring's brain, independently from the infection itself (Boulanger-Bertolus et al., 2018).

It is thought that the maternal activation of the innate and adaptive immune systems due to infection, stress, autoimmunity, asthma, allergies, or inflammation can lead to several neuropathologies in the progeny, particularly ASD and schizophrenia (Jiang et al., 2018). This is because MIA may influence the developing fetal CNS through the increased production of inflammatory cytokines acting as a disease primer or first ''hit'' and predisposing susceptible individuals for further exposures or ''hits'' later in life (Jiang et al., 2018; Bilbo et al., 2018).

Furthermore, immune abnormalities seem to be more common in individuals and first degree relatives with ASD (e.g., Gładysz et al., 2018). For instance, fetal brainspecific antibodies have been identified in mothers of autistic children in several different studies (Keil et al., 2010; Nordahl et al., 2013; Fox-Edmiston and Van de Water, 2015; Hughes et al., 2018).

In line with this hypothesis, we found that almost a third of the mothers of the enrolled patients suffered from Hashimoto's thyroiditis during pregnancy. This prevalence is almost thrice higher than the estimated prevalence (13%) of the disorder in the United States (Staii et al., 2010).

Studies conducted in our geographic area found that between 5% and 20% of female and between 1% and 5% of male are affected by Hashimoto's thyroiditis in the general population (Chiovato et al., 1993).

In the last decades, the disease has become even more common than it was until the early 1990s (Benvenga and Trimarchi, 2008).

It could be inferred that our children have been exposed to elevated levels of circulating antibodies during their intrauterine life.

The association between autoimmune thyroiditis and psychiatric disorders in offspring has been poorly studied.

A family history of autoimmune disorders was described in children with ''regressive'' autism. Regression was significantly associated with a family history of autoimmune disorders (adjusted OR= 1.89) and particularly with autoimmune thyroid disease (adjusted OR= 2.09) (Molloy et al., 2006).

A recent systematic review showed a significant association between maternal thyroid dysfunction during early pregnancy, including low and high thyroid hormone level and autoimmune thyroiditis, and several offspring behavioral and psychiatric disorders such as attention-deficit/ hyperactivity disorder (ADHD), autism, pervasive developmental problems, and externalizing behavior, in addition to epilepsy and seizures (Fetene et al., 2017). In particular, the odds of autism were increased by nearly 80% among offspring of mothers who were Thyroid peroxidase antibody (TPO-Ab) positive during pregnancy (OR = 1.78), compared to mothers negative for this autoantibody (Brown et al., 2015). Gestational immune activation and the presence of maternal autoantibodies are thought to be directly contributing to abnormal brain development mechanisms and thus involved in the pathogenesis of ASD (Hughes et al., 2018).

By the analogy with ASD and other neurodevelopmental disorders, we underline the strong presence of familial autoimmunity in our PANS sample arguing that maternal infective and immunological factors may play an important role also on the PANS phenotypic expression.

In parallel, it appears interesting that among the children of our sample, anti-TPO, Thyroglobulin antibodies, TSH receptor antibodies (anti-TRAbs), and Thyroid Stimulating Hormone Receptor Antibody (anti-TSH receptor) are above the upper reference limit in the 36% of our sample, even though none of our patients had overt hypothyroidism (TABLE 8).

This rate is much higher than the prevalence of thyroid antibodies reported in other studies where TPO-Ab positive rates ranged between 11 and 13% in different areas of the world (Hollowell et al., 2002; Amouzegar al., 2017). Longitudinal studies on general population (e.g., Li et al., 2008) demonstrated that positive thyroid antibodies were associated with an increased risk of developing hypothyroidism later in life.

In particular, thyroid peroxidase antibody (TPO-Ab) measurement has been considere appropriate to identify patients at risk of developing hypothyroidism (Zelaya et al., 2010).

Nevertheless, according to our data, the positive antithyroid antibodies in euthyroid children with PANS are probably an expression of a condition arising from a more general abnormal immune response. Recurrent infections are also frequently reported in the medical history of our patients. Recurrent infections are very common in children, mostly in healthy preschool children, who can experience up to six to eight respiratory tract infections per year (Gruber et al., 2008).

However, the recent findings of a population-based cohort study using Danish nationwide registers provide evidence for the involvement of infections and the immune system in the etiology of a wide range of mental disorders in children and adolescents (Ko¨hler-Forsberg et al., 2018).

The role of infectious diseases and immune dysregulation has been recently studied also in specific populations, such as individuals with fragile X syndrome (Yu et al., 2020). In PANS/PANDAS, the infection history of children is of particular interest because most PANS are suspected to be postinfectious in origin, although no single microbe, other than GABHS, has yet been consistently associated with the onset of PANS (Chang et al., 2015).

Reliably, half of our sample had different germ antibodies (IgG and/or IgM Antibodies against Mycoplasma pneumoniae, Chlamydia pneumoniae, Epstein–Barr virus, Haemophilus influenzae, and Herpes Simplex Virus –HSV- Type 1) at the clinic presentation.

The most common antibodies were IgG and/or IgM against Mycoplasma and Chlamydia that are considered important causative pathogens of community-acquired infections in school-age children and adolescents. Therefore, our results are consistent with the PANS Collaborative Consortium's hypothesis that other infectious agents, particularly those with characteristically prolonged colonization, have the potential to activate PANS (Chang et al., 2015).

In addition, the nasopharyngeal culture results were often positive for different germs such as Staphylococcus aureus, Streptococcus pneumoniae, and Haemophilus influenzae (see Description in Results section).

Group A beta-hemolytic streptococcus (GABHS) was found in 21% of the subjects. Therefore, the percentage of patients with an active GABHS infection as a triggering agent in our sample appears lower than that reported in previous reports (Murphy et al., 2015; Calaprice et al., 2017), although the 39% of our study subjects were positive for the antistreptolysin O antibody (ASO). However, among our 15 children with ASO >250 IU, only in 9 of them (23% of whole sample) the value exceeded the laboratory's stated upper limit of normal by twofold.

In addition to recurrent infections, allergy and atopic diseases should be considered. Atopy causes chronic inflammation of the airways that facilitate the adherence of pathogens to the respiratory epithelium and the development of respiratory infections (Mucha and Baroody, 2003).

In our sample, allergic and atopic diseases were described almost in one in two children (46%). However, this rate of prevalence is not far fromthe prevalence of any atopic diseases (40%) in general pediatric population (Christiansen et al., 2016).

Notably this high atopy prevalence is mostly due to a high prevalence of rhinoconjunctivitis (33%), with lower prevalence of asthma (13%) and atopic dermatitis (8%) (Christiansen et al., 2016).

Conversely, in our sample atopic dermatitis appears as the most frequent atopic disease (59%), largely more represented than in normal pediatric population. This finding is consistent with the accumulating evidence on the association between atopic dermatitis and several children mental health disorders (es. Kandelaki et al., 2015; Catal et al., 2016).

In particular, attention-deficit/ hyperactivity disorder and autism (Chen et al., 2014; Lee et al., 2016; Liao et al., 2016) and anxiety and depression (Cheng et al., 2015; Becker-Haimes et al., 2017) have been associated with atopic dermatitis.

In a recent Japanese study, eczema and children mental health problems (emotional symptoms, conduct problems, hyperactivity/inattention, and peer problems) were found to be significantly related, and the mean Strengths and Difficulties Questionnaire total difficulties score was significantly increased with worsening eczema status (Kuniyoshi et al., 2018).

To the best of our knowledge, there are few reports on the putative association between PANS and atopic dermatitis.

A recent study (Rosa et al., 2018) described a prevalence of allergic and immune-mediated food disorders similar to the general population in a group of 69 subjects with PANS, with the exception of a higher rate of allergic rhinitis and a lower rate of atopic dermatitis than the general population. However, studies showed that different allergic diseases, not limited to atopic dermatitis, have been associated with an increased hazard of psychiatric disorders both in adults (Perugi et al., 2015; Tzeng et al., 2018) and in children (Nanda et al., 2016; Miyazaki et al., 2017).

ESR and CRP are widely used laboratory markers of systemic inflammation. A very small portion of our patients (18%) had values above the upper reference limit. However, these tests have a low index of specificity and are influenced by numerous disease factors (Bray et al., 2016). Their utility in providing valuable information in PANS has not been established yet. These inflammatory markers could have low specificity for conditions characterized by brain inflammation. Also the ANA values resulted above the upper reference limit in a relatively small part of our subjects (33%), even though the rate exceeds the estimated prevalence of positive ANAs (12–13%) in healthy pediatric population (Satoh et al., 2012).

Likewise, our data coincide with the assumption of PANS Consortium (Chang et al., 2015) that the rate of positive ANAs in patients with PANDAS and PANS is higher than the baseline.

In the present sample, a low NK cell count appears strongly associated with PANS symptoms.

The recommendations from the 2013 PANS Consensus Conference (Chang et al., 2015) suggest that immune evaluation should encompass the study of lymphocyte subsets.

Seventy two percent of the sample showed a percentage of NK cells below the reference limit. NK cells are a component of the innate immune system and one of the first effectors on sites of inflammation. The regulatory function of NK cells is to limit and prevent autoimmunity by killing of autologous immune cells.

Their implication in neurotoxicity and neuroprotection following CNS pathology, as well as the cross talk between NK cells and brain-resident immune cells, has been recently linked to CNS and mental disorders (Poli et al., 2013). A deranged Th17/T regulator balance and a reduced NK cell number are considered to be associated intermediat biological factors in childhood trauma, psychosis liability, and social stress reactivity in psychotic patients (Counotte et al., 2018).

A few studies have documented a decreased regulatory T cell count among children with Tourette syndrome compared with healthy controls (e.g., Kawikova et al., 2007; Bos-Veneman et al., 2011).

Some studies analyzed the NK cells and white blood cell counts and activity in OCD, although with contrasting results (Denys et al., 2004; Rodriguez et al., 2017). An altered number and function of NK and T cells have been shown also in patients with schizophrenia, psychotic disorders (Karpin´ski et al., 2016; Vasilyeva et al., 2016), and posttraumatic stress disorder (Bersani et al., 2016).

Furthermore, a consistent decrease in cytotoxic activity of NK lymphocytes (NKCA) and in lymphocyte proliferation by mitogens has frequently been reported in patients with major depressive disorder (MDD) (Ravindran et al., 1999; Zorrilla et al., 2001).

A more recent research (Jeon et al., 2018) found that the NKCA was more closely related to depressive and anxiety factor scores in their 49 patients with MDD. Furthermore, the CD8-positive cell number increased and CD4/CD8 ratio decreased after 4 weeks treatment with selective serotonin reuptake inhibitors.

All these findings suggest that PANS is a complex syndrome encompassing many psychiatric symptoms (anxiety, obsessive compulsive behaviors, and irritability/depression) and potentially arising from immune abnormalities, where the reduced NK cell counts could be a potential biomarker.

Finally, a large portion of our sample (56%) had overnight EEG evaluation that showed intermittent or persistent focal or generalized slow wave activity both at rest (mainly localized in temporal–frontal regions) and in vigilant state, suggesting a focal or generalized cerebral dysfunction. Notably, none of these subjects had ever suffered from epilepsy.

The Consensus guideline (Chang et al., 2015) suggests that EEGs, particularly overnight evaluations, may be helpful in demonstrating focal or generalized slowing and/or epileptiform activity.

Very few reports, however, have been published on EEG evaluations of subjects with PANS/PANDAS.

One study describes the results of a polysomnographic investigation of 11 children with PANDAS showing periodic limb movements and abnormalities of rapid eye movement (REM) sleep, including REM behavior disorder and nonspecific REM motor disinhibition (Gaughan et al., 2016). One more polysomnographic study on 15 subjects meeting criteria for PANS revealed that 87% of them had evidence of various forms of REM sleep motor disinhibition (excessive movement, laughing, hand stereotypes, moaning, or the continuation of periodic limb movements during sleep into REM sleep) (Gaughan et al., 2016).

No further data are yet available for results of EEG evaluations in PANS. Accordingly, improving the awareness on EEG patterns and sleep characteristics associated with PANS could be helpful to obtain a comprehensive and multidisciplinary clinical management.

The whole familial, clinical, and laboratory variables, summarized in TABLE 9, appear very compound and describe the PANS as a complex syndrome supported by different clinical conditions. Genetic, metabolic, infective, and environmental risk factors seem to have important implications for the assessment of such children.

The lines of evidence derived from the available studies suggest to be particularly comprehensive in searching for specific laboratory biomarkers of PANS taking into account the variety of factors potentially affecting not only the mental function but also the general health of the affected children.

2.7. LIMITATIONS

The present work presents an analysis of correlation between a limited laboratory dataset of pertinent PANS signs and relevant family history conditions: a semantic connectivity map analyzing a larger set of objective parameters could be more informative about PANS construct. Another limitation of the study is the lack of a control group, as only PANS patients were included. Nevertheless, the statistic model that we have used consent to analyze a single group dataset.

Comparison with patients presenting a limited association of two or more PANS criteria, but not the complete disorder may help to verify the strength of the observed associations. Finally, an enlarged sample size will be useful to confirm the present results.

2.8. FORTHCOMING ISSUES

Further studies are needed to investigate the relationships and potential diagnostic values of autoimmune/inflammatory markers. It would be meaningful to check for possible relationship between maternal autoimmune activation and PANS in offspring.

In particular, a possible association between maternal thyroid autoimmune disease and PANS in offspring would be detected.

Furthermore, the existence of antithyroid antibodies in clinically euthyroid PANS children could represent an indicator of a larger abnormal immune response. In particular, TPO-Ab measurement may be appropriate to help identify patients at risk of developing true autoimmune hypothyroidism.

It could be also relevant to study the possible implication of human leukocyte antigen (HLA) genes in PANS because of the increasing data on the implication of HLA genes in psychiatric and neurodevelopmental disorders (Nudel et al., 2019). To the best of our knowledge, no specific studies on NK cell counting in children with PANS are still available.

Our data suggest a putative role of the NK cell counting as biomarkers in the diagnosis of PANS. EEG features are understudied in relation to their importance in lending insight into the diagnosis of localized CNS or systemic immunemediated inflammation.

Finally, the large prevalence of sleep disorders in PANS children suggests to investigate the polysomnographic features to contribute in outlining the qualitative and quantitative aspects of the sleep in this population.

2.9. CONCLUSION AND CLINCAL IMPLICATION

Our study could be considered a statistical validation of the existence of the still controversial clinical entity named PANS and describes it as a clinical complex constellation of psychiatric symptoms and adventitious movements, as well as the expression of different serological variables of an autoimmune/inflammatory disease.

By a data mining approach, we describe the PANS as a very specific pattern of clinical variables, each of them having a low diagnostic meaning per se, but with significant predictive values as a whole. PANS, as well as the broad spectrum of autoimmunemediated inflammatory brain diseases, represents a rapidly developing area of medical science.

This condition challenges clinicians to find reliable biomarkers facilitating the recognition of the brain susceptibility to autoimmunity and the accurate diagnosis and treatment of children presenting with new onset neuropsychiatric symptoms. The coherence among PANS symptoms may suggest to consider the syndrome as clinical entity per se, stimulating clinicians to search for a specific combination of symptoms and signs helpful in identifying this condition.

Finally, PANS should represent for clinicians a stimulus to assume a new perspective looking to the brain as an organ strictly linked with the rest of the body and potentially influenced by some general pathological conditions such as inflammatory and autoimmune diseases.

3. STUDY N. 2. SLEEP ALTERATIONS IN PEDIATRIC ACUTE-ONSET NEUROPSYCHIATRIC SYNDROME (PANS): A POLYSOMNOGRAPHIC STUDY

3.1. RATIONAL AND OBJECTIVES

Sleep disorders represent one of the most frequent manifestations of PANS, involving around 80% of patients, often emerging at the onset of the psychiatric symptoms and subsequently regress after the acute phase or follow the course of the disease (Chang et al., 2015; Gagliano et al., 2020).

Despite this frequent finding, to date few studies have been conducted analyzing the sleep characteristics of these patients, and only two polysomnographic studies have been published in patients diagnosed with PANS (Gaughan et al., 2016; Santoro et al., 2018).

Both these studies report a higher prevalence of sleep disorders in PANS patients than in the general pediatric population.

Among the sleep disorders described in PANS, parasomnias (nightmares, nocturnal *pavor*, sleepwalking or somnambulism) as well as difficulties in falling asleep or maintaining sleep (early or intermediate insomnia), early awakenings (terminal insomnia), REM sleep disorders such as REM Sleep Without Atonia (RSWA), and sleep movement disorders such as Periodic Limb Movement Disorder (PLMD) are the most frequently mentioned (Swedo, 2012; Santoro et al., 2018).

It should be taken into account, however, that some sleep disorders, such as REM-sleep Behavior Disorder (RBD), may be secondary to the concomitant intake of drugs such as Selective Serotonin Reuptake Inhibitors (SSRI) or Dopamine-receptors antagonists and, therefore, it should be necessary to exclude an iatrogenic condition (Lloyd et al., 2012).

Actually, 6 out 9 patients of the Gaugan series were taking benzodiazepines and no mention on medication status was reported in the Santoro series (Gaughan et al., 2016).

Aim of the present study is to describe the clinical and polysomnographic features of sleep in a group of children diagnosed with PANS with no medications nor nutraceutics for at least 4 weeks before enrollment.

The main purpose is to identify putative relationships between sleep disorders and other PANS symptoms by using a data mining approach with fourth-generation Artificial Neural Networks, a computational adaptive systems able to discover subtle trends and associations among variables. Starting from the analysis of these relationships, the authors intend to suggest an interwoven pathophysiological mechanism underling both sleep and PANS symptoms.

3.2. METHODS

3.2.1. Participants

The participants are children diagnosed with PANS during a time interval of six months (from March 1, 2019 to August 31, 2019), consecutively enrolled from the outpatient-service at the Clinic of Child and Adolescent Neuropsychiatry of the Hospital "A. Cao - G. Brotzu" of Cagliari.

Inclusion criteria for their retrospective selection were: diagnosis of PANS according to the criteria proposed by the 2015 Consensus Conference (Chang et al., 2015); absence of previous sleep disturbances and disorders; drug-free period of at least 4 weeks; interruption of nutraceutical supplements (included Melatonin) at least 4 weeks before admission to the study.

All participants underwent a comprehensive clinical evaluation including cognitive assessment, blood chemistry examination (tests listed in TABLE 10), a nasal and pharyngeal swab and polysomnography.

Beside PANS, specific DSM-5 disorders (Tic or Tourette Disorder, Obsessive-compulsive disorder, Mood Disorders, Anxiety Disorder, Attention Deficit Hyperactivity Disorder and Oppositional Defiant Disorder, Learning Disorders) were also considered and evaluated by Kiddie SADS PL (Kaufman et al., 1997) and specific assessment scales.

In particular:

- Yale Global Tic Severity Scale (YGTSS; Leckman et al., 1989) for the evaluation of tics (Score $\geq$20 as threshold)
- Children's Yale-Brown Obsessive-Compulsive Scale (CY-BOCS; Scahill et al., 1997) for the evaluation of obsessive-compulsive symptomatology (Score $\geq$7 as threshold)
- Pediatric Acute-onset Neuropsychiatric Syndrome Scale (PANSS; Murphy et al. 2015) for the evaluation of specific PANS symptoms (Score $\geq$20 as threshold with a possible maximum score of 54).

The level of impairment of global functioning was evaluated by Children's Global Assessment Scale (C-GAS; Shaffer et al., 1983), cognitive profile by Wechsler series scales (WISC-IV and WPPSI-III) considering Full Scale IQ, Verbal Comprehension Index (VCI), Perceptive Reasoning Index (PRI), Working Memory Index (WMI), Processing Speed Index (PSI); cognitive levels were dichotomized based on a cut-off of 85 (1 SD below the average).

3.2.2. The Polysomnography (PSG)

Each subject underwent a full-night ambulatory polysomnography (PSG) recording (Nox A1 – Medical®).

Sleep stages, respiratory and legs activity were scored manually by a clinical neurophysiologist expert in sleep medicine (PC), following standard AASM criteria (Berry et al., 2018) on 30-seconds epochs.

The characteristics of polysomnographic study (EEG channels and sensors) and specific sleep parameters are presented below.

The following parameters were included in the polysomnographic study:

- ✓ EEG (3 channels: one frontal, one central, and one occipital, referred to the contralateral earlobe)

- ✓ Electrooculogram (electrodes placed 1 cm above the right cantus and 1 cm below the left cantus and referred to A1)

- ✓ Electromyogram (EMG) of submental muscle

- ✓ EMG of bilateral tibialis anterior muscle (bipolar derivations with two electrodes placed 3 cm apart on the belly of the anterior tibialis muscle of each leg)

- ✓ One single-lead ECG.

The sleep respiratory pattern was assessed by means of nasal airflow (nasal pressure cannula), thoracic and abdominal respiratory effort (strain gauge) and oxygen saturation (pulse-oximetry) during the study night.

Sleep signals were stored on hard disk in European data format for further analysis.

The polysomnographic parameters evaluated were: Total Sleep Time (TST), Sleep Efficiency (SE), Sleep Latency (SL), REM Latency, N1% TST, N2% TST, N3% TST, REM% TST, Wake After Sleep Onset % (WASO%), Awakenings, Periodic Limb Movement Index (PLMI), RSWA (REM Sleep Without Atonia), and the presence of Frequent change position.

According to the standard AASM criteria (Berry et al., 2018) and to the existing paediatric literature (Marcus et al., 2014; Berry et al., 2018), the parameter "Ineffective Sleep" has been defined by values of Sleep Efficiency < 85%; the parameter "Fragmented Sleep" has been defined by values of WASO > 10% and the diagnosis of Periodic Limb Movement Disorder (PLMD) has been formulated if the PLMI was ≥ 5.0 events/hr.

The diagnosis of paediatric obstructive sleep apnea syndrome (OSAS) was defined by an AHI >1/hr.

The parameter "Abnormal PSG" includes all the sleep diagnosed disorders (Ineffective Sleep, Fragmented Sleep, PLMD and RSWA).

3.2.3. Artificial Neural Networks Analysis

For the analysis of the collected data, the Auto Contractive Map (Auto-CM) system has been used.
Artificial Neural Networks (ANNs) are computational adaptive systems inspired by the functioning processes of the human brain: they are considered particularly useful to solve non-linear problems and to discover subtle trends and associations among variables.
The Artificial Neural Networks (ANNs) analysis have been described in detail in Chapter 2.

3.3. ETHICAL APPROVAL

The independent Ethical Committee of Cagliari University Hospital approved the study. All the parents were given a full explanation of the study methods and purposes and gave their written consent.

3.4. RESULTS

Between March 2019 and August 2019, a total of 43 individuals with PANS were retrospectively screened for enrolment. Of these, 20/43 (46.5%) were excluded as they did not meet inclusion criteria (12/20 were assuming melatonin or vitamin D supplementation and 8/20 were assuming SSRI or Dopamine-receptors antagonist drugs).

In the remaining 23 patients (19 males, 4 females; gender ratio of 5:1), mean age at enrollment was 9.8 years (SD: 2.6; range: 4.8 - 15.4), mean age at onset of PANS symptoms was 6.4 years (SD: 2.6; range: 2.5 - 11.1), mean age at the time of diagnosis was 8.7 years (SD: 2.8; range: 3.8 - 15.1), corresponding to a mean diagnosis delay of 2.3 years (SD: 1.7; range: 0 - 6.5).

At the time of the clinical assessment, 9/23 subjects (39.1%) were in the acute phase of the disease (onset or relapsing phase), and the remaining 14/23 (60.8%) had a chronic PANS presentation or were on a "wane" phase of a "wax and wane" PANS course.

Among the diagnoses associated formulated according to the DSM-5 criteria, Tic Disorder/Tourette Disorder was the most frequent (19/23; 82.6%).
Also OCD (10/23; 43%), ADHD/ODD (12/23; 52%) and Learning Disorder (8/23; 34%) were highly recurring: Less frequent were Anxiety 4/23 (17%) and Mood Disorders: 2/23 (8,6%).
The descriptive characteristics of the sample are summarized in TABLE 11.

As expected, polysomnography show abnormality in 17 out 23 (73.9%). In particular, in accordance with AASM criteria, 8/17 children (47%) had an ineffective sleep, 10/17 (58.8%) fragmented sleep, 8/17 (47.1%), Periodic Limb Movement Disorder (PLMD) and 11/17 (64.7%) REM-Sleep Without Atonia (RSWA). As suggested by the distribution of percentages, most patients received more than one diagnosis of sleep disorder ("Abnormal PSG"). Similarly, to the whole sample, among the19/23 patients diagnosed with Tic Disorder/Tourette Disorder, 8/19 (42.1%) show PLMD and 10/19 (52.6%) and RSWA polysomnographic features (TABLE 12).

The respiratory parameters were available only for 18/23 (78.2%) patients, due to the difficulties for 5 children to accept the pulse-oximeter. Among them, 14/18 (77.8%) subjects had a respiratory pattern characterized by snoring and 6/18 (33%) had a condition of paediatric obstructive Sleep Apnea Syndrome (OSAS) with a severity score between mild (4/6) and severe (2/6) (see TABLE 12).
These 2 patients with a severe OSAS underwent a tonsillectomy and reported an improvement of the clinical condition after surgery.

Biological parameters were out of the normal range in a large portion of patients. Serology and blood chemistry showed high antibody titers against Chlamydia Pneumoniae in 15/23 participants (65.2%) of which 10/15 were IgM and 5/15 IgG-mediated, and against Mycoplasma pneumoniae in 6/23 participants (26%) of which 4/6 were IgM and 2/6 IgG-mediated. Elevated ASLO (Anti Streptolysin-O; 11/23, 47.8% of the sample) and Anti-Nuclear Antibodies (ANA) titers (5/23, 21.7%) were also found. Neuron-specific enolase (NSE), an enzyme detected in serum following structural damage of neuronal brain cells, was elevated in 15/23 subjects (65.2%).
Severe lower levels of vitamin D (14/23, 60.8%) and of serum iron (sideraemia, 8/23, 34.7%), were also detected. Erythrocyte Sedimentation Rate (ESR) faster-than-normal rate and a high level of C-Reactive Protein (CRP) were measured only in 4/23 (17.3%) and 1/23 (4.3%) patients.

To statistic purposes, we converted some continue variables in dichotomous measures. In particular, the ASLO patient's serum titers have been pooled in two groups (ASLO titer > or < of 200 IU/ml), based on the upper reference limit.

The ANA patient's serum titers have been pooled in two groups (titers < or > of 1: 120), based on the upper reference limit. ESR, NSE and CRP were evaluated as abnormal if above the upper reference limit of 15 mm/h, 18 ug/L and 2.0 mg/dl, respectively. Vitamin D was considered insufficient under the lower reference limit of 25 ng/ml.

3.4.1. Semantic connectivity maps

The semantic connectivity maps (Auto-CM method) - Minimum spanning tree (MST) graphs show the strength of association across the clinical, neuropsychological, biological and polysomnographic variables visualized by the concept of "closeness": the variables whose connection weights are higher get relatively nearer and vice versa. The more the links strength values are close to 1 the more the variable are related. In our maps, all the links strength values were above 0.8 and most of them were very close to 1. As shown in all maps, the variable "Abnormal PSG", which includes all the diagnosed sleep disorders, acts as a hub having a considerable number of connections with the other clinical, neuropsychological and biological variables.

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The Map 11 (FIGURE 11) shows the connections between all sleep parameters and the clinical variables, expressed by the standardized scores at the evaluation scales (YGTSS; CY-BOCS; PANSS; C-GAS). The same map includes the diverse diagnoses, formulated according to DSM-5 criteria (in add-on to PANS diagnosis). The "Maximally Regular Graph" (red diamond, for details see Supplementary Material S2) encompasses the variables with higher number of connections.

These are represented by the DSM-5 diagnosis of Tic disorder/Tourette disorder, the total YGTSS score >20, the PANSS total score >20, the CGAS <60, the "Abnormal PSG" and the presence of snoring.

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The Map 12 (FIGURE 12) explores the connections between all the sleep parameters and both the clinical dimensions assed by PANSS and neuropsychological parameters assessed by Wechsler intelligence scales.

This map is aimed to highlight the hidden connection between sleep characteristics and cognitive and behavioural symptoms. Both "Abnormal PSG" and "sleep disturbance" represent hubs with a high number of links. The "Maximally Regular Graph" (red diamond) includes also the motor symptoms, the learning cognitive symptoms, anxiety, irritability and OCD symptoms.

Among PSG variables, the "Maximally Regular Graph includes snoring.

The Map 13 (FIGURE 13) explores the connections between all sleep parameters and the laboratory variables exceeding the normal range. This Map reveals that the variable "Abnormal PSG" represents the hub with the highest number of links, receiving convergence from both sleep and biological parameters.

The "Maximally Regular Graph" (red diamond) includes the D3 vitamin low level, the sideraemia low level, the positive anti-chlamydia antibodies and the ASLO high title as well as other clinical sleep variables (RSWA, PLMD, frequent position changes and Snoring).

3.5. DISCUSSION

The results of the present study confirm the key qualitative and quantitative aspects of sleep disturbances in children diagnosed with PANS, and strongly suggest that sleep disorders may play a central role in the clinical presentation of the syndrome.

A growing scientific attention has recently focused on PANS in children. On one hand, several cohort studies demonstrated associations between different microbial infections and the abrupt emergence of psychiatric symptoms.

On the other hand, biological studies disagree in showing PANS to have a clear immune basis and strong consistency for treatment with antimicrobials or immunotherapy is still lacking, with the only randomized, controlled clinical trial suggesting little outcome difference between placebo and immunotherapies (Wilbur et al., 2019).

However, given the severe and sometimes disabling nature of PANS and the serious effects on family and child functioning, we feel mandatory to understand the biological basis of this syndrome and to distinguish this entity from others.

3.5.1. Polysomnographic study

Polysomnography was abnormal in 73.9% of these patients and this rate is consistent with the finding of PANS consortium reporting sleep alterations in about 80% of patients with PANS at the onset of symptoms and after the acute phase (Chang et al., 2015).

A strength point of our study is the complete pharmacological and nutraceutical wash-out condition of all enrolled patients.

Compared to previous PANS polysomnographic studies (Gaughan et al., 2016; Santoro et al., 2018), this study has no biases related to the potential drug-driven interferences on the sleep-wake rhythm and the intrinsic structure of sleep.

The subjects of this sample showed sleep disorders both in acute and chronic phase of the disease.

Among the sleep disorders diagnosed, PLMD and RSWA were largely represented in our sample Notably, among the 19/23 patients diagnosed with Tic Disorder / Tourette Disorder, 10/19 (52.6%) had a condition of RSWA and 8/19 (42.1%) had a condition of PLMD.

Rate of RSWA were similar to those reported by Gaughan (2016).

Unfortunately, we could not have compared our RSWA rate to that of the general paediatric population, since, to the best of our knowledge, it is not available in the literature. Nonetheless, we infer that the prevalence rate of PLMD in our sample was significantly higher than that in the general paediatric population (2-4%; Rulong et al., 2017).

The prevalence of PLMD in our Tic/Tourette's PANS patients was also significantly higher than that among paediatric patients with isolated Tourette's Disorder (6% described by Kostanecka-Endress et al., 2003).

This difference might represent a possible distinction-key between a "pure" Tourette's Disorder and a Tourette's Disorder diagnosed in the context of a PANS.

The high prevalence of PLMD among PANS patients may suggest a specific involvement of dopaminergic circuits, in the disorder: PLMD is thought to be sustained by a deficit of motor inhibition during sleep (Congiu et al., 2019; Manconi et al., 2007; Turjanski et al., 1999; Staedt et al., 1993; Ruottinen et al., 2000).

Periodic Limb Movements (PLMs) are associated with a hypofunction of dopaminergic system as suggested by their suppression after treatment with L-dopa or dopaminergic agonist drugs, whereas they may be may induced or worsened by dopamine-receptor antagonists (Aggarwal et al., 2015).

In patients with PLMs in sleep or restless legs syndrome, PET and SPECT studies have shown a mild presynaptic nigrostriatal and postsynaptic striatal dopaminergic hypofunction.

In patients with Parkinsons's disease reduced striatal [(123)I] beta-CIT binding correlated with the number of periodic limb movements in sleep (Happe et al., 2003; Puligheddu et al., 2014); parkinsonian patients off of dopaminergic treatment had a significantly higher rates of PLMs that patients under treatment (Bliwise et al., 2012).

3.5.2. Semantic Connectivity Maps

Artificial Neural Network methodology and the Auto-Contractive Map was used to investigate the putative relationships among the frequent PSG abnormalities and clinical, neuropsychological and laboratory parameters.

Exploiting all not obvious links among the full spectrum of variables, the Auto-Contractive Map method revealed the simultaneous connections among them, facing the complexity of PANS phenotype.

The semantic connectivity Map 11 (FIGURE 11) shows the connections between sleep parameters, DSM-5 diagnoses, and all the behavioral parameters assessed by scales and checklists.

The clearest evidence of this map is that the variable "Abnormal PSG" is related to higher PANS total score, suggesting a strong impact of sleep disturbance on PANS phenotype. Interestingly, "Tic/Tourette" diagnosis is the variable closer to the "Abnormal PSG", confirming the strong connection between sleep abnormalities and the presence of daily uncontrolled movement as tics.

An extensive nationwide population-based study showed as Tourette Disorder per se is an independent risk factor for sleep disorders in children with associated neurodevelopmental disorders (Lee et al., 2017).

However, the overall incidence rate of sleep disorders in TD subjects reported in Lee's study (7.24%) is largely lower than the incidence rate of sleep disorders in our PANS series, suggesting a wider involvement of the sleep regulation mechanisms in PANS than in TD.

The "Maximally Regular Graph" (red diamond) encompassing the variables with higher number of connections, also includes the variable "snoring".

This is an interesting finding since snoring is usually related in children with adeno-tonsillar hypertrophy, which, in turn, represents a potential cause of OSAS (Friedman et al., 2009).

In this sample the 33% of the 18 patients for which oximeter data were available had OSAS. A previous study, aimed to describe the otorhino-laryngologic findings in patients with Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections (PANDAS), found a high rate (65.7%) of adenoid and tonsil hypertrophy (Cocuzza et al., 2018).

The authors stated that not all children with adenotonsillar hypertrophy suffer also from sleep breathing disorders and that the correlation between the degree of lymphatic structures hypertrophy and the severity of obstructive respiratory symptoms is not linear. However, the same authors discuss the efficacy of adenotonsillectomy for pediatric OSAS and, according to the guidelines for tonsillectomy (American Academy of Otolaryngology-Head & Neck Surgery; Ryan et al., 2020), suggest to consider the surgical therapeutic option also for PANDAS/PANS patients.

The semantic connectivity Map 12 (FIGURE 12) shows the connections between sleep parameters, clinical dimensions assed by PANSS and neuropsychological parameters assessed by Wechsler intelligence scales.

"Abnormal PSG" and "sleep disturbances" variables represent hubs with a high interconnectivity with motor symptoms, learning cognitive symptoms, anxiety, irritability and OCD. Therefore, the "Maximally Regular Graph" (red diamond) includes both cognitive and psychiatric symptoms strictly linked to sleep disturbances, suggesting a central role of these last in defining the PANS phenotype.

Among PSG variables, snoring is the closest to learning and cognitive dimension of PANS. A potential impact of sleep disordered breathing symptoms on daily cognitive performances has already been described by many authors (e.g., Pietropaoli et al., 2015; Kheirandish-Gozal, 2016).

A similar impairment of both sleep and cognitive aspects also occurs in other sleep disturbances such as narcolepsy and Kleine-Levin syndrome.

In these conditions, sleep alterations are often associated with a certain degree of cognitive dysfunction as confusion and fogging (Iranzo, 2019).

Interestingly, the semantic connectivity Map 12 (FIGURE 12) shows a close link between the sleep variables, encompassed in the red diamond, and the cognitive indices "Processing Speed Index (PSI)" and "Working Memory Index (WMI)" of the Wechsler intelligence scales for children. Processing speed is thought as the ability to rapidly process novel information and to identify, discriminate, integrate, make a decision about information while working memory accounts for higher order thinking processes and, in particular, for the ability to temporary store and manipulate new information to ensure successful task execution (Kauffman et al., 2006).

We can assume that both these functions are impaired in the condition known as "brain fog" described as a weakening in memory, attention, executive function, and the speed of cognitive processing. This condition has been clinically observed in a large part of the children of our sample and confirmed by the result of the cognitive test, showing relatively low mean scores in PSI and WMI index (84.6 and 90.5 respectively).

At the same time, the "*brain fog*" condition is largely described among different autoimmune and inflammatory diseases. For instance, people with Systemic Lupus Erythematosus experience a range of cognitive difficulties ('Lupus fog') such as confusion, difficulty in articulating thoughts and memory impairment (Mackay, 2015).

A mild degradation of cognitive functions, referred to as "brain fog", has been also reported in patients with coeliac disease (Yelland, 2017).

Such deficits associated with sleep disturbance, also occur in patients with Crohn's disease, particularly in association with systemic inflammatory activity (Van Langerberg et al., 2017).

Taken together, these data could indicate a possible link between sleep disorders and brain fog and the association of both these conditions with systemic inflammation.

Furthermore, the symptomatic overlap of PANS with other autoimmune diseases and other sleep disorders of well-known immune-mediated pathogenesis is consistent with the explanation of PANS as a complex syndrome encompassing different symptoms, epiphenomena of a common inflammatory/autoimmune condition.

The analysis of the connections between sleep and biological parameters by semantic connectivity Map 13 (FIGURE 13), shows that the node "Abnormal PSG" acts as a hub, receiving a great number of convergences.

This evidence stresses, once again, the central role of sleep disturbances in PANS phenotype. The "Maximally Regular Graph" (red diamond) links the PSG variables (PLMD, Sleep fragmentation, RSWA, frequent position changes and Snoring) with some metabolic and infective variables linked to PANS.

In particular, some infectious markers such as a high ASLO titer and the presence of anti-Chlamydia antibodies, appears closely linked to sleep variables suggesting a shared disimmune basis, possibly triggered by an infectious episode.

This concept has already been proposed by some authors which have stressed the bidirectional relationship between sleep and immunity against infections, starting from the evidence of a large proportion of patients with narcolepsy having antibodies (ASLO) against Group A Beta-Hemolytic Streptococcal (GABHS) (Ibarra-Coronado et al., 2014; Silber et al., 2016).

The authors' hypothesis was that this bacterium might trigger narcolepsy through an autoimmune mechanism. Furthermore, starting from the observation of a high density of cytokines' receptors (particularly interleukins' receptors) in hypothalamic neurons, some authors propose that the wake-sleep rhythm and immune system modulate each-other (Ibarra-Coronado et al., 2014).

However, not only GABHS but also other bacterial pathogens frequently causing sore throat (e.g., Chlamydia pneumoniae and Mycoplasma pneumoniae) are supposed to be triggers for PANS symptoms (Chang et al., 2015) and, specifically for sleep disorders related to PANS, as suggested by the strong link between the anti-Chlamydia and anti-Mycoplasma antibodies and sleep variables, in this sample.

A second significant correlation described by the semantic connectivity Map, FIGURE 13, is the link between sleep parameters and the condition of hypo-sideraemia.

Systemic iron deficiency is described in about two-thirds of children with restless legs syndrome (RLS) and it seems to be related to a more severe symptomatology (Kotagal, 2017, Howard et al., 2018).

In the Wisconsin Sleep Cohort, a PLM index ≥ 15 was associated with low (≤ 50 ng/ml) serum ferritin levels suggesting a role for low iron stores in the pathogenesis of Periodic Limb Movements (Congiu et al., 2019).

A current explanation is that iron deficiency may result in a dopaminergic dysfunction supporting the RLS and PLMD, since iron is a necessary cofactor for dopamine production via tyrosine hydroxylase (Earley et al., 2014).

Iron deficiency adversely affects the basal ganglia function potentially leading to cognitive and motor dysfunctions, condition which could represent an intersection between PANS, Tourette Disease and PLMD (Trotti et al., 2017).

Finally, in the Map 3 (FIGURE 13), we observed a strength connection between vitamin D deficiency and "Abnormal PSG".

This is a somehow expected result given the role of vitamin D both as immunomodulatory agent and fundamental factor for a balanced neurotransmitter setting in the central nervous system (Rulong et al., 2018).

A recent meta-analysis indicates that serum 25(OH)D <20 ng/mL could significantly increase the risk of unhealthy sleep and sleep disorders (Gao et al., 2018).

At the same time, a large amount of literature supports the central role of vitamin D in modulating immunological functions (e.g. Yang et al., 2013).

Insufficient vitamin D levels are thought to be linked to a higher susceptibility for infectious and autoimmune diseases (Peelen et al., 2011; Hewison, 2012; Gunville, 2013). Within this framework the connection between sleep disorder, vitamin D deficiency and PANS is an expected condition.

3.5.3. A hypothetical dysfunctional model

Obsessive-compulsive, tic and sleep disorders have in common varying degrees of dysfunction in attentional vigilance and motor disturbances. Starting from this consideration, we propose a hypothetical dysfunctional model explaining the co-occurrence of sleep, motor, obsessive-compulsive and cognitive symptoms in PANS, graphically explained in the FIGURE 14.

Basal ganglia play a crucial role in sleep physiology and pathophysiology (Qiu et al., 2010).
The midbrain dopamine system via the dorsal and ventral striatum tunes cortical inputs, regulating a wide range of functions including cognition, motor behavior, and sleep–wake states (Bjorklund and Dunnett, 2007; Schultz, 2007, Malenka et al., 2009; Nambu, 2008).

Recent evidence from sleep animal models indicate that stimulation of nigrostriatal dopaminergic pathways, activating the external globus pallidus externa (GPe) increases sleep and EEG delta power, while stimulation of mesolimbic dopaminergic, activating the nucleus accumbens, induces arousal, decreasing sleep and reducing EEG power (Oishi and Lazarus, 2017; Volkow et al., 2008)
Recent neuroimaging studies indicate that microstructural differences (i.e. apparent diffusion coefficient, but not regional brain volume nor cerebral blood flow) may be observed in the basal ganglia, thalamus, and amygdala of children and adolescents with PANS (Zheng et al., 2020).

Interestingly, recent evidence indicates that also thalamus, and amygdala play an important role in sleep regulation (Chenyan et al., 2019).

Considering the complex microstructure of the Basal Ganglia (Crittenden et al., 2011), PANS neuro-inflammation may heterogeneously impact specific sub regions modulated by midbrain dopaminergic pathways, leading to apparently opposite symptoms (i.e. tics and PLMD).

Interestingly, high prevalence of a PLMD (a *hypo*-dopaminergic condition) in patient also showing motor and vocal tics (a *hyper*-dopaminergic condition) may appear contradictory and of puzzling explanation: in fact, dopaminergic regulation of sleep-wake cycle may be of help (Alexander et al., 1990; Schultz, 2007; Nambu, 2008; Haber, 2003).

The thalamus and Basal Ganglia interconnect distant parts of the cerebral cortex via cortico-thalamo-cortical and cortico-striato-thalamic loops.

In recent year, the parallel and segregated model of Basal Ganglia modulation of cortical motor, cognitive and limbic function (Alexander et al., 1990), evolved into a model of multiple spatially distant cortical projections to the subcortex, describing the circuits as "parallel and integrative" rather than purely parallel (Haber, 2006).

Integrative model may explain as even small lesions in the thalamus or basal ganglia can be neurologically devastating, but similarly sized lesions in the cerebral cortex may go unnoticed (see as example Siegel et al., 2014; Greene et al., 2020).

Immune-mediated impairment of basal ganglia may explain the specific cognitive relative impairment of cognition (WISC IV Processing Speed Index and, more in general "Brain fog") observed in our PANS patients and other case series.

3.3. LIMITATIONS

One major limitation of our study is the lack of a control group, as only PANS patients were included. However, our chosen statistic model allowed us to analyze a single group data set. Comparison with typical neurodevelopmental subjects and/or with patients presenting a limited association of PANS criteria, but not the complete disorder, may help to verify the strength of the observed associations.

Another limitation is due to the intrinsic retrospective nature of the study.

Data collection was not always exhaustive for all selected patients, neuropsychological examinations were not uniformly performed and relied on the skill of the treating neuropsychologist.

A third limitation is the relatively small number of subjects. An enlarged sample size will be useful to confirm the present results.

Further studies including more subjects and a control cohort are warranted to confirm these findings, leading to the inclusion of sleep abnormalities among PANS major diagnostic criteria.

3.4. CONCLUSIONS AND CLINICAL IMPLICATIONS

Also considering the above mentioned limitations, the present study underlines that sleep disorders represent, for prevalence and impact on quality of life, a cardinal symptom in patients with PANS. Thus, considering the role that of sleep disrupstions on diagnosis and prognosis of PANS, it could be considered the opportunity to include them among the major diagnostic criteria.

Taken together the results of present study strongly suggest that the inflammatory/disimmune impairment of the thalamus, amigdala and basal ganglia (Zheng et al., 2020) underlying the PANS core symptom may also induce sleep disorders.

Several lines of evidence support this hypothesis:

- the symptomatic overlaps with other sleep disorders (narcolepsy and Klein-Levine syndrome) of established immune-mediated pathogenesis;
- the strong link between some infectious markers (anti-Chlamydia Pneumoniae and ASLO titers) and sleep disorders, supporting the infective trigger causative model via abnormal activation of the immune system;
  - the association between a deficit of vitamin D, a known immunomodulatory agent, and the polysomnographic alterations
  - the association with PLMD and with serum iron deficiency suggesting an impairment of dopaminergic system in the pathogenesis of both PLM and tics in PANS.

In conclusion, a complete evaluation of sleep by means of a comprehensive history should be performed for all PANS patients since the onset of the disorder, and a polysomnographic investigation should be suggested in all PANS patients for whom the parents report sleep disturbance. A PSG could be suggested also for patients with strong indicators of cognitive alterations, as brain fog condition, daily sleepiness, confusion or attentive difficulties.

4. STUDY N.3. METABOLOMICS CHARACTERIZATION OF THE PEDIATRIC ACUTE-ONSET NEUROPSYCHIATRIC SYNDROME (PANS)

4.1. RATIONALE AND OBJECTIVES

In PANS, various aetiological agents, including viruses (Hoekstra et al., 2005), Mycoplasma pneumoniae (Müller et al., 2004) and Haemophilus influenzae, are supposed to act, in predisposed subjects, as triggers for the activation of the immune response, in turn, responsible for the inflammatory cascade and the consequent release of chemical mediators of inflammation at the CNS level (Swedo et al., 2012; Frankovich et al., 2015).

Functional and structural abnormalities of the cortico-basal ganglia circuitry, similar to that seen in those with acute Sydenham's chorea, have been described in PANDAS.

In particular, an enlarged striatal volume (Giedd et al., 2000; Elia et al., 2005) and an inflammatory state of the striatum, confirmed by positron emission tomography using a marker of microglial activation (Kumar et al., 2015), has been reported in PANDAS.

A recent diffusion-weighted magnetic resonance imaging study identified cerebral microstructural differences in children with PANS in multiple brain structures (including the deep grey matter structures as the thalamus, basal ganglia, and amygdala) putatively related to a neuroinflammatory state (Zheng et al., 2020).

In cohorts of rigorously selected subjects with PANS, evidence of post-infectious autoimmune processes and/or a condition of neuroinflammation was observed in over 80% of cases (Swedo et al., 2017).

A very recent paper showed that antibodies from children with PANDAS bind specifically to striatal cholinergic interneurons and alter their activity, sustaining the pathophysiology of rapid-onset of obsessive-compulsive symptoms (Xu et al., 2020).

Despite these accumulating evidences, PANS is still regarded as controversial clinical entity, consisting of a clinical complex constellation of psychiatric symptoms and adventitious changes, as well as the expression of a variety of serological variables of an autoimmune/inflammatory disease (Gagliano et al., 2020).

In this scenario, the research for new specific biomarkers is strongly desirable.

Currently, there are no systematic metabolomics datasets available of subjects who have received PANS diagnosis. In a single case of a 10 year-old girl with PANS, the involvement of several metabolic pathways concerning microbial activity and protein biosynthesis, as well as energy and amino acid metabolism, has been suggested (Piras et al., 2019).

The present study aims to identify and describe the specific serum metabolomics profiles in a sample of children with highly clinically characterized PANS, measured through NMR spectroscopy.

4.2. MATERIALS AND METHODS

Consecutive patients referred for PANS, between May 2019 to May 2020 to the outpatient clinics of Child & Adolescent Neuropsychiatric Unit, "G. Brotzu" Hospital Trust, Cagliari were enrolled in this observational study. Furthermore, the study recruited an age matched sample of healthy subjects (CG).

All participants were assessed by a panel of standardized scales and questionnaires encompassing the Paediatric Acute Neuropsychiatric Symptom Scale (PANSS) with the purpose to screen the symptoms and their severity.

Diagnosis of PANS was confirmed by two child psychiatrists (AG and FC), according to PANS working criteria defined by experts convened at the National Institute of Mental Health (NIH) in July 2010 (Swedo et al., 2012).

Exclusion criteria were the following: I) occurrence of immunologic diseases or cancer; II) presence of other medical or neurological/psychiatric diseases; III) active treatment with psychoactive substances, non-steroidal anti-inflammatory drugs, or corticosteroid agents; IV) unwillingness of the patients to participate in the study.
A control group (CG) of healthy subjects matched for age and sex, living in the same geographic area of the clinical group, was also recruited.

All participants were assessed by a battery of standardized scales and questionnaires aimed to screen the symptoms and their severity.
The clinical instruments used for the evaluation of the enrolled patients, were:

- Pediatric anxiety Rating Scale (PARS)
- Pediatric Acute Neuropsychiatric Symptom Scale (PANSS)
- Children's Yale-Brown Obsessive-Compulsive Scale (CYBOCS)
- Yale Global Tic Severity Scale score (YGTSS)
- Children's Global Assessment Scale (C-GAS)
- UFMG (Universidade Federal de Minas Gerais) Sydenham's Chorea Rating Scale (USCRS)
- Wechsler Intelligence Scale for Children 4th Edn. Full Scale Intelligence Quotient (FSIQ)

The details of the standardize clinical scales and questionnaires are described below.
✓ Pediatric anxiety Rating Scale (PARS): Clinician-rated instrument for assessing the severity of anxiety symptoms associated with anxiety disorders (social phobia, separation anxiety disorder, and generalized anxiety disorder) in children (the research units on pediatric psychopharmacology anxiety study group, 2002).

- ✓ Pediatric Acute Neuropsychiatric Symptom Scale (PANSS): a parent self-report form to assess common PANDAS/PANS symptoms with a possible maximum score of 54 (Murphy et al., 2015).

- ✓ Children's Yale-Brown Obsessive-Compulsive Scale (CYBOCS): a semistructured measure of obsessive-compulsive symptom severity in children and adolescents with obsessive-compulsive disorder (OCD) (Scahill et al., 1997).

- ✓ Yale Global Tic Severity Scale score (YGTSS): a commonly used measure to document the intensity, complexity and the frequency of motor and phonic tics, performed by a clinician interview providing; the total score is a measure of overall tic severity (Leckman et al., 1989).

- ✓ Children's Global Assessment Scale (C-GAS): a useful measure of overall severity of disturbance recommended to both clinicians and researchers as a complement to syndrome-specific scales (Shaffer et al, 1983).

- ✓ UFMG (Universidade Federal de Minas Gerais) Sydenham's Chorea Rating Scale (USCRS): a scale designed to provide a detailed quantitative description of the performance of activities of daily living, behavioral abnormalities, and motor function of subjects with SC; the scale comprises 27 items and each one is scored from 0 (no symptom or sign) to 4 (severe disability or finding) (Teixeira et al., 2005).

- ✓ Wechsler Intelligence Scale for Children, 4th Edn (WISC-IV): the most widely used measure of intellectual ability for children from 6 to 16 years.
  It was developed to provide an overall measure of general cognitive ability (Full Scale Intelligence Quotient FSIQ), and also measures of intellectual functioning in Verbal Comprehension (VC), Perceptual Reasoning (PR), Working Memory (WM) and Processing Speed (PS) (Wechsler, 2004).

4.2.1. Participants

Among a total of 52 consecutive outpatients referred for PANS, the metabolomic study recruited 34 outpatients diagnosed with PANS.

A total of 18 subject were excluded according with the exclusion criteria of the research study; most of them because on active treatment with psychiatric drugs or anti-inflammatory agents.

Furthermore, a control group (CG) of 25 healthy subjects matched for age and sex, living in the same geographic area of the clinical group, was also recruited.

The summary of the demographic is reported in TABLE 13.

Exclusion criteria were the following:

1. Occurrence of immunologic diseases or cancer
2. Presence of other medical or neurological/psychiatric diseases
3. Active treatment with non-steroidal anti-inflammatory drugs, or corticosteroid agents
4. Opposition to sign the consent form for participating to the study/ unwillingness of the patients to participate in the study.

4.2.2. The Metabolomics

Currently, the holistic view represents the dominant and innovative perspective for solving many medical questions.

In this panorama, omics disciplines, and among them Metabolomics, have become particularly important in many fields of Biomedicine.

Specifically, the use of Genomics, Proteomics and Metabolomics analyses have contributed to the better knowledge concerning biochemical and biological mechanisms in complex systems, also determining the possibility of discovering new diagnostic biomarkers of pathological states, response to therapies and patient stratification.

Metabolomics is the youngest of the omics disciplines, and is based on the analysis of low molecular weight molecules (amino acids, carbohydrates, lipids, etc.) which represent the product of all biological processes influenced by endogenous and exogenous factors, such as physiological states, pathologies, therapies, lifestyles, etc.

The possibility of comparing different perturbed biological systems (e.g. pathological patients) with control systems (e.g. healthy patients) has led to the possibility of identifying any alterations in the metabolic composition, making metabolomics a versatile technique in many biomedical sectors, having the goal is to identify new diagnostic or response markers to therapy and to better interpret pathophysiological mechanisms.

Since the measurable metabolic composition in biofluids and tissues is extremely varied and cannot be determined with a single technique, it is essential to use different analytical platforms for a vision as complete as possible.

Metabolomics allows the simultaneous and relative quantification of different metabolites within a given biological sample (Mamas, et al., 2011) through the application of two sensitive and specific methodologies such as Nuclear Magnetic Resonance (NMR) (Smolinskaa et al., 2012) and Mass Spectrometry (MS) (Dettmer et al., 2007).

4.2.2.1. The Metabolome

The goal of Metabolomics is the study of the composition of the metabolome.
The Metabolome can be defined as the set of all metabolites that participate in the metabolic processes of a biological system.

This discipline describes the chemical profile of low molecular weight molecules, such as lipids, sugars and amino acids deriving from the metabolic reactions present in cells, tissues, organs and biological fluids.

The components of the Metabolome (metabolites) can be seen as the end product of gene expression and protein activity, thus giving rise to the biochemical phenotype of a biological system as a whole.

In response to the environment, drugs and diet, the integration of the various "omics" sciences (genomics, proteomics, metabolomics) constitutes Systems biology, a discipline that evaluates the interactions between all the different components of a system rather than each component individually (FIGURE 15) (Kitano, 2022; Hood, 2003; Hood et al., 2004) (quoted in Murgia, 2016).

4.2.2.2. Analytical techniques for Metabolomic studies

The most common techniques used in metabolomics for data acquisition are Nuclear Magnetic Resonance (^{1}H-NMR) and Mass Spectrometry (MS).

They allow simultaneous measurement of multiple metabolites of a single biological sample during a single experiment.

NMR, penalized by a low sensitivity, allows the measurement of a general metabolic fingerprint, while MS is characterized by high sensitivity and the possibility of measuring a large amount of metabolites (in the hundreds), but it is "destructive" and therefore the sample after the analysis cannot be recovered.

Both techniques can produce large amounts of data (up to hundreds of metabolites for each individual sample/ patient) which are organized into matrices which are treated by multivariate analysis techniques.

This application allows to identify, within the measured variables (metabolites), those actually responsible about presence of the phenomenon being studied.

A lot of studies have highlighted the usefulness of metabolomics for the discovery of new biomarkers in various diseases regarding the Central Nervous System, whose anatomical-functional correlates are difficult to investigate in vivo with other methods (Kaddurah-Daouk R. et al., 2009).

Metabolomics is an approach that allows a total analysis of low PM metabolites (<1500) in a biological sample that can be obtained with very low invasive methods, such as the collection of plasma and urine.

Metabolomics uses techniques of magnetic resonance spectroscopy, mass spectrometry and biofluid chromatography for the characterization of various pathologies of the nervous system.

4.2.2.3.   Sample preparation for ^{1}H-NMR

10 mL of blood were collected from each sample and were centrifuge at 2500 g for 10 min at 4°C and the obtained serum were stored at -80°C until analysis.

Subsequently, samples were thawed and 400 µL were treatedwith a modified Folch method (Bligh et al., 1959) to extract and separate hydrophilic and lipophlic metabolites. 400 µL of each serum sample were mixed with 600 µL of methanol, 600 µL of chloroform and 175 µL of Milli-Q water.

The samples were vortexed for 1 min and centrifuged for 30 min at 1700g at room temperature. Aliquots (10 µL) from each sample were used to create a pool for quality control (QC) samples.

The QC sample was analyzed at the beginning and at the end of the analysis. The hydrophilic and hydrophobic phases were obtained.

The water-phase was divided in 2 aliquots, concentrated overnight using a speed vacuum centrifuge.

4.2.2.4. ^{1}H-NMR analysis

For the NMR analysis, 700 µl of the water-phase containing low-weight molecules (amino acids, sugars, etc.) for each sample, was concentrated overnight in a speed-vacuum. The concentrated water-phase was resuspended in 630 µl of D_2O phosphate buffer (pH 7.4) and 70 µl trimethylsilyl propanoic acid (TSP) 5.07 mM.
TSP was added to provide an internal reference for the chemical shifts (0 ppm), and 650 µl of the solution were transferred to a 5 mm NMR tube.

The samples were analyzed with a Varian UNITY INOVA 500 spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA), which was operated at 499 MHz equipped with a 5 mm triple resonance probe with z-axis pulsed field gradients and an auto-sampler with 50 locations.
One-dimensional ^{1}H-NMR spectra were collected at 300 K with a pre-sat pulse sequence to suppress the residual water's signal.

The spectra were recorded with a spectral width of 6000; a frequency of 2 Hz; an acquisition time of 1.5 s; a relaxation delay of 2 ms; and a 90° pulse of 9.5 µs. The number of scans was 256.
Each Free Induction Decay (FID) was zero-filled to 64 k points and multiplied by a 0.5 Hz exponential line-broadening function. The spectra were manually phased and baseline corrected. By using MestReNova software (version 8.1, Mestrelab Research S.L.) each NMR spectrum was divided into consecutive "bins" of 0.04 ppm. The spectral area investigated was the region between 0.6 and 8.6 ppm.

The regions between 4.60 and 5.2 ppm and between 5.24 and 6.6 ppm were excluded to remove variations in the pre-saturation of the residual water resonance and spectral regions of noise.
To minimize the effects of the different concentrations of serum samples, the integrated area within each bin was normalized to a constant sum of 100.
The final data set consisted of a 150 x 96 matrix.

The columns represent the normalized area of each bin (variables), and the rows represent the samples (subjects).

4.2.2.5. *Multivariate statistical analysis*

Multivariate statistical analysis was performed on NMR data by using SIMCA-P software (ver.15.0, Sartorius Stedim Biotech, Umea, Sweden) (Eriksson et al., 2013).

The variables were Pareto scaled to emphasize all metabolite signals and reduce the spectral noise for the ^{1}H-NMR analysis.

The initial data analyses were conducted using the Principal Component Analysis (PCA), which is important for the exploration of the sample distributions without classification. In particular, PCA analysis was performed to observe intrinsic clusters and find outliers.

For this aim, the DmodX and Hotelling's T2 tests were applied, The PCA model was performed including the QC samples to evaluate the good quality of the analysis.

Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) were subsequently applied. OPLS-DA maximizes the discrimination between samples assigned to different classes, in this case discriminating between patients with PANS and healthy subjects.

The OPLS-DA model removes variability not relevant to class separation (Rousseau et al., 2007). The variance and the predictive ability (R^2X, R^2Y, Q^2) were established to evaluate the suitability of the models. In addition, a permutation test (n = 400) was performed to validate each single the model.

This rigorous test compares the goodness of fit of the original model with that of randomly permuted models (Lindgren et al., 1996). In particular, the permutation test evaluates model validity in terms of the explained variance parameter (R^2) and the cross-validation parameter (Q^2) that indicates respectively the goodness of fit and accuracy of prediction in the supervised model.

Simultaneously, CV-ANOVA (analysis of variance testing of cross-validated predictive residuals) test was performed to determine significant differences between Pans and the other classes in the OPLS-DA models (p < 0.05).

Variables corresponding to a VIP (Variables Important in the Projection) value of >1 (a measure of their relative influence on the model) from the OPLS-DA models together with the relative S-plot were selected as the most important. Indeed, VIPs of >1 are the most relevant for explaining Y (assignment of two classes).

The selected variables were identified using the Chenomx NMR Suite 7.1 (Chenomx Inc., Edmonton, Alberta, Canada (Weljie et al., 2006).

GraphPad Prism software (version 7.01, GraphPad Software, Inc., San Diego, CA, USA) was used to perform the univariate statistical analysis of the data.

To verify the significance of the metabolites resulting from multivariate statistical analysis U-Mann Whitney test was performed, followed by ROC curves to test the sensitivity and specificity of the metabolites with p-values <0.05. ROC curves are conventionally used to evaluate diagnostic performance in clinical research.

4.2.2.6. Pathway analysis

Metabolic pathways were generated using MetaboAnalyst 4.0 (Chong et al., 2018) a web server designed to obtain metabolomic data analysis, visualization and biological interpretation [www.metaboanalyst. ca].

In particular, the pathway analysis module of MetaboAnalyst 4.0 helps researchers identify the most relevant pathways involved in the conditions under study using high-quality Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways as the backend knowledgebase.

4.3. ETHICAL APPROVAL

The study was conducted with the approval of the independent Ethical Committee of Cagliari University Hospital (Prot. PG/2019/7413 on 29/05/2019). All the parents and all children older than 12 were given a full explanation of the study's method and purpose. The parents signed the consent form, agreeing to participate, and to the data being published. Furthermore, the study was conducted in accordance with the Declaration of Helsinki, V edition (2000).

4.4. RESULTS

Among a total of 52 consecutive outpatients referred for PANS, 34 were considered for the metabolomics analysis. Eighteen subjects were excluded according to the study exclusion criteria described above.

The serum samples of the affected patients were compared to the serum of 25 neurotypical subjects matched for age, gender, and intelligence quotient (see TABLE 13).

Through the ^{1}H-NMR technique, it was possible to identify a total of 44 hydrophilic metabolites that were tested as possible biomarkers for PANS disease (FIGURE 16). Principal Component Analysis (PCA) was performed using the bins dataset.

The Hotelling's T^2 test did not identify any outliers (not shown).

Orthogonal-Partial Least Square-Discriminant Analysis (OPLS-DA) models were performed, comparing the classes (FIGURE 17). Separation of the samples, in line with the presence of PANS diagnosis, was observed by the application of the supervised model between PANS and C classes. (FIGURE 17). The model was validated through the respective permutation test. The statistical parameters of the model were: R^2X=0.44, R^2Y=0.54, Q^2 =0.44, p-value <0.0001, permutation test Intercept $R^2\backslash Q^2$= 0.17/-0.28.

Subsequently, the most important variables were identified for each model through the analysis of the S-plot and using the corresponding VIP-value.

These variables, with a VIP-value of >1, were identified and underwent univariate statistical analysis with the U-Mann Whitney test. 2-OH butyrate, acetone, alanine, asparagines, dimethylamine, glycerol, glycine, glutamine, histidine, isoleucine, tryptophan, tyrosine were the metabolites that exhibited the greatest differences between PANS and CG (FIGURE 18), according to a p-value of <0.05, and were selected to create the ROC curve. The bar-graphs and the corresponding ROC curves of these metabolites are reported in FIGURE 18 and the corresponding statistical parameters are reported in TABLE 14.

MetaboAnalyst was used to characterize the altered pathways in the PANS group. Compared to CG, the most altered pathways were alanine, aspartate and glutamate metabolism, phenylalanine, tyrosine and tryptophan metabolism, nitrogen metabolism, glutamine and glutamate metabolism (FIGURE 19).
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4.5. DISCUSSION

The metabolomics approach applied in this study allowed the identification of a set of hydrophilic metabolites which appears to represent a specific pattern characterizing the PANS condition.
The current lack of solid biomarkers for PANS leads us to consider our data as an important starting point in understanding the pathological features of this complex syndrome. 2-OH butyrate, acetone, asparagines, dimethylamine, glycerol, glycine, glutamine, histidine, isoleucine, tryptophan, tyrosine were the metabolites that exhibited the greatest differences between the PANS and healthy children.

The pathways analysis indicated alanine, aspartate and glutamate metabolism, phenylalanine, tyrosine and tryptophan metabolism, nitrogen metabolism, glutamine, and glutamate metabolism as the most altered biochemical pathways.

Metabolites are molecular products of cellular metabolic functions, and they serve as direct signatures of biochemical activity (Patti et al., 2012).

Since metabolomics profiles are not directly encoded in the genome, they are considered the omics layer most proximal to the phenotype (Haas et al., 2017): in contrast to genetic variants, metabolomics profile can be either a cause or a consequence of the phenotype of interest. Following this reasoning, analysis of the putative role of specific metabolites and altered pathways may help to define a comprehensive metabolic pattern of the disorder, which, in turn, may be of help in defining specific biomarkers.

The amino acid tryptophan (Trp) was found at significantly lower serum concentrations in patients with PANS compared to controls; the pathways analysis confirmed an alteration in tryptophan metabolism. In recent years, tryptophan metabolism has been increasingly recognized as central to the pathogenesis of many neuropsychiatric disorders.

In particular, a decrease in tryptophan level was found in major depressive disorder (Liu et al., 2015; Moreno et al., 2010), schizophrenia (Yao et al., 2010; Xuan et al., 2011), exhaustion disorder (Hadrévi et al., 1971).

These findings are in line with present results suggesting that involvement of tryptophan metabolism may be common to several neuropsychiatric conditions, including PANS, sharing several clinical features.

The predominant pathway of Trp metabolism in humans is the Kynurenine Pathway (KP) (Dantzer, 2016): several tryptophan-kynurenine pathway metabolites have being increasingly recognised as a key immune-regulatory mechanism, brain inflammation (Mándi and Vécsei, 2012) and microglial activation, potentially related to severe neuropsychiatric disorders (Horikawa et al., 2010), such as depression (Dantzer, 2016), psychosis and Autism Spectrum Disorder (ASD) (Olloquequi et al., 2018).

Supporting this putative mechanism, in animal models symptoms such as severe involuntary movement, increased locomotor activity, persisting impaired active avoidance learning, suggestive of a PANS-like phenotype, can be induced by administering quinolinic acid (QA) a metabolite of the KP) into the striatum (Vécsei and Flint Beal, 1991).

As well as products of the KP pathway, tryptophan is also the precursor to 5-hydroxytryptamine (5-HT) (Wichers and Maes, 2004).

Given this, it is reasonable to assume a direct link between the two biomolecules. 5-HT has diverse roles in memory, mood, anxiety, aggression, pain, sleep, and eating behavior (Sandyk, 1992).

Accordingly, 5-HT deficit has been implicated in several neuropsychiatric illnesses (Bell et al., 2001) including major depressive disorder, obsessive-compulsive disorderandanxiety (Colle et al., 2020; Baumgarten and Grozdanovic, 1998). Interestingly, several conditions involving Trp malabsorption (e.g., Hartnups disease) are often characterized by symptoms such as psychosis or depression (Oyanagi et al., 1967), as well as dietary Trp deprivation having been shown to exacerbate ASD symptoms (McDougle, 1996).

Moreover, serotonin is the substrate in melatonin synthesis, and consequently, serotonin deficiency reduces the synthesis of melatonin (Zimmermann et al., 1993). This suggests a possible explanation for the sleep disruption frequently occurring in PANS.

Combined, these data strongly suggest that abnormal tryptophan metabolism may play a role in the pathophysiology of PANS, since the presentation of PANS symptoms encompasses tics, as well as obsessive, psychotic, and anxiety symptoms.

Glycine is another metabolite significantly decreased in concentration (p=0.0048) in the serum of patients with PANS compared to healthy controls. Glycine's biomolecular role is notable in several psychiatric disorders (Rujescu and Giegling, 2016; Humer et al., 2020; Erjavec et al., 2018).

Glycine is widely distributed in the mammalian CNS, functioning as an inhibitory or excitatory neurotransmitter, depending on its localization. Glycine is the main neurotransmitter in inhibitory interneurons of the spinal cord, brainstem, and in other brain regions involved in the processing of sensorimotor information and locomotor behavior (Chatterton et al., 2002).

Glycine can activate two classes of distinct ligand-gated ion channels: chloride-permeable inhibitory GlyRs (Gielen et al., 2015), and cation-selective excitatory NMDARs.

Electrophysiological, immunocytochemical, and in situ hybridization studies have shown that GlyRs are prominent in the brainstem and spinal cord (Altschuler et al., 1986; Alvarez et al., 1997), and detectable also in the following brain regions: prefrontal cortex, hippocampus, amygdala, hypothalamus, cerebellum, nucleus accumbens, ventral tegmental area, and substantia nigra (Chattipakorn and McMahon, 2002; McCool and Botting, 2000; Ye et al., 1998).

As an excitatory neurotransmitter, glycine acts as a co-agonist of NMDAR, allowing for depolarization, removal of the magnesium blockade, and Na+/Ca2+ passage through the ion channel, which ultimately enhances the glutamatergic excitatory tone that is critical for learning and neuronal plasticity (Nakazawa et al., 2004; Collingridge et al., 2013).

Notably, the affinity of glycine for NMDARs is significantly higher than that of GlyRs (EC50 = 134 nM vs. EC50 = 270 mM) (Chattipakorn and McMahon, 2002; Cubelos et al., 2014) , thus, under physiological conditions, endogenous glycine may exert a mainly excitatory effect in the hippocampus, where both GlyRs and NMDARs are expressed.

Interestingly, an increase in serum glycine levels measured by ^{1}H NMR spectra has been shown to differentiate schizophrenia (with higher glycine levels) from bipolar patients (with glycine plasma levels similar to controls; Tasic et al., 2019), suggesting and important role for glycine in regulating cognition and affective regulation, also supported by evidence from animal models (Humer et al., 2020) in neuropsychiatric disorders.

Irritability, emotional lability, behavioral regression has been shown a crucial component of the PANS clinical presentation (Gagliano et al., 2020), rendering the involvement of glycine metabolism in PANS biological plausible.

Glutamine levels and the associated pathways (glutamine and glutamate metabolism and aspartate and glutamate metabolism) were also found to be altered in PANS patients compared to healty CG. Glutamine in the CNS plays an important role in the glutamate/GABA-glutamine cycle; it is transferred from astrocytes to neurons, where it replenishes the inhibitory and excitatory neurotransmitter pools (Leke and Schousboe, 2016).

Decreased glutamine plasma concentrations may be determined by several factors, including acute inflammatory activity and consecutive distribution abnormalities, and therefore is not, *per se*, an indicator of actual shortage (Soeters et al., 2012).

Glutamine is an essential nutrient in all rapidly proliferating cells including immune cells; it provides many different building blocks for these cells and simultaneously maintains redox balance by providing reducing equivalents, which are also necessary to allow the appropriate functioning of the immune system.

Glutamine supplementation may be beneficial in patients with a long-standing inflammatory activity that is not producing sufficient quantities of glutamine either due to malnutrition or because they cannot meet the demands of the extremely severe inflammatory illnesses of patients (i.e. ICU patients). More precise information on glutamine role in PANS could open innovative therapeutic approaches.

Histidine, which was also found to be at a significant deficit in PANS patients, is a precursor of the ubiquitously distributed neurohormone neurotransmitter histamine.

In the CNS, histamine is known to regulate sleep and wakefulness, learning and memory, feeding, and energy (He et al., 2012; Hu and Chen, 2017).

A decrease in plasma histidine level has been considered a metabolomic signature of schizophrenia, although contrasting results have been reported on plasma and brain histamine levels (Takahashi et al., 2006).

Taken together, the significant decrease of histidine observed in the present study indicates the need for further investigation on a putative role of histamine in the pathophysiology of PANS; histamine levels were not measured in the present study, but the increasing availability of histaminergic receptor modulators supports interest in this neurotransmitter.

Finally, 2-Hydroxybutyrate, was found to have significantly increased concentration in the PANS serum. It is normally produced as a result of excessive glutathione anabolism Gall et al., 2010).

Glutathione is an antioxidant whose synthesis has been demonstrated to undergo compensatory upregulation in the blood of individuals experiencing increases in oxidative stress, such as smokers (Agarwal et al., 2019), as well as in the aging brain (Tong et al., 2016). Combined with our analysis, this increase may indicate that oxidative stress may be considered a crucial feature of PANS.

Overall, the evidence provided by the present study is consistent with the accumulating data supporting the presence of a neuroinflammatory component in several psychiatric illnesses (Najjar et al., 2013).

Inflammatory biomarkers have been found in common neuropsychiatric outcomes (Yuan et al., 2019), such as ADHD (Dunnet al., 2019), ASD (Xu et al., 2015), bipolar disorder (Muneer, 2016), depression (Dowlati et al., 2010), and schizophrenia (Na et al., 2014).

4.6. LIMITATIONS

The present work presents data collected from an omogeneus group of children and adolescents diagnosed with PANS; a comparison with patients presenting a limited association of two or more PANS criteria, but not the complete disorder may help to verify the strength of the observed associations. Finally, an enlarged sample size will be useful to confirm the present results.

4.7. CONCLUSIONS

PANS is currently conceptualized as a complex syndrome with a number of aetiologies and disease mechanisms, encompassing psychiatric symptoms and arising from inflammatory/immune abnormalities triggered by a variety of agents (Swedo, 2012; Frankovich et al., 2015).

The specific plasma metabolites observed in the present study might reflect specific changes in metabolic pathways induced by inflammation, blood-brain barrier breakdown, and dysregulation of energy metabolism, processes that may represent a common final pathway triggered by different agents.

In conclusion, the results of the present study suggest unique plasma metabolite profiles in PANS patients, significantly differing from healthy children, while potentially involving specific patterns of neurotransmission (tryptophan, glycine, histamine/histidine) as well more general mechanisms of neuroinflammation and oxidative stress (glutamine, 2-Hydroxybutyrate and, potentially, the tryptophan-kynurenine pathway).

Further studies on larger cohorts of patients with PANS, using specific methods, such as MS, able to identify other metabolites left undetected by NMR, are needed to confirm these findings and support their interpretation.

The present work presents data collected from an omogeneus group of children and adolescents diagnosed with PANS; a comparison with patients presenting a limited association of two or more PANS criteria, but not the complete disorder may help to verify the strength of the observed associations. Finally, an enlarged sample size will be useful to confirm the present results.

However, the results of the present metabolomics study can be considered a strong suggestion to enhance PANS biomarker research in metabolomics field and to plan more detailed analysis on putative disease mechanisms for PANS.

5. GENERAL CONCLUSIONS AND FUTURE PROSPECTS

Using a mathematical approach based on Artificial Neural Networks, the putative associations between PANS working criteria, as defined at the NIH in July 2010 (Swedo et al., 2012), were explored by the Auto Contractive Map (Auto-CM) system, a mapping method able to compute the multidimensional association of strength of each variable with all other variables in predefined dataset.

The PANS symptoms were strictly linked to one another on the semantic connectivity map, shaping a central "diamond" encompassing anxiety, irritability/oppositional defiant disorder symptoms, obsessive-compulsive symptoms, behavioral regression, sensory motor abnormalities, school performance deterioration, sleep disturbances, and emotional lability/depression.

The semantic connectivity map also showed the aggregation between PANS symptoms and laboratory and clinical variables.

In particular, the emotional lability/depression resulted as a highly connected hub linked to autoimmune disease in pregnancy, allergic and atopic disorders, and low Natural Killer percentage.

Also anxiety symptoms were shown to be strongly related with recurrent infectious disease remarking the possible role of infections as a risk factor for PANS.

The data mining approach showed a very specific constellation of symptoms having strong links to laboratory and clinical variables consistent with PANS feature.

Furthermore, polysomnography showed abnormality in 17 out 23 recruited subjects (73.9%).

In particular, in accordance with AASM criteria, 8/17 children (47%) had ineffective sleep, 10/17 (58.8%) fragmented sleep, (47.1%) Periodic Limb Movement Disorder (PLMD) and 11/17 (64.7%) REM-Sleep Without Atonia (RSWA).

Most patients had more than one sleep disorder.

Notably, among the 19/23 patients diagnosed with Tic/Tourette Disorder, 8/19 (42.1%) show PLMD and 10/19 (52.6%) RSWA.

Artificial Neural Network methodology and the Auto-Contractive Map exploited the links among the full spectrum of variables revealing the simultaneous connections among them, facing the complexity of PANS phenotype.

Sleep disorders represent, for prevalence and impact on quality of life, a cardinal symptom in patients with PANS. Thus, considering the weight of sleep disorders on diagnosis and prognosis of PANS, it could be considered the opportunity to include them among the major diagnostic criteria.

Finally, 34 outpatients referred for PANS (mean age 9.5 yrs; SD 2.9, 71% male) and 25 neurotypical subjects matched for age, gender and intelligence quotient were considered in the metabolomics analysis.

Separation of the samples, in line with the presence of PANS diagnosis, was observed by the application of a supervised model (R^2X=0.44, R^2Y=0.54, Q^2=0.44, p-value <0.0001).

The most important variables were 2-OH butyrate, glycine, glutamine, histidine, tryptophan, while most altered pathways were phenylalanine, tyrosine and tryptophan metabolism, and glutamine and glutamate metabolism.

This study (Study N.3) found a unique plasma metabolic profile in PANS patients, significantly different from healthy children.

This metabolomics study offers new insights into biological mechanisms underpinning the disorder and encourages the research on further potential biomarkers implicated in PANS.

In conclusion, PANS, as well as the broad spectrum of autoimmune-mediated inflammatory brain diseases, represent a rapidly developing area of medical science.

This condition challenges clinicians to find reliable biomarkers facilitating the recognition of the brain susceptibility to autoimmunity and the accurate diagnosis and treatment of children presenting with new onset neuropsychiatric symptoms.

Further studies are needed to investigate their relationships and potential diagnostic values of autoimmune/ inflammatory markers.

We believe that the clinical, neurophysiological and metabolomics data we presented in this work could be considered powerful predictive markers of PANS phenotypes, and points towards further biochemical investigation which could provide further and more specific biomarkers, improving the understanding of acute-onset neuropsychiatric symptoms in paediatric patients.

The simultaneous identification of diverse biomarkers may assist the clinician in making an accurate and timely diagnosis of PANS, potentially allowing for effective treatment strategies to be implemented.

5.1.   ACKNOWLEDGMENTS

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Study N. 2

The Study N. 2 has already been submitted for publication:
Gagliano A., Puligheddu M., Ronzano N., Congiu P., Tanca M.G., Cursio I., Carucci S., Sotgiu S., Grossi E., Zuddas A. Sleep alterations in pediatric acute-onset neuropsychiatric syndrome (PANS): a polysomnographic study.

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Study N. 3

The Study N. 3 has already been submitted for publication:

Murgia F., Gagliano A., Tanca M.G., Hendren A.J., Pintor M., Cera F., Carucci S., Cossu F., Sotgiu S., Atzori L., Zuddas A. Metabolomics Characterization of the Pediatric Acute-Onset Neuropsychiatric Syndrome (PANS).

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7. TABLES

TABLE 1. PANS Scale-R – lifetime paediatric acute-onset neuropsychiatric syndrome (PANS) symptoms [a] (Hesselmark and Bejerot, 2019, pag. 6).

	Interview-confirmed PANS (*n* = 28)	Suspected PANS (*n* = 29)	Never suspected PANS (*n* = 32)	Missing (*n*)	Comparison between group frequencies			
					χ^2	F	d.f.	P
Obsessive–compulsive symptoms,[b] *n* (%)								
Any obsessive–compulsive symptom	25 (89)	24 (83)	14 (44)	1	16.736		2	<0.001
Contamination	17 (61)	16 (55)	7 (23)	1	10.276		2	0.006
Causing harm	13 (46)	9 (31)	4 (13)	2	7.598		2	0.022
Sexual or religious	7 (25)	3 (10)	4 (13)	2	2.524		2	0.283
Symmetry	16 (57)	20 (69)	9 (29)	1	10.155		2	0.006
Hoarding	7 (25)	10 (34)	4 (13)	1	3.869		2	0.144
Other	7 (26)	5 (18)	2 (7)	6	3.488		2	0.175
Eating disorder, *n* (%)	17 (61)	17 (59)	8 (26)	1	9.243		2	0.010
Separation anxiety, *n* (%)	22 (79)	19 (66)	8 (26)	1	18.294		2	<0.001
General anxiety, *n* (%)	16 (62)	14 (48)	19 (61)	3	1.352		2	0.509
Phobias, *n* (%)	13 (46)	13 (45)	3 (10)	1	11.753		2	0.003
Panic episodes, *n* (%)	12 (43)	14 (50)	10 (34)	4	1.409		2	0.494
Emotional lability, *n* (%)	20 (71)	25 (86)	15 (52)	3	8.228		2	0.016
Depression, *n* (%)	20 (71)	19 (66)	20 (67)	2	0.256		2	0.880
Irritability or aggression, *n* (%)	18 (64)	24 (83)	15 (50)	2	7.032		2	0.030
Behavioural regression, *n* (%)	13 (46)	19 (66)	3 (10)	2	19.561		2	<0.001
Personality change, *n* (%)	17 (61)	19 (66)	7 (23)	2	12.602		2	0.002
Attention deficit, *n* (%)	20 (71)	25 (89)	22 (73)	3	3.154		2	0.207
Loss of academic skills, *n* (%)	17 (63)	13 (52)	3 (10)	8	17.933		2	<0.001
Sensory sensitivity, *n* (%)	23 (82)	25 (86)	16 (53)	2	9.757		2	0.008
Hallucinations, *n* (%)	9 (36)	9 (33)	11 (39)	9	0.212		2	0.900
Dilated pupils, *n* (%)	13 (46)	14 (48)	1 (4)	5	15.741		2	<0.001
Urinary symptoms, *n* (%)	21 (75)	17 (59)	9 (31)	3	11.389		2	0.003
Dysgraphia, *n* (%)	12 (44)	15 (56)	6 (20)	5	7.976		2	0.019
Choreiform movements, *n* (%)	11 (42)	13 (46)	3 (11)	8	9.103		2	0.011
Complex motor tics, *n* (%)	10 (37)	11 (38)	3 (10)	3	7.348		2	0.025
Motor hyperactivity, *n* (%)	13 (46)	20 (69)	9 (30)	2	9.023		2	0.011
Simple motor tics, *n* (%)	20 (71)	13 (45)	7 (23)	2	13.511		2	0.001
Sleep problems, *n* (%)	25 (89)	23 (79)	19 (63)	2	5.64		2	0.060
Global severity 0–100, *n* (mean) s.d.	27 (48.74) 26.2	27 (65.74) 22.0	27 (42.17) 29.7	8		F = 5.83	2, 78	0.004

a. PANS Scale-R is an unpublished structured interview (Leckman, 2014, personal communication) based on the diagnostic criteria for PANS.
b. All obsessive–compulsive items of the scale ask about both obsessions and related compulsions

TABLE 2. Diagnoses according to Mini International Neuropsychiatric Interview (MINI) and MINI-KID (Hesselmark and Bejerot, 2019, pag. 5).

	n (%)			Missing (n)
	Interview-confirmed PANS	Suspected PANS	Never suspected PANS	
Major depressive episode (current)	1 (4)	11 (41)	9 (28)	3
Major depressive episode (recurrent)	19 (68)	17 (63)	16 (50)	2
Suicidality	3 (11)	6 (23)	7 (23)	5
Dysthymia	3 (16)	6 (30)	3 (21)	36
Mania (current)	0 (0)	2 (7)	0 (0)	2
Mania (past)	1 (4)	3 (11)	0 (0)	1
Hypomania (current)	1 (4)	0 (0)	0 (0)	3
Hypomania (past)	3 (11)	6 (21)	1 (3)	2
Panic disorder (current)	2 (7)	8 (30)	1 (3)	2
Panic disorder (past)	8 (30)	9 (33)	9 (28)	3
Agoraphobia	3 (11)	5 (18)	6 (19)	1
Separation anxiety disorder	1 (10)	5 (22)	4 (19)	25
Social phobia (current, generalised)	4 (11)	10 (37)	9 (29)	4
Social phobia (current, not generalised)	1 (4)	0 (0)	0 (0)	4
Specific phobia (current)	4 (20)	3 (14)	2 (12)	31
Obsessive–compulsive disorder (current)	20 (71)	17 (61)	9 (28)	1
Post-traumatic stress disorder (current)	1 (4)	0 (0)	3 (9)	1
Alcohol dependence	0 (0)	0 (0)	2 (6)	1
Alcohol misuse	0 (0)	0 (0)	1 (3)	1
Substance dependence	0 (0)	0 (0)	0 (0)	1
Substance misuse	0 (0)	0 (0)	1 (3)	1
Tourette syndrome	10 (37)	4 (14)	4 (13)	4
Motor tics	7 (26)	5 (18)	3 (10)	5
Vocal tics	4 (15)	6 (21)	6 (20)	5
Transient tics	1 (4)	2 (7)	0 (0)	7
ADHD, combined	6 (22)	12 (44)	9 (29)	4
ADHD, inattentive	8 (30)	7 (26)	8 (28)	6
ADHD, hyperactive	0 (0)	0 (0)	0 (0)	6
Conduct disorder	3 (12)	3 (13)	2 (7)	12
Oppositional defiant disorder	8 (33)	15 (58)	4 (14)	11
Psychotic disorders (current)	1 (4)	6 (29)	4 (13)	10
Psychotic disorders (past)	9 (33)	8 (38)	7 (22)	9
Mood disorder with psychotic features (current)	0 (0)	2 (10)	1 (3)	12
Mood disorder with psychotic features (current)	2 (7)	3 (15)	2 (7)	12
Anorexia nervosa (current)	1 (4)	0 (0)	0 (0)	2
Bulimia nervosa (current)	0 (0)	1 (4)	1 (3)	1
Generalised anxiety disorder (current)	10 (36)	9 (36)	14 (44)	4
Antisocial personality disorder	0 (0)	0 (0)	2 (8)	44
Adjustment disorders	0 (0)	0 (0)	0 (0)	42
Pervasive developmental disorder	6 (22)	17 (59)	12 (40)	3

PANS, paediatric acute-onset neuropsychiatric syndrome; ADHD, attention-deficit hyperactivity disorder.

TABLE 3. General approach to using induction corticosteroids and/ or non-steroidal anti-inflammatory drug therapies in PANS/ PANDAS (Frankocivh et al., 2017, pag. 580).

	Mild-to-moderate flare	Moderate-to-severe flare	Severe-to-extreme flare[a]
Early in flare or early in initial presentation (<14 days). *Early application of corticosteroids (once infection is ruled out) and NSAIDs may abort or limit duration of disease flares.*	(A) Refer to CBT and supportive therapy. Or (B) NSAIDs+(A). or if no improvement or deteriorating baseline then (C) ↓	(A) Refer to CBT and supportive therapy. Or (B) prednisone 1–2 mg/kg/day · 5 days+ (A). or (C) oral dexamethasone pulse (20 mg/m^2 divided twice daily for 3 days) +(A). or (D) IV MP pulse · 1 (30 mg/kg/dose) + (A).	(A) Refer to CBT and supportive therapy. Or (B) oral dexamethasone pulse (20 mg/m^2 divided twice daily for 3 days) alone or in combination with adjunct therapy + (A). (C) IV MP one to three consecutive daily pulses (30 mg/ kg-dose-day · 3 days) alone or in combination with adjunct therapy + (A).
Late in flare (2–4 weeks). Very delayed care (>4 weeks). *Application of corticosteroids late into the disease often requires higher dosing and/or more prolonged tapers.* *Steroid bursts may be followed by NSAIDS, with caution.*	(A) Refer to CBT and supportive therapy. Or (B) NSAIDs+A. or (C) prednisone 1–2 mg/ kg-dayX5 days+(A). If no response, re-evaluate for underlying infection per guidelines. If no infection and baseline worsening, go to next column.	Same as above box, except: (B) consider adding a 1-month prednisone taper (see Appendix B2 for taper) to oral prednisone burst. The mentioned pulse therapy approaches do not need tapers. (A) Refer to CBT and supportive therapy. Or (B) prednisone 1–2 mg/kg-dayX5 days+(A). Consider adding a 1–2-month prednisone taper. Or (C) oral dexamethasone pulse (20 mg/m^2 divided twice daily for 3 days) +(A). or (D) IV MP one to three consecutive daily pulses (30 mg/kg-dose-dayX3 days) +(A). patient may need weekly or monthly pulses to maintain effect. Add steroid-sparing agent if patient is responsive to steroids but does not hold.	(A) Refer to CBT and supportive therapy. Or (B) oral dexamethasone pulse (20 mg/m^2 divided twice daily for 3 days) alone or in combination with steroid-sparing agent +(A). Long-standing disease will likely need more persistent corticosteroids. (C) IV MP one to five consecutive daily pulses (30 mg/kg-dose-day for up to 5 days) alone or in combination with adjunct therapy. Consider weekly IV MP pulses for up to 6 weeks (if tolerated) +(A).

Optimal dosing approaches and utilization of adjunct immunomodulation have not been determined for PANS, but the approaches outlined in this table serve as a starting point for clinicians and academicians who treat patients with PANS and who are planning trials.

Important steroid warning: Most patients have transient worsening of psychiatric symptoms while on corticosteroids. If patient has rage/violence, life- threatening impulsivity, mood instability, suicidality, etc. and caregivers (including medical personnel) are unable to manage potentiation of these behaviors, give corticosteroids in psychiatric unit or medical-psychiatric unit or bypass corticosteroids and go straight to IVIG or other steroid-sparing agent. If no response to initial corticosteroid burst/pulse or relapse after steroid burst/pulse, consider reassessing for underlying infection per guidelines (Chang et al., 2015; Cooperstock et al. 2017) with attention to the possibility of sinusitis or close contact with GAS or asymptomatic acquisition of GAS.

If no infection, repeat steroid bursts/pulses and/or give corticosteroid sparing agent.

[A]If patient meets criteria for another brain inflammatory disease, use said treatment protocol.

AE, autoimmune encephalitis; CBT, cognitive behavioral therapy; GAS, group A _Streptococcus_; IV, intravenous; IVIG, intravenous immunoglobulins; MP, methylprednisolone; NSAIDs, nonsteroidal anti-inflammatory drugs; PANS, pediatric acute-onset neuropsychiatric syndrome.

TABLE 4. General strategies for management of PANS based on disease trajectory (Frankocivh et al., 2017, pag. 578).

Disease trajectory	*Recommendations*
New-onset or acute flare	(1) Work-up infections and other causes of acute neuropsychiatric deteriorations per guidelines[a] (Van Mater 2014; Chang et al. 2015; Graus et al. 2016; Cooperstock et al. 2017; Dale et al. 2017). (2) Refer for CBT and provide other supportive therapies (Thienemann et al., 2017). (3) Consider early use of corticosteroids (oral bursts or IV pulses) to abort or shorten flares. (4) Consider high-dose IVIG or other immunomodulatory therapies in moderate-to-severe cases.

Relapsing-remitting	(1)-(4) as above.
	(5) Evaluate for possibility of recurrent infections/exposures triggering flares.
	  (a) If GAS infection is a frequent trigger for relapses, evaluate/treat close contacts and consider prophylaxis according to guidelines (Cooperstock et al. 2017).
	  (b) Keep in mind that most flares are viral triggers. See (2)-(4) above for treatment of each flare.
	  (c) Evaluate immune system competency: pursue immunodeficiency work-up if patient has recurrent sinopulmonary disease or fevers per guidelines (Chang et al. 2015). If immunodeficiency is present, IVIG may reduce the number and severity of intercurrent infections (Cooperstock et al. 2017).

Chronic-static or chronic-progressive
 	
Initial therapy is proposed in the box to the right. Patients with chronic-static or progressive disease may respond to corticosteroids or other induction immunotherapies but then relapse if therapy is stopped. Some patients need repeated doses of steroids and/or other immunotherapies (IVIG or other steroid-sparing agent).
	(1)-(4) as mentioned.
	
	(5) Pursue immunomodulatory therapies according to symptom categories below: Mild-to-moderate neuropsychiatric symptoms:
	  NSAIDs.
	  Oral corticosteroid burst to see whether baseline improves.
	  Caution: use of combination NSAIDs + corticosteroids may result in gastritis; but these medications can be used safely in tandem.
	
	Mild-to-moderate neuropsychiatric symptoms with no response to NSAIDs and/or short burst of corticosteroids:
	  (Repeat) oral prednisone – prolonged taper.
	  Pulse corticosteroids (oral dexamethasone or IV methylprednisolone).
	Moderate-to-severe neuropsychiatric symptoms:
	  Oral prednisone–taper or pulse corticosteroids.
	  High-dose IVIG or other induction steroid-sparing agent.
	Severe-to-extreme neuropsychiatric symptoms:
	  Refer to subspecialists for further evaluation for AE, NPSLE, CNS vasculitis, and consideration of using established (published and institutionally based) treatment protocols.
	  Consider high-dose IV corticosteroids and/or other immunotherapies.
	Refractory disease course (i.e., psychiatric symptoms not responsive to initial immunomodulatory approaches already mentioned and no improvement in neurological signs):
	Refer to subspecialist for consideration of additional agents[b] and/or combination therapy (up to four immunomodulatory therapies are used simultaneously to treat inflammatory brain diseases; that is, corticosteroids + TPE + IVIG + rituximab).
	Consider possibility of injured neurocircuitry and need for shifting to primary rehabilitation mode.

TABLE 5. Frequency of use and effectiveness of psychotherapy (Calaprice et al., 2018, pag. 99).

Type of psychotherapy	Received in the past			Currently receiving		
	N reporting treatment effect	Very effective	Somewhat effective	N reporting treatment effect	Very effective	Somewhat effective
CBT	159	21 (34)	33 (53)	139	17 (23)	56 (78)
CBT + ERP	71	39 (28)	28 (20)	44	27 (12)	43 (19)
Habit Reversal Therapy	13	8 (1)	23 (3)	9	11 (1)	33 (3)
Behavior management	48	4 (2)	46 (22)	62	13 (8)	63 (39)
Counseling	143	8 (12)	36 (51)	124	8 (10)	59 (73)
Other	22	9 (2)	41 (9)	27	26 (7)	52 (14)

CBT, cognitive behavioral therapy; ERP, exposure response prevention.

TABLE 6. Mean Scores and SD at a 4-Point Likert Scale of the Main and Additional Pediatric Acute Onset Neuropsychiatric Syndrome Neuropsychiatric Symptoms and Number and Proportion of Patients Who Had Each Symptom

PANS neuropsychiatric symptoms	Description	Mean Score and SD	Number of patients/39 and percentage
Obsessive-compulsive symptoms	Intrusive and repetitive thoughts (e.g. obsessional worries about contamination, order or symmetry, harm or danger, etc) and/or compulsions (e.g. repeated checking, counting, tapping, arranging, repeating certain words/actions/questions, etc; red ring around the mouth from excessive lip-licking, chapped hands from excessive washing or irritation of the external genitalia from excessive wiping)	1.87(0.95)	34/39 (87%)
Restricted food intake	Refusal to eat or marked decrease in food or fluids intake; worries about consequences of eating or avoidance based on the sensory characteristics of food; nausea or severe lack of interest in eating or food; secondary dehydration or emaciation	1.26(1.14)	26/39 (66%)
Anxiety	Hyperalert, anxious, terrified or in the "fight or flight" mode; separation anxiety (need to maintain proximity to familiar members or locations); irrational fears or phobias; general anxiety; panic episodes	1.97(1.03)	35/39 (89%)
Emotional lability/depression	Emotional lability characterized by involuntary and uncontrollable episodes of crying or laughing that are mood incongruent; depression with a flat or depressed affect, auditory or visual hallucinations; self-injurious or aggressive behaviours	1.79(1.23)	30/39 (76%)
Irritability/Oppositional defiant disorder	Severe impulsivity and agitation, oppositional behaviour, irritability, aggression, and temper tantrums episodes, defiant/ irrational demands; reactive aggressive behaviour, rage attacks	2.15(1.08)	35/39 (89%)
Behavioural regression	Developmental regression with "baby talk", paucity of speech or mutism, memory impairments; severe changing in personality	1.36(1.06)	30/39 (76%)
School performance deterioration	Meaningful changing in school achievement with a deterioration of school performance and behaviour (difficulties in paying attention on cognitive tasks and in remaining seated). Deterioration of handwriting with mixtures of printing and cursive writing, irregular sizes, shapes, or slant of letters and inconsistent position of letters on the page; difficulties in drawing even simple figures	1.59(1.20)	29/39 (74%)
Sensory or motor abnormalities	Motor or phonic tics (grunting, squeaking, etc); choreiform or "piano playing fingers" movements, mildly reduced proximal muscle weakness and slouched posture, adventitious movements, developmental motor regression. Heightened sensitivity to sounds, light, smell or taste; shape or spatial distortion of objects vision; visual or auditory hallucinations; dilated pupils – "terror-stricken look"	1.59(1.11)	32/39 (82%)

| Sleep disturbances | Insomnia, inability to sleep, lengthy bedtime rituals, parasomnias (e.g., sleepwalking, night terrors), periodic limb movement, etc | 1.67(1.13) | 31/39 (79%) |
| Enuresis/urinary frequency | Increased urinary frequency or urge to urinate; urinary incontinence or inability to urinate. | 0.95(1.07) | 24/39 (61%) |

(Likert scale from 0 = absence to 3 = extremely severe)
SD, standard deviation.
ODD, oppositional defiant disorder.

TABLE 7. Proportion of Patients Who Had a Family and Pregnancy-Related/Intrapartum History

Family history and Maternal diseases	*N/39*	*%*
Family History of Autoimmune Diseases [1]	31	80
Family psychiatric history [2]	28	72
Maternal pregnancy-related/intrapartum complications [3]	25	64
Maternal infections in pregnancy [4]	6	15
Maternal autoimmune diseases in pregnancy[1]	23	59
Maternal Hashimoto's disease in pregnancy[5]	13	33

Legend

1 Family History of Autoimmune Diseases in first-degree relatives: Sydenham chorea, Systemic lupus erythematosus (SLE), Sjögren syndrome, Kawasaki's disease, Myasthenia gravis, Asthma, Guillain–Barre´ syndrome, Multiple sclerosis, Hughes syndrome, type 1 diabetes, Celiac disease, Crohn's disease, Vitiligo, Psoriasis, Scleroderma, Familial Mediterranean fever and Behçet's Disease.
2 Psychiatric history in first-degree relatives of OCD, eating disorders, emotional lability, mood disorders, ADHD, anxiety disorders, tic disorders, psychosis, disruptive disorders, autism spectrum disorder, language and communication disorders, intellectual disability and specific learning disorders.
3 Pregnancy/delivery complications: high blood pressure, gestational diabetes, preeclampsia, placenta previa, preterm labour, low birth weight, fetal distress, perinatal asphyxia.
4 Infections in pregnancy: urinary tract infection, bacterial vaginosis, toxoplasmosis, cytomegalovirus, hepatitis B virus, influenza, Epstein-Barr virus infection. Group B Streptococcus infections.
5 Hashimoto's thyroiditis, or chronic lymphocytic thyroiditis in which the thyroid gland is gradually destroyed.

OCD, obsessive compulsive disorder; ADHD, attention-deficit/hyperactivity disorder.

TABLE 8. Proportion of patients who had diseases and abnormal laboratory test results

CHILDREN VARIABLES	*N/ 39*	*%*
CLINICAL VARIABLES		
Recurrent infections[1]	32	82
Allergic and atopic disease[2]	18	46
Atopic dermatitis[3]	23	59
EEG alterations[4]	22	56
LABORATORY TESTS		
ASO test>250[5]	15	39
ASO test<250[5]	24	61
Positive other germs antibodies[6]	19	49
Positive antithyroid antibodies[7]	14	36
ANA> 1:120[8]	13	33
ANA<1: 120 [8]	26	67
Natural killer cells <3 % [9]	28	72
Natural killer cells ≥ 3 % [9]	11	28
Positive inflammatory markers [10]	7	18
Positive nasopharyngeal culture [11]	23	59
Brain MRIs abnormalities [12]	8	21

Legend

1 Recurrent child's infections: sinusitis, chronic otitis, pharyngitis or tonsillitis, pneumonia, skin infections (i.e. staph) and/or signs of GAS infection (i.e., pharyngitis, anal or vulvar redness, skin lesions); this variable has been estimated as a dichotomic condition; a cut-off has been established on three or more respiratory infections (e.g., sinusitis, otitis, bronchitis) in one year, or the need for antibiotics for two months/year (Ballow et al. 2008).
2 Frequent episodes of asthma, allergic rhinitis (AR), Immunoglobulin E (IgE)-mediated food allergies (Fas), and other immune-mediated food disorders requiring food avoidance.
3 Common evidence for cracked and itching skin or red and brownish-grey patches on the hands, feet, knees, wrists, upper chest, face, scalp, etc.
4 Standard and sleep EEG alterations.
5 The antistreptolysin O (ASO) patient's values in the blood plasma have been pooled in two groups (antistreptolysin titer > or < of 250 IU), based on the upper reference limit.
6 One or more IgG and IgM Antibodies against other germs (Anti- Mycoplasma pneumoniae, Chlamydia pneumoniae, Epstein Barr virus, Borrelia Burgdorferi, and Herpes Simplex Virus –HSV- Type 1).
7 One or more antithyroid antibodies (Anti-thyroid peroxidase[anti-TPO], Thyroglobulin antibodies TSH receptor antibodies [anti-TRAb], Thyroid Stimulating Hormone Receptor Antibody [anti-TSH receptor]) above the upper reference limit.

8 The Antinuclear Antibodies (ANA) patient's values in the blood plasma have been pooled in two groups (titers < or > of 1: 120), based on the upper reference limit.
9 Among the Lymphocyte subset (T, B, natural killer [NK] cells) values we considered the NK cells (CD3+/CD56+) % value and we pooled them in two groups (NK ≥ 3% or NK <3%), based on the lower reference limit of 3%.
10 One or more inflammatory markers (erythrocyte sedimentation rate [ESR] and C-reactive protein [CRP]) above the upper reference limit.
11 Nasopharyngeal culture positive for Group A beta-hemolytic streptococcus (GABHS) or Staphylococcus aureus (MRSA), Streptococcus pneumoniae (pneumococcus), Haemophilus influenzae, or other upper respiratory tract pathogenic germs
12 Evidences for changes in brain morphology at MRI with T-2 weighted images or contrast enhancement.

TABLE 9. Summary of Pediatric Acute Onset Neuropsychiatric Syndrome Familial, Clinical, and Laboratory Variables

- A high prevalence rate of pregnancy/delivery complications, active infections, and autoimmune diseases during pregnancy is associated with PANS phenotype

- Hashimoto's thyroiditis is very common among PANS children's mothers

- A large part of PANS children has high titers of Anti-thyroid peroxidase (anti-TPO), Thyroglobulin antibodies, TSH receptor antibodies (anti-TRAbs), and Thyroid Stimulating Hormone Receptor Antibody (anti-TSH receptor), with no overt hypothyroidism

- Recurrent infections are frequently reported in the medical history of PANS patients

- Different germ antibodies (IgG and/or IgM against Mycoplasma pneumoniae, Chlamydia pneumoniae, Epstein–Barr virus, Haemophilus influenzae, and Herpes Simplex Virus –HSVTyp 1) are present in the serum of PANS patients

- Nasopharyngeal culture is often positive for Staphylococcus aureus, Streptococcus pneumoniae, and Haemophilus influenza, Group A beta-hemolytic streptococcus (GABHS)

- Atopic dermatitis is described in more than half of PANS children

- ANA values are positive in around one-third of PANS patients

- ESR and CRP are usually normal

- Low Natural Killer (NK) cell count appears strongly associated with PANS

- Almost 50% of patients with PANS shows EEG alterations both at rest and in vigilant

TABLE 10. Laboratory variables measured in the study sample (PANS-panel) (Sleep study).

ROUTINE BLOOD TEST	Blood counts, sideraemia, Erythrocyte sedimentation rate (ESR), C-Reactive Protein (CRP), D-vitamin, Free Triiodothyronine (Ft3), Free thyroxine (Ft4), Thyroid-stimulating hormone (TSH), Neuron-specific Enolase (NSE).
IMMUNOLOGICAL PARAMETERS	Lymphocyte subpopulation, immunoglobulin and complement profile, Anti-TSH Receptor Antibodies (AbTR), Anti-thyroid peroxidase (AbTPO), Anti-thyroglobulin Antibodies (AbTG), Lupus Anti-Coagulant (LAC), Anti-Nuclear Antibodies (ANA), anti-cardiolipin Antibodies, Anti-Glicoprotein B2 Antibodies, anti-deamidated gliadin, transglutaminase and endomysial Antibodies.
INFECTIOUS PARAMETERS	Anti-streptolysin O (ASLO), Herpes Simplex Virus 1 (HSV-1), Epstein Barr Virus (EBV), Mycoplasma Pneumoniae and Chlamydia Pneumoniae, Nasal and Pharyngeal swab.

TABLE 11. Demographic and clinical patients' characteristics (Sleep study)

CONTINUOUS VARIABLES			
	MEAN VALUE (years)	**RANGE (years)**	**SD (years)**
Mean age at study enrolment	9.8	4.8 – 15.4	2.6
Mean age at full symptom onset	6.4	2.5 – 11.1	2.6
Mean age at diagnosis	8.7	3.8 – 15.1	2.8
Diagnostic mean delay	2.3	0 – 6.5	1.7

	MEAN VALUE	RANGE	SD
Full Scale Intelligence Quotient (Full Scale IQ)	94.6	55 – 116	15.9
Verbal Comprehension Index (VCI)	99.2	60 – 124	18,9
Perceptive Reasoning Index (PRI)	102.4	71 – 124	15,5
Working Memory Index (WMI)	90.5	70 – 118	12,3
Processing Speed Index (PSI)	84.6	65 – 109	12,7

DISCRETE VARIABLES		
Gender	Females: 4/23 (17%)	Males: 19/23 (83%)
Phase of disease	Acute: 9/23 (39%)	Chronic: 14/23 (61%)

DSM-5 ASSOCIATED DIAGNOSIS	
OCD 10/23 (43%)	**ADHD/ODD**: 12/23 (52%)
Anxiety 4/23 (17%)	**Tic /TD**: 19/23 (82%)
Learning Disorder 8/23 (34%)	**Mood Disorders**: 2/23 (8,6%)

Acute = symptoms onset or wax phase of a relapsing/remitting course; Chronic = Chronic course or wane phase of a relapsing/remitting course. OCD: obsessive-compulsive disorder. ADHD: attention deficit hyperactivity disorder. ODD: oppositional defiant disorder. Wechsler intelligence scale indices. Notably, both the Processing Speed Index (PSI) and the Working Memory Index (WMI) show a mean value relatively low, if compared to the other indices' scores.

TABLE 12. Polysomnographic parameters

	Mean	Std. Deviation	95% confidence interval	D'Agostino & Pearson normality test	
				P value	Passed normality test (alpha=0.05)?
Total sleep time. Min	473,20	59,31	447,6 - 498,90	0,81^{ns}	Yes
Sleep efficiency. %	89,57	5,86	87,04 - 92,11	0,67^{ns}	Yes
Sleep Latency (min)	14,53	12,79	8,995 - 20,06	0,17^{ns}	Yes
REM latency (min)	144,90	66,85	116 - 173,80	0,74^{ns}	Yes
Sleep stage N1, %	7,94	5,47	5,574 - 10,30	0,00**	No
Sleep stage N2, %	46,69	6,19	44,01 - 49,36	0,64^{ns}	Yes
Sleep stage N3, %	24,74	4,10	22,97 - 26,51	0,53^{ns}	Yes
Sleep stage R, %	21,14	5,74	18,66 - 23,62	0,72^{ns}	Yes
Wakefulness after sleep onset. %	10,16	5,43	7,813 - 12,51	0,87^{ns}	Yes
WASO min	50,87	29,84	37,97 - 63,77	0,80^{ns}	Yes
Awakenings	10,70	7,41	7,49 - 13,90	0,05*	No
PLMS index, n/hour	5,70	6,56	2,86 - 8,53	<0,0001****	No
Apnea-Hypopnea index, n/hour[#]	2,79	4,56	0,45 - 5,14	<0,0001****	No
Oxygen Desaturation Index n/Hour[#] <3%	2,01	2,84	0,55 - 3,47	0,00***	No
SpO2mean (%)[#]	96,31	0,57	96,01- 96,60	0,30^{ns}	Yes
SpO2min (%)[#]	92,13	1,54	91,3 - 92,95	0,89^{ns}	Yes
Heart rate[#]	68,28	8,95	63,51 - 73,05	0,07^{ns}	Yes

PLMS = periodic limb index.

Polysomnographic parameters. [#] respiratory parameters were available only for 18/23 patients, due to the difficulties for 5 children to accept the pulse-oximeter. Test for Gaussian distribution. GP: 0,1234 (ns); 0.0332 (*), 0.0021(**), 0.0002(***), <0.0001(****)

TABLE 13. Demographics of the enrolled patients (Metabolomics)

Demographic								
Classes	N	Female/Male	% Female/Male	Age				
				Mean value	Median	Mode	SD	Range
PANS	34	10/24	29/71	9,15	9	10	2,90	5-16
Controls	25	9/16	36/64	12,12	11	11	2,17	8-17

TABLE 14. Statistical parameters of the univariate analysis from the comparisons between PANS and Controls (Metabolomics)

METABOLITES	PANS	*p-*value	*p*-value corrected	AUC	Std. Er	CI	p-Value
				ROC-CURVE			
2-OH-Butyrate	+	0.009	0.06	0.71	0.07	0.6-0.8	0.01
Acetone	+	0.04	0.1	0.66	0.07	0.5-0.8	0.04
Alanine	-	0.04	0.1	0.66	0.07	0.5-08	0.04
Asparagine	-	0.01	0.06	0.70	0.07	0.6-0.8	0.01
Dimethylamine	-	0.04	0.1	0.66	0.07	0.5-0.8	0.04
Glycerol	+	0.01	0.06	0.69	0.07	0.5-0.8	0.01
Glycine	-	0.001	0.01	0.72	0.06	0.6-0.9	0.002
Glutamine	-	0.03	0.1	0.66	0.07	0.5-0.8	0.03
Histidine	-	0.003	0.03	0.73	0.06	0.6-0.9	0.003
Isoleucine	-	0.03	0.1	0.67	0.07	0.5-0.8	0.03
Tryptophan	-	0.003	0.03	0.73	0.07	0.6-0.8	0.004
Tyrosine	-	0.006	0.04	0.72	0.07	0.6-0.8	0.007

U-Mann Whitney test and ROC curves were performed.

8. FIGURES
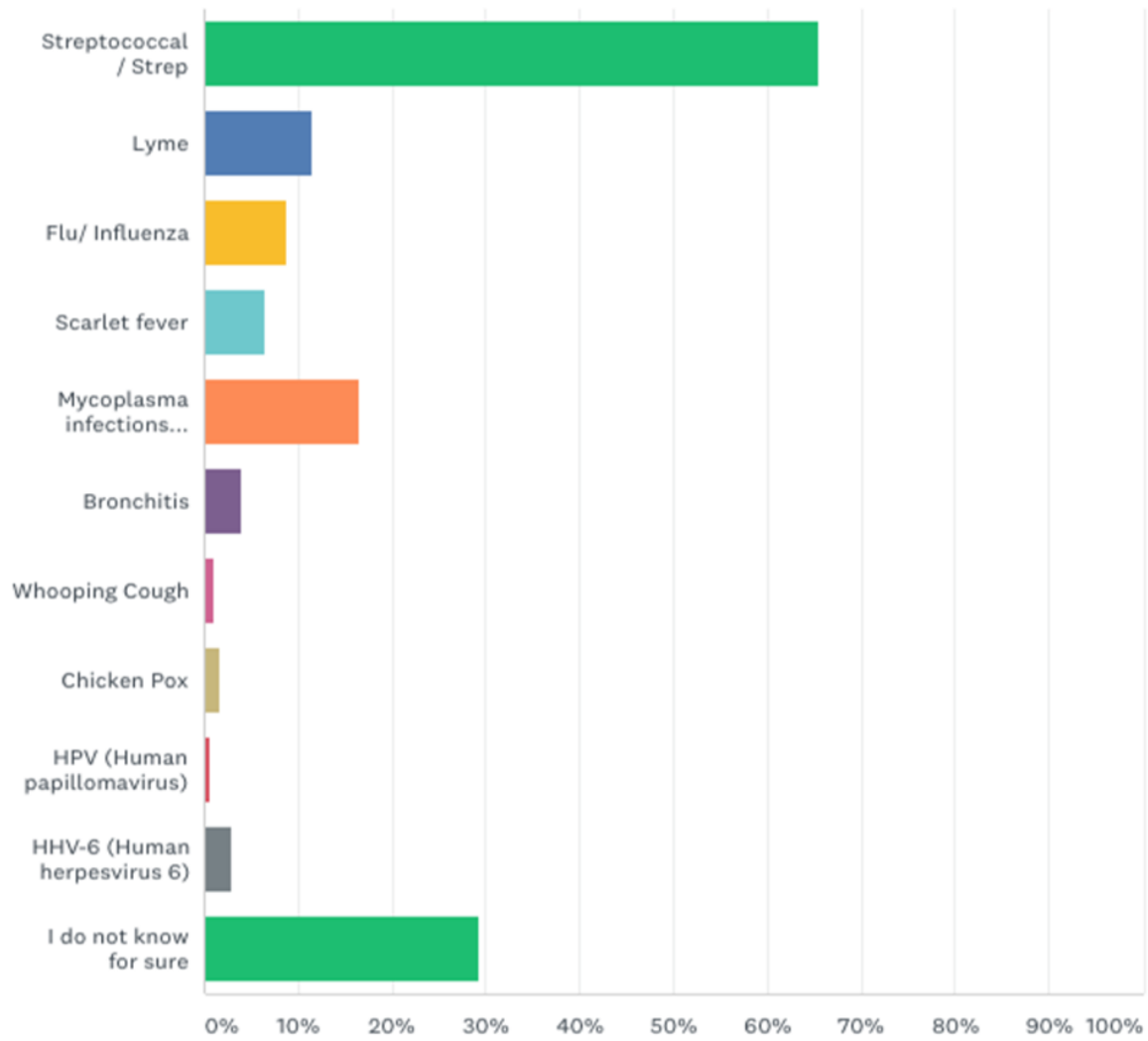
FIGURE 1. Survey aimed at parents of children with PANS, concerning the disorders present at the time of onset (PN State of Our Children SURVEY, 2018).
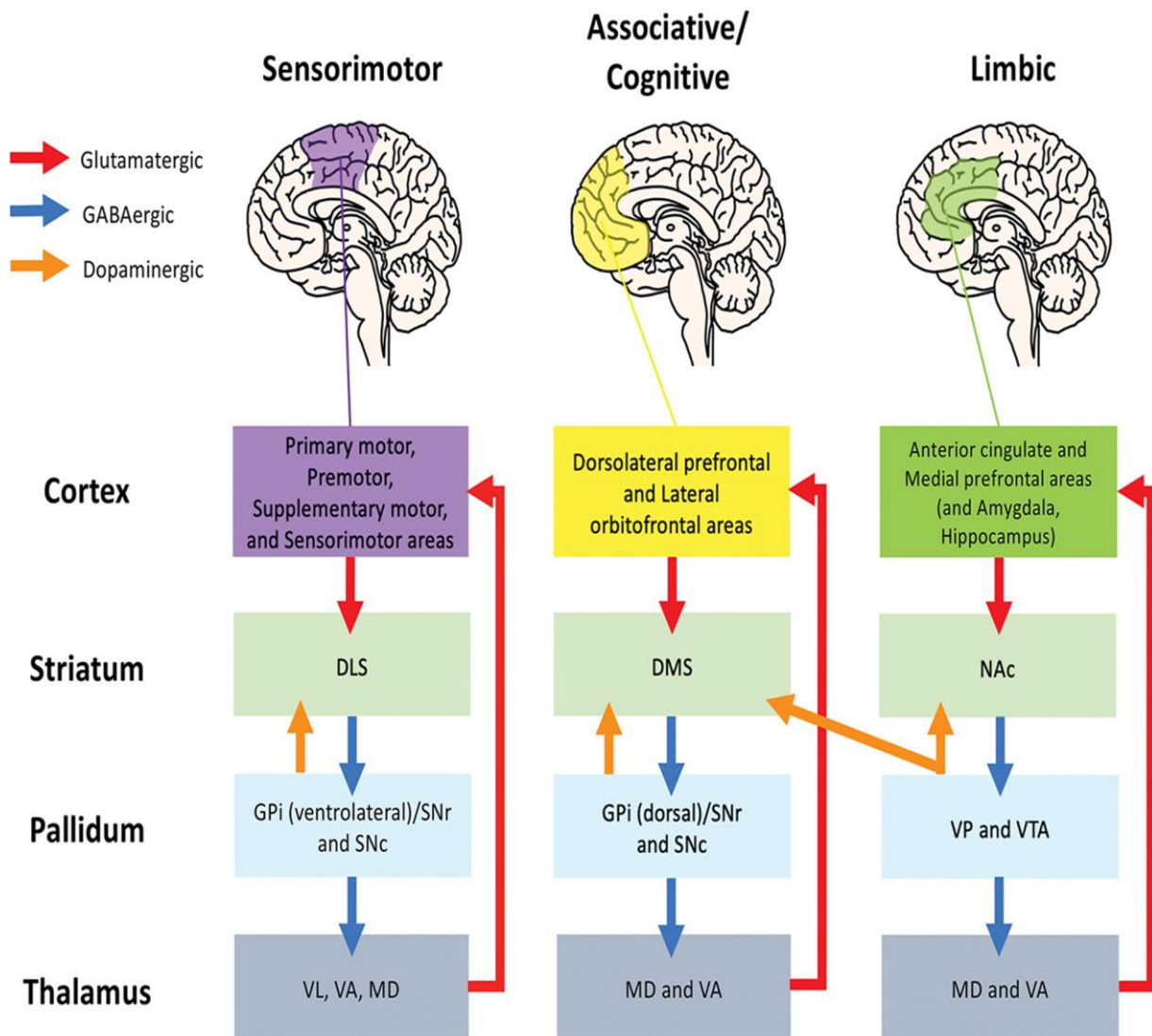
FIGURE 2. Basal Ganglia Neurocircuits (Alexander et al., 1986; Alexander et al., 1990; Haber, 2003; Macpherson and Hikida, 2019, pag. 290).

Basal Ganglia Neurocircuits can be broadly divided into three functional loops: the sensorimotor, associative/cognitive, and limbic neurocircuits. DLS, dorsolateral striatum; DMS, dorsomedial striatum; GPi, globus pallidus internal section; MD, medial dorsal thalamus; NAc, nucleus accumbens; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VA, ventral anterior thalamus; VL, ventrolateral thalamus; VP, ventral validum; VTA, ventral tegmental area.
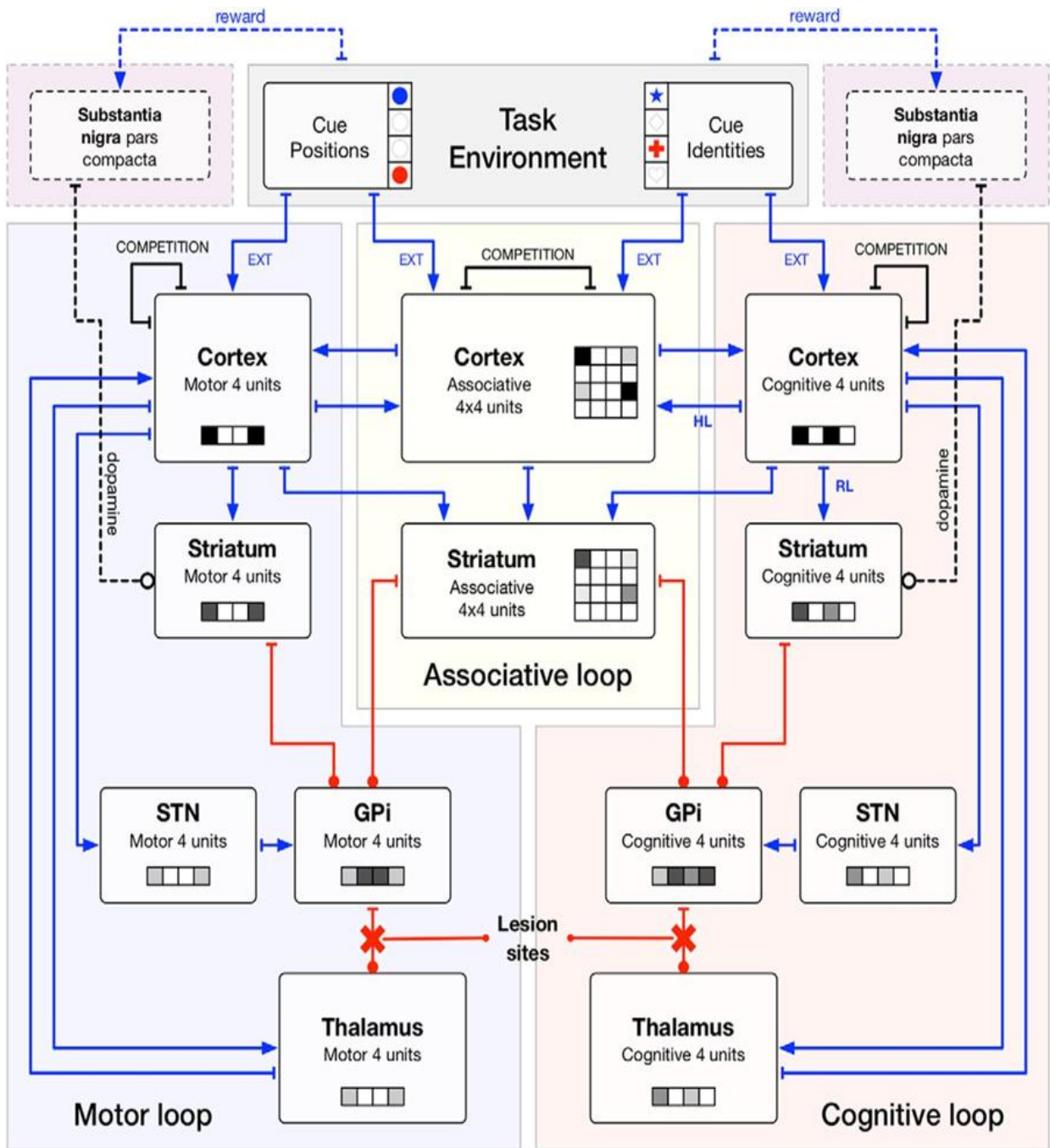
FIGURE 3. Architecture of the model of Dual Competition between the Basal Ganglia and the Cortex (Topalidou et al., 2018, pag. 5).
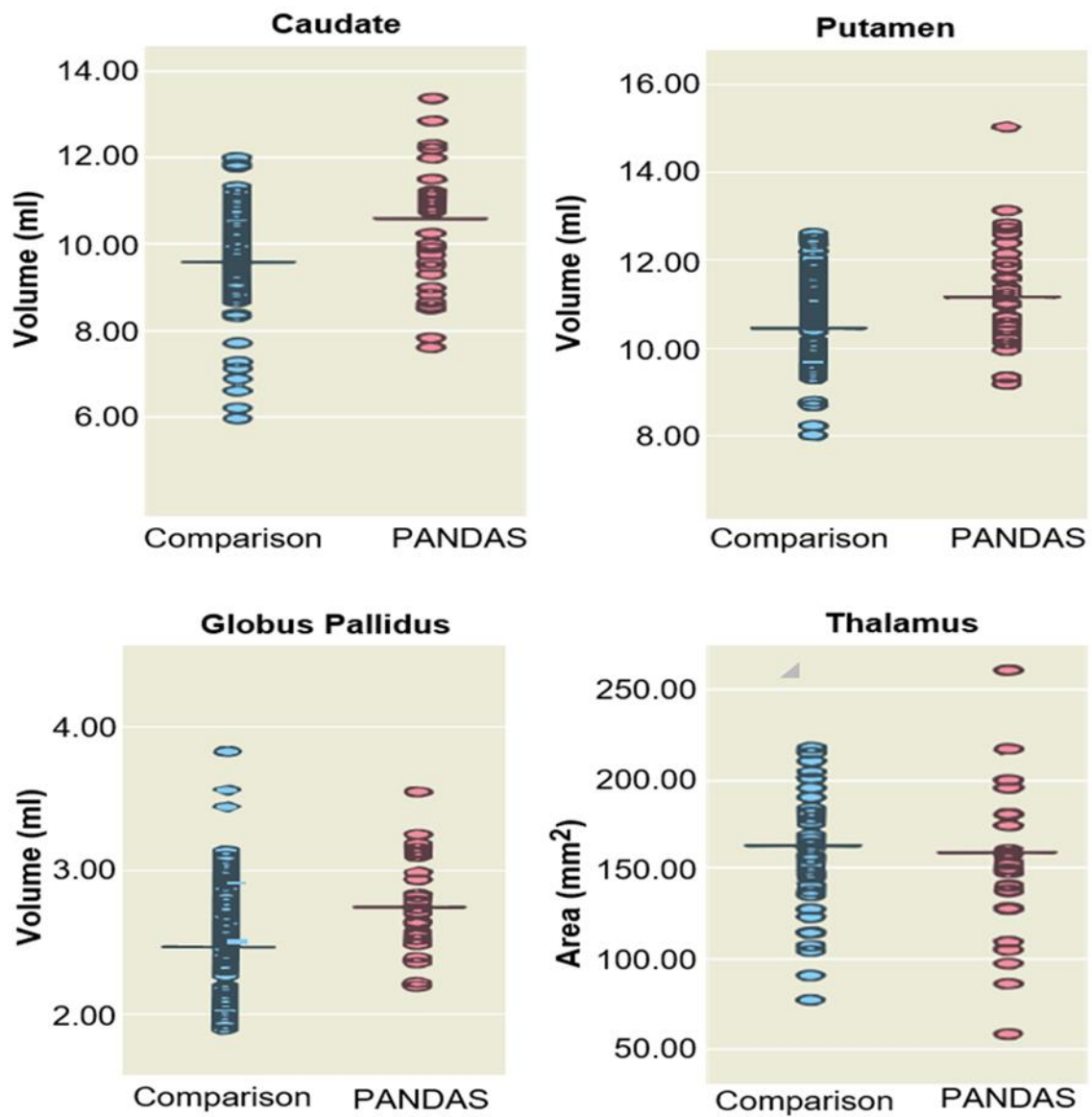
FIGURE 4. Basal Ganglia' s volumetric differences between PANDAS and controls (Giedd et al., 2000, pag. 282).
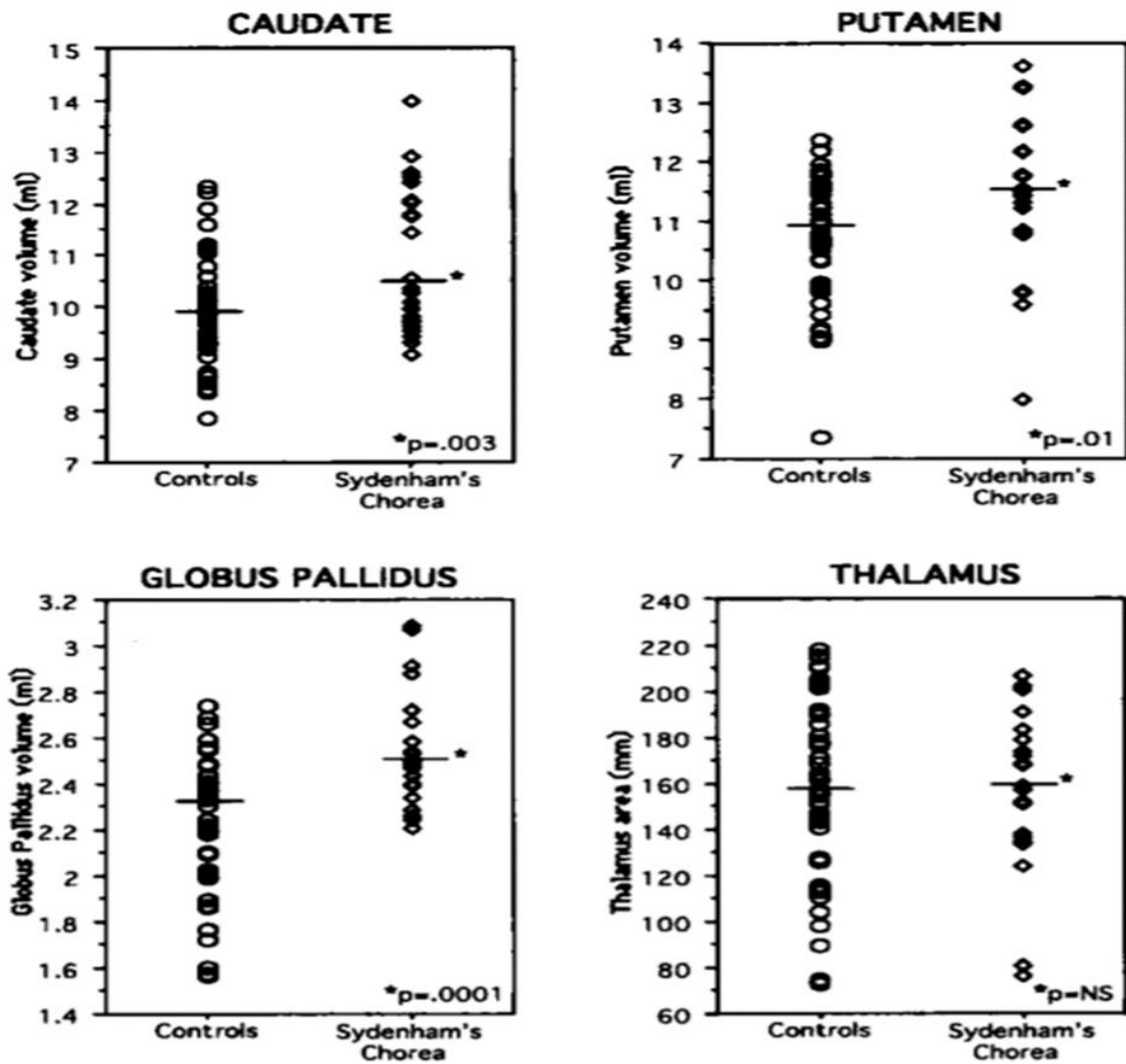
FIGURE 5. Basal Ganglia' s volumetric differences between Sydenham's Chorea and controls (Giedd et al., 1995).
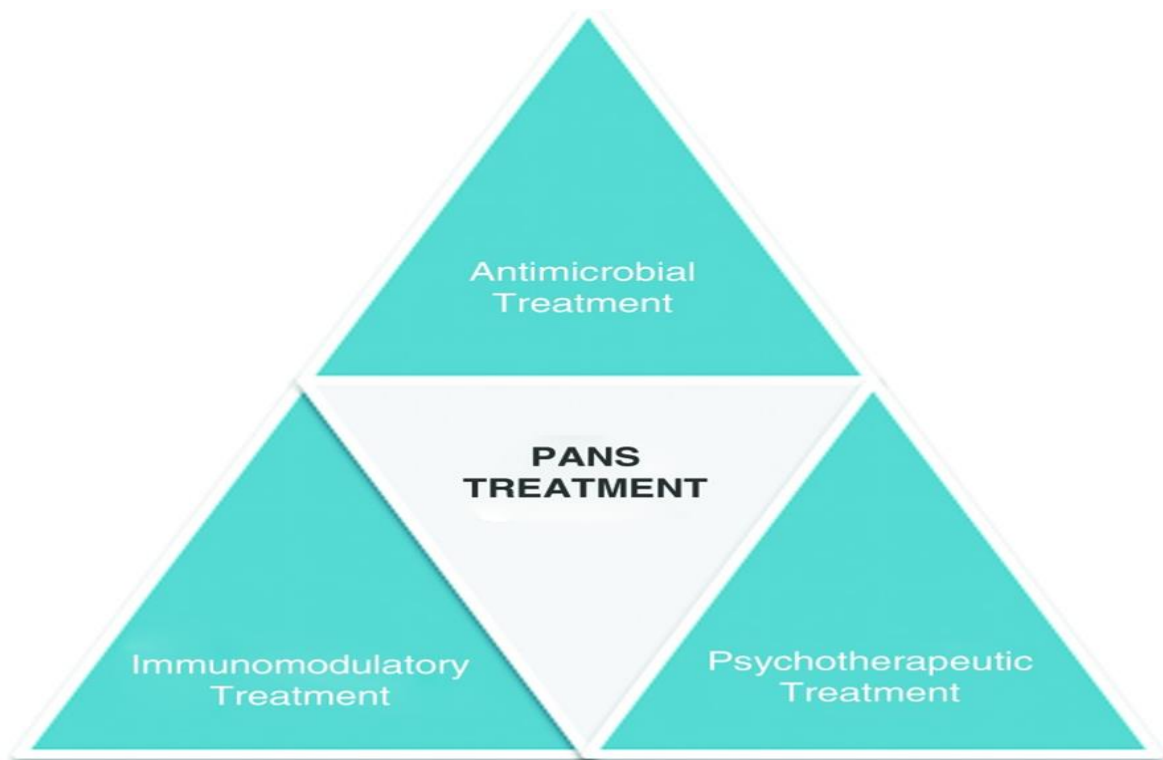
FIGURE 6. The PANS treatment triangle. PANS, pediatric acute-onset neuropsychiatric syndrome (Swedo et al., 2017, pag. 563).
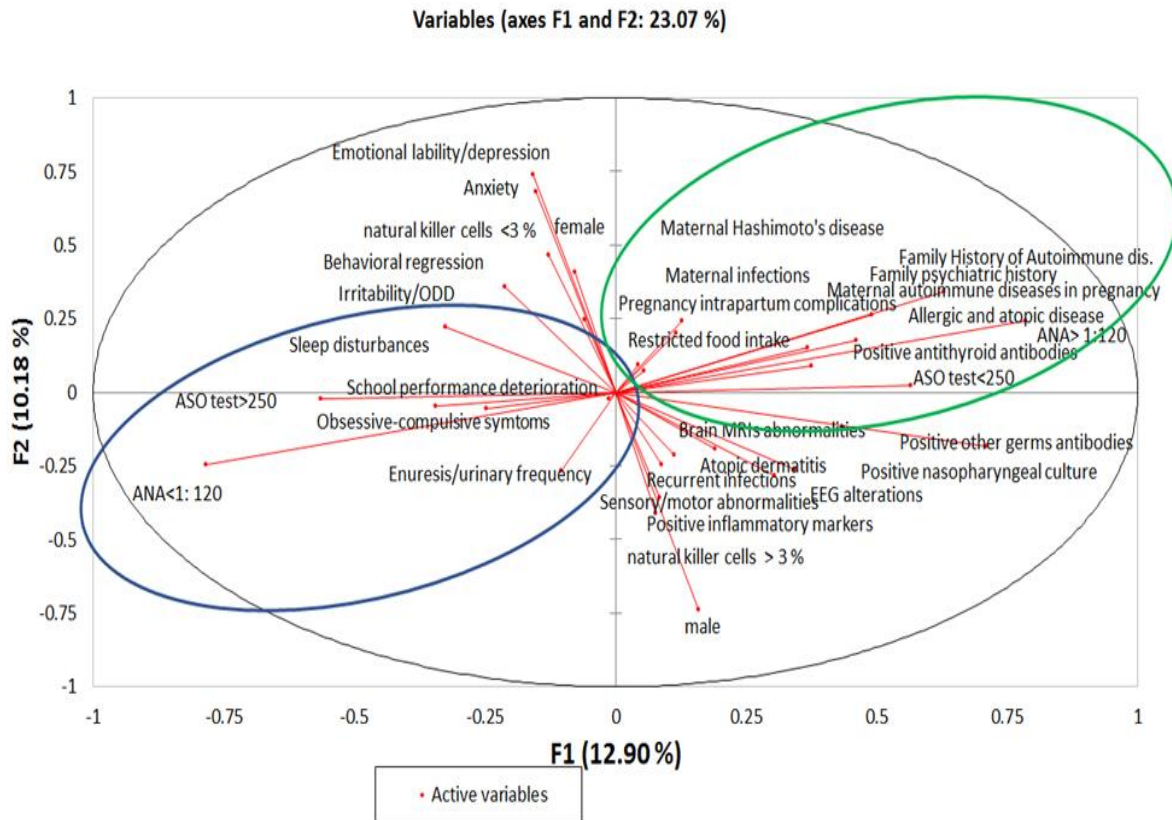
FIGURE 7. Principal Component Analysis (FIRST Two Components) regarding the constellation of clinical variables

Principal component analysis (PCA). The lines represent variable vectors. When two vectors are close, forming a small angle, the two variables they represent are positively correlated. Example: emotional lability/depression and anxiety. When they diverge and form a large angle (close to 180°), they are negatively correlated. Example: recurrent infection and natural killer < 3. There are two main clusters, the first circled in blue and the second circled in green. See text for detailed description.
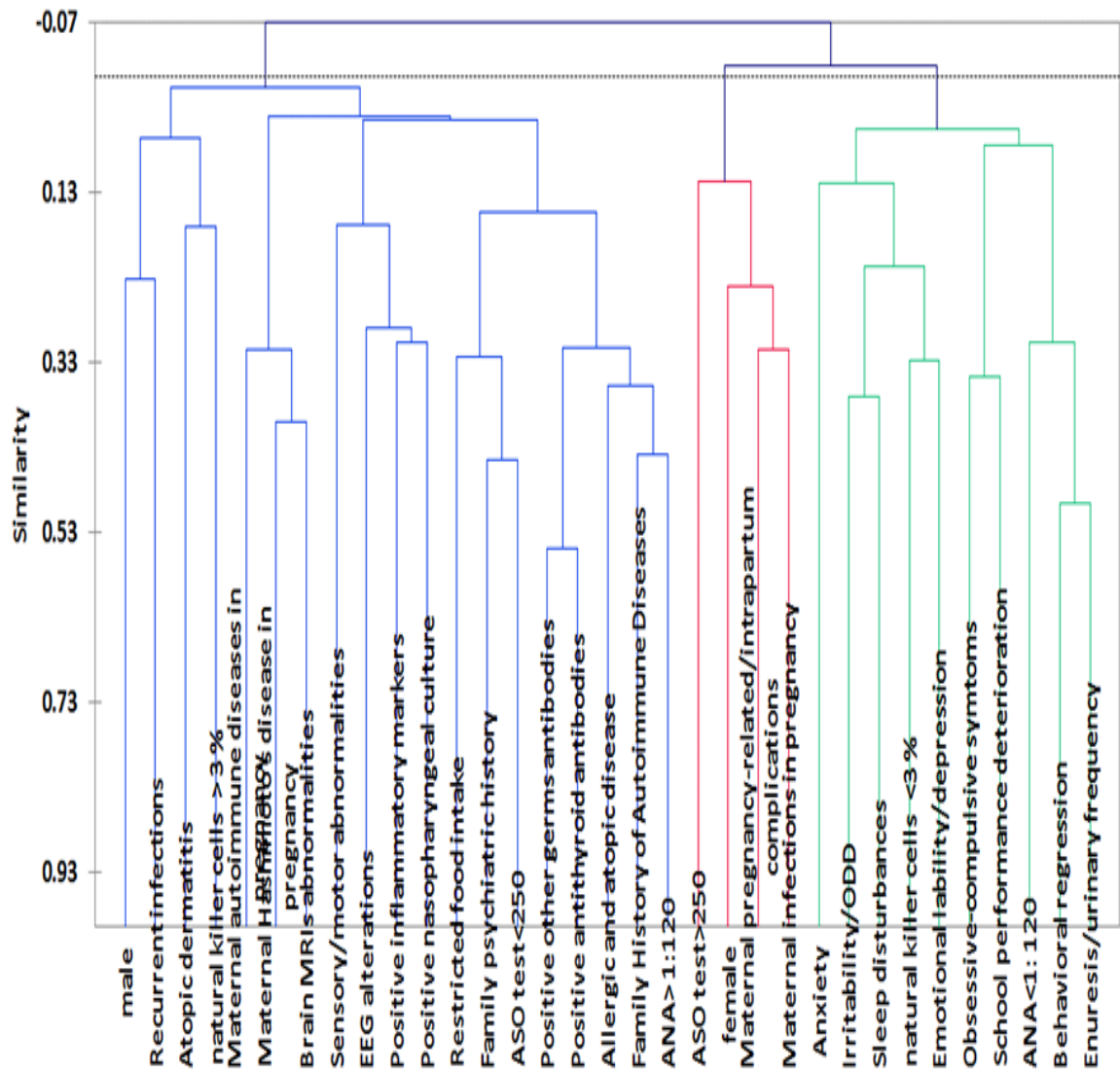
FIGURE 8. Agglomerative hierarchical clustering (AHC) regarding the constellation of clinical variables

Agglomerative hierarchical clustering (AHC). There are three clusters of variables marked in red, blue and green. See text for detailed description. In the ordinate axis the degree of similarity is gradually decreasing, going up.
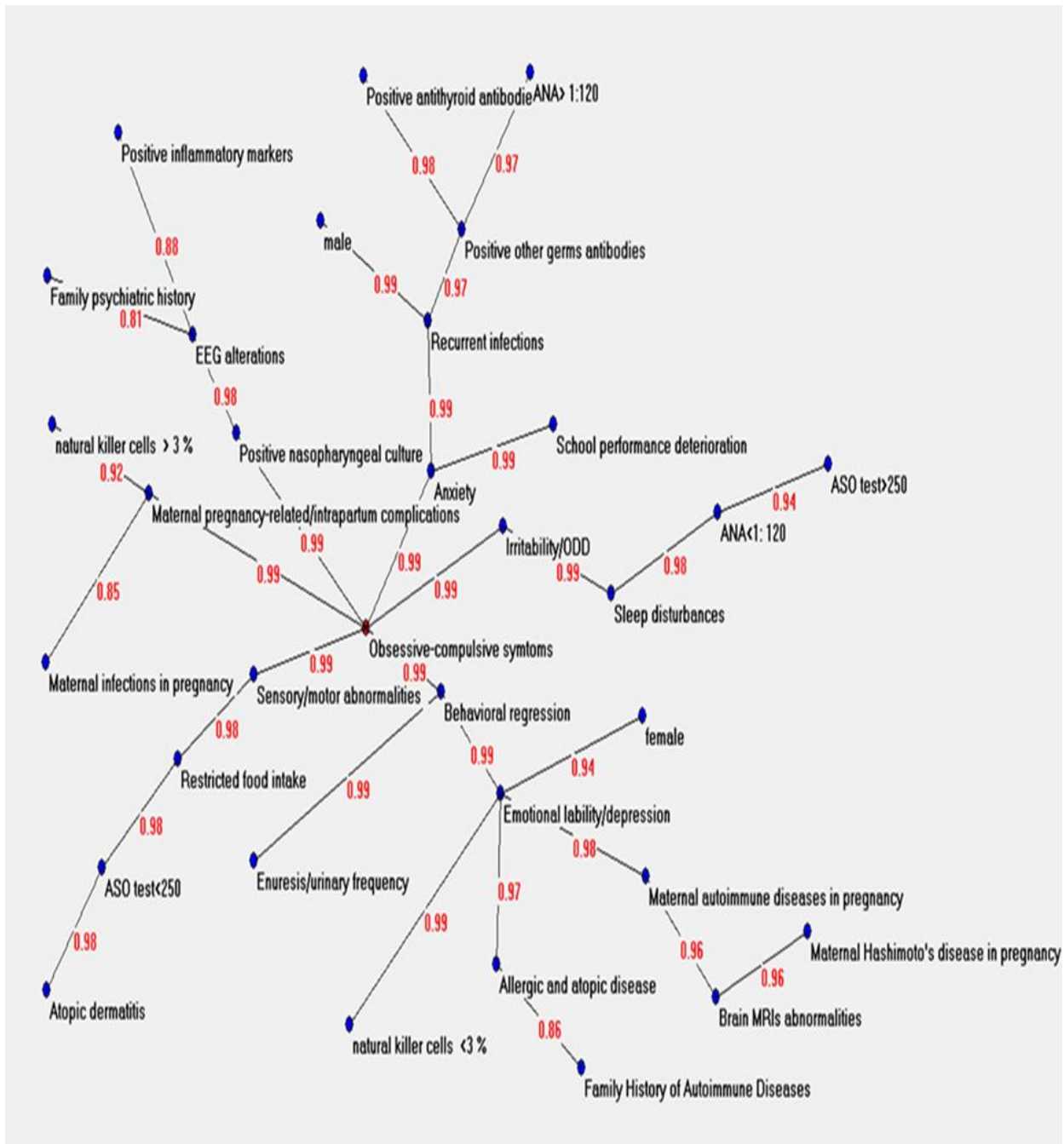
FIGURE 9. Sematic connectivity map (Auto-CM method), Minimum spanning tree whit links strength values regarding the constellation of clinical variables
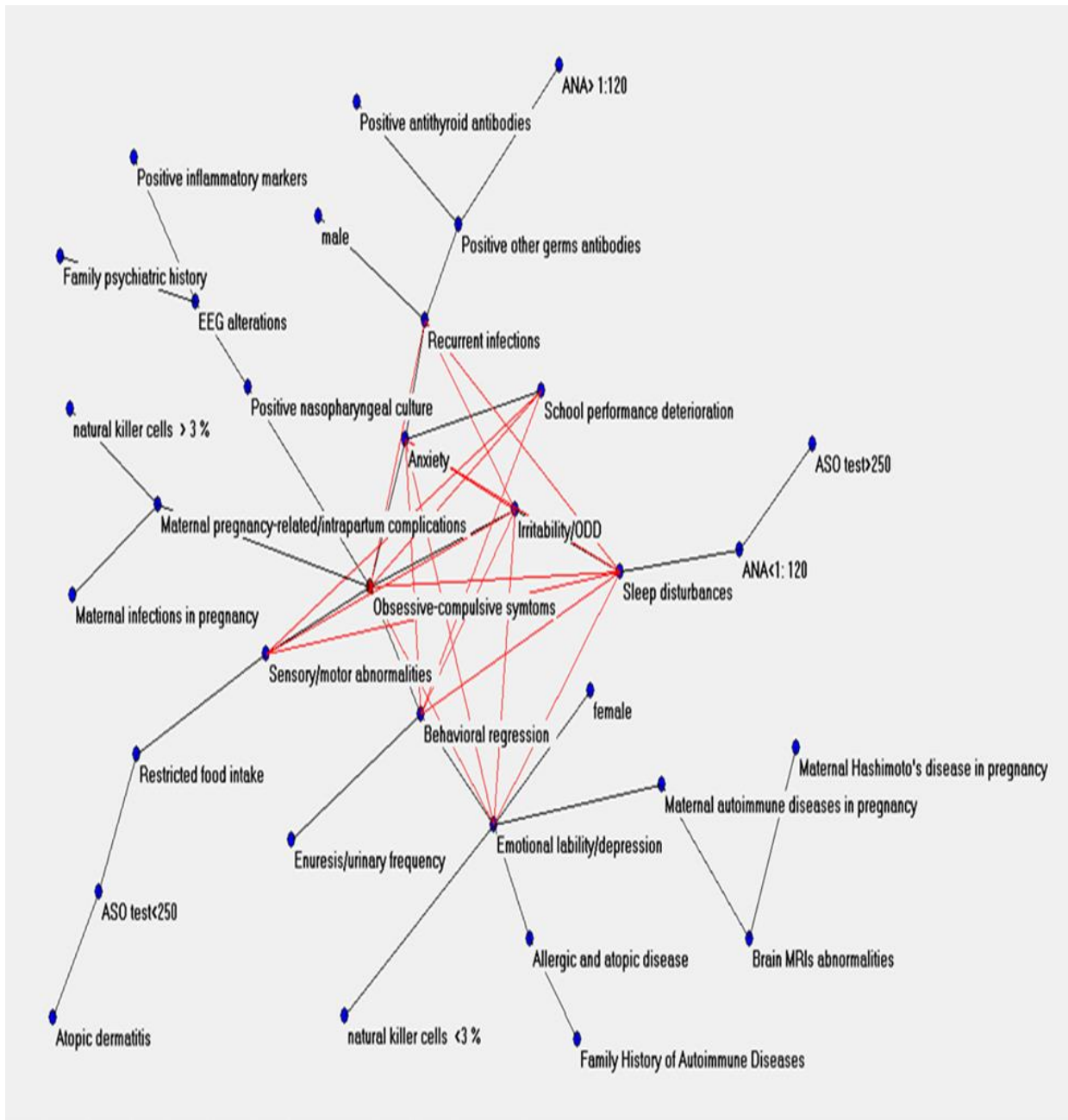
FIGURE 10. Semantic connectivity map (Auto-CM method), Minimum spanning tree and Maximal Regular Graph regarding the constellation of clinical variables
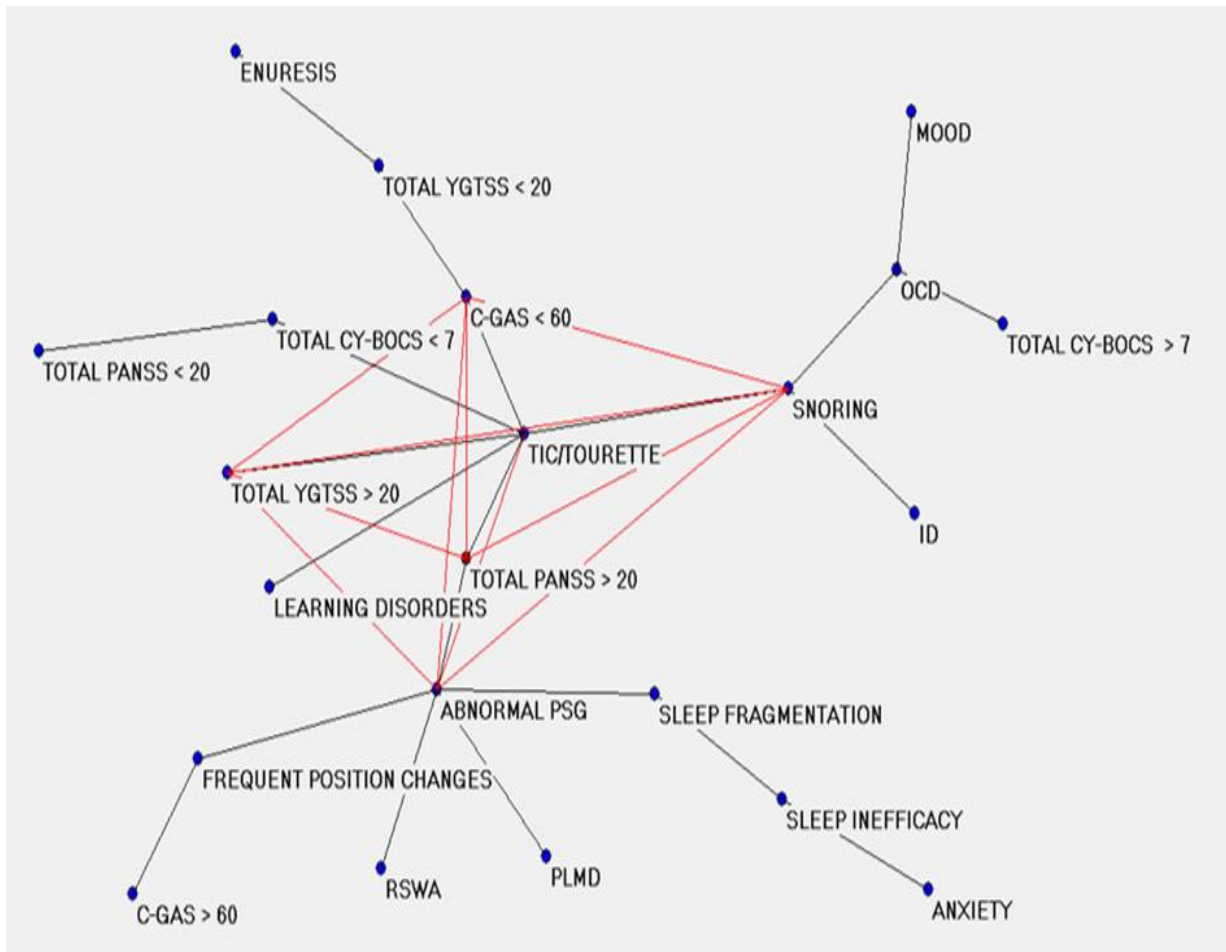
FIGURE 11 (Map). Connections between Sleep Parameters inferred by Polysomnographic Study, DSM-5 diagnoses, and all the behavioural parameters assessed by scales and checklists

Legend: PSG: polysomnography. OCD: obsessive-compulsive disorder. ID: intellectual disability. PLMD: periodic limb movement disorder. RSWA: REM sleep without atonia. Mood: mood disorders. Anxiety: anxiety disorders. Tic/Tourette: Tic/Tourette disorder. Total YGTSS: Total score in Yale Global Tic Severity Scale. Total CY-BOCS: Total score in Children's Yale-Brown Obsessive-Compulsive Scale. Total PANSS: Total score in Pediatric Acute-onset Neuropsychiatric Syndrome Scale. C-GAS: score in Children's Global Assessment Scale.
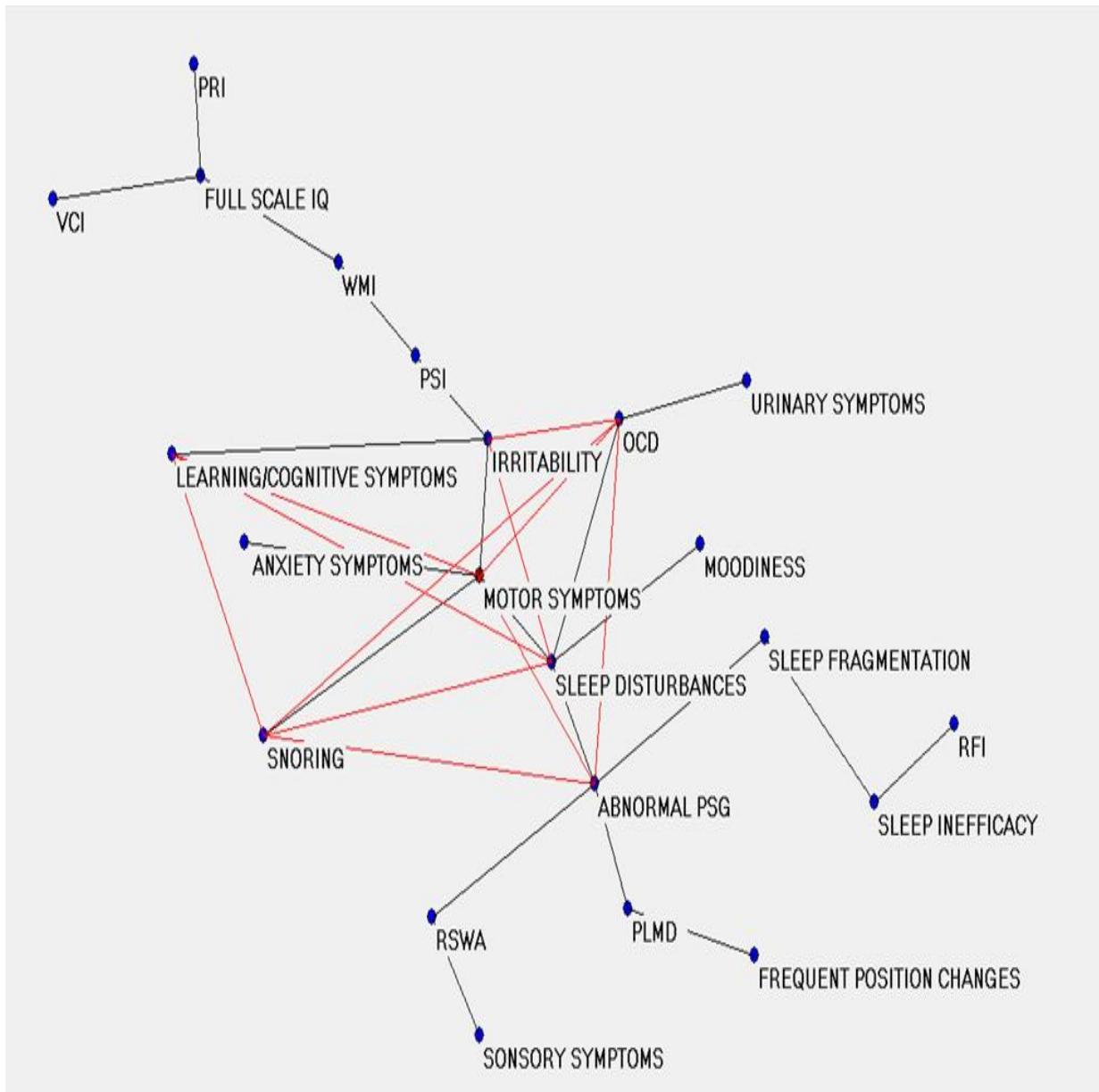
FIGURE 12 (Map). Connections between Sleep parameters inferred by Polysomnographic study, clinical dimensions assessed by PANSS and Neuropsychological parameters assessed by Wechsler Intelligence Scales

Legend: PSG: polysomnography. PLMD: periodic limb movement disorder. RSWA: REM sleep without atonia. PRI: Perceptive Reasoning Index. VCI: Verbal Comprehension Index. PSI: Processing Speed Index. WMI: working memory index. Full-Scale IQ: Full-Scale Intelligence Quotient.
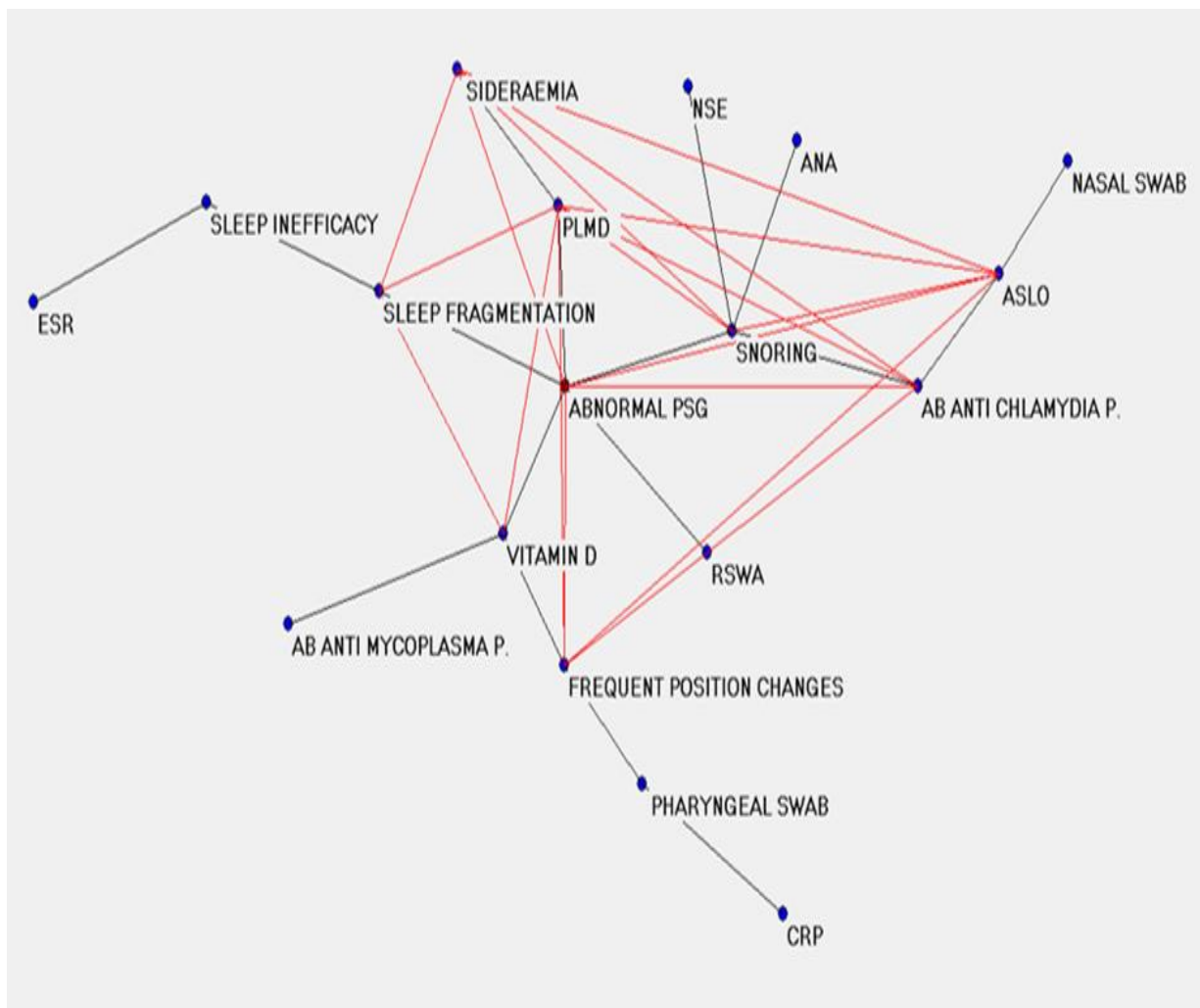
FIGURE 13 (Map). Connections between clinical sleep parameters inferred by polysomnographic study and biological parameters which exceeded the normal range

Legend: PSG: polysomnography. PLMD: periodic limb movement disorder. RSWA: REM sleep without atonia. ESR: Erythrocyte sedimentation rate. CRP: C-Reactive Protein. ANA: Anti-Nuclear Antibodies. NSE: Neuron-specific Enolase. ASLO: Anti Streptolysin O. Nasal and pharyngeal swab: culture positive for Group A beta-hemolytic streptococcus (GABHS) or Staphylococcus aureus (MRSA), Streptococcus pneumoniae (pneumococcus), Haemophilus influenzae or other upper respiratory tract pathogenic germs.
The antistreptolysin O (ASLO) patient's values in the blood plasma have been pooled in two groups (antistreptolysin titer > or < of 200 IU/ml), based on the upper reference limit. One or more IgG and IgM Antibodies against other germs (Anti Mycoplasma pneumoniae and Anti Chlamydia pneumoniae).
The Antinuclear Antibodies (ANA) patient's values in the blood plasma have been pooled in two groups (titers < or > of 1: 120), based on the upper reference limit. One or more inflammatory markers (erythrocyte sedimentation rate [ESR] and C-reactive protein [CRP]) above the upper reference limit.
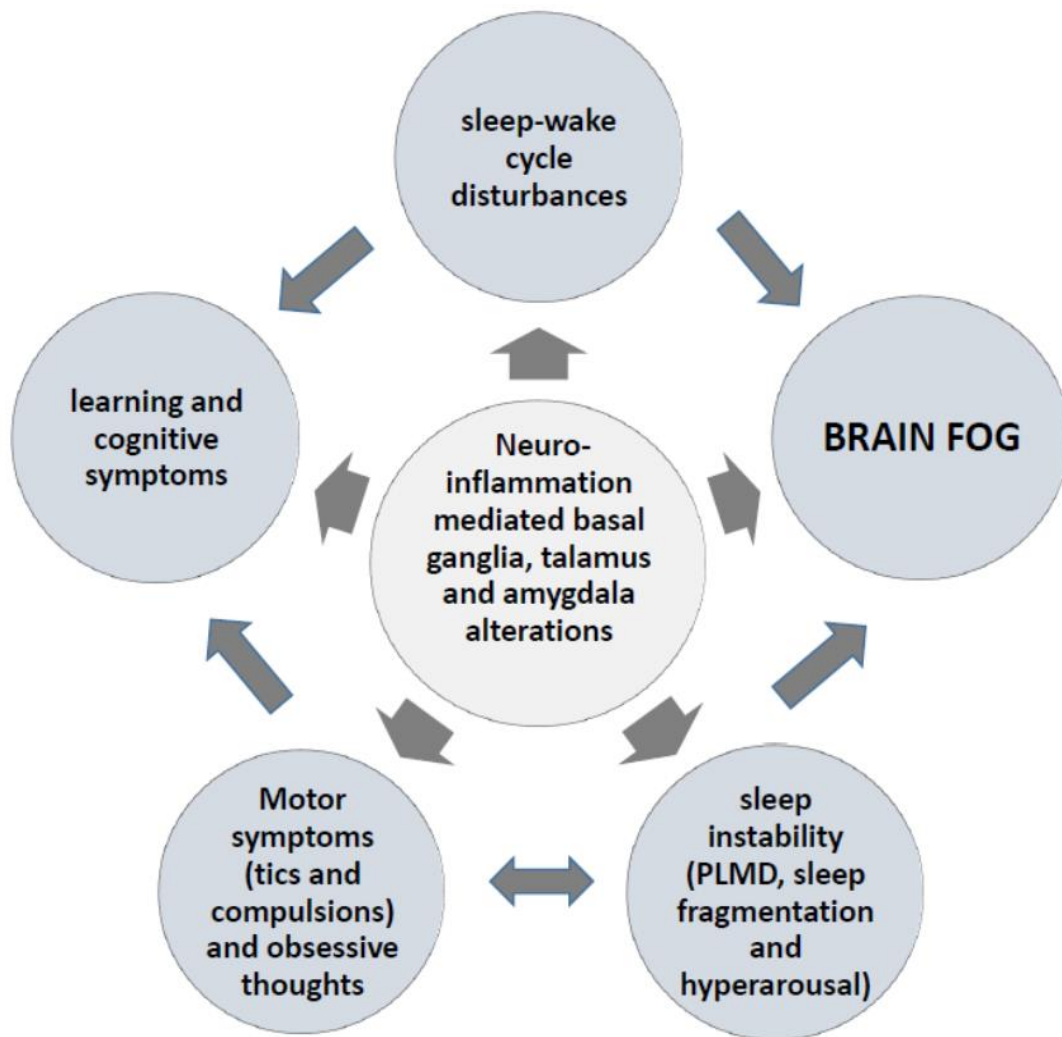
FIGURE 14. A putative dysfunctional model explaining the co-occurrence of sleep, motor and cognitive symptoms in PANS

PANS-related obsessive-compulsive and tic symptoms, putatively following insults to the basal ganglia and to the consequently functional alterations of CSTC motor circuit. Inflammatory-driven alteration of basal ganglia may explain the reduction of the mental capacity to concentrate or to think or reason clearly ("Brain fog") observed in PANS patients and, at the same time, may favour the sleep instability and sleep-wake cycle disturbances.
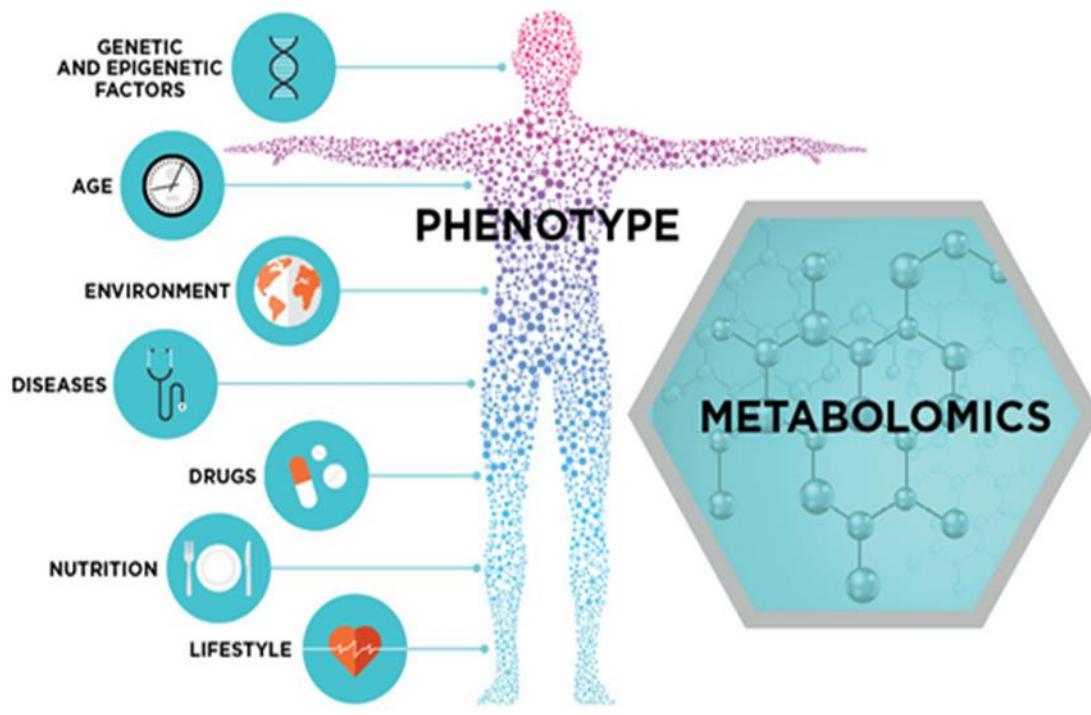
FIGURE 15. Metabolomics faithfully describes the phenotype being the result of the interaction between genetic and epigenetic factors, with stimuli from the external environment
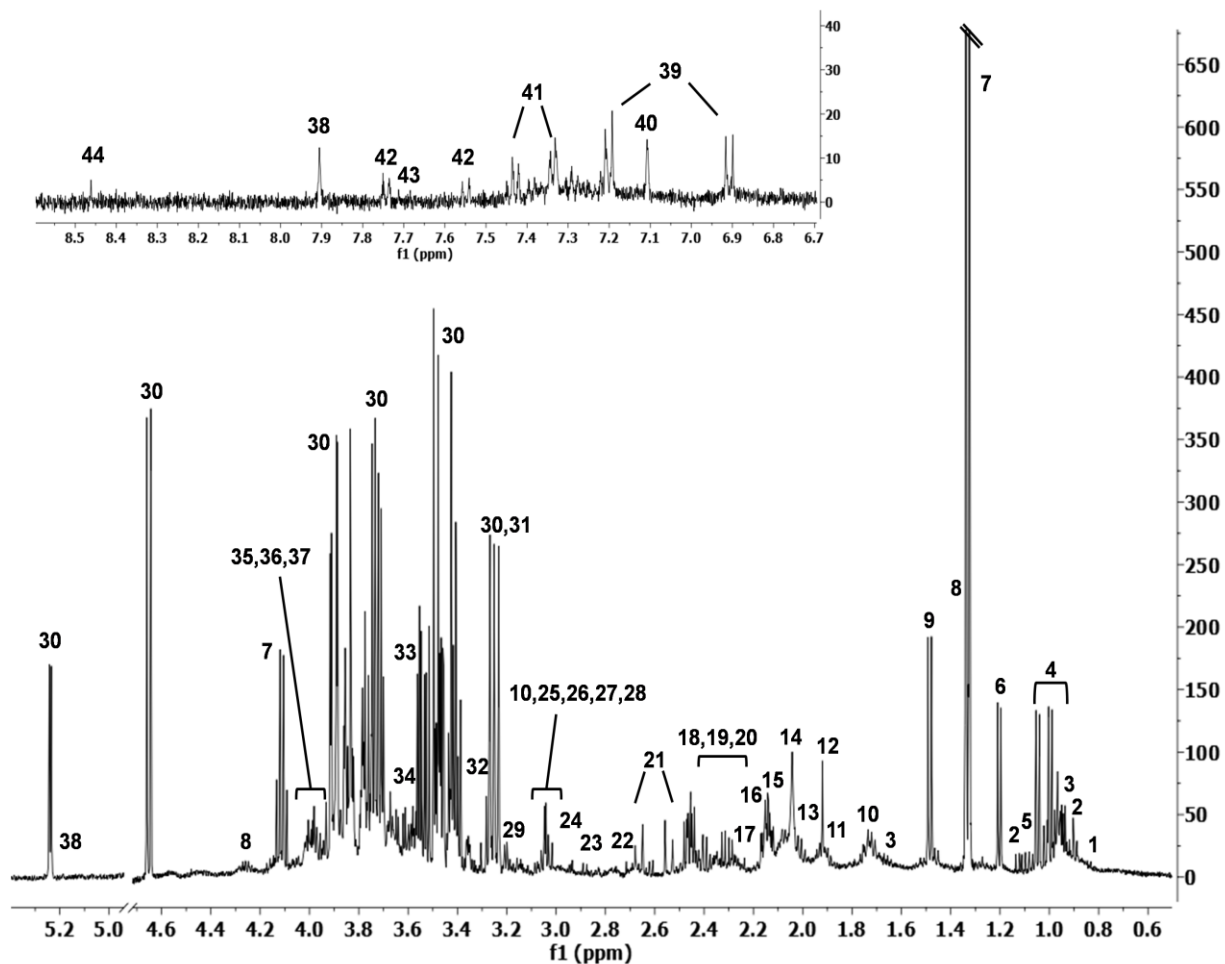
FIGURE 16. Signal's assignments of the serum metabolites in an ^{1}H-NMR spectrum (Metabolomics)

1. 2-Hydroxyisovalerate; 2. 3-methyl-2-Oxoglutarate; 3. 2-Hydroxybutirate; 4. Branched Aminoacids: Valina, Leucine, Isoleucine, 5. 2-Methylglutarate; 6. 3-Hydroxybutirate; 7. Lactate; 8.Threonine; 9. Alanine; 10. Lysine; 11. Arginine; 12. Acetate; 13. Proline; 14. N-acetyl-Groups; 15. Methionine; 16. Glutamine; 17. Acetone; 18. Glutamate; 19.Pyruvate; 20. Pyroglutamate; 21. Citrate; 22. Dimethylamine; 23. Aspartate; 24. Asparagine; 25. Creatine; 26. Creatine phosphate; 27. Creatinine; 28: Ornithine; 29. Choline; 30. Glucose; 31. Betaine; 32.TMAO; 33. Glycine; 34. Glycerol; 35. Serine; 36. Fructose; 37. Myo-Inositol; 38. Mannose; 39.Tyrosine: 40. Histidine; 41. Phenylalanine; 42. Tryptophan; 43. T methyl-Histidine; 44. Formate.
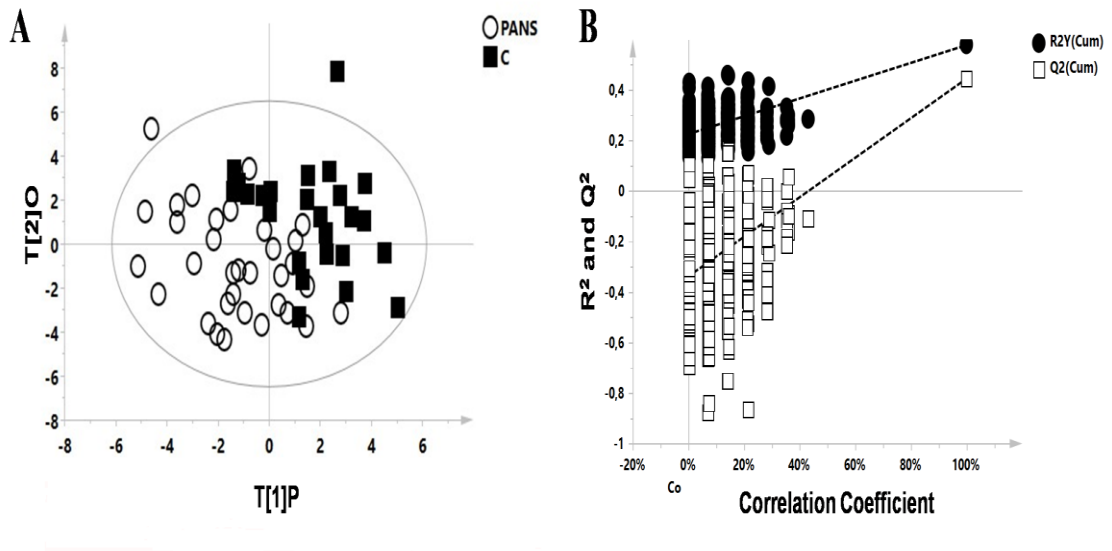
PANS vs Controls

FIGURE 17. OPLS-DA models of the analysed classes (Metabolomics)

PANS (white circles) vs Controls subjects (black boxes) with the respective permutation test
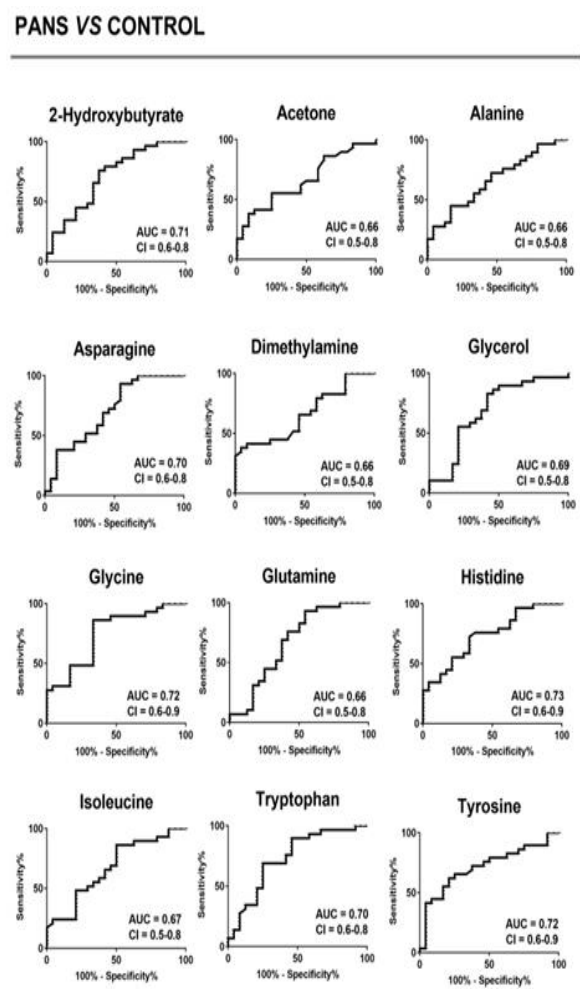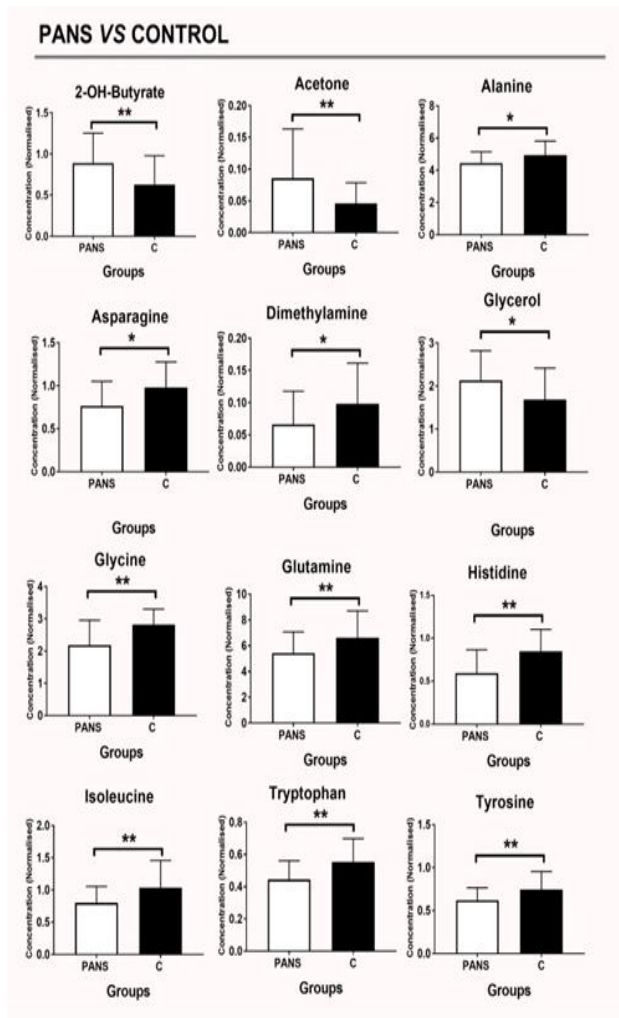
FIGURE 18. Comparison between PANS and Controls patients (Metabolomics)

Bar graphs and ROC curves of the metabolites exhibiting a p-value of < 0.05. U-Mann Whitney analysis was used. White bars represent the PANS class while black bars represent the control patients.
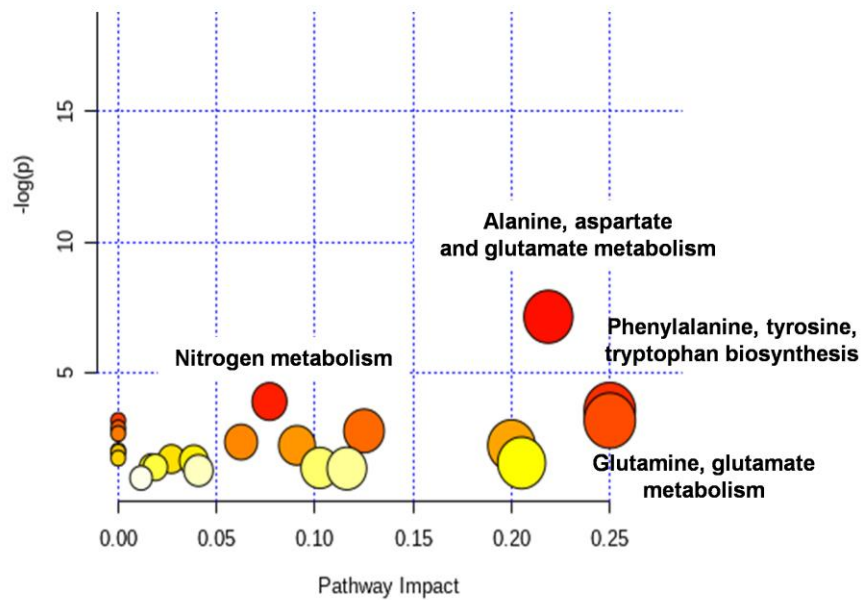
Figure 19. The metabolic pathways most altered in PANS patients (Metabolomics)

The metabolic pathways most altered in patients with PANS diagnosis were alanine, aspartate and glutamate metabolism, phenylalanine, tyrosine and tryptophan metabolism, nitrogen metabolism, glutamine and glutamate metabolism. Glycine, serine and threonine metabolism, arginine biosynthesis, nicotinate and nicotinamide metabolism were the most altered pathways in PANS patients compared to ASD class.