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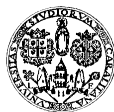

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A Flavia e Antonio,

Mamma, Babbo ed Elisa

This thesis is based on the following scientific papers:

1) Murgia, F.; Lorefice, L.; Poddighe, S.; Fenu, G.; Secci, M.A.; Marrosu, M.G. et al. Multi-Platform Characterization of Cerebrospinal Fluid and Serum Metabolome of Patients Affected by Relapsing-Remitting and Primary Progressive Multiple Sclerosis. J Clin Med. 2020,9,863.

2) Federica Murgia, Lorefice Lorefice, Antonio Noto, Martina Spada, Jessica Frau, Giuseppe Fenu, Giancarlo Coghe, Antonella Gagliano, Luigi Atzori, Eleonora Cocco. Metabolomic changes in patients affected by Multiple Sclerosis and treated with Fingolimod. Under review: European Journal of Neurology.

3) Federica Murgia, Florianna Giagnoni, Lorena Lorefice, Paola Caria, Tinuccia Dettori, Maurizio N. D'alterio, Stefano Angioni, Aran J Hendren, Pierluigi Caboni, Monica Pibiri, Giovanni Monni, Eleonora Cocco, Luigi Atzori. Sex hormones as key modulators of the immune response in Multiple Sclerosis: a review. Biomedicine.

Other publications of the Author related to MS and metabolomics

1) Cocco, E.; Murgia, F.; Lorefice, L.; Barberini, L.; Poddighe, S.; Frau, J.; Fenu, G.; Coghe, G.; Murru, M. R.; Murru, R.; Carratore, F. D.; Atzori, L.; Marrosu, M. G. ^{1}H-NMR Analysis Provides a Metabolomic Profile of Patients with Multiple Sclerosis. *Neurology - Neuroimmunology Neuroinflammation* 2016, *3* (1).

2) Lorefice, L.; Murgia, F.; Fenu, G.; Frau, J.; Coghe, G.; Murru, M. R.; Tranquilli, S.; Visconti, A.; Marrosu, M. G.; Atzori, L.; Cocco, E. Assessing the Metabolomic Profile of Multiple Sclerosis Patients Treated with Interferon Beta 1a by 1H-NMR Spectroscopy. *Neurotherapeutics* 2019, *16* (3), 797–807.

3) Poddighe, S.; Murgia, F.; Lorefice, L.; Liggi, S.; Cocco, E.; Marrosu, M.G.; Atzori, L. Metabolomic analysis identifies altered metabolic pathways in Multiple Sclerosis. Int. J. Biochem. Cell Biol. 2017, 93, 148–155.

Abstract

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system with a strong autoimmune component. MS is the most common cause of non-traumatic acquired neurological disability in young adults with a particularly high incidence in Sardinia. Several aspects still represent an active challenge in the clinical management of MS, such as the early diagnosis, the classification of the patients, the response to the therapy and the influence of the hormonal alteration in the progression of the disease. Finding potential biomarkers useful to address all these points, represents the purpose of this thesis, in which I used the metabolomics approach to investigate three main points: 1) Classification of patients affected by different forms of MS (relapsing remitting, RRMS, and primary progressive, PPMS); 2) Monitoring of the response to the Fingolimod therapy; 3) To study the protective role of pregnancy on MS progression.

Plasma, serum and cerebrospinal fluid (this only for the first study) were collected from selected MS patients and healthy subjects, and Nuclear Magnetic Resonance and Mass Spectrometry were used to measure the metabolic profile. Multivariate and univariate statistical tools were exploited to find specific patterns of metabolites based on the different MS aspect.

Aim 1: statistical models allowed to define different metabolic profiles in RRMS or PPMS patients. These differences could be extremely important to explain the specific pathophysiological mechanism behind the two different MS forms (e.g. neuroinflammation *vs* neurodegenerative).

Aim 2: treatment of MS patients with FINGO influences aminoacidic and energy metabolisms, and reduces oxidative stress and the activity of the immune system, both typical features of the disease.

Aim 3: the known pregnancy's protective role can also be evinced from the metabolic point of view. Most of the metabolites changed their concentration with the same trend in healthy and MS women during pregnancy, indicating that the presence of the pregnancy is predominant in the MS.

To date, metabolomics represents an innovative approach to discover putative biomolecules that, in a specific pathological contest, can open new avenues for the deep investigation of target mechanisms.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS) with a strong autoimmune component, characterized by demyelination and variable degrees of axonal loss (Ruiz et al. 2019). The clinical correlate of the irreversible neuronal damage is represented by accumulation of irreversible disability.

From an anatomopathological and radiological point of view, MS is characterized by the presence of focal demyelinated plaques disseminated in the white matter of the brain and spinal cord (Frischer at al. 2015). The mechanisms responsible for the formation of this type of lesions in different patients and different stages of the disease as well as those involved in the induction of diffuse brain damage are multifaceted and heterogeneous (Lassmann et al. 2007). The disease shows a variable constellation of symptoms depending on the localization of the plaques (Lemus at al. 2018, Thompson et al. 2018).

MS epidemiology

MS is the most common cause of non-traumatic acquired neurological disability in young adults with an average age at onset ranging between 29 and 40 years, affecting approximately 2.5 million people worldwide (Oh at al. 2018). Considering difficulties in surveillance, MS seems to be inexistent in black Africans as well as native populations of the Oceania and Americas while, with some exceptions, MS is prevalent in geographic areas farther away from the equator, in high latitude regions, and northern European populations (Simpson et al. 2011). In Asia the prevalence of MS is around 2 patients per 100.000 inhabitants against 1 per 1000 inhabitants of Western countries where it can reach values of 1 per 400 (Koch-Henriksen et al. 2010, Walton et al. 2020) (Figure 1).

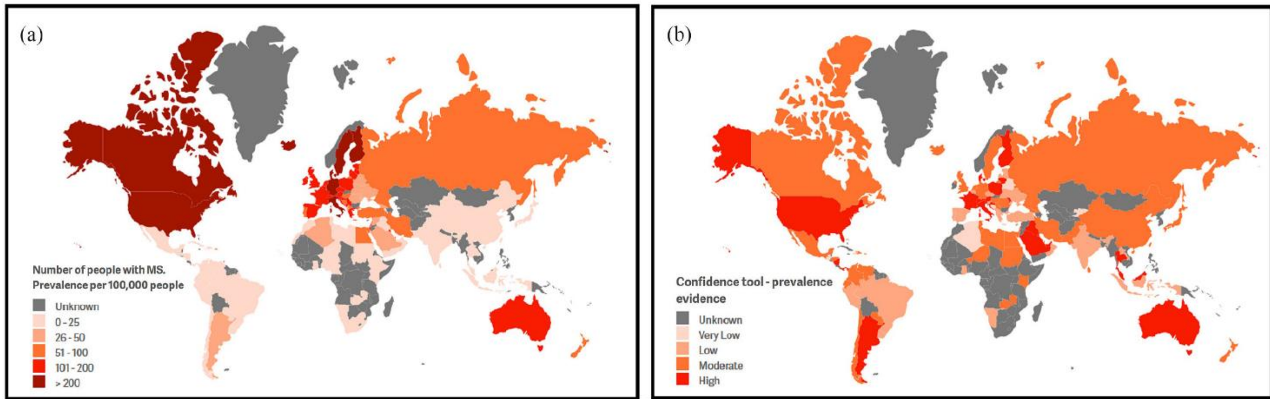

Figure 1. Map showing geographic variation in MS prevalence and in data confidence scores (this parameter considers four variables: 1) size of the population covered by data source by country; 2) year of data collection, from 0, before 2009, to 5, in 2017–2019; 3) type of data source, from 0 if unknown to 5 if peer-reviewed journal article; 4) additional points were assigned for using certain methodological criteria: for example using the 2017 McDonald Criteria, using multiple consistent data sources for the estimate. Confidence ratings were given based on the total scores using the following thresholds: $\leqslant 5$ = very low, 6–10 = low, 11–15 = moderate, $\geqslant 16$ = high). A) MS prevalence per 100,000 population by country. B) The confidence score is assigned to each country based on the prevalence data sources provided (Walton et al. 2020)

In these regions, the incidence of MS appears to have increased noticeably over the last century, primarily in women. This type of variability suggests that environmental factors may play a crucial role in the pathogenesis of the disease. As proof of this, people who migrate from high to low-incidence areas do not maintain the same risk but acquire the risk of the country to which they moved (Thompson et al. 2018). Considering the European continent, the highest frequency is present in Sweden, and moving to the Mediterranean areas, the frequency decreases, albeit with some exceptions: In Italy, the prevalence of MS is estimated at 100 cases per 100.000 inhabitants, while the incidence stands at 3.9 cases per 100.000. In Italy, there are about 70,000 people affected by this pathology (Howard et al. 2016).

Despite the geographical position placed in a lower-risk latitude, Sardinia is notoriously considered a high-risk area, ranking in the first places in terms of incidence and prevalence, both nationally and globally (Pugliatti et al. 2002).

In Sardinia the prevalence is about 200 people out of 100.000; furthermore, the incidence seems to be higher in the island's inland areas. The prevalence of the disease on the island has been the subject of several studies that have focused on different areas: a prevalence of 157/100.000 inhabitants in the central area, 102/100.000 inhabitants in the north-west and 210/100.000 inhabitants has been identified in the southwest area confirming the island as one of the highest risk areas in the world (Montomoli et al. 2002. Pugliatti et al. 2002, Cocco et al. 2011, Pugliatti et al. 2006).

As indicated, MS is characteristically a young adult disease, but up to 10% of patients show their first demyelinating event in childhood or adolescence. Most patients with onset in advanced adulthood (after 40 years) tend to present from the onset the progressive form.

Aetiology and Immunopathogenesis

The exact cause of MS remains still elusive, but its aetiology is certainly multifactorial (Dendrou et al. 2015): among the environmental factors, Epstein bar virus infection, low vitamin D status, and cigarette smoking, contribute to MS development. Moreover, despite MS is not classified as an inherited disease, is observed a strong genetic component in to its aetiology: the risk of MS in first-degree relatives of MS patients is 10–50 times higher compared to the general population (absolute risk 2–5%) (Garg et al. 2015). Genetic studies indicated more than 50 gene loci associated with the major histocompatibility complex (MHC) HLA DR15/DQ6 (MHC is a gene-dense region including the HLA genes, with numerous immune response loci) as the most vulnerable to variants and a total of 110 polymorphisms in 103 distinct loci outside the MHC have been also associated with susceptibility (Sawcer et al. 2014, Hollenbach et al. 2016). A summary of the risk factors for MS is reported in Table 1.

Table 1. Summary of the risk factors for Multiple Sclerosis.

Probable risk factors for Multiple Sclerosis
Demographic factor
Caucasian ethnicity
Female gender
Genetic
HLA DR15/DQ6, IL2RA and IL7RA alleles
Environmental/lifestyle
Low vitamin D/Lack of sunlight exposure
Cigarette smoking
Temperate climate
Infections
Epstein–Barr virus (EBV) infection

Although the pathogenesis has been the focus of MS research for several decades, it is not fully understood yet and only partially unravelled. Several pieces of evidence point to autoimmune pathogenesis with the persistent peripheral activation of autoreactive T-cell-mediated directed against CNS antigens such as myelin and other proteins with a possible role for B cells (Gonsette at al. 2012). MS occurs on a background of inflammatory reaction, composed of lymphocytes, and activated macrophages or microglia and it is widely thought that an inflammatory process of autoimmune nature is the driving force of tissue injury (Hohlfeld et al. 2004). In this immune attack, the main involved actors are the auto-reactive CD4+ T cells that are activated in the periphery and cross the blood-brain barrier to reach the CNS (Trapp et al. 2008). The transmigration process is mediated by the interaction of different adhesion molecules, chemokines, and matrix

metalloproteinases (Gold et al. 2011, Garg et al. 2015). After entering the CNS, the autoreactive peripherally activated T cells can be reactivated and come into contact with the autoantigenic peptides in the brain parenchyma in the context of MHC class II molecules which are expressed by local antigen-presenting cells (APCs, dendritic cells, macrophages, and B cells) prompting an inflammatory cascade leading to release of cytokines and chemokines, recruitment of other inflammatory cells including T-cells, monocytes, and B cells and persistent activation of microglia and macrophages resulting in myelin damage (Selter et al. 2013, Goverman et al. 2009). The activation of the glial cells of the CNS leads to persistent inflammation even in the absence of further infiltration of exogenous inflammatory cells. Moreover, the local inflammatory environment and demyelination result in the exposure of sequestered myelin autoantigens which are an additional target for self-reactive T cells (“epitope spreading”) (Lubetzki et al. 2014).

In this context, several animal studies evidenced the central role of the CD4+ T-helper 1 (Th1) cells

(which releases pro-inflammatory cytokines such as interferon-gamma IFN-γ, interleukin-2 IL-2, tumour necrosis factor-α TNF-alfa), in mediating inflammation in MS. Also, the class of the CD4+ T-helper-17 (Th17) which secrete IL-17 seems to play a pivotal pro-inflammatory role. On the other hand, lymphocytes CD4+ T-helper 2 (Th2) secrete interleukin-4,5, and 10 showing a counter-regulatory role in inhibiting the inflammatory process and limiting damages TH1-mediated (Yadav et al. 2015).

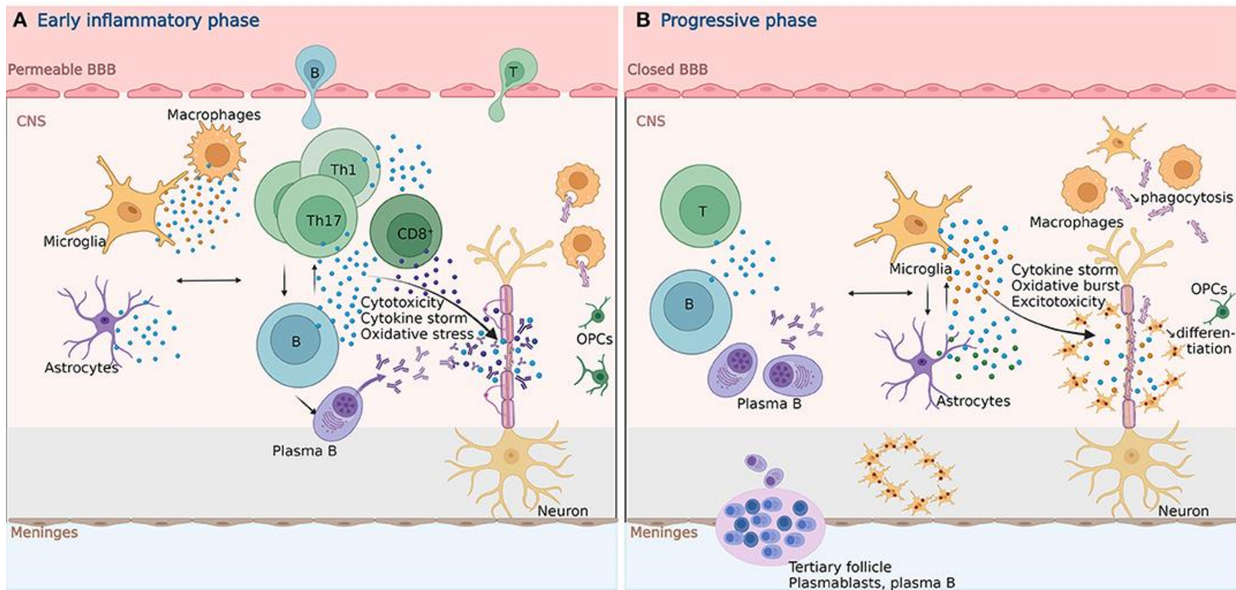

Figure 2. Pathophysiology of MS. Peripheral adaptive immune cells penetrate the CNS through a damaged BBB. In the brain parenchyma these cells, once re-activate, secrete cytokines and cytotoxic molecules, and activate macrophages and microglia. This cytotoxic pro-inflammatory environment leads to the damage of the myelin sheaths around axons and induces energy failure in the axon (Perdaens et al. 2022).

Neuropathology

The characteristic pathological sign of MS are focal plaques, or areas of demyelination where it is possible to observe inflammatory infiltrates associated with activated T-lymphocytes CD4+ e CD8+, activated macrophages, and microglia, plasma cells, and B-lymphocytes (Induruwa et al. 2012). The plaques can affect the entire central nervous system but more frequently are located in the optic nerves, the spinal cord, the brainstem, the cerebellum, and regions of white matter close to the cerebral ventricles or near the basal ganglia (Popescu et al. 2013).

MS plaques can be classified from the histological point of view as active, chronic, and remyelinated. Active lesions are common in RRMS form, MS and are characterized by myelin degradation and inflammatory environment with macrophage infiltration, reactive astrocytes, etc. While in the initial phases of the lesion formation, axons and neurons are partly preserved, a substantial axonal loss became evident during the chronic phase of the disease, which represents an

important pathological substrate for an irreversible neurological deficit. Chronic or inactive plaques are frequent in patients affected by a progressive MS form and are characterized by more extensive demyelination, often with important axonal and oligodendrocytes loss, and absence of active inflammation (Garg et al. 2015). Based on these considerations, the possibility to find active plaques decreased significantly after about 15 years of disease, while, conversely increasing the probability to observe inactive or chronic plaques (Lassmann et al. 2013).

Looking at close to the active plaques could be possible to see remyelinated plaques, which contain finely myelinated axons with an increased number of oligodendrocyte precursors (Frischer et al. 2015). During the past decades, particular attention has been paid to the spontaneous phenomenon of remyelination, which ensures axonal survival rather than restores neuronal fibres conduction (Thompson et al. 2018).

These plaques also called "shadow plaques", have been found both in RR and progressive forms of MS and demonstrate that MS plaques can be partially repaired by remyelination. They appear as areas of reduced myelin density compared to the normal appearance of white matter; the entity of remyelination is variable among patients and partly depends on the location of the lesion (Lassmann et al. 2013).

The localization of the plaques is extremely important because it strongly influences symptomatology. In general, considering the inflammatory nature of MS, the onset of symptoms of an attack in the RRMS form is typically gradual and can evolve over days. A clinical attack lasts a minimum of 24 hours in absence of fever or infections. In PPMS, symptoms have a gradual and insidious onset over at least 12 months by the moment of diagnosis (Dobson et al.2018, Ford et al. 2020).

There is no single clinical sign of MS (Figure 3), but it can lead to sensory disturbances (hemianesthesia, paraesthesia), motor disturbances (decreased strength, contractility disturbances, increased tone or spasticity) often link with partial myelitis due to inflammatory lesions in the

spinal cord, cerebellar disturbances (walking disturbances, dizziness, balance problems), pain, vegetative disorders (especially urinary disorders, such as urinary incontinence), retrobulbar optic neuritis (with gradual monocular visual loss, pain on moving the eye and altered colour vision), depression and fatigue.

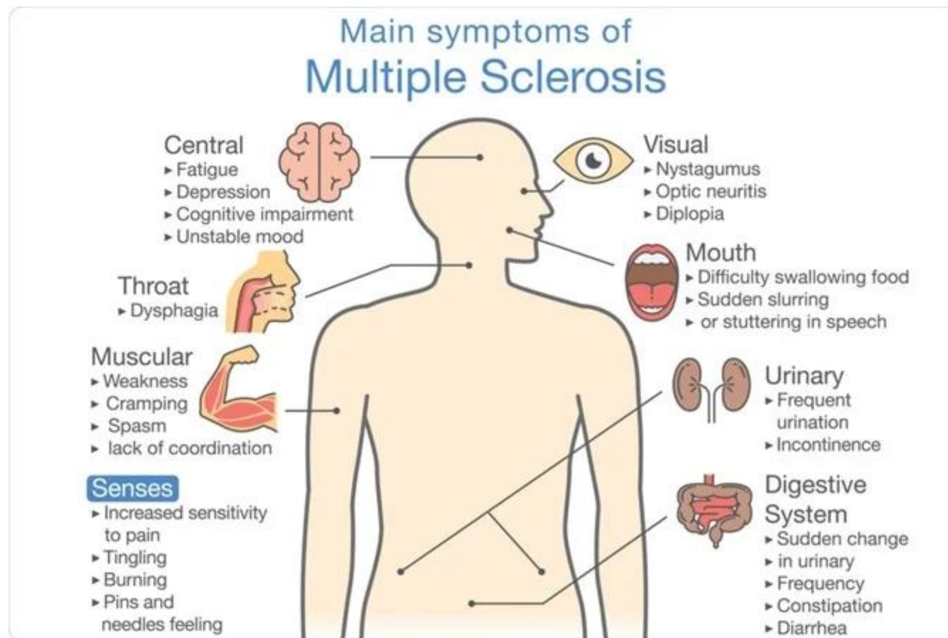

Figure 3. Summary of the symptoms of Multiple Sclerosis (https://www.news-medical.net/health/Early-signs-of-Multiple-Sclerosis.aspx)

Regards the cognitive sphere, impairment occurs in all multiple sclerosis phenotypes and regards mainly cognitive processing speed, memory and learning as well as deficits in executive function and visuospatial processing while basic language, semantic memory, and attention span are rarely compromised (Benedict et al. 2020).

Multiple Sclerosis subtypes

There are basically four clinical forms of MS:

- Relapsing-remitting (RRMS) (60-70%): the course of this form is characterized by outbreaks of the disease with neurological dysfunction, defined as the appearance of new

neurological symptomatology or the worsening of already present symptomatology (relapses), lasting some days or sometimes few weeks and followed by a more or less complete recovery with relative clinical stability free of new neurological symptoms (remission).

- Secondarily Progressive (SPMS) (20-30%): represents a late phase of the RR form, after an extremely variable disease period of more or less 10-15 years. Initially, the attacks are followed by a restoration of the symptoms, but over time the new ones tend to get closer, and the symptomatology does not regress. Phenotypically, the course of SPMS is not uniform, with periods of progression plus possible superimposed relapses as well as periods of relatively stable disability.
- Primarily Progressive (PPMS) (10%): in this form, the clinical symptomatology begins and never regresses. The disease from its onset shows a progressively worsening course without acute attacks. This form of MS typically affects people of advanced age and is the most resistant to the drugs typically used in the treatment of the disease.
- Relapsing-progressive (RPMS): the course of this form is characterized by a progressive *ab initio* decline and periodic acute attacks of the disease. Clinical symptoms start without regressing totally, on the contrary, the level of disability tends to grow more and more. On this level of basal disability that there are phases of exacerbation (Klineova et al. 2018) (figure 4).

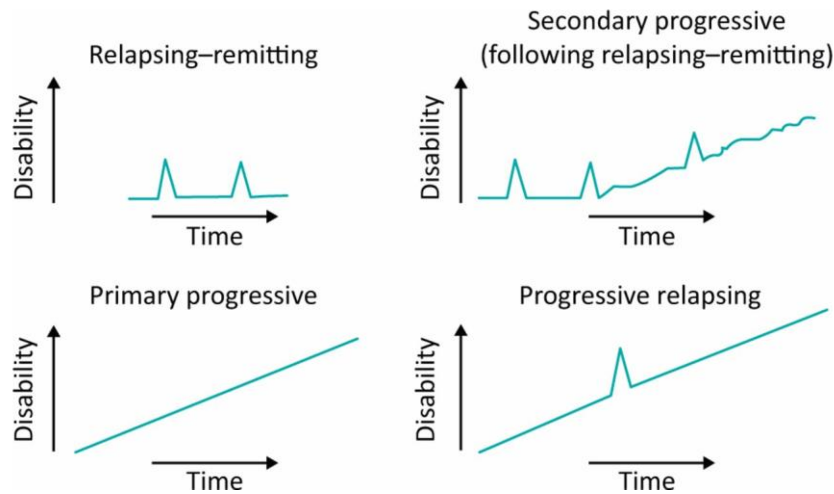

Figure 4. Disease courses of Multiple Sclerosis (Ford et al. 2020).

Clinical and MRI data suggest that in the RRMS form, the characterizing features are inflammation and the formation of new white matter lesions, while in the progressive forms new inflammatory demyelinating lesions seem to be rare but appear to be more prominent the neurodegenerative effect with diffuse atrophy of the grey and white matter (Dobson et al. 2018, Ford et al. 2020). This fact can be deduced considering also that anti-inflammatory therapies have limited efficacy in the progressive phase of the disease. The link between the RRMS and the progressive forms is complicated: the number and the severity of new relapses during the early stage of MS can in part define the time at which the progressive stage is reached, but there isn't clear evidence about its influence on the rate of progression once the progressive phase is reached (Ransohoff et al. 2015). Based on this consideration, relapse can be a surrogate marker to predict the time of onset and degree of future progression, but the research in this field showed conflicting results, and this point represent a fundamental challenge in the MS scenario. To date, there are no available imaging or immunological markers of progression, which is estimated based on clinical evidence over a period of at least 6-12 months. Moreover, the pathology involved in SPMS and PPMS is poorly understood and complex, involving some degree of persistent inflammation (less than in RRMS) combined with neurodegeneration with resultant axonal damage.

Therapies

The treatment of MS includes three categories: 1) acute relapse management; 2) disease-modifying treatments (DMTs); 3) symptomatic treatments. The first category aims to treat an acute episode, a true relapse with the priority to exclude and treat any concomitant infection (e.g. urinary tract infections), which can cause or influence such perturbations. If the relapse is of moderate functional severity or worse, then high-dose methylprednisolone therapy should be considered as well as the adrenocorticotropic hormone (ACTH) (Berkovich et al. 2013), as corticosteroids tend to shorten the duration of the relapse, with the addition of plasma exchange if the relapse is rapidly progressive or severe (Doshi et al. 2016).

The most significant advance in MS treatment over the past two decades has been the development of immunomodulatory therapy. Since the introduction of the first immunomodulating drug, namely Interferon beta-1b in 1993, several other drugs have become available with different mechanisms of action, methods and frequency of intake (Jakimovski et al. 2018).

The mechanism of action of the immunomodulatory therapy in the treatment of MS is linked directly to its pathophysiology: DMTs show an extensive suppression of the immune response mediated by autoreactive lymphocytes. Indeed, most of these drugs are effective in relapsing-remitting MS where inflammatory demyelination is the main process, while their benefit has been questioned in patients with the progressive form (Damal et al. 2013). DMTs are a fundamental component of the “long-term management” of patients with MS. This class of drugs aim to reduce the early clinical and subclinical disease activity in terms of frequency of relapses and number of lesions that are thought to contribute to long-term disability (Dobson et al 2018). Treatment is highly variable and differs based on disease severity, cost, adverse effect profiles, and patient and prescriber preference. To date, we have numerous first and second-line DMTs available, based on their effectiveness and their safety profile (Dobson et al. 2018, Hart et al. 2016).

Finally, the treatment of MS includes a series of symptomatic drugs, aimed at controlling and reducing neurological symptoms or otherwise at improving and enhancing certain functions. The use of these treatments is to be tailored to the patient, depending on the symptoms presented, tolerability, and general health conditions. The management of the so-called invisible symptoms is also of central importance, such as chronic fatigue, mood problems, and the control of painful symptoms, which lead to a negative impact on the quality of life of patients (Sailer et al. 2019) .

Despite the great development in the field of MS treatment, this aspect remains still a strong challenge. MS shows particularly complex pathophysiology that hinders the identification of effective therapy, which may have several significant side effects (Loma et al.2011). Moreover, the response to treatment is quite heterogeneous because many patients continue to experience MS disease activity despite the treatment (Tedeschi et al. 2005). It is very important in clinical practice to identify the best patient candidate for each DMT, as well as predictors of a better response to the drug.

These aspects represent a crucial challenge in managing MS and highlight the need to identify biomarkers that allow the classification of patients based on their potential responsiveness to different drugs and risk of severe adverse events (Ziemssen at al. 2019, Harris et al. 2014).

Multiple sclerosis and gender

MS affects more frequently women than men, with a ratio going from 2:1 to 3:1, suggesting that sex-related factors probably have an impact on MS susceptibility (Ysrraelit et al. 2019).

Also in physiological conditions, there are great differences between male and female immune responses, and these differences are emphasized in pathological conditions, especially in autoimmune diseases, such as multiple sclerosis (Klein et al. 2016). In a heterogeneous disease such as MS, it would be extremely important to understand the existing differences between male and female immune responses.

Differences between male and female immune response

Sex is a biological factor that influences the immune response both toward self-antigens and non-self-antigens (Klein et al. 2016).

Male immune system and female immune system show differences in anatomy, cytology, immunoglobulins levels, and responsivity. Compared to men, women develop stronger innate and adaptive immune responses (Fish et al. 2008): they develop stronger responses to different kinds of vaccines and are less susceptible to infections caused by bacteria, viruses, parasites, and fungus (vom Steeg et al. 2016), but at the same time they are more prone to autoimmune diseases (Jaillon et al. 2019) as more than 80% of autoimmune diseases predominantly affects women (Klein et al. 2016) (Figure 5).

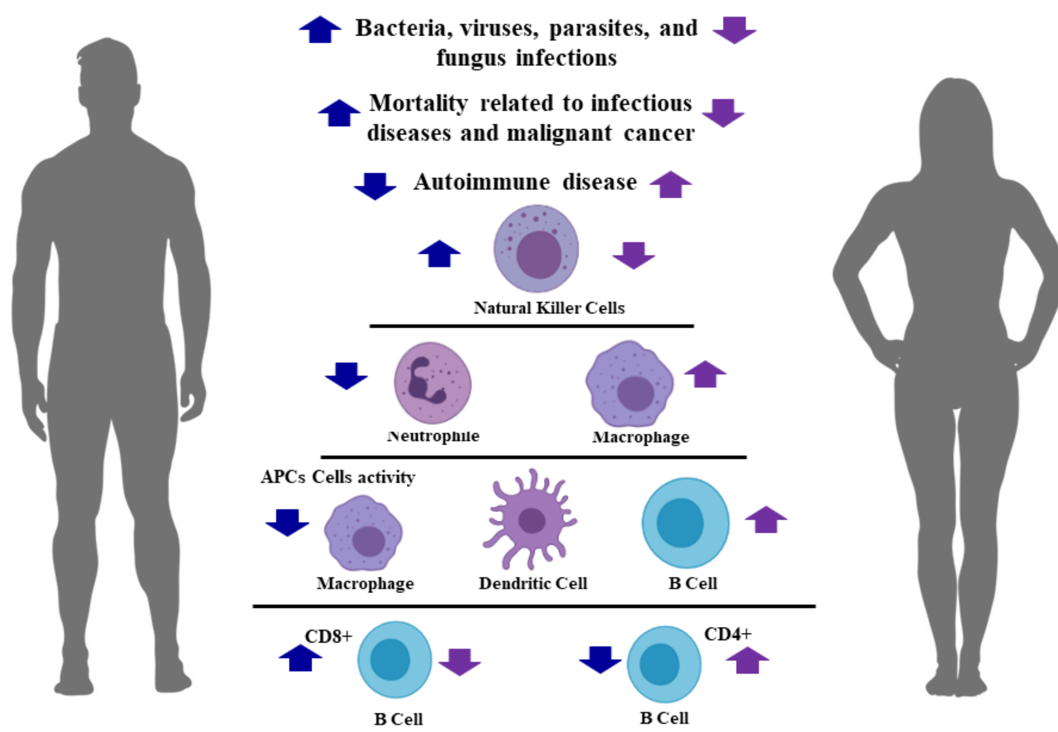

Figure 5. Main differences in the immune response between men and women. Blue arrows represent the trend in men while violet arrows represent the trend in the n. Innate immunity cell number and activity differ between males and females. Males present a higher number of natural killer cells compared to females, while the phagocytic activity of

neutrophils and macrophages is higher in females. Also, antigen-presenting cells (APCs) are more efficient in females than males. Sex hormones and an XY karyotype also influence adaptive immunity; differences between the two sexes are present in B lymphocytes, CD4+ and CD8+ lymphocytes. For example, females present with a higher lymphocyte count and a higher CD4/CD8 ratio compared to males of the same age, while males exhibit a higher CD8+ lymphocytes count. Women showed higher numbers of activated CD4+ and CD8+ lymphocytes and a higher number of proliferating T lymphocytes compared to men. Furthermore, women show higher levels of immunoglobulins and higher numbers of B lymphocytes compared to men (Murgia et al. 2022).

Innate immunity cells' number and activity differ between males and females. Males present a higher number of natural killer cells while the phagocytic activity of neutrophils and macrophages, instead, is higher in females. Also, antigen-presenting cells (APCs) are more efficient in females than males (Abdullah et al. 2012).

It has been demonstrated that sex hormones contribute to the innate immune response (Jaillon et al. 2019), but sex also influences multiple aspects of adaptive immunity. Differences between the two genders concern lymphocyte subgroups, including B lymphocytes, CD4+, and CD8+ lymphocytes. Females present a higher CD4+ lymphocyte count and a higher CD4/CD8 ratio compared to males of the same age, while males have a higher CD8+ lymphocytes count (Uppal et al. 2003).

In conclusion, between males and females, there are evident differences in immune responses, from infection response to autoimmunity, suggesting the role of sex hormones in immune reaction modulation (Kovats et al. 2015). Estrogens and testosterone modulate the differentiation, maturation, and function of the immune cells, including neutrophils, macrophages, NK cells and dendritic cells.

In general, the reaction to various immunogenic stimuli is stronger in females than males. This also happens when we compare the reaction to specific myelin autoantigens in women affected by MS compared to men with MS (Voskuhl et al. 2012).

Pregnancy and post-partum disease activity

Hormonal and/or genetic factors are presumably involved in regulating the course and the progression of MS, and sex hormones probably play a role in these complex mechanisms. It has been widely documented that different hormone-related physiological conditions corresponding to different life phases of women, such as puberty, pregnancy, puerperium, and menopause, significantly impact the frequency and course of the disease (Ysrraelit et al. 2019) (Figure 6).

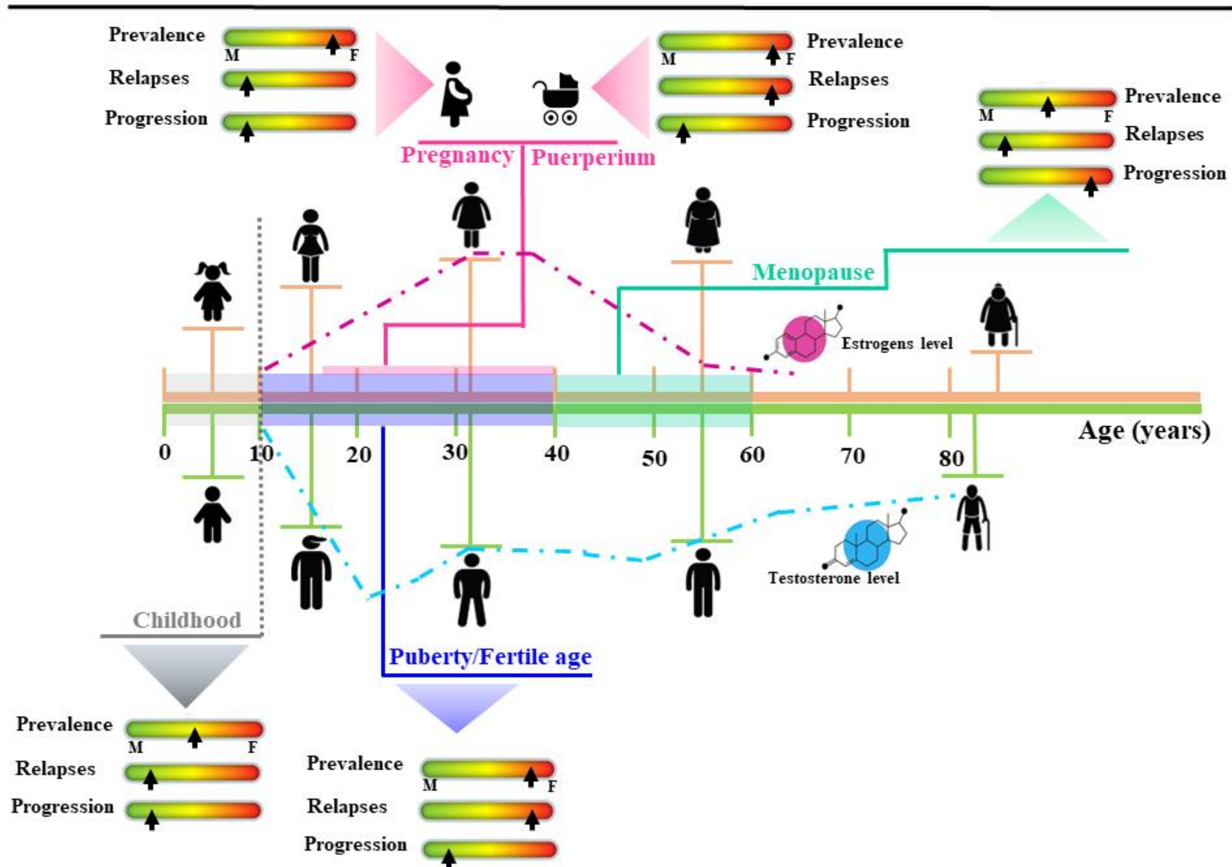

Figure 6. Summary of the effects of hormonal changes on the prevalence, relapses, and progression of the iple Sclerosis (MS) during the different life phases. Before puberty (age 10 years), MS prevalence is similar between males and females. After menarche, in line with the increase in estrogen the prevalence in girls is three times higher. During the fertile age, female predominance remains, and relapses are shown in both sexes but during pregnancy, a significant decrease in female relapse rates (about 70%) is observed especially in the third trimester. After delivery during the puerperium, relapse rates increase three times higher than pre-pregnancy levels. Worsening symptoms and progression of MS have been shown in menopause (Murgia et al. 2022).

Pregnancy is a protective condition against cell-mediated autoimmune disorders, such as MS, rheumatoid arthritis, and psoriasis, but it's not protective against other autoimmune disorders, such as systemic lupus erythematosus (Soldan et al. 2003, Sicotte at al. 2002). This observation suggests that the immune alterations taking place during pregnancy don't suppress the whole immune system, but they rather cause an immune shift that results to be helpful in cell-mediated diseases, but not in antibodies-mediated ones (Voskuhl et al. 2012, Murgia et al. 2022).

Studies focused on MS showed a 70% reduction of relapses in the third trimester of pregnancy when compared to the clinical situation before pregnancy (Voskuhl et al. 2016, Soldan et al. 2003, Sicotte at al. 2002). The reduction in relapses during pregnancy is remarkable considering that the current MS therapies induce a 40-70% reduction in relapses (Ysrraelit et al. 2019).

This was found for the first time in a big multinational study conducted by Confavreux and colleagues, who followed MS women not only in the year before conception but also during pregnancy and in the postpartum period. In this group of patients, the annual relapse rate went from 0.7 in the pre-pregnancy period to 0.2 in the gestational trimester, meaning a reduction higher than 70% (Confavreux et al. 1998). The same study showed an increased relapse frequency in the first 3 months after childbirth, reaching levels that were three times higher than before pregnancy: in particular, relapse frequency changed from 0.2 in the third trimester of pregnancy to 1.2 in the first three months after delivery. Part of the participants of the PRISM study was followed also for the next 2 years and it was detected that three indices were significantly correlated to the risk of a post-partum relapse: 1) an increased relapse rate in the year before pregnancy 2) an increased relapse rate during pregnancy and 3) a higher EDSS score (Vukusic et al. 2004).

A recent observational retrospective study including women affected by MS analysed demographic and clinical data (disease course and duration, age at the onset, EDSS disability levels, RMN evaluations) and the obstetrical history (Lorefice et al. 2021). What stands out from the results is

that there was an increased relapse frequency in the year following childbirth compared to the year preceding the conception.

From the immunological point of view, during a physiological pregnancy, there's an increase in the circulating levels of regulatory T cells (Treg); these cells can develop either in the periphery or in the placenta, where their job is to suppress the allogenic response against the fetus. Indeed, it's generally assumed that the estrogens' protective action against MS is partially based on estrogen-mediated anti-inflammatory cytokines production and Treg cell proliferation (McCombe et al. 2013). Pregnancy seems to modify the balance between Th1 and Th2 responses by inhibiting Th1 responses, it reduces INFγ and matrix metal-proteinases production, while it increases Th2 anti-inflammatory cytokines production (Airas et al. 2008). On the other hand, the post-partum period with its hormonal changes and its increased relapse rate is associated with an immunological shift toward Th1 responses and estrangement from Th2 responses.

The increased disease activity in the period after childbirth seems to be correlated to the sudden reduction of oestrogen levels that takes place after delivery and to the loss of the immunosuppressive condition that characterizes pregnancy. In addition, elevated pro-inflammatory cytokines concentration in the last phases of pregnancy has been associated with a higher relapse rate in the post-partum period (Airas et al. 2015).

As for now, the administration of DMA (disease-modifying agents), even when given immediately after delivery, can rarely prevent post-partum relapses because of their delay of action.

Exploring the mechanisms responsible for the protective role hormones-related of the pregnancy on MS progression could be a very interesting and promising point regarding the possibility to find new potential therapeutic targets.

Diagnosis

Nowadays, there is no single diagnostic test for MS. The diagnosis of MS is based on the history of the patient with clinical features supported by neuroimaging (especially Magnetic Resonance Imaging, MRI) to demonstrate the presence of typical demyelinating lesions of the CNS disseminated in space (DIS) and time (DIT) (Brownlee et al. 2017). MRI is often sufficient to confirm the diagnosis when typical lesions complement a characteristic clinical syndrome but, in some cases, further supportive information is gained by Cerebral Spinal Fluid (CSF) analysis, where is possible to find inflammatory markers as oligoclonal bands present in up to 85% of the patients with MS and/or elevated IgG index which, however, is less specific and sensitive (Link et al. 2017). Supportive diagnostic evidence can be provided by paraclinical tests such as evoked potentials which identify clinically silent lesions in the visual, brainstem and spinal cord pathways.

Several diagnostic criteria have been proposed considering both clinical and supporting data, but the most used one is the McDonald criteria, revised in 2017 (Thompson et al. 2017). The basic concept of these criteria is the demonstration of DIT and DIS using the diagnostic tools described above: the definitive diagnosis of MS requires ≥2 attacks or objective clinical evidence, of ≥ 2 lesions or objective clinical evidence of 1 lesion with historical evidence of a prior attack.

The criteria for PPMS include 1 year of disease progression plus two of the following criteria: 1. evidence of DIS in the brain, 2. DIS in the spinal cord (≥ 2 T2 lesions in the cord), 3. positive CSF oligoclonal bands and/or elevated IgG index.

Despite the presence of diagnostic criteria used to have the MS diagnosis, research has shown that up to 60–70% of patients with a first clinical experience do not meet the criteria for MS (Miller at al. 2012,Kuhle et al. 2015) . Interestingly, up to 85% of these patients will develop typical MS in the future. Currently, existing treatment can delay the progression of MS especially if used at the beginning of the disease. This is the main thing that makes the early diagnosis of MS becoming a crucial clinical challenge.

Biomarkers and precision medicine

Considering all the statements treated before, it is clear the need for new biomarkers which could be helpful in several aspects of MS such as the early diagnosis, the classification of the patients the response to the therapy as well as the elucidation of pathophysiological aspects still unclear.

Biological biomarkers must have specific properties for identifying an MS subtype or predicting an MS course (Sapko et al. 2020). As for every biomarker, the common expectations are standardized analysis techniques, validation in large independent cohorts of patients, and accessible cost for clinical practice. The ideal biomarker should be complementary to information already provided by MRI imaging and give a different spectrum of information (Klineova et al. 2018).

During the last decades, many serum and CSF promising biomarkers have been identified and studied but very few were also validated, and unfortunately, none of those has been used in clinical practice yet.

Among them, for the first diagnosis analysing the CSF emerged, for example, CSF C–X–C motif chemokine 13 (CXCL13), CSF chitinase-3-like protein 1 (CHI3L1), and CSF neurofilament light chain (NfL) which are cytoskeletal proteins released from damaged axons into the CSF and the blood (Yang et al. 2022). Increased cNfL levels are correlated with increased CD4+ T lymphocytes and progression of RRMS to SPMS (Salzer et al. 2010). Talking about MS subtypes, specifically the identification of PPMS versus RRMS was supported by the serum microRNAs miR-223 and miR-15b. Decreased levels of CSF *N*-acetyl aspartate (NAA) were found in patients with SPMS compared to RRMS. CSF-restricted IgM OCBs were found in patients with high relapse rates and early progression to SPMS patients (Teunissen et al. 2015).

Development of new biomarkers is a long process and deep collaborative studies are essential for the validation of results before any biomarker makes it into clinical practice if we consider the complexity of the MS this process appears unattainable.

Precision medicine represents a valid chance to improve and ameliorate the management of the patients affected by MS. The rapid evolution of pioneering, high-throughput technologies, and the huge improvements in knowledge on the molecular basis of human biology and physiopathology changed the viewpoint on patient care toward the holistic approach (Fiandaca et al. 2017). The main goal of precision medicine is to shape an individual medical map, based on multiple data acquired from a patient's history and social conditions, clinical examination, therapeutic path, imaging, and from a variety of omic technologies (Topol et al. 2014) (Figure 7).

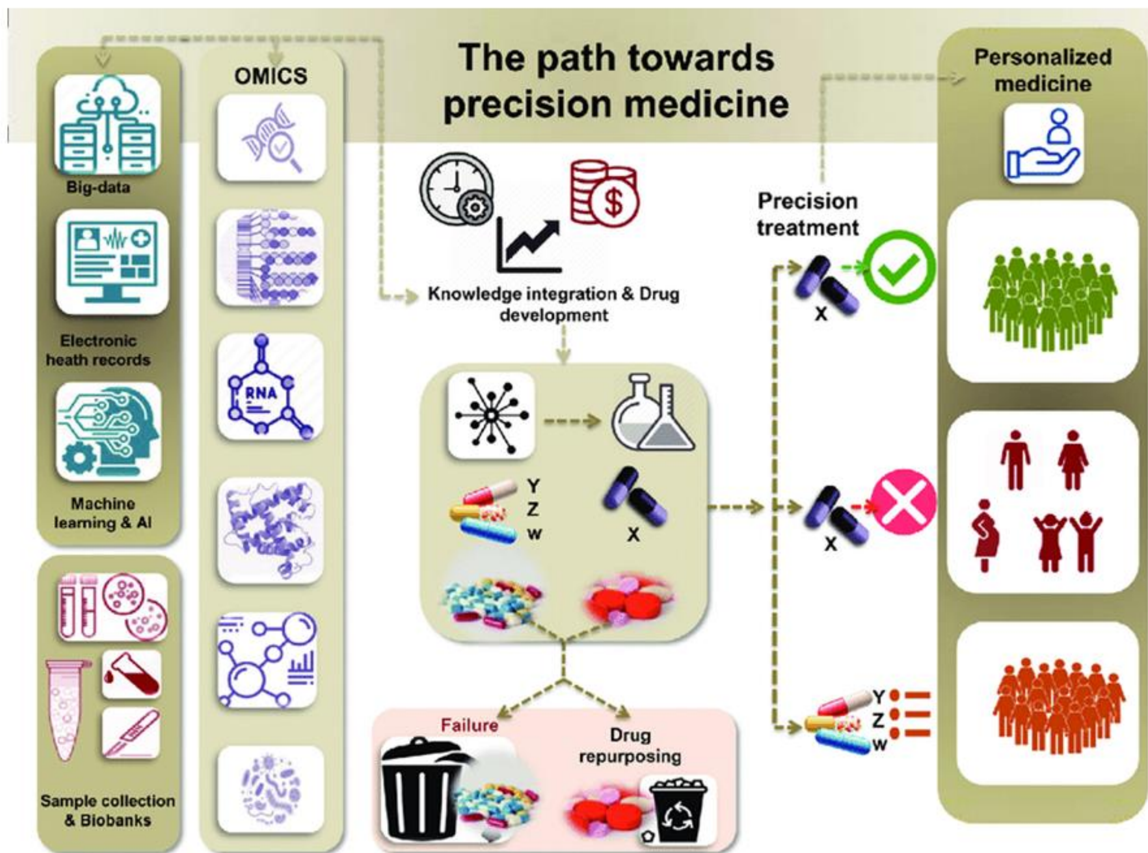

Figure 7. Milestones in the path of precision medicine. The path includes the integration of knowledge from big data, electronic health records, collections of biological material in biobanks, and machine learning strategies which are linked to high-throughput OMICs experiments (Sookoian et al. 2020).

In this perspective, diseases are categorized as the full spectrum of associated phenotypic alterations due to multiple factors, such as genetic and epigenetic changes, pathogenesis, the host immune response, the gut microbiota, and both the beneficial and adverse effects of the therapeutic interventions (Mussap et al. 2021). All these variables contribute to improving the disease delineation and stratification, detection of disease symptoms as early as possible, and, very importantly, identification of pre-symptomatic individuals. Moreover, a strong contribution can be given to individual surveillance measures and pharmacological treatments to significantly delay disease onset and prevent it (Beckmann et al. 2016).

We can define personalized medicine as a medical model of personalization of health, with clinical decisions, practices, and/or products tailored to the patient. Applying precision medicine means personalizing the therapeutic strategy, bringing the best available science in terms of data and modern technologies, directly to patient care to obtain a better and earlier diagnosis and follow-up than the generic model, resulting in individual benefits for patients, families and the health system optimizing the use of resources. With the perspective of promoting a treatment path that considers the biological profile of the patient but with equal attention also his personal, social and cultural dimension the concept of precision medicine marries the approach of translational medicine which has the task of quickly and effectively translating the discoveries in clinical practice, through a path of qualification, verification and validation of biomarkers, proposing a strong collaboration between scientists, producers, and clinicians.

The combination of omics data, from whole-genome, proteome, metabolome and microbiome, reflects the system biology approach and offers computational modelling of the complex cellular activity (Karczewski et al. 2018). Data integration represents a crucial point for developing personalized therapeutic strategies targeted to different classes of patients but with a common clinical presentation and biological basis of disease.

The bridge between lifestyle, clinical and omics information generates complex and heterogeneous big data also referred to as a “digital phenotype” (Jain et al. 2015). It is reasonable to expect that this amount of high-resolution data analyzed by high sophisticate methods, can and will result in a profound revolution in healthcare with clinical benefits for patients (Hawgood et al. 2015).

Metabolomics

In the scenario of personalized medicine, metabolomics plays a fundamental role as part of the omics world. It presents a wide range of applications and has improved extensive progress in the field of health and disease research (Holmes et al. 2008), pharmaceutical sciences (Ufer et al. 2017), personalized medicine (Koen et al. 2016), microbiome research (Li et al. 2008), and so on. The metabolomics approach is based on the profiling of metabolites, small molecules generally weighing under 1200 Da (amino acids, lipids, fatty acids, sugars, biogenic amines, etc.) present in complex biological samples, which are the biological substrates and products of essential cellular functions (energy production and storage, signal transduction, etc.). Cellular functions are influenced by several factors correlated to genomics and proteomics, the individual lifestyle, presence of the pathological state, therapy treatment, age, etc. so it is expected associated the measure of the levels of the metabolites to the accurate description of the individual phenotypes (figure 8).

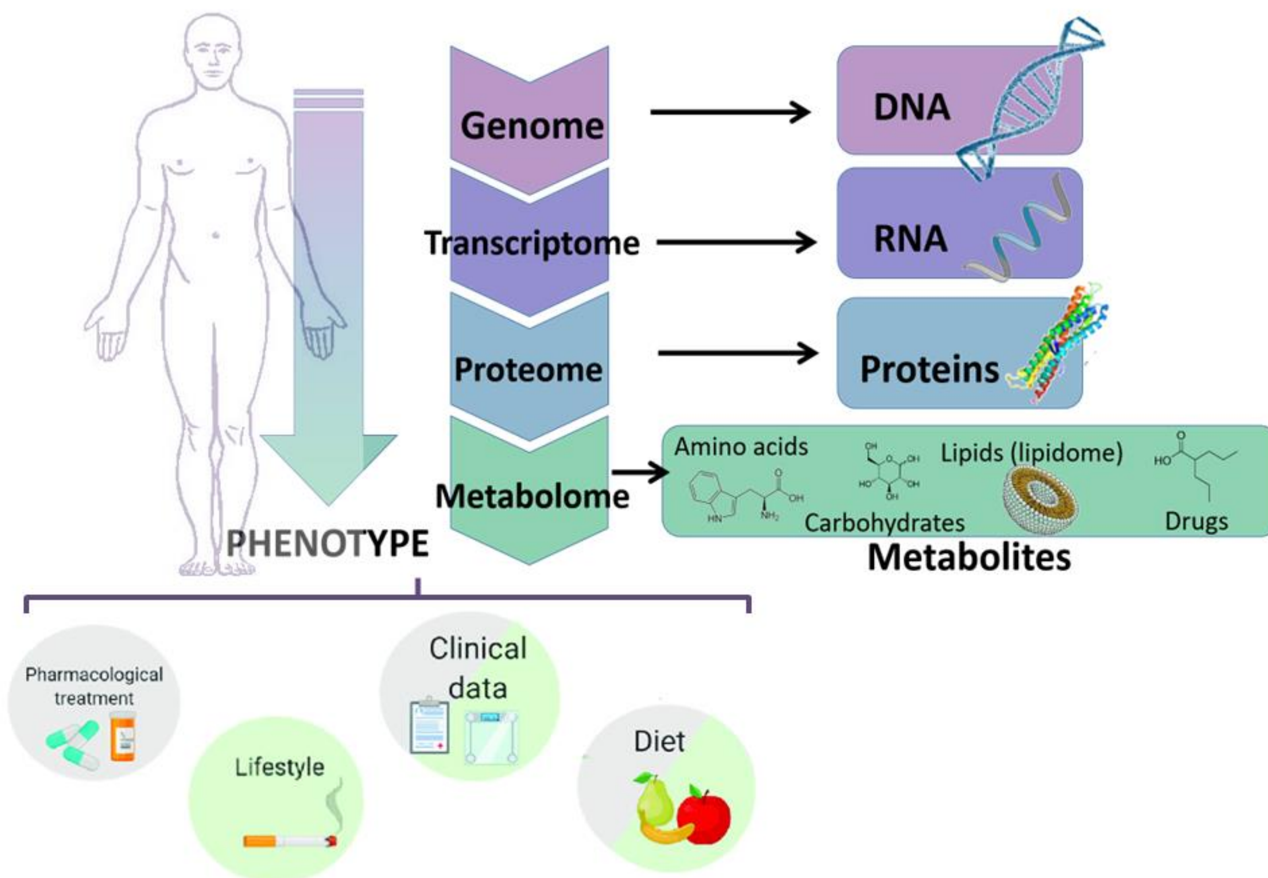

Figure 8. Metabolomics and system biology.

Therefore, the application of metabolomics to MS would have the aim not only of identifying the metabolic circuits involved in the pathogenesis of the disease, but also the discovery of new non-invasive diagnostic techniques and predicting the response to the therapy.

Typically, a metabolomics workflow started with an experimental design well-defined on the topic of interest (Figure 9).

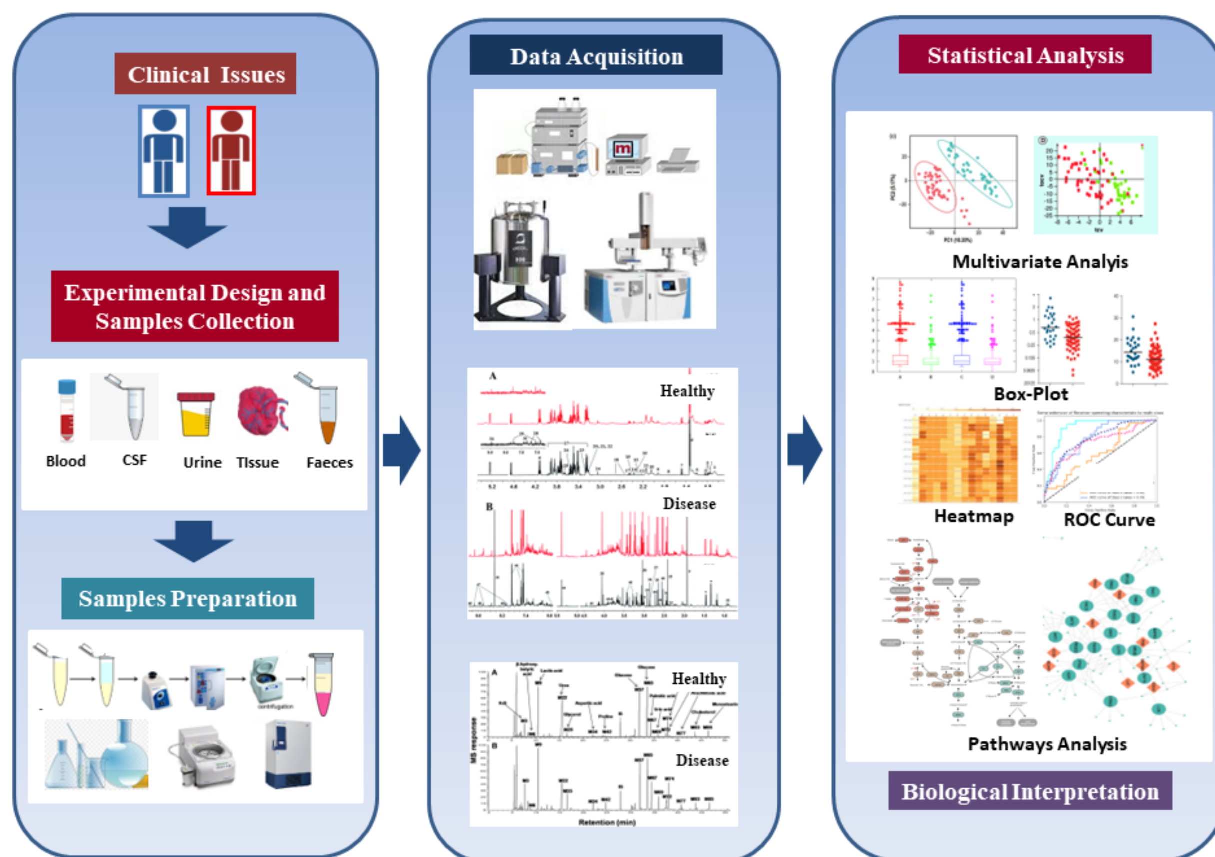

Figure 9. Experimental workflow in metabolomics.

The essential point is the choice of the best biospecimen to analyse, which could be cells, tissues, and every type of biofluid (urine, blood, CSF, saliva etc.). Tissue analysis, in particular, is a powerful approach for investigating localized and specific responses to stimuli and pathogenesis, and it provides explicit biochemical information about the mechanisms of disease (Johnson et al. 2016). The best choice is the paired approach where, in a given experiment, the analysis is structured on both tissue and biofluid to have information about the metabolite uptake/release patterns across the tissue of interest and therefore gives insight into tissue homeostasis. This approach correlated biological information from both localized and systemic responses to a given phenomenon such as the presence of a pathological condition, treatment etc. The sample should be treated by optimizing the extraction of the maximum amount and classes of metabolites often pre-concentrating the extracts and removing the interfering compounds (Jacob et al. 2017).

Sample preparation depends on the instrumentation used in metabolomics. Currently, two main analytical platforms as Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) are used to measure several metabolites for a subject in a single experiment. In general, is possible to use an untargeted approach or a targeted approach: untargeted or global metabolomics aims to measure the widest range of metabolites present in a given sample without a priori knowledge of the metabolome revealing novel and unanticipated perturbations. However, it is not possible to gain all metabolite classes simultaneously, as many factors (often linked to the different chemical nature of the metabolites) can affect metabolite recovery. Additionally, there are a large number of unknown metabolites that remain unannotated in metabolite databases, making challenging the interpretation of the final result (Zamboni et al. 2015). However, the types of metabolites result in a complex data set that requires advanced computational tools to identify and correlate metabolites between samples and to investigate their interconnectivity in metabolic pathways about the phenotype or pathological states (Johnson et al. 2016). Conversely, targeted metabolomics provides higher sensitivity and selectivity than untargeted metabolomics because it is possible to analyse metabolites based on a priori information, whereby methods are developed, optimized and proved for the analysis of specific patterns of metabolites and metabolic pathways of interest. Standard curves for a concentration range of the metabolite of interest are organised to gain an accurate quantification and to obtain exact concentrations of metabolites identified by untargeted metabolomics, providing analytical validation. Targeted analysis constitutes a fundamental part of a metabolomics workflow as it is essential to validate and expand results from the untargeted analysis (Johnson et al. 2016).

As previously written, NMR and MS approaches are the most commonly used in metabolomics: NMR spectroscopy is a non-invasive technique, and the sample can be recovered and used in the following experiment. Conversely, MS analysis, because of the nature of the technique, is destructive but, relatively small volumes of samples are required. NMR spectroscopy is a robust and

reproducible technique (Ward et al. 2010) and presents the advantage of providing the accurate quantitation of numerous analytes with a single internal or external reference (Holzgrabe et al. 2005). However, NMR has relatively low sensitivity, and its biological sample spectrum shows ubiquitous signal overlapping that limits the identification of metabolites and the subsequent discovery of potential biomolecular changes and biomarkers.

On the other hand, MS-based techniques are undoubtedly the most extensively implemented strategies for metabolomics purposes thanks to the high sensitivity that this technique ensures. However, these techniques are less robust than NMR ones, and although targeted analyses are generally comparable between laboratories (Nye et al. 2019), untargeted methods require very careful quality control (QC, biological pool sample) procedures specially to assess and check repeatability over time (Gika et al. 2016). Despite metabolomics greatly benefiting from the incredible progress made in both MS and NMR in the past decades (sensitivity, resolution, and rapidity) most of these are costly, time-consuming, and require advanced technical skills, making these techniques not easily accessible (Letertre et al. 2021). Currently, none of the analytical platforms allows a complete capture of the metabolome. A summary of the advantages and disadvantages of the two analytical platforms is reported in Table 2.

Table 2. summary of the advantages and disadvantages of NMR ah MS approaches.

	Nuclear Magnetic Resonance	**Mass Spectrometry**
Selectivity	It is generally used for nonselective analysis (untargeted analysis)	Can be used for both selective and nonselective (targeted and nontargeted) analyses
Sensitivity	Low but can be improved with higher field strength	High and detection limit reach nanomolar
Reproducibility	Very high	Moderate, depending on the targeted or untargeted approach
Detection limits	Low micromolar, but nanomolar using cryo- and microprobes, and dynamic nuclear polarization	Picomolar with the standard methods but can be lower with special techniques
Sample preparation	Minimal, the addition of buffer, deuterated solvent and chemical shift reference	Often needs extraction protocols, protein precipitation and, in the case of GC-MS, derivatization

Sample measurement	All metabolites can be detected in one measurement	Usually need different separative techniques for different classes of metabolites
Sample recovery	Non-destructive; sample can be recovered and stored for a long time	Destructive technique but need a a small amount of sample
Amount of sample used	Typically, 200-400 µL but less with specifics probes	Low µL range
Number of detectable metabolites in biofluids samples	40–200 depending on spectral resolution	Could be more than 500 using different MS techniques
Molecular identification	Easy	Difficult
Robustness of the instruments	High	Low

The NMR and the MS analysis report signals emitted by the various molecules contained in the sample under analysis. The various signals of the different molecules are represented as Lorentzian peaks whose area expresses the concentration of the metabolite in relative units. The analyses produce MxN matrices where generally the M lines represent the observations (the subjects), while the N columns represent the metabolic variables (for example the concentrations of the metabolites or the normalized areas of the regions of the NMR spectrum called bins).

The interpretation of the data or matrices resulting from the analytical analysis of the patient samples is highly complex and requires computational methods to convert the raw data into biological knowledge and connect them in a meaningful way (Jacob et al. 2017). Now, novel tools that accelerate and automate computational workflows are available and accessible for both novice and expert bioinformaticians. Data mining and various statistical techniques are required to manage the data and multivariate and univariate analyses are usually performed: the first one to simultaneously analyses all the variables, the second to test potential biomarkers and interpret the biological mechanisms of the metabolomics data.

Multivariate methods are fundamental to metabolomics since one biomarker often will not be sufficiently specific for a given condition by itself. The multivariate analysis consists of:

Non-supervised analysis: PCA (Principal Component Analysis) represents the preliminary phase that precedes any type of analysis or can even represent the analysis itself (Wold et al. 1987, Trygg et al. 2007). The primary purpose is the reduction of a high number of variables (representing the characteristics of the phenomenon analysed) in some latent variables. This occurs through a linear transformation of the variables that projects the original ones into a new Cartesian system in which the variables are sorted in decreasing order of variance. The goal of the PCA is to identify appropriate linear transformations of the observed variables, easily interpretable and capable of highlighting and synthesizing the information inherent in the initial matrix without a priori hypotheses. This survey tool is useful to extract as much information as possible with a small set of variables. The objectives that can be achieved with its application are: 1) the identification of characteristic trends in the observations; 2) evidence of groupings between observations; 3) the identification of the dominant variables for the different observations; 4) the search for possible outliers.

Among the various tests aimed at identifying the presence of outliers (observations that differ excessively from the distribution of the others) that could somehow affect the goodness of the data analysis, two are of great importance: Hoteling and DModX test. Hotelling's T^2 test highlights strong outliers, while the DModX test highlights moderate ones. Strong outlier means that the observation is different from the others in relation to the model and they can affect the suitability of the model and should be eliminated; for the moderate outliers, on the other hand, the difference in behaviour occurs in the residuals and is not observable within the model so they don’t affect the suitability of the model.

Supervised analysis: additional information on the data allows the classification of the samples a priori, moving on to the construction of the models using supervised classification methods, such as DA (Discriminant Analysis), and supervised regression methods, such as PLS-DA (Partial Least Squares Discriminant Analysis) and OPLS-DA (Orthogonal Partial Least Squares Discriminant

Analysis). These statistical models make it possible to identify internal clusters of data corresponding to groups or phenotypes of subjects analysed (for example pathological vs controls) and it is possible to evaluate, within the same group, which are the variables that influence more than the others on the distribution of samples: the set of VIPs is thus obtained, the metabolic variables that most contribute to the characterization of groups whose presence has been hypothesized (Worley et al. 2013). Ultimately, the PLS-DA technique can be used to build models for the classification, prediction, and interpretation of data (Madsen et al. 2010).

The significant variables are extracted from the loading or S-plot analysis of each model (NMR and MS) and identified and quantified for the NMR analysis using Chenomx NMR suite 7.1 (Weljie et al. 2006). The concentrations were used for further statistical analysis.

Univariate statistical analysis: once the list and the concentrations of the most discriminant variables (metabolites) have been obtained from the multivariate analysis, univariate statistical methods allow to compare and test the significance between means of metabolites belonging to different classes of patients (for example controls and pathological). According to the distribution of the values (normal or not), it is possible to choose the appropriate test (parametric T-*test,* or non-parametric *U-Mann Whitney test).*

• ROC curves (Receiver operating characteristic curve): this kind of analysis can be used to test the discriminant metabolites as potential biomarkers. The ideal diagnostic tests (the so-called golden standards) perfectly discriminate the pathological patients from the healthy, individuals are classified with absolute certainty as affected or not affected by the disease. The ROC curve is a statistical technique that measures the accuracy of a diagnostic test along the entire range of possible values. Since the ROC curve measures the agreement between the test of interest and the presence/absence of a specific disease, it represents the method of choice for validating a diagnostic test. The ROC curve also allows to identify of the optimal threshold value (the so-called best cut-off), that is the value of the test that maximizes the difference between the true positives (i.e. the

proportion of individuals who have an altered value of the test among all those who affected by the disease) and false positives (i.e. the proportion of individuals who, despite having an altered test value, are not affected by the disease of interest) (Obuchowski et al. 2018).

Finally, network modelling and pathway-mapping software can help to understand the role that metabolites play in relation to each other and pathological contexts (Khatri et al. 2012). Pathways analysis could be performed using specific software that allows, through the use of several libraries, to correlation metabolites into the main physiological processes, indicating which could be affected in a given pathological context.

2. Metabolomics and Multiple Sclerosis: aims of the thesis

The potential of metabolomics has captured the attention of neurologists and neuroscientists experts in MS. Indeed, MS is strongly associated with changes in the metabolome. Several studies employing several metabolomics platforms have examined a range of biological material, from brain tissue to urine demonstrating consistent alterations in multiple metabolic pathways in MS (Bhargava et al. 2020).

What emerged is that metabolomics research in MS is intricate because of the complex nature of the disease itself. The clinical course of MS is heterogeneous, patients have different phenotypes at different time points, and their metabolic profile probably differs before and after a relapse episode. However, the increased number of publications about MS and metabolomics and the promising results obtained are something strongly stimulating.

The identification of a specific MS metabolic fingerprint could improve knowledge of disease mechanisms by identifying altered metabolic pathways (Regenold et al. 2008, Lim et al. 2017). It could help to find biomarkers for early diagnosis (Mehrpour et al. 2013, Senanayake et al. 2015) , classification of the patients, and monitoring of disease progression (Dickens et al. 2014, Stoessel et al. 2018) and treatment response (Lorefice et al. 2019, Signoriello et al. 2020).

Considering all the statements treated in the above sections, during these years we focused our attention on several critical points regards the management of patients affected by MS and previously we applied the metabolomics approach to define the metabolic profile of patients affected by MS at the baseline compared to control subjects (Cocco et al. 2016). As previously discussed, early treatment is considered the best option to prevent long-term disability, so a correct and early diagnosis is extremely important. Currently, the lack of a single predictive or diagnostic test represents a great obstacle in the management of MS at most stages. We compared the plasma metabolic profile of patients affected by MS with a group of healthy controls to find a specific

pattern of metabolites by using ^{1}H-NMR technique. We found a specific MS pattern of metabolites that significantly changed their concentrations compared to healthy controls: in particular glucose, 5-OH-Tryptophan, and tryptophan were lower in patients with MS, whereas 3-OH-butyrate, acetoacetate, acetone, alanine, and choline had higher concentrations in the patients with MS. These metabolites suggested alterations on pathways related to the tryptophan metabolism and energetic homeostasis.

This study represented a milestone in our experimental path and in the present thesis, I investigated other aspects to have a complete framework of the metabolic alteration in MS. In detail, I addressed the following aims:

1) Classification of patients affected by different forms of MS: Difficulties in the diagnosis of progressive forms (especially PPMS), both in terms of distinguishing it from other progressive neurological disorders and excluding patients with previous relapse activity, remain a challenge point in the MS scenario. The lack of a single predictive or diagnostic test for the phenotypic classification constitutes an obstacle to personalized MS care. The addition of markers of activity and progression enhances the description of the ongoing disease dynamic in a given period ameliorating prognostication, treatment decisions, and outcomes in clinical care as well as research. We proposed a metabolomics investigation of a cohort of patients affected by RRMS and PPMS to find potential biomarkers to distinguish between the two forms of MS. For this aim, both CSF and blood samples (serum) were collected, and ^{1}H-NMR and MS (GC-MS and LC-MS) were used as analytical techniques.

2) Monitoring of the response to the therapy: the complex MS pathophysiology hinders the identification of effective therapy, which may have several significant side effects. Current treatment for MS consists of a multidisciplinary approach including DMTs which decrease the frequency of relapses and reduce short-term disability. However, the response to treatment is quite heterogeneous because many patients continue to experience MS disease activity. An important

challenge in clinical practice is to identify the best patient candidate for each DMT, as well as predictors of a better response to the drug. Fingolimod (FINGO) (Gilenya; 1.25 mg) is an oral drug approved for RRMS by the FDA in 2010 as a first-line treatment, and by the EMA in 2011 as a second-line treatment. Several trials and real-world studies have been conducted, indicating the efficacy/effectiveness of FINGO on MS activity as well as on disease progression in adult and pediatric MS patients[8-10]. After the first dose, rare transient FINGO-associated bradycardia, and heart conduction abnormalities, usually asymptomatic, have been reported, while there is a risk of infections during treatment, even if severe opportunistic infections are rarely observed. We evaluate the ^{1}H-NMR plasma metabolic modifications in a group of naïve RRMS patients starting FINGO treatment, also analysing whether specific metabolomic characteristics present at baseline could predict the FINGO therapeutic response as well as the possible side effects.

3) Investigation of the protective role of pregnancy on the MS progression from the metabolic point of view: female MS patients experience pathophysiological changes during reproductive phases (pregnancy, puerperium and menopause). The presence of hormone receptors on immune cells allows sex hormones (e.g. testosterone, estrogens, and progesterone) to influence the immune system, potentially enabling them to modify MS risk, activity, and progression and to play a role in treatment. This process can influence the metabolic profile, which, in turn, can reflect the protective role of the pregnancy. We analysed the serum metabolic profile of women affected by MS in different reproductive life phases: before and during pregnancy and puerperium. We also collected samples from healthy women during the same phases. For this aim, ^{1}H-NMR was used.

3. Aim 1: Classification of patients affected by different forms of MS

Materials and Methods

From Murgia et al. 2020.

Thirty-four CFS and blood samples were collected at the Multiple Sclerosis Centre of the Binaghi Hospital, Cagliari, from patients affected by MS (according to 2017 revisions of the McDonald criteria) (Thompson et al. 2017) with RR (22) and PP (12) course. CSF and blood samples were obtained at diagnosis time therefore, no influence of DMDs drugs on the metabolite profile of MS patients occurred. In addition, all patients exposed to steroid therapy in the previous 30 days were excluded from the study to avoid possible confounding factors. Mean values for age and disease duration were 36.8 (SD ± 11.1) and 2.1 (SD ± 1.5) years respectively, while mean expanded disability status scale (EDSS) (Kurtzke et al. 1983) at sampling time was 1.5 (SD ± 1.1). Table 3 shows the demographic and clinical features of MS patients examined in the study.

Table 3. Demographic and clinical features of multiple sclerosis (MS) patients included in the study.

	MS Patients (n=34)	**Relapsing (n=22)**	**Progressive (n=12)**	***p*-value**
Male Gender	14 (41.2%)	6 (27.2%)	8 (66.6%)	ns
Age (mean ± SD) years	37.3 ± 12.8	32±8.4	47.1 ± 12.8	<0.05
MS Disease Duration (mean ± SD) years	2.1 ± 1.5	1.2 ± 1.4	3.7 ± 1.2	<0.05
Expanded Disability Status Scale (EDSS) score	2.1 ± 1.1	1.1 ± 1.5	3.9 ± 1.7	<0.05

Of the 22 patients with a relapsing course, 12 showed clinical or neuroradiology activity in the last 3 months, while no disease activity was observed in progressive patients. The study was conducted in accordance with the Declaration of Helsinki and the institutional ethics committee of the

University of Cagliari approved the study (n° 20/2015). Written informed consent was also obtained from each participant. For each patient, 1 ml of CSF and 10 mL of blood were previously collected through the lumbar puncture and venous sampling. The blood samples and CSF samples were firstly centrifuged at 2500g for 10 min at 4°C, and divided into different aliquots for the different analytical analysis (^{1}H-NMR, Gas Chromatography-MS and Liquid Chromatography-MS). All the samples were stored at −80 °C until analysis.

Biocrates Absolute IDQ p180 Kit

CSF and serum samples were analysed by Biocrates Life Sciences AG, Innsbruck, Austria. Biocrates is a company that provides phenotyping products and quality-controlled targeted metabolomics services by using platforms that combines basically MS innovative technology including sample preparation, metabolite identification, and data analysis. An aliquot of 50 μL of serum and 30 μL of CSF of each sample was sent to Biocrates: samples were analysed using the Absolute IDQ p180 kit Biocrates (BIOCRATES Life Sciences AG, Innsbruck, Austria). Samples were investigated using all the pre-analytical and analytical procedures documented and reviewed according to the ISO 9001:2008 certified in-house quality management rules and guidelines. Samples were centrifuged, and the supernatant was used for the analysis. A single instrumental analysis containing all the samples placed randomly in the plates was performed. The KIT plates were used for the quantification of 180 metabolites including amino acids, acylcarnitines, sphingomyelins, phosphatidylcholines, hexoses, and biogenic amines. The fully automated assay was based on PITC (phenylisothiocyanate)-derivatization in the presence of internal standards for the LC/MS analysis (amino acids, biogenic amines). Samples underwent LC-MS and FIA-MS/MS analysis (acylcarnitines, lipids, and hexose) using an AB SCIEX 4000 QTrap® mass spectrometer (AB SCIEX, Darmstadt, Germany) with electrospray ionization. The amino acids and biogenic amines were analyzed quantitatively by LC−ESI-MS/MS, with the use of external calibration standards in seven different concentrations and isotope-labeled internal standards for most analytes.

The acylcarnitines, glycerophospholipids, sphingolipids, and sum of hexoses were analyzed by FIA-ESI-MS/MS, using a one-point internal standard calibration with representative internal standards (nine isotope-labeled acylcarnitines, one isotope-labeled hexose, one non-labeled lyso-PC, two non-labeled PCs, one non-labeled SM, a total of 14 internal standards). The experimental metabolomics measurement technique is described in detail by patent US 2007/0004044 (Ramsay et al. 2007). The accuracy of the measurements (determined with the accuracy of the calibrators) was in the normal range of the method (deviations from target ≤ 20%) for all analytes. For sample analysis, validated analytical methods were applied. Quality control samples were within the pre-defined tolerances of the method. Biocrates' in-house MetIDQ™ software was applied for data export and mapping of measurements with chemical and biochemical background information.

NMR and GC-MS Analysis

Sample Preparation

CSF. 800 µL of each CSF sample were used for the analysis; 600 µL of sample were lyophilized overnight for NMR analysis, 200 µL of CSF were lyophilized overnight for GC-MS analysis. For the NMR analysis, the lyophilized samples were resuspended in 630 µL D_2O + 70 µL of 1.5 mM phosphate buffer with 5.85 mM trimethylsilylpropanoic acid (TSP), and 650 µL of the solution was transferred in the NMR tube. For the GC-MS analysis, dried extracts were derivatized with 50 µL of methoxyamine dissolved in pyridine (10 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) at 70 °C. After 1 h 100 µL of N-Methyl-N-(trimethylsilyl)-trifluoroacetamide, (MSTFA, Sigma-Aldrich, St. Louis, MO, USA) were added and left at RT for 1 hr. The samples were successively diluted in 100 µL of hexane (Sigma-Aldrich, St. Louis, MO, USA).

Serum: Serum samples were thawed and centrifuged at 2500g for 10 min at 4°C. An 800 µL aliquot was added to 2400 µL of Folch solution (chloroform/methanol 1:1) plus 350 µL of distilled water. The samples were vortexed for 1 min and centrifuged for 30 min at 1700× g at RT. The

hydrophilic and hydrophobic phases were obtained. The first one was divided into 2 aliquots, concentrated overnight using a speed vacuum centrifuge for GC-MS and ^{1}H-NMR analysis. For the NMR analysis, the concentrated hydrophilic phase was resuspended in 630 µL of D2O, 70 µL of 5.07 mM trimethylsilylpropanoic acid (TSP). 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. For the GC-MS analysis derivatization was undertaken by adding 100 µL of methoxyamine hydrochloride in pyridine solution (10 mg/mL) to dried samples for 17 h. Subsequently, 100 µL of N-trimethylsilyltrifluoroacetamide (MSTFA) were added and vortexed atRT, 1 hr. Samples were then diluted in hexane (600 µL) with an internal standard (undecane at 25 ppm). Diluted samples were then filtered (PTFE 0.45 µm) and transferred into glass vials. By following the same procedure, samples blanks were used to avoid background noise resulting from the chemicals used for the preparation and the laboratory instruments.

NMR Analysis and Data Processing

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 NOESY pulse sequence for the CSF samples and PreSat sequence for the serum samples to suppress the residual water signal. The spectra were recorded with a spectral width of 6000.2; a frequency of 2 Hz; an acquisition time of 1.5 s; a relaxation delay of 2 ms; and a 90° pulse of 9.2 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 Mestre Nova software (version 8.1, Mestrelab Research S.L. Santiago de Compostela, Spain)) each NMR spectrum was divided into consecutive “bins” of 0.04 ppm. The serum 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. The CSF spectral area investigated was the region between 0.64 and 6.4 ppm. To minimize the effects of the different concentrations of serum/CSF samples, the integrated area within each bin was normalized to a constant sum of 100.

GC-MS Analysis and Data Processing

One microliter of derivatized sample underwent splitless injection into a 7890A gas chromatograph coupled with a 5975C Network mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a 30m×0.25mm ID, fused silica capillary column, with a 0.25 μM TG-5MS stationary phase (Thermo Fisher Scientific, Waltham, MA, USA). The injector and transfer line temperatures were at 250 °C and 280 °C, respectively. The gas flow rate through the column was 1 mL/min. The column initial temperature was kept at 60 °C for 3 min, then increased to 140 °C at 7 °C/min, held at 140 °C for 4 min, increased to 300 °C at 5 °C/min and kept for 1 min. Identification of metabolites was performed using the standard NIST 08 ((http://www.nist.gov/srd/mslist.cfm), Fiehn 2013 (http://fiehnlab.ucdavis.edu/Metabolite-Library-2007) and GMD (http://gmd.mpimp-golm.mpg.de) mass spectra libraries (match ≥ 40%) and, when possible, by comparison with authentic standards. Data processing was performed by using a pipeline in Knime (Liggi et al. 2018). In brief, peak detection and deconvolution were performed in a R-XCMS package, and filtering was performed using blank samples and keeping features present in ≥50% of the samples. Missing value imputation was conducted using a random forest algorithm. Relative concentrations of the discriminant metabolites were obtained by the chromatogram area and then normalized by total area (=100).

Statistical Analysis

A multivariate statistical analysis was performed on the matrix resulting by LC-MS/MS, FIA-MS/MS, ^{1}H-NMR, and GC-MS using SIMCA-P software (ver. 15.0, Umetrics, Sweden). The

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

The initial statistical analyses were conducted using the Principal Component Analysis (PCA), which is important for the exploration of the sample distributions without classification. To identify potential outliers, the DmodX and Hotelling's T2 tests were applied.

Partial least square discriminant analyses (PLS-DA) were subsequently applied. PLS-DA maximize the discrimination between samples assigned to different classes. The variance and the predictive ability (R^2X, R^2Y, Q^2) were established to evaluate the suitability of the models. PLS-DA models were performed by using only variables corresponding to VIP (Variable Influence on Projection) value > 1, as a quantitative estimation of the discriminatory power of each metabolite. Variables with VIP > 1 are the most relevant for explaining Y (assignment of two classes).

In addition, a permutation test (n = 400) was performed to validate the models. The scores from each PLS-DA model were subjected to a CV-ANOVA to test for significance ($p < 0.05$).

The most significant variables were extracted by the loading plot from each model and for the ^{1}H-NMR data were identified using the Chenomx NMR Suite 7.1 (Chenomx Inc., 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 and Holm–Bonferroni test, to correct for multiple comparisons were performed. Subsequently, to test the sensitivity and specificity of these metabolites, Receiver Operating Characteristic ROC curves were built; this is generally considered the standard method for performance assessment of target biomolecules (Xia et al. 2013). ROC curves were built using the concentrations of the metabolites with p-value < 0.05 as input, to test their sensitivity and specificity in classifying the patients by using GraphPad Prism software (version 7.01, GraphPad Software, Inc., San Diego, CA, USA).

Pathways Analysis

Metabolic pathways were generated by using MetaboAnalyst 4.0 (MetaboAnalyst 4.0, Xia Lab. Ste. Anne de Bellevue, Quebec) a web server designed to obtain a comprehensive metabolomic data analysis, visualization and interpretation (Chong et al.2018) . In particular, the pathway analysis module of Metaboanalyst 4.0 combines results from powerful pathway enrichment analysis with pathway topology analysis to help researchers identify the most relevant pathways involved in the conditions under study correlating metabolites changes. MetaboAnalyst performs in-house mapping of common compound names to a wide variety of database identifiers including KEGG, HMDB, ChEBI, METLIN, and PubChem before performing any functional analysis. In our analysis, metabolites having p-value<0.05 were used. Only pathways having p-value<0.05 were considered for the discussion and biological interpretation.

Results

All the data generated by the different analytical technique used for the analysis of the CSF and serum were organized in a matrix that underwent multivariate statistical analysis. Firstly, a multivariate analysis was performed on the results from NMR and GC-MS. For the NMR analysis, the total number of variables obtained (bins) was 103 for the CSF, and 155 for the serum, while for the GC-MS analysis of the CSF, the total number of variables obtained was 35, and 40 for the serum. The generated models did not show any significant difference between RRMS and PPMS (low Q2 and $p > 0.05$, figure 10), and for these reasons the VIPs were not taken into consideration.

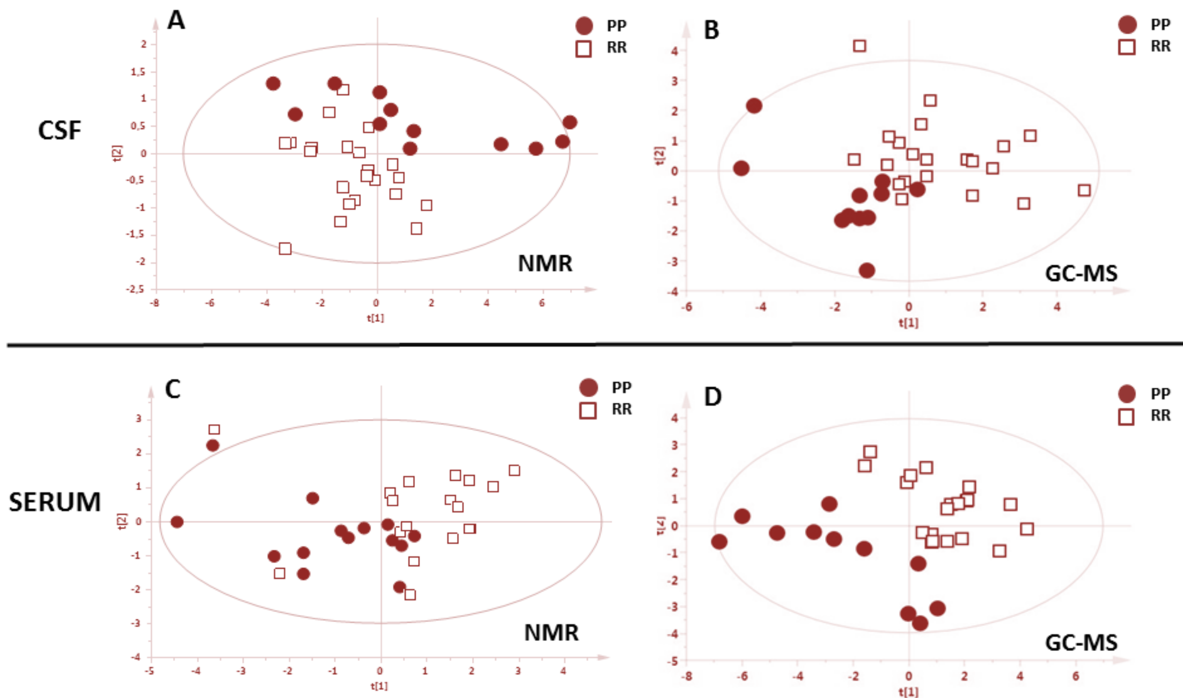

Figure 10. Multivariate analysis. A-B: models resulted from the analysis of CSF samples with NMR and GC-MS. The statistical parameters were not significant. C-D: models resulted from the analysis of serum samples with NMR and GC-MS. Black circles indicate PPMS patients while white boxes indicate RRMS patients. The statistical parameters were not significant.

The summary of the relative statistical parameters is reported in Table X.

Table 4. Statistical parameters of the multivariate models resulting from the analysis of the matrix generated by NMR and GC-MS analysis.

	CSF		SERUM	
	Q^2	p-value	Q^2	*p*-value
NMR	0.01	0.8	-0.001	1
GC-MS	0.12	0.21	0.223	0.13

Subsequently, multivariate analysis of the matrix from FIA-MS/MS (acylcarnitines, lipids, and hexose) and LC/MS (amino acids, biogenic amines) was applied to test the possible differences between RRMS vs PPMS. A separation of the samples, in line with the presence of the different

types of MS, was observed by the application of the supervised model PLS-DA (Figure 11) with good statistical parameters.

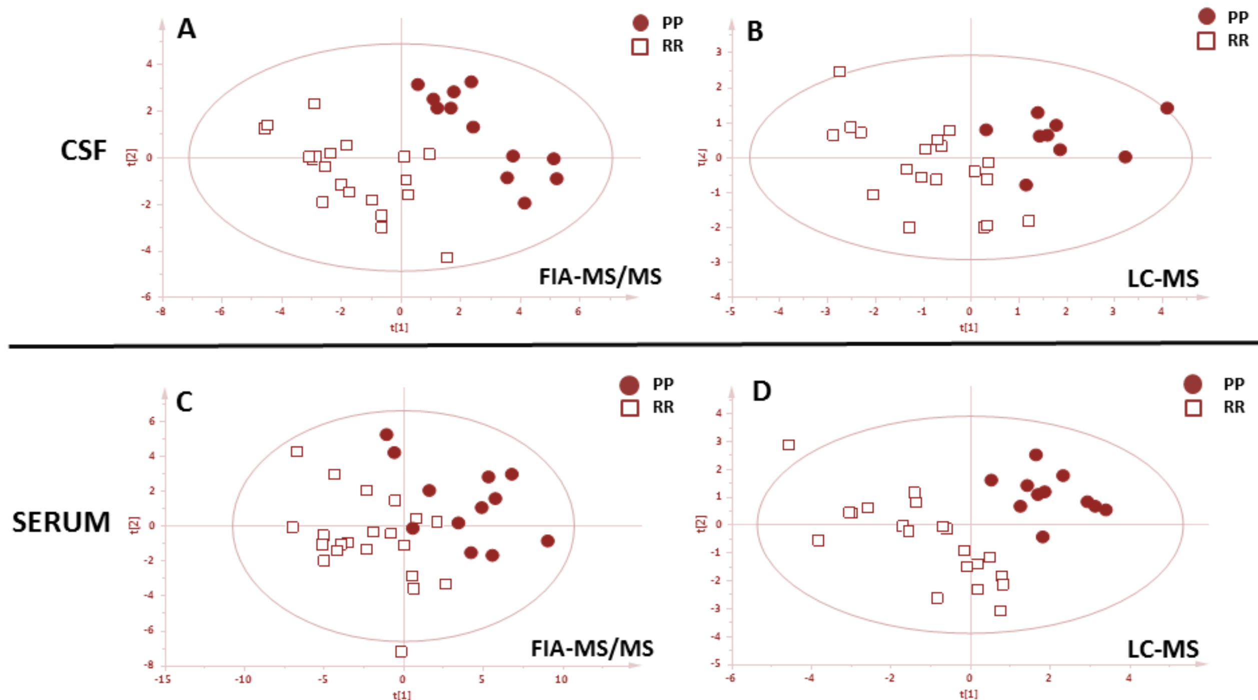

Figure 11. Multivariate analysis. A–**B**: PLS-DA models resulted from the analysis of cerebrospinal fluid (CSF) samples with FIA-MS/MS and LC-MS. **C**–**D**: PLS-DA models resulted from the analysis of serum samples with FIA-MS/MS and LC-MS. Black circles indicate PPMS patients while white boxes indicate RRMS patients.

All the parameters of the models were reported in Table 5.

Table 5. Summary of the statistical parameters of the multivariate analysis on the matrices FIA-MS/MS and LC-MS/MS.

	CSF					SERUM				
	R^2X	**R^2Y**	**Q^2**	***p*-value**	**Permu test:** Intercept R^2\\Q^2	**R^2X**	**R^2Y**	**Q^2**	***p*-value**	**Permu test:** Intercept R^2\\Q^2
FIA-MS/MS	0.272	0.862	0.634	2,6e-05	0.59/-0.23	0.523	0.666	0.512	0.0004	0.33/-0.19
LC-MS	0.395	0.697	0.496	0.002	0.29/-0.28	0.224	0.846	0.514	0.0002	0.35/-0.26

Based on the value of the VIP (>1), 49 metabolites belonging to the classes of acylcarnitines, glycerophospholipids, sphingolipids and 12 metabolites belonging to the classes of the amino acid and biogenic amines were included for the CSF's PLS-DA model.

Similarly, for the serum, based on the value of the VIP (>1), 50 metabolites belonging to the classes of acylcarnitines, glycerophospholipids, sphingolipids, and 16 metabolites belonging to the classes of amino acids and biogenic amines were included in the PLS-DA models.

The goodness of fit of the models allowed the possibility of identifying the discriminant variables responsible for RRMS and PPMS by exploiting the information coming from the loadings plot and VIPs values. Box plots of the most important metabolites (having VIPs values > 1) are represented in Figure 12.

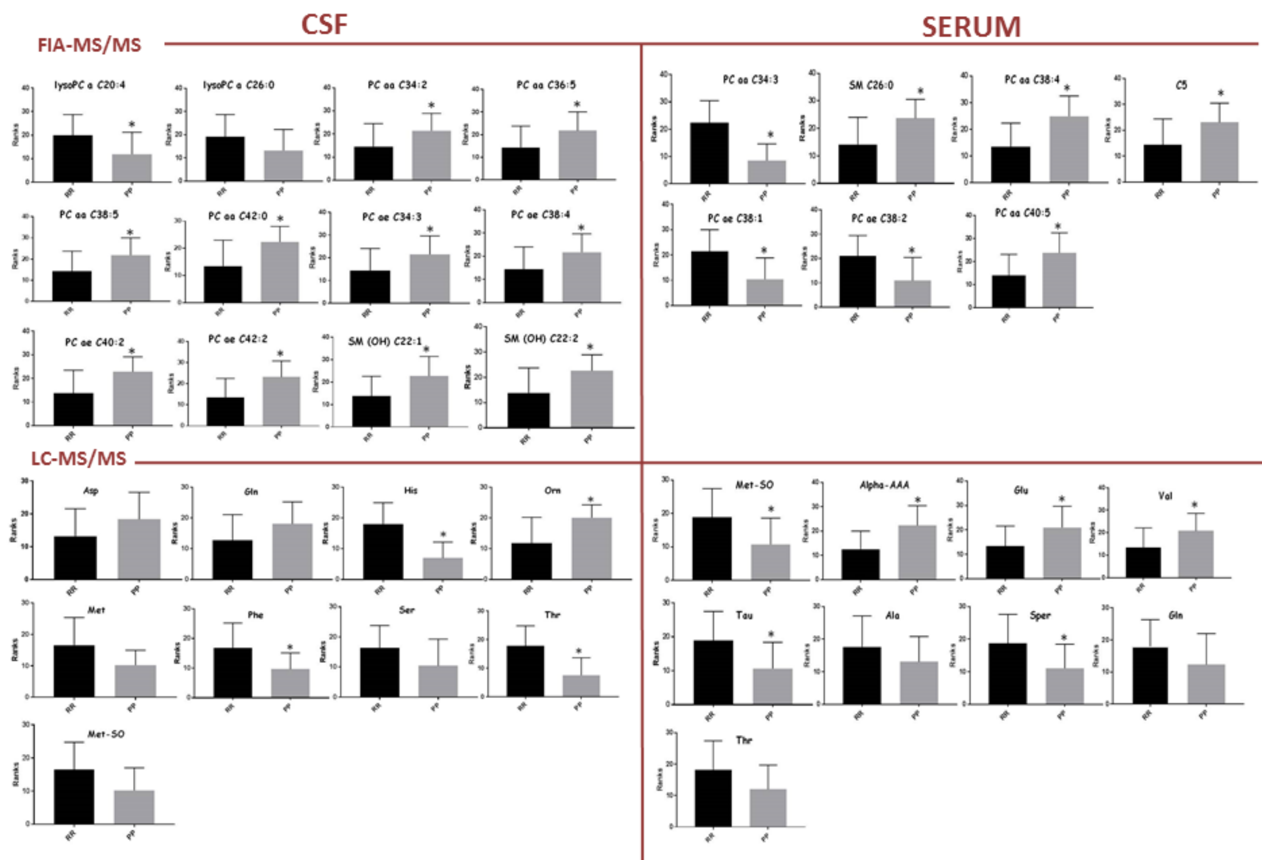

Figure 12. Univariate analysis. Bar-plot of the most discriminant metabolites resulting from the analysis of CSF and serum analyzed with FIA-MS/MS and LC-MS/MS. The black bar indicates the average concentration of the relapses and remissions (RRMS) class expressed as ranks, while the grey bar indicates the average concentration of the PPMS class expressed as ranks. Stars indicate significant change in the concentration of the metabolite

Moreover, the different concentrations of the discriminant metabolites for each class were tested through the U-Mann–Whitney test and subsequently, for those metabolites having p-value < 0.05, a Holm–Bonferroni correction for multiple comparisons was applied. Furthermore, metabolites that exhibited the greatest differences between the studied groups according to a p-value < 0.05 were selected to create the ROC curve. The univariate analysis revealed that all the metabolites from the analysis of the serum by LC-MS/MS and FIA-MS/MS resulting significantly different after U-Mann–Whitney test, passed the Holm–Bonferroni correction (PC aa C34:3, PC aa C38:4, PC ae C38:1, PC ae C38:2, PC aa C40:5, SM C26:0, C5, Methionine-Sulfoxide, alpha-Aminoadipic acid, glutamate, valine, taurine, spermidine). In the case of CSF analysis, 1 phosphocholine (PCae C42:2)

from the FIA-MS/MS analysis and histidine, ornithine, phenylalanine and threonine from the LC-MS/MS passed the correction for multiple comparison. These metabolites have been considered suitable as biomarkers for the classification of the two types of MS. In this light, ROC curves were performed to test their sensitivity and sensibility. A synthesis of the significantly altered metabolites resulting from the analysis of the CSF and the serum is reported in Table 6 and Table 7, respectively. The tables indicate: trends of metabolites in the RR and PP classes (+ or -), *p*-value after U-Mann–Whitney test, *p*-value after Holm–Bonferroni correction and statistical data of the ROC-curve (area under the curve, standard error, confidential interval, *p*-value).

Table 6. Trend, univariate analysis, and ROC curve analysis of the discriminant metabolites in CSF samples.

	CSF								
	METABOLITES	**RR**	**PP**	**P-value**	**Holm-Bonf. correction**	**ROC-CURVE**			
						AUC	**Std. Error**	**CI**	**P-value**
FIA-MS/MS	-lysoPC a C20:4	+	-	0.02	ns	0,74	0,09	0,55-0,93	0,02
	-PC aa C34:2	-	+	0.04	ns	0,71	0,08	0,53-0,88	0,04
	-PC aa C36:5	-	+	0.03	ns	0,72	0,09	0,54-0,90	0,03
	-PC aa C38:5	-	+	0.02	ns	0,73	0,09	0,55-0,90	0,03
	-PC aa C42:0	-	+	0.009	ns	0,78	0,08	0,61-0,94	0,01
	-PC ae C34:3	-	+	0.04	ns	0,71	0,09	0,53-0,89	0,04
	-PC ae C38:4	-	+	0.03	ns	0,72	0,08	0,55-0,89	0,03
	-PC ae C40:2	-	+	0.007	ns	0,78	0,08	0,62-0,93	0,008
	PC ae C42:2	-	+	0.004	0.04	0,79	0,07	0,64-0,95	0,005
	SM(OH) C 22:1	-	+	0.010	ns	0,77	0,08	0,6-0,93	0,01
	SM(OH) C 22:2	-	+	0.01	ns	0,76	0,08	0,6-0,92	0,01
LC-MS/MS	HIS	+	-	0.0004	0,001	0,89	0,06	0,77-1	0,0009
	ORN	-	+	0.01	0,010	0,79	0,08	0,63-0,96	0,03
	PHE	+	-	0.03	0,010	0,75	0,09	0,57-0,93	0,03
	THR	+	-	0.001	0.002	0,86	0,07	0,71-1	0,002

Table 7. Trend, univariate analysis, and ROC curve analysis of the discriminant metabolites in serum samples

	SERUM								
	METABOLITES	**RR**	**PP**	**P-value**	**Holm-Bonf. correction**	**ROC-CURVE**			
						AUC	**Std. Error**	**CI**	**P-value**
FIA-MS/MS	PC aa C34:3	+	-	<0,0001	0,001	0,91	0,05	0,81- 1,00	<0,0001
	PC aa C38:4	-	+	0,0010	0,005	0,83	0,07	0,70-0,97	0,001
	PC ae C38:1	+	-	0,0016	0,006	0,82	0,08	0,67-0,97	0,002
	PC ae C38:2	+	-	0,0036	0,011	0,80	0,08	0,62-0,97	0,004
	PC aa C40:5	-	+	0,0059	0,012	0,78	0,08	0,61-0,95	0,007
	SM C26:0	-	+	0.006	0,012	0,79	0,08	0,63-0,93	0,008
	C5	-	+	0,0149	0,012	0,75	0,08	0,6- 0,92	0,015
LC-MS/MS	MET-SO	+	-	0,010	0,040	0,76	0,08	0,59- 0,94	0,01
	ALPHA-AAA	-	+	0,002	0,010	0,81	0,08	0,65-0,98	0,003
	GLU	-	+	0,020	0,040	0,74	0,09	0,56-0,93	0,02
	VAL	-	+	0,02	0,040	0,74	0,09	0,56-0,92	0,02
	TAU	-	+	0,01	0,040	0,77	0,08	0,59-0,94	0,01
	SPER	-	+	0.02	0,040	0,75	0,08	0,57-0,92	0,02

ROC curves of the discriminant metabolites in CSF and serum were reported in Figure 13 and Figure 14, respectively.

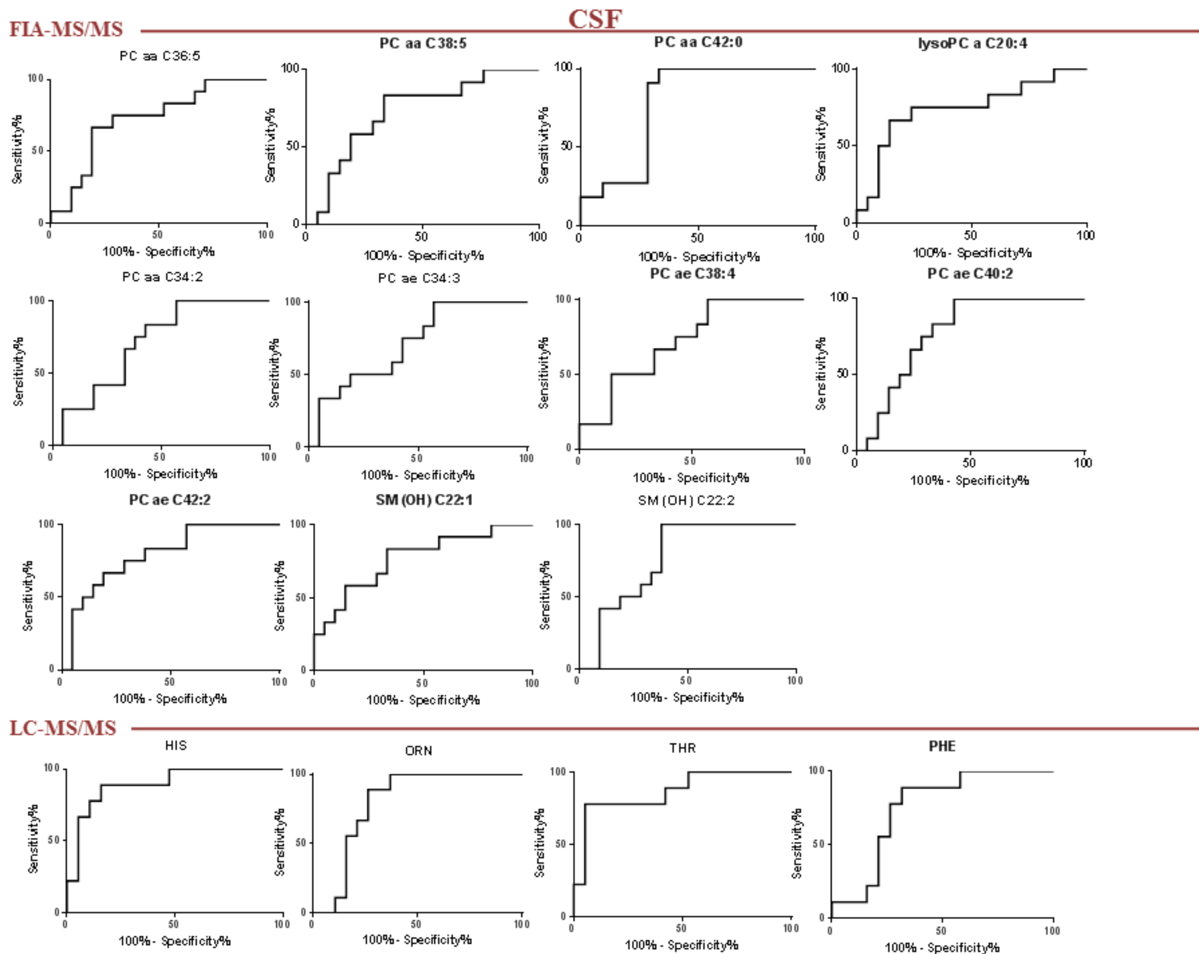

Figure 13. CSF Biomarkers evaluation. Roc curves of the most important metabolites resulting from the multivariate analysis of the CSF matrix, generated with FIA-MS/MS and LC-MS/MS respectively.

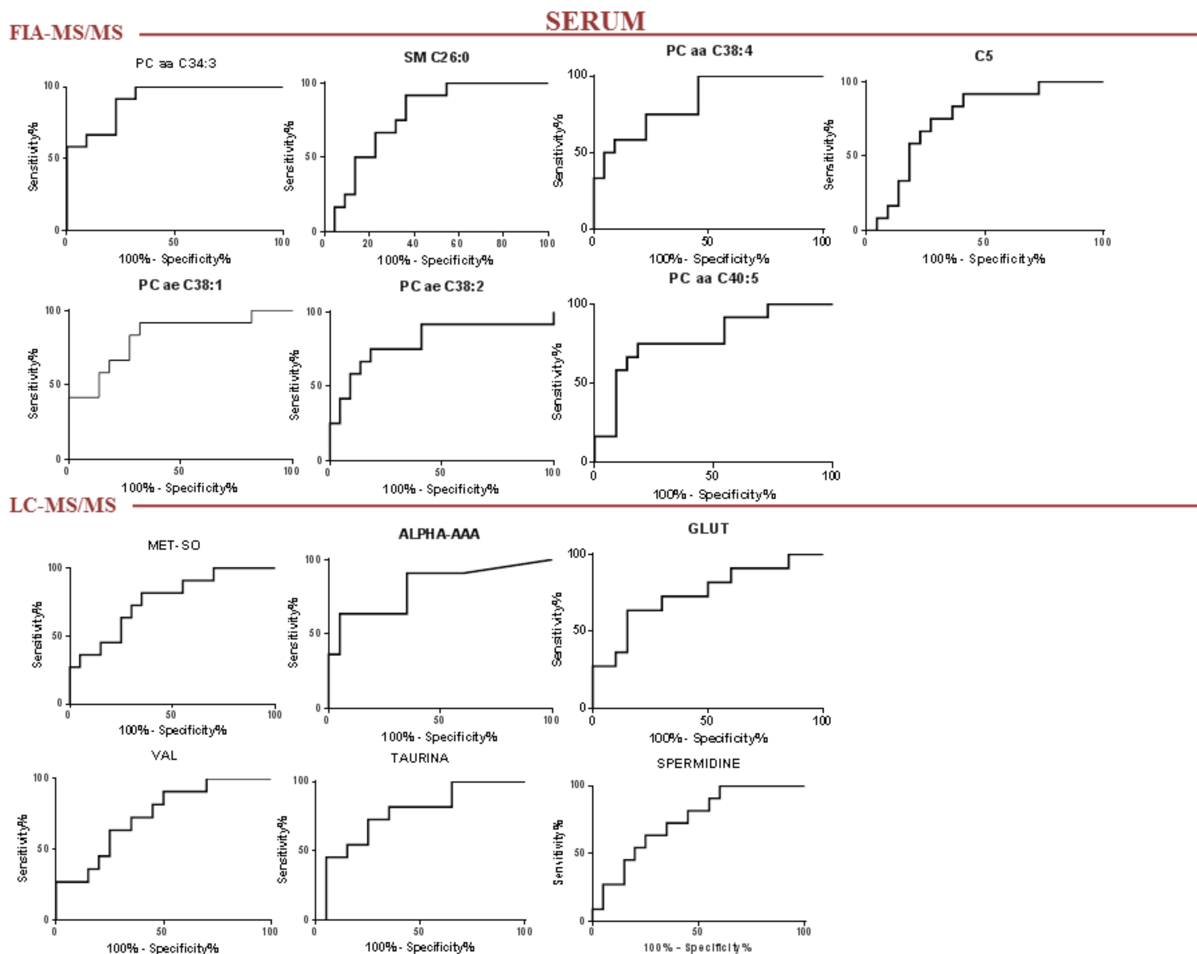

Figure 14. Serum biomarkers evaluation. Roc curves of the most important metabolites resulting from the multivariate analysis of the serum matrix, generated with FIA-MS/MS and LC-MS/MS respectively.

To avoid the confounding effect, due to the different age of the patients of the two classes, Spearman Correlation was performed relating the selected metabolites and the age of the patients. A weak correlation was found for the ornithine ($R^2 = 0.5$), PC ae C42:2 ($R^2 = 0.46$) and histidine ($R^2 = -0.44$) in the CSF, while for the serum metabolites PC aa C34:4 ($R^2 = -0.48$), taurine ($R^2 = -0.55$), alpha AAA ($R^2 = 0.41$) and spermidine ($R^2 = -0.41$) showed a weak correlation. All the results are shown in Figures 15 (A-B).

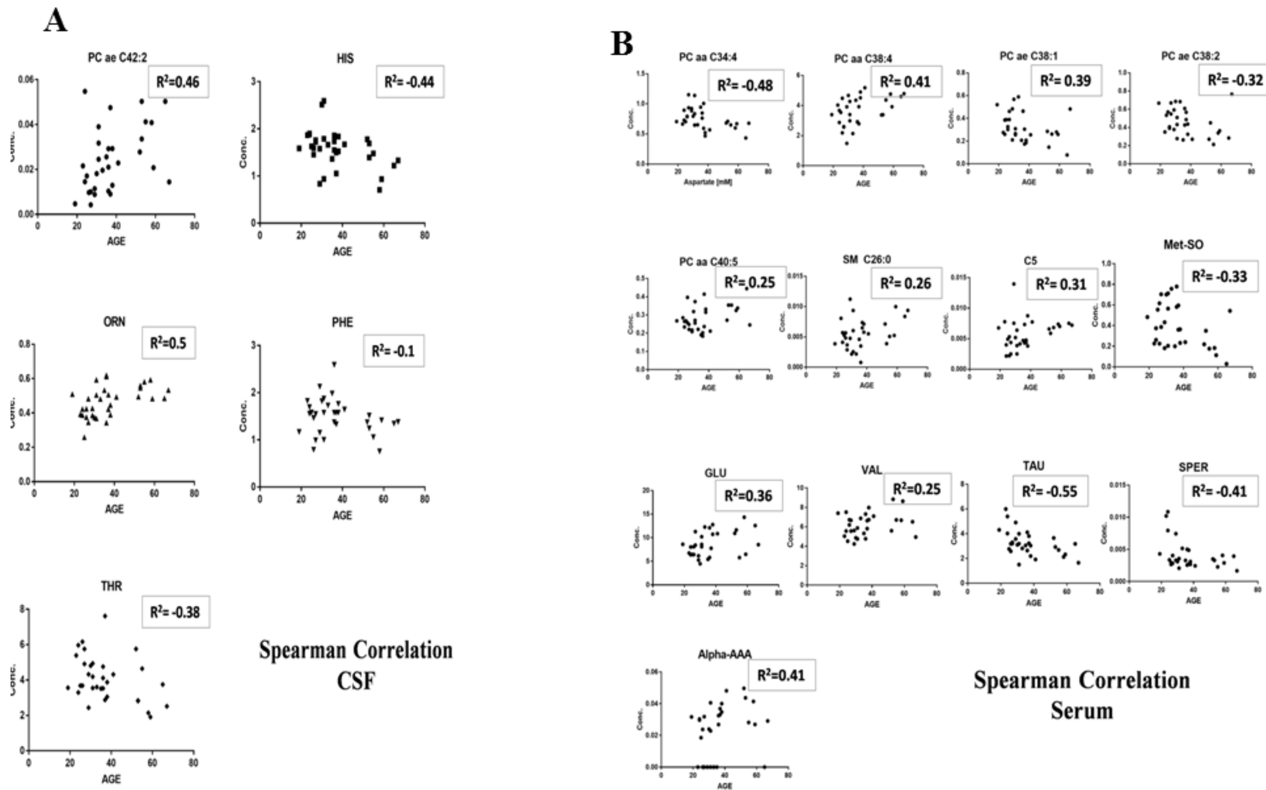

Figure 15. A) Graphs of the Spearman Correlation of the metabolites of CSF passing the Holm-Bonferroni correction. Concentrations were correlated with the age of the patients. R^2 is reported for each graph. B) Graphs of the Spearman Correlation of the metabolites of serum passing the Holm-Bonferroni correction. Concentrations were correlated with the age of the patients. R^2 is reported for each graph.

The summary of the statistical parameters is reported in Table 8.

Metabolites	R^2	*p*-value
CSF		
PC ae C42:2	0,4626	0,007
His	-0,4444	0,009
Orn	0,5162	0,002
Phe	-0,1909	0,287
Thr	-0,392	0,024
Serum		
PC aa C34:4	-0,48	0,005

PC aa C38:4	0,41	0,009
PC ae C38:1	0,39	0,024
PC ae C38:2	-0,32	0,035
PC aa C40:5	0,25	0,052
SM C26:0	0,26	0,056
C5	0,31	0,033
Met-SO	-0,33	0,035
GLU	0,36	0,020
VAL	0,25	0,053
TAU	-0,55	0,001
SPERM	-0,41	0,009
Alpha-AAA	0,41	0,009

The metabolites passing the Holm–Bonferroni correction were considered the most relevant for the classification of the RRMS and PPMS classes. The subsequent step was to investigate the biological meaning of the selected metabolites by using the Metaboanalyst tool. The pathway analysis algorithm was based on Fisher's Exact Test and Out-degree Centrality for the Pathway Topology Analysis. As shown in Figure 16, nitrogen metabolism, arginine and ornithine metabolism, branched chain amino acid (BCAAs) biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis and histidine metabolism were the most altered pathways between the two classes of patients in CSF. The most altered pathways between RR and PP resulting from the analysis of the serum metabolites were glutathione metabolism, nitrogen metabolism, arginine and proline metabolism, glutamine and glutamate metabolism, linoleic acid metabolism, taurine and hypotaurine metabolism and, finally, alanine, aspartate, and glutamate metabolism.

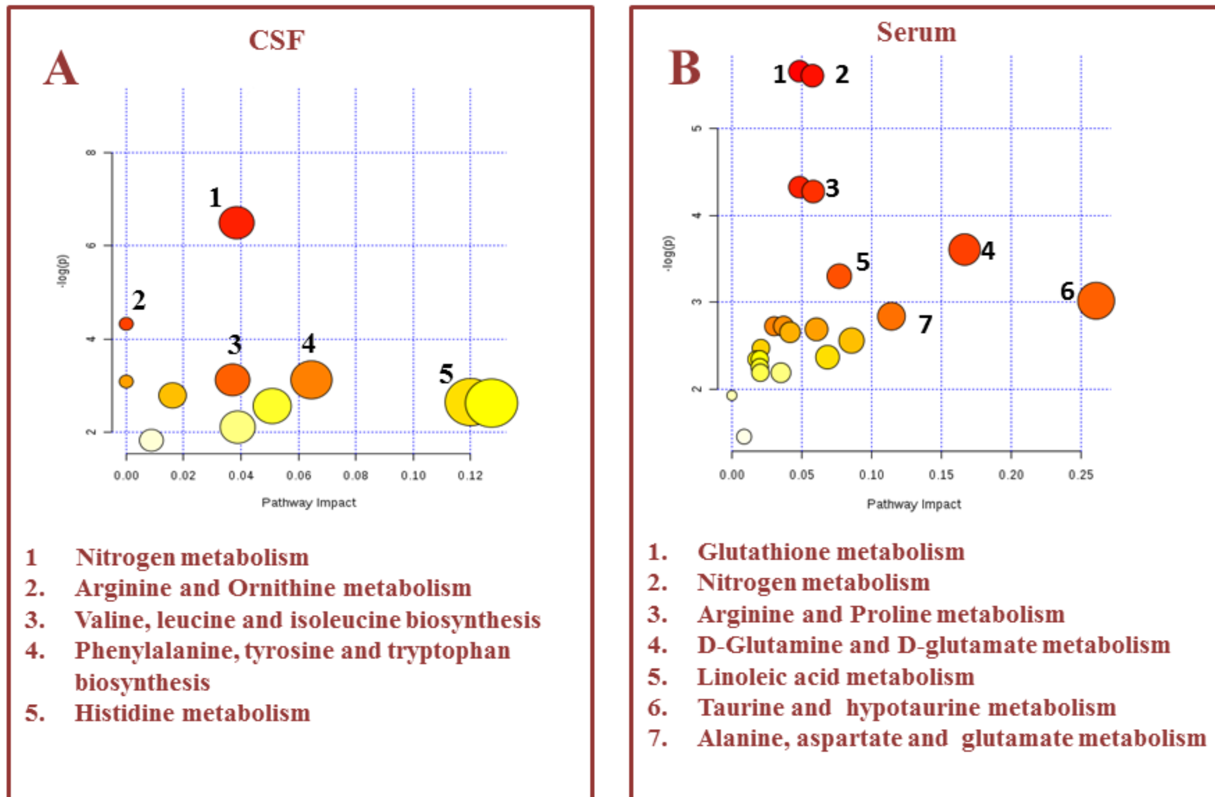

Figure 16. Pathways analysis of the most discriminant metabolites passing the Holm–Bonferroni correction in CSF and serum. (**A**) Most altered pathways between RR and PP patients in CSF samples: nitrogen metabolism, arginine and ornithine metabolism, branched chain amino acid (BCAAs) biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis and histidine metabolism. (**B**) Most altered pathways between RR and PP patients in serum samples: glutathione metabolism, nitrogen metabolism, arginine and proline metabolism, glutamine and glutamate metabolism, linoleic acid metabolism, taurine and hypotaurine metabolism and, finally, alanine, aspartate, and glutamate metabolism.

The best similarities of the results between CSF and blood were common altered pathways such as pathways linked the oxidative stress (Glutathione metabolism and nitrogen metabolism) and arginine metabolism, while differences were characterized by changes in different amino acid pathways (glutamine and glutamate metabolism, taurine, and hypotaurine metabolism and alanine, aspartate metabolism in serum; branched chain amino acid, phenylalanine, tyrosine, tryptophan biosynthesis and histidine metabolism in CSF).

Discussion

Until the advent of “-Omics” technologies, the identification of reliable biomarkers for MS was very difficult due to the clinical and pathophysiological complexity of the disease. Therefore, this study aimed to use the opportunity of large scale-analysis offered by metabolomics to characterize the metabolomics profile of patients affected by different MS sub-types: RR and PP.

We chose to investigate the metabolome of both CSF and blood samples. While the sampling of the CSF metabolites, directly reflecting brain activity, may provide crucial information about neurological damages induced by MS progression (Kuenz et al. 2008), blood analysis represents a non-invasive method to investigate peripheral pathological alterations.

Among the several analytical techniques used in this study to determine the concentration of a large number of potential biomarkers in CSF and serum, both ^{1}H-NMR and GC-MS did not allow the identification of a specific metabolic fingerprint for MSRR and MSPP patients. The serum of a similar cohort of patients (three subtypes of MS, RRMS, SPMS, and PPMS) was previously analyzed by NMR by Dickens et al underlining, in line with our data, the lack of a predictive model between the MSPP and MSRR patients (Dickens et al. 2014).

Thus, we focused our attention on the results from the MS/MS analysis. Subsequently, multivariate analysis of the matrix from FIA-MS/MS (acylcarnitines, lipids, and hexose) and LC/MS (amino acids, biogenic amines) was applied to test the possible differences between RRMS vs PPMS.

FIA-MS/MS and LC-MS/MS analysis led to the identification of PC aa C34:3 (AUC = 0.91. p-Value after Holm–Bonferroni correction 0.001) and ae C42:2 (AUC = 0.79, p-value after Holm–Bonferroni correction 0.04) as the best lipid compounds to classify the two groups in serum and CSF, respectively. In addition, alpha-AAA (AUC = 0.81, p-Value after Holm–Bonferroni correction 0.01) and histidine (AUC = 0.89, p-Value after Holm–Bonferroni correction 0.001) resulted the most discriminant between the amino acids and biogenic amines in serum and CSF, respectively.

To better understand the pathophysiology of the two sub-types of MS, the discriminant metabolites were studied by identifying the changes in metabolic pathways in RRMS and PPMS. Unfortunately, due to the limited availability of metabolomics studies based on the different subtypes of MS in literature, it is not easy to fully understand our results. In serum, we found that the most altered metabolisms between the two classes were those of glutathione, nitrogen, arginine and proline, glutamine and glutamate, linoleic acid, taurine and hypotaurine, and alanine, aspartate and glutamate. In CSF, nitrogen metabolism, arginine and ornithine metabolism, BCAAs biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, and histidine metabolism represented the most altered pathways. In line with our results, Stoessel et al. comparing the plasma of PPMS with RRMS patients and controls found the alteration of linoleic acid metabolism, arginine and proline metabolism, phenylalanine, tyrosine and tryptophan biosynthesis and nitrogen metabolism (Stoessel et al. 2018). Moreover, in the same study, they found a decline in LysoPC (20:0) levels during the disease course of PPMS, while we did not find any significant differences of this metabolite comparing the two classes of patients.

Glutathione and nitrogen metabolism, which metaboanalyst suggested to be altered in both CSF and blood of SM patients, are closely related to oxidative stress. Oxygen and nitrogen free radical production may be crucial in the pathogenesis of MS. Radical oxygen species (ROS) are bio-products of the metabolism of excitatory amino acids and neurotransmitters and are particularly active in the brain and neuronal tissue. They are generated in mitochondria during oxidative phosphorylation and by the activation of many enzymatic and non-enzymatic pathways (Gilgun-Sherki et al. 2004). ROS can target glia and neurons leading to neuronal damages, such as demyelination and axonal injury. Similarly, reactive nitrogen species may promote myelin and oligodendrocyte destruction by exerting cytotoxic effects on nerve and glial cells (Bizzozero et al. 2005). In addition, free radicals can activate transcription factors (such as transcription factor-kappa B, NF-κB) involved in the up-regulation of many genes involved in MS, (tumor necrosis factor-α,

nitric oxide synthase, iNOS, intracellular adhesion molecule 1, ICAM-1, etc.) (Winyard et al. 1997). The key role of oxidative stress in MS has been proved by several studies evidencing lipid peroxidation in the CSF and in the plasma of MS patients (Naidoo et al. 1992). In line, increased free radical activity, and/or deficiencies in important antioxidant enzymes have been found in MS patients compared with healthy controls (LeVine et al. 1992). As to metabolomics, different studies have described a key role of metabolites linked to oxidative stress in MS (Bhargava et al. 2017, Calabrese et al. 2002). Interestingly, Koch et al. analyzed the serum and peripheral blood leukocytes from patients with benign relapsing MS, secondary progressive MS, and primary progressive MS finding increased ROS formation compared to healthy controls in all subgroups, with the highest production found in patients with PPMS (Koch et al. 2006).

Pathways found altered in the serum of our MS patients were glutamine and glutamate metabolism, as well as alanine, aspartate and glutamate metabolism. These are excitatory amino acids (EAA), fundamental for synaptic connection. Glutamate is the major EAA and both intracellular and extracellular physiological concentrations are precisely regulated (Gonsette et al. 2008). Glutamate overload causes an imbalance between excitatory and GABA/glycine-mediated inhibitory processes leading to brain excitotoxicity. Glutamate overload most frequently is determined by glutamate–glutamine cycle dysfunction leading to neurological tissue damage, due to overstimulation of glutamate receptors, and subsequent excitotoxic injury of neurons and glial cells (Groom et al. 2003, Pitt et al. 2003). Considering the pivotal role exerted by glutamate in the CNS, it is not surprising its relevance in MS (Pieragostino et al. 2015, Villoslada et al. 2017). Interestingly, Sarchielli et al. demonstrated increased CSF levels of glutamate and aspartate in RRMS patients both during relapse and a stable clinical phase. Moreover, increased CSF levels of glutamate and aspartate also were evidenced in SPMS patients compared with controls, particularly in patients with a progression of neurological impairment (Sarchielli et al. 2003).

We also observed the alteration of the arginine metabolism in both serum and CSF of SM patients which has been shown to represent a typical feature in MS (Stojanovic et al. 2012, Poddighe et al. 2017), in both humans and animal models (Mangalam et al. 2013). The pathogenesis of MS is explained based on two major theories: an autoimmune and a neurodegenerative mechanism. The neurodegenerative hypothesis involves metabolic changes in the myelin constituents resulting in the destabilization of membrane architecture and myelin degradation (Pritzker et al. 2000). One of the modifications is represented by an enzymatic reaction called deamination (Moscarello et al. 2007, Finch et al. 1971), resulting in the conversion of peptide-bound arginine to peptide-bound citrulline. Moreover, as arginine represent the precursor of nitric oxide in a reaction catalyzed by the nitric oxide synthase family, alteration of arginine metabolism could affect nitric oxide synthesis and be involved in oxidative stress.
Our findings also demonstrate the involvement of several amino acid systems in MS. In particular, BCAAs participate in many important biochemical functions in brain tissue, both directly and indirectly, including protein synthesis, energy production, and glutamate compartmentalization (Fernstrom et al. 2005). In addition, BCAAs represent a known source of pyruvate for energy metabolism, and de novo synthesis of macromolecules within neural and immune cells (Hutson et al. 2007, Li et al. 2007) .

In CSF samples of MS patients, we also found the alteration of the phenylalanine, tyrosine, and tryptophan biosynthesis pathway. Tryptophan is closely linked to the kynurenine pathway (KP), which is activated in several inflammatory and neurodegenerative diseases, including MS, thus representing a common pathological mechanism highly relevant for the understanding of MS pathology (Schwarcz et al. 2004). Increasing evidence demonstrates the activation of the KP during neurocognitive disorders under CNS inflammatory conditions, such as MS (Rajda et al. 2007, Lovelace et al. 2016, Watzlawik et al. 2016). Moreover, this net was also analysed in the comparison between RRMS, and the progression disease subtypes SPMS and PPMS, revealing how

the kinurenic acid/quinolic acid ratio was increased in SPMS and PPMS compared to the RRMS, and this was directly linked to an excitotoxicity effect. This finding has shown the key role exerted by the metabolites involved in the tryptophan metabolism in SM.

4. Aim 2: Monitoring of the response to the therapy

Materials and Methods

Patients

The study included a group of RRMS patients, who had been therapy-free for at least 90 days, to be initiated on therapy with FINGO, and a healthy control group. The blood samples of MS patients were collected at four time points: 1) before starting the therapy with FINGO -Time 0 (T0); 2) six months after FINGO initiation - Time 6 (T6); 3) twelve months after FINGO initiation - Time 12 (T12); and twenty-four months after FINGO initiation - Time 24 (T24). The patient's clinical features (disease duration and level of disability evaluated using the Expanded Disability Status Scale, EDSS, and MRI data, indicating the presence of Gadolinium (Gd)-enhancing lesions) were recorded before FINGO initiation, whereas the number of clinical relapses, EDSS variations, and the presence of new/enlarging T2 or T1 Gd-enhancing lesions on MRI was collected at T12 and T24.

Patients were categorized into two groups: responders (R) and not responders (NR), according to the NEDA 3 definition (absence of clinical relapses, no confirmed disability progression (EDSS) sustained for 6 months, and no new/enlarging T2 or T1 Gd-enhancing lesions on MRI). The comparisons of the metabolomic profiles were performed at baseline and during treatment.

Sample preparation and ^{1}H-NMR analysis

In this study we choose to perform firstly the ^{1}H-NMR analysis at the University of Cagliari, since the use of the Biocrates platform, being an external service, requires huge economic resources. Samples were prepared and analysed with this platform as described in the previous study discussed in this thesis as the extraction of the hydrophilic phase for serum and plasma is exactly the same. We adjusted and optimized the protocol using half of the quantity. Briefly 10 mL of blood were collected from each sample, and the plasma samples were stored at -80°C until analysis. Plasma

samples were thawed and centrifuged at 2500 g for 10 min at 4°C. Then, 400 µl aliquot was added to 1200 µl of chloroform/methanol 1:1 plus 175 µl of distilled water. The samples were vortexed for 1 min and centrifuged for 30 min at 1700*g* at room temperature. The hydrophilic and hydrophobic phases were obtained. The hydrophilic phase was concentrated overnight using a speed vacuum centrifuge for the subsequent ^{1}H-NMR analysis. The sequence and the parameters of the ^{1}H-NMR analysis were the same of the previous study and the final dataset consisted of a 146x82 matrix. A multivariate statistical analysis was performed using an up-grade of SIMCA-P software (ver. 16.0, Umetrics, Sweden) (Eriksson et al. 2013). The variables were Pareto scaled to emphasize all metabolite signals and reduce the spectral noise. Also, in this case the initial data analyses were conducted using the PCA. To study a possible linear relationship between a matrix Y (dependent variables, for example, increasing therapy time) and a matrix X (predictor variables, e.g., metabolites) Partial Least Squares projection to latent structures regression (PLS) model was performed (Wold et al. 2001). Finally, the supervised models were built to maximizes the discrimination between samples assigned to different classes. The variance and the predictive ability (R^2X, R^2Y, Q^2) and the CV-ANOVA were established to evaluate the suitability of the models. In addition, a permutation test (n=400) was performed to validate the models (Lindgren et al. 1996). The S-plot extracted the most significant variables from each model, and the ^{1}H-NMR data were identified using the Chenomx NMR Suite 7.1.

GraphPad Prism software (version 7.01, GraphPad Software, Inc., 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, two tests were used: 1) Wilcoxon test, a non-parametric statistical hypothesis test used to compare two related samples, matched samples, or repeated measurements on a single sample to assess whether their population mean ranks differ; 2) U-Mann Whitney test, a non-parametric statistical hypothesis test used to compare two independent samples (this test was used to compare all the groups at different time point with the control class).

Results

The study included 42 RRMS patients (54.8% female), of which 30 (71.43%) were classified as R after two years of FINGO treatment, whereas 12 patients were NR. Blood samples collected at T0, T6, T12 and T24 were available for the 15 patients who fully completed the experimental protocol; thus, only these patients were used to analyze the metabolic variation during the FINGO treatment. Twenty-two control subjects were also included in the study. The summary of the demographic data is reported in table 9.

Table 9. Demographic characteristics of MS patients and healthy controls.

Characteristics of Patients and Controls						
MS patients						
Patients	**Age ± SD Range**	**F/M**	**MS duration (mean years)**	**EDSS score (mean)**	**Inclusion criteria**	**Exclusion criteria**
42 SM-RR	39 ± 8.7 (22-56)	23/19	10 ± 6	3 ± 1.7	Adults ≥ 18 years of age	Corticosteroids exposure in the previous 30 days
					MS diagnosis according to McDonald 2010 criteria	Presence of other chronic comorbidities
					Relapsing remitting course	Use of other chronic medications
					Scheduled Fingolimod treatment	
Healthy controls						
22 C	40.8 ± 13.8 (20-67)	17/5	Adults ≥ 18 years of age		No family history of MS	Presence of chronic disease
						Use of chronic medications

Differences between age was not significant.

Samples were analyzed through the ^{1}H-NMR, which led to correctly identify forty-four hydrophilic compounds (Figure 17).

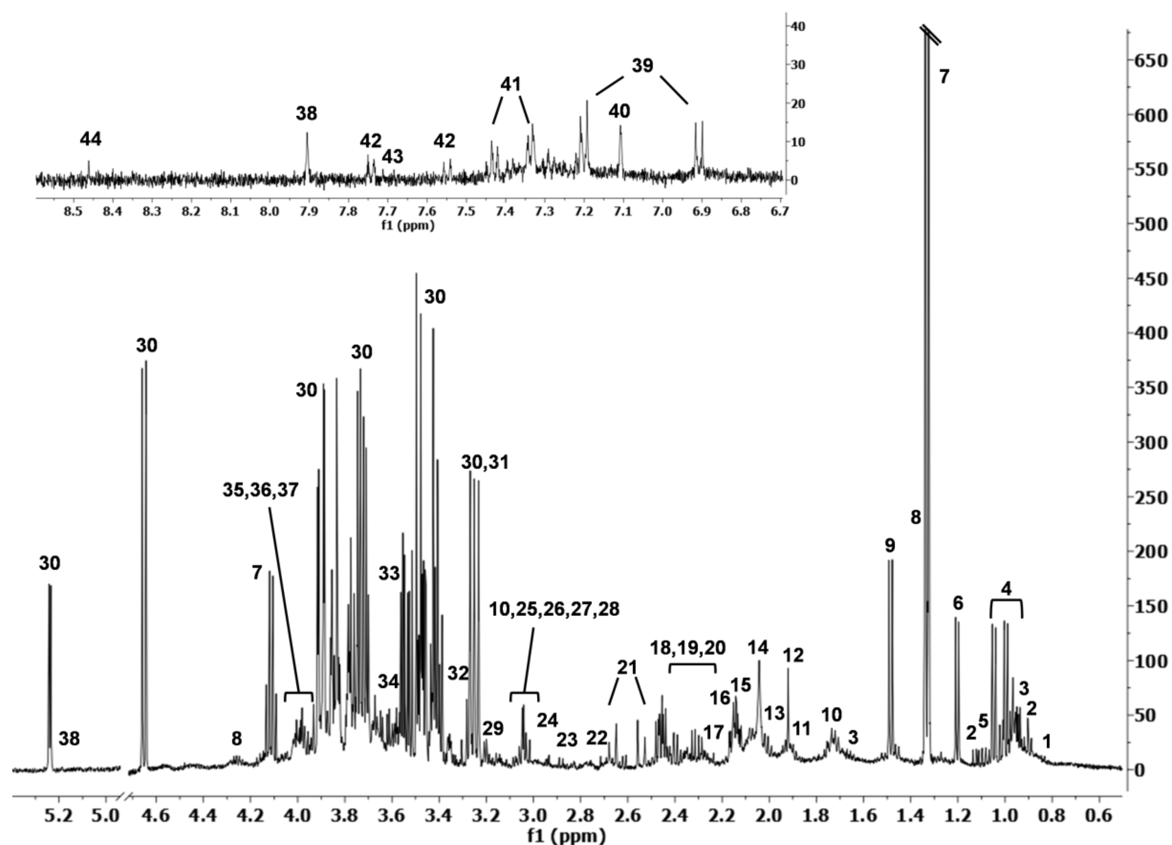

Figure 17. Signal's assignments of the serum metabolites in an ^{1}H-NMR spectrum: 1. 2-Hydroxyisovalerate; 2. 3-methyl-2-Oxoglutarate; 3. 2-Hydroxybutirate; 4.Branched Aminoacids: Valina, Leucine, Isoleucine, 5. 2-Methylglutarate; 6. 3-Hydroxybutirate; 7. Lactate; 8.Threonine; 9. Alanine; 10. Lysine; 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. τ methyl-Histidine; 44. Formate.

The non-supervised multivariate PCA was firstly applied using the bins dataset to examine clustering or separation trends between samples and find potential outliers. The obtained score plots and the result of the T^2 Hotelling test did not indicate the presence of outliers (figure 18).

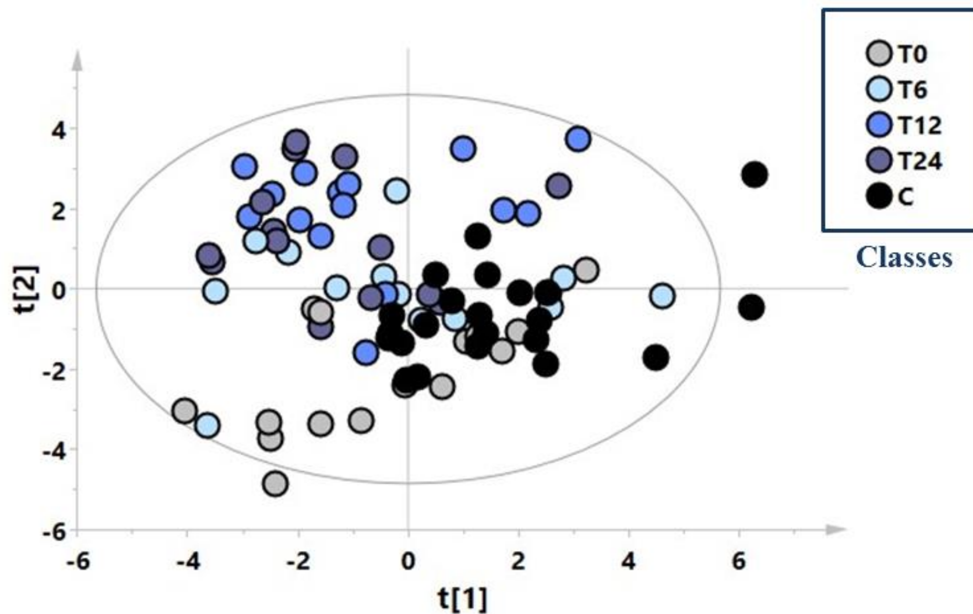

Figure 18. Unsupervised Principal Component Analysis model of the samples enrolled in the study. Grey dots represent the patients before the treatment, light blue dots represent the patients after 6 months of Fingolimod treatment, blue dots represent the patients after 12 months of Fingolimod treatment, violet dots represent the patients after 24 months of Fingolimod treatment, black dots represent the control subjects.

A longitudinal grouping of the samples was observed based on the period of the therapy intake and was subsequently observed by the application of the supervised model OPLS-DA.

Firstly, a supervised model was built considering all the samples, and as shown in figure 19A, a clear distribution of the samples at the different time points (T0, T6, T12, T24) was observed.

Furthermore, the close correlation between changes in metabolic profile and the duration of FINGO treatment (considered as months) is also represented in figure 19B, where PLS analysis resulted in an R^2=0.65.

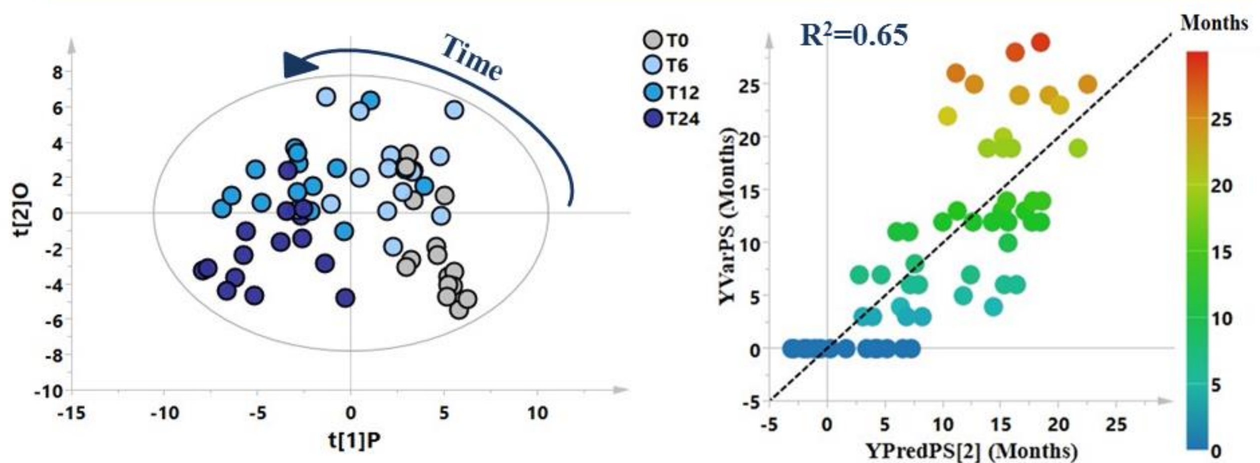

Figure 19. Multivariate models generated comparing all the groups of patients. Blood samples were collected at baseline (T0) and then at 6 (T6), 12 (T12), and 24 (T24) months of FINGO treatment. A) OPLS-DA model including all the samples; B) PLS correlation model generated using the x-variables (bins) and the y-variable as months of therapy (R^2=0.65).

Subsequently, to better explore metabolic changes occurring at T24, supervised single models were built comparing T0 with T6, with T12, and with T24. Figure 20 shows the results of the single comparisons between the classes.

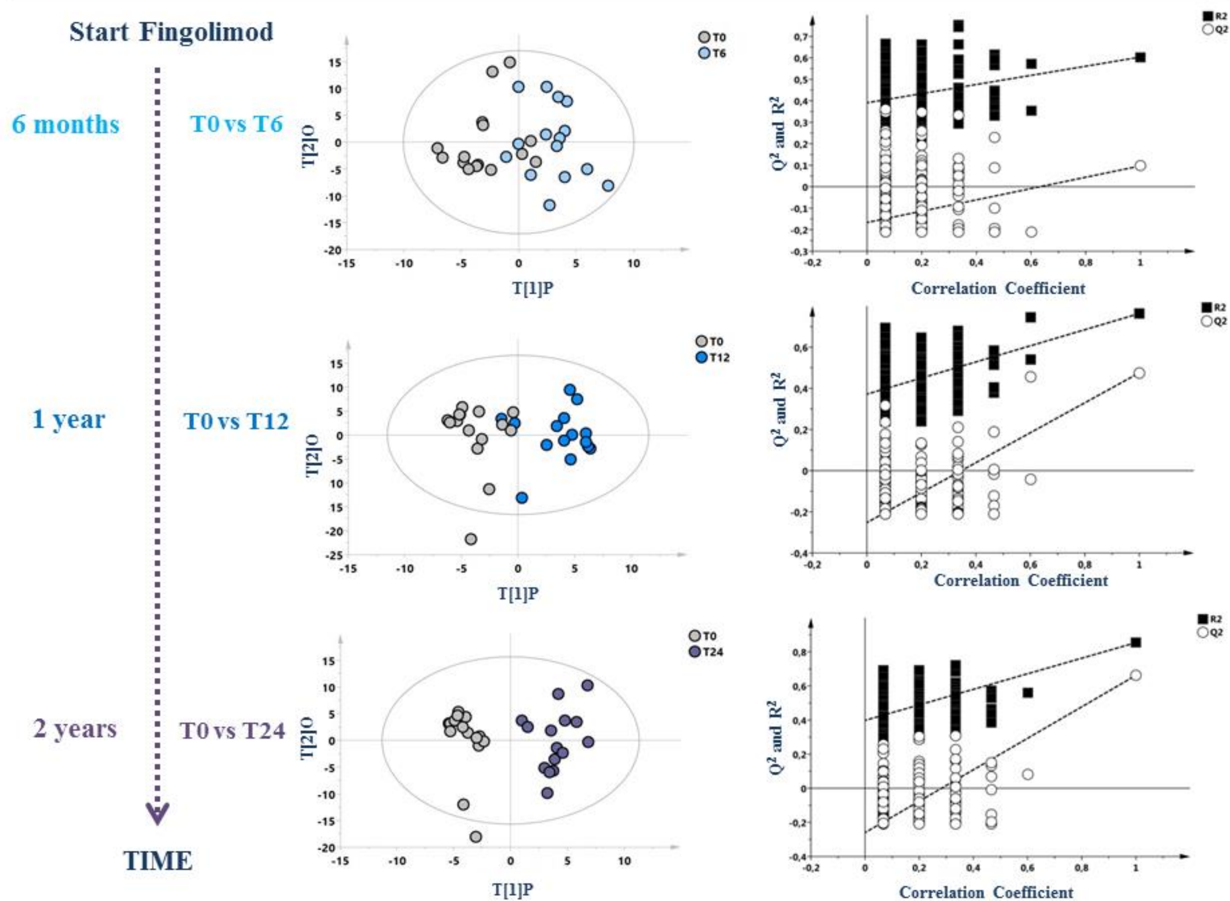

Figure 20. Supervised OPLS-DA models generated comparing the different classes of patients. Blood samples were collected at baseline (T0) and then after 6 (T6), 12 (T12), and 24 (T24) months of FINGO treatment. A-B) OPLS-DA model between T0 and T6 and the respective permutation test. C-D) OPLS-DA model between T0 and T12 and the respective permutation test. F-G. OPLS-DA model between T0 and T24 and the respective permutation test.

The statistical parameters (R^2X, R^2Y, Q^2, *p*-value, and data relative to the permutation tests) of the models are reported in table 10.

Table 10. Summary of the statistical parameters of the multivariate models of the comparisons between the classes of subjects. Blood samples were collected at baseline (T0) and then at 6 (T6), 12 (T12), and 24 (T24) months of FINGO treatment.

	SUPERVISED MODELS					
	N	**R^2X**	**R^2Y**	**Q^2**	***p*-value**	**Permutation test: Intercept R^2\\Q^2**
T0 *vs* T6 *vs* T12 *vs* T24	60	0.523	0.393	0.173	0.02	0.2/-0.33
T0 *vs* T6	30	0.40	0.60	0.08	ns	0.38/-0.4
T0 *vs* T12	30	0.42	0.76	0.52	<0.001	0.39/-0.55
T0 *vs* T24	30	0.51	0.90	0.72	<0.0001	0.56/-0.7
R vs NR	42	0.60	0.70	0.49	0.002	0.38/-0.6

By analysing the VIPs list and the S-plot relative to each model, it was possible to identify a set of metabolites changing their concentrations depending on the drug intake. Metabolites were identified and quantified by using the Chenomx NMR Suite.

In figure 21 the concentrations' graphs, showing almost one comparison with a *p*-value <0.05, are represented. The amino acids alanine, phenylalanine, glycine, pyroglutamic acid, and tryptophan showed a linear increase during the two years of treatment with FINGO. The same trend was also observed for fructose, glucose, 2-hydroxisovalerate and creatinine. By contrast, lactate, isoleucine, and glutamate, showed a decreased trend during the two years of treatment.

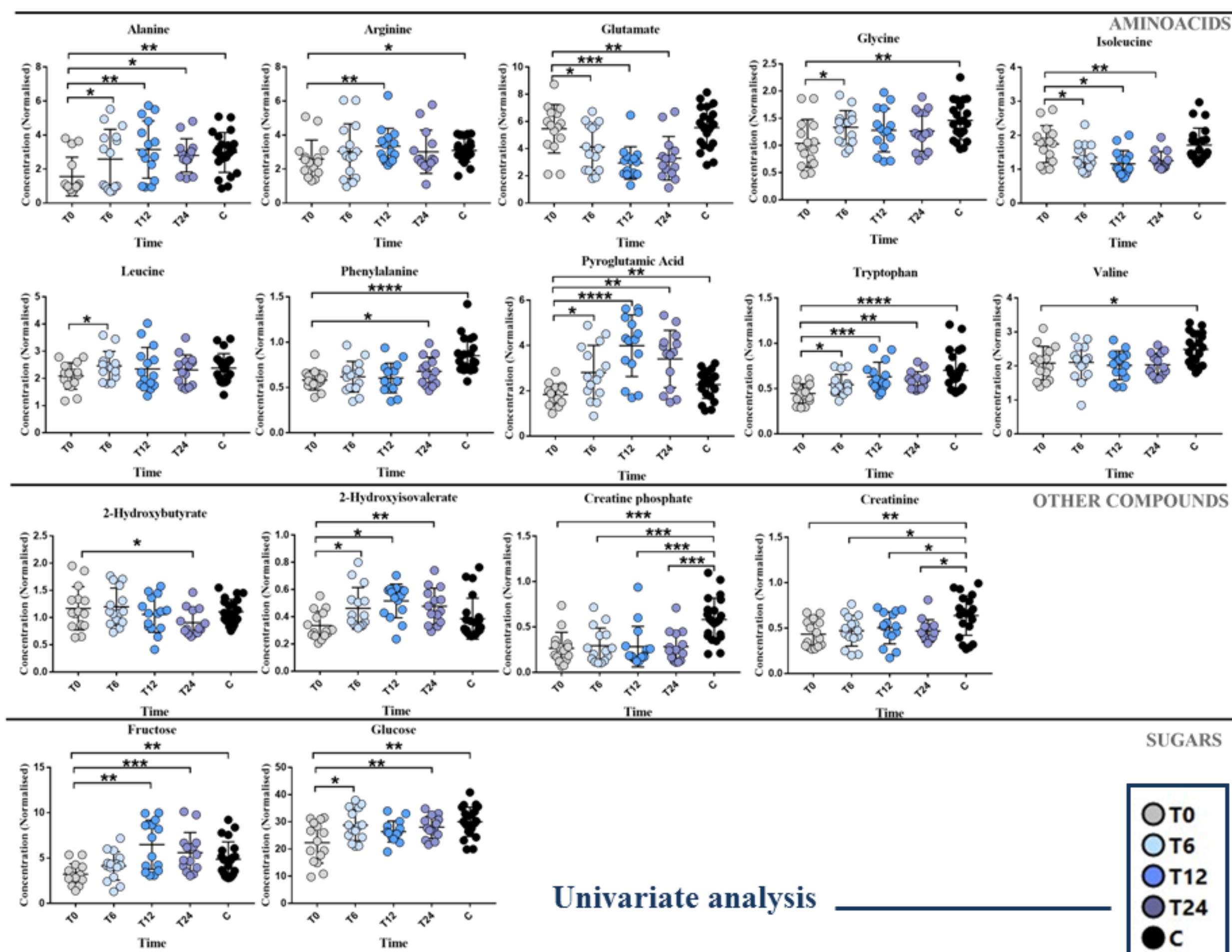

Figure 21. Most important metabolites identified by the analysis of the multivariate models (T0 *vs* T6, T0 *vs* T12, and T0 *vs* T24) and the control group. Graphs indicating trends of the most important metabolites having p-value <0.05 in at least one comparison of the different groups after the application of the Wilcoxon test (T0 *vs* T6, T12, T24) or U-Mann Whitney test (SM *vs* C). * means < 0.05.

Figure 22 shows an OPLS-DA model with three classes, including each patient's T0 and T24 samples and the controls' samples. Data obtained demonstrated that the metabolomic profile of patients treated for 24 months was different from the basal profile (T0) and more similar to the control one ($R^2X = 0.44$, $R^2Y = 0.67$, $Q^2 = 0.\ 49$, $p < 0.001$), although constituting a distinct class, as shown in the scores plot of the model.

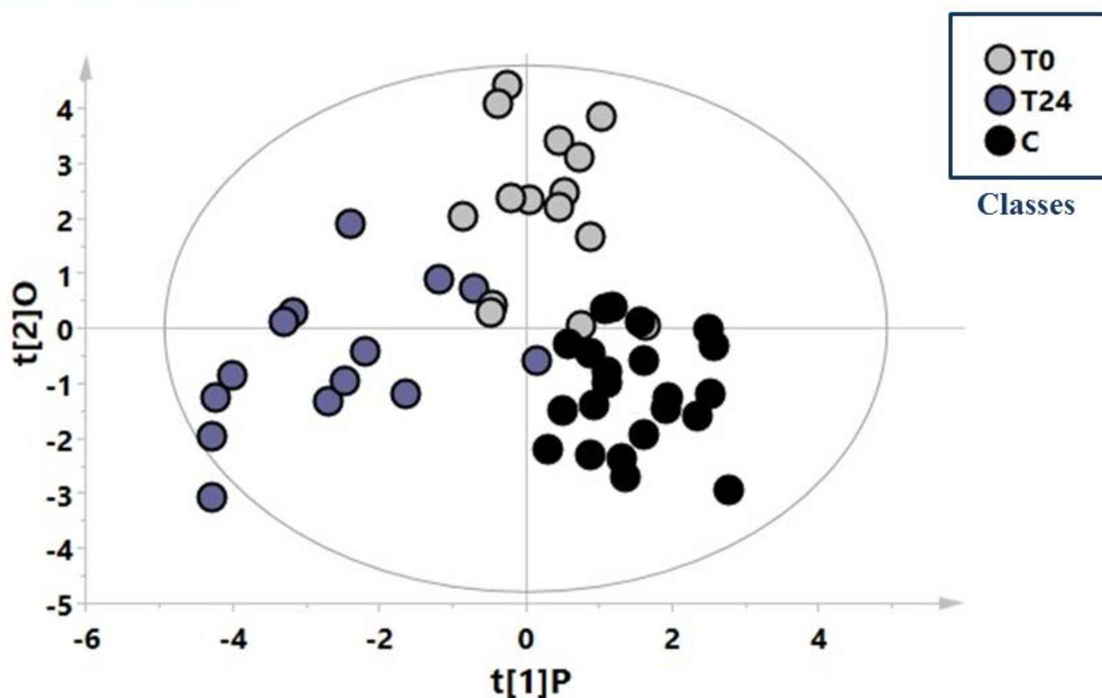

Figure 22. OPLS-DA model with 3 classes, including the T0 (grey circles) and T24 (violet circles) samples of each patient and the samples from healthy controls (black circles). The blood samples included in the OPLS-DA model were collected at baseline (T0) and 24 months (T24) of FINGO treatment.

Moreover, the supervised OPLS-DA model shows the comparison between not-responders (NR) and responders (R) patients (according to NEDA 3 definition) considering only the T0 samples (Figure 23A, R^2X=0.6, R^2Y=0.7, Q^2=0.493, p = 0.002). Characteristics of the patients are reported in Table 11.

Table 11. Baseline characteristics of the responder (R) and not-responder (NR) patients.

Baseline Characteristics	R patients (n=30)	NR patients (n=12)
Age (mean) ± SD	37 ± 8.6	43 ± 7.8
Gender F/M	18/12	5/7
MS duration (mean years)	9	13
EDSS score (mean)	3 ± 1.8	3 ± 1.6
MRI activity (Gd + lesions)	0	8 (67%)

p-value age <0.05, *p*-value MS duration <0.05, *p*-value MRI activity <0.05.

The model was validated through the respective permutation test (figure 23B), and the most important variables were identified by analysing the V-plot and using the corresponding VIP value. Variables with a value > 1 were considered the most relevant (Figure 23C). The R group was characterized by an increased concentration of lactate, and lysine, while the NR group showed a high glucose level.

Multivariate model

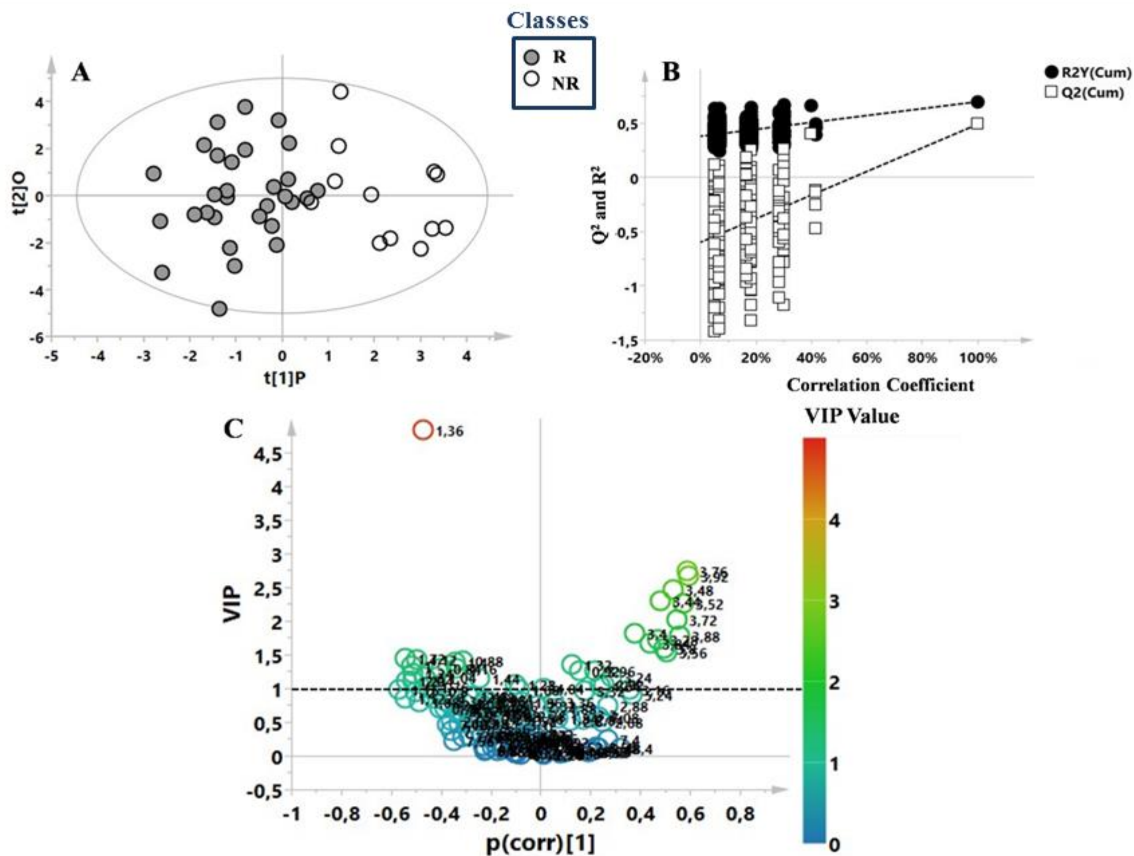

Figure 23. A) OPLS-DA model obtained from 42 plasma samples of MS patients at time point T0. Patients were classified as responders (R) and non-responders (NR) after FINGO treatment, according to NEDA 3 definition. Statistical parameters were $R^2X = 0.592$, $R^2Y = 0.70$, $Q^2 = 0.493$, $p = 0.002$. B) The model was validated through the respective permutation test. C) Volcano plot indicating the most important variables responsible of the separation of the samples.

Finally, a supervised model was built between patients who showed cardiac-adverse events after the FINGO treatment (n=3) and patients who did not show any cardiac-adverse events. For this aim, only the T0 samples were considered (figure 24). The statistical parameters were R^2X=0.53, R^2Y=0.86, and Q^2=0.49, but the *p*-value was not significant, probably due to the small size of the group.

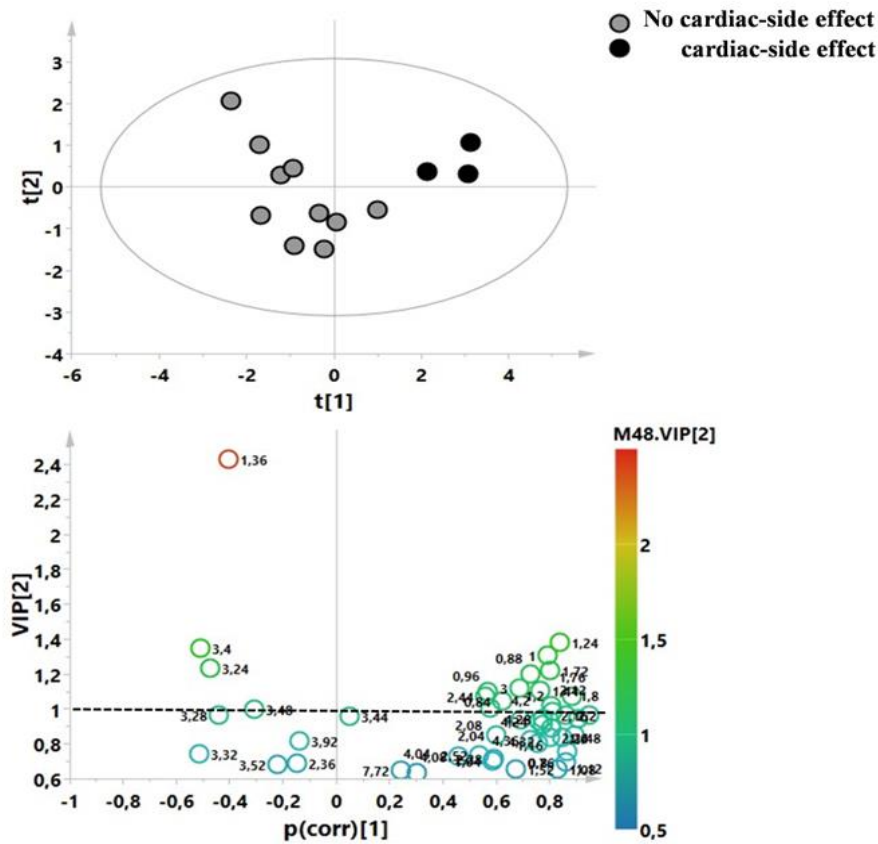

Figure 24. Supervised model of patients who showed cardiac-adverse events after the FINGO treatment and patients who did not show any cardiac-adverse events. The statistical parameters were $R^2X=0.53$, $R^2Y=0.86$, and $Q^2=0.49$, but the *p*-value was not significant.

Discussion

Fingolimod (FINGO) (Gilenya; 1.25 mg) is an oral drug approved by the FDA in 2010 as a first-line treatment for RRMS, and by the EMA in 2011 as a second-line RRMS treatment. FINGO acts by modulating sphingosine-1-phosphate (S1P) receptors, inhibiting the egress of T and B cells from lymph nodes into blood, and thus their circulation in the CNS, with protective effects on MS inflammatory damage. Several trials and real-world studies have demonstrated the efficacy/effectiveness of FINGO on MS activity as well as on disease progression in adult and

pediatric MS patients (Kappos et al. Cohen et al. 2010, Fonseca et al. 2015). After the first dose, rare transient FINGO-associated side effects have been reported, mainly consisting in bradycardia, and heart conduction abnormalities, usually asymptomatic, while the risk of infections during treatment was evidenced, even if severe opportunistic infections were rarely observed. The active form of FINGO binds a G protein-coupled receptor subtype (S1P1), thus inducing the seizure of lymphocytes inside the lymphoid organs. This determines the strong reduction of the number of circulating CD4+ and CD8+ cells (Ingwersen et al. 2012) which results in an effective reduction of inflammatory disease activity. A recent Cochrane revision by La Mantia et al. on 3531 RRMS patients indicated that a 24-month (T24) FINGO treatment had an increased probability of being relapse- free compared with 6 (T6)- and 12-month (T12) treatments (La Mantia et al. 2016) , although a substantial variability in the drug response was observed among RRMS patients. On these bases, in the first phase of this second aim of the study we analysed the plasma metabolic modifications in a group of naïve RRMS patients (T0) treated with FINGO and monitored periodically (T0, T6, T12, T24) for two years. As expected, FINGO treatment induced significant changes in the patient's metabolomic profile depending on the time of drug exposure (see Fig 19). Interestingly, the metabolic profile related to T24 time-point resulted almost similar to that identified in the healthy control group, possibly indicating a reapproaching of health's normality (Fig 20,21). This is suggested by the series of OPLS-DA models shown in figure 20, where naïve patients (T0) are compared with T6, T12 and T24 patients. As discussed below, the metabolic phenotypes identified indicated a shared pattern of metabolites involved in two main processes: 1) the reduction of the inflammatory response and 2) the reduction of oxidative stress, both typical features of RRMS patients.

AAs metabolism is highly involved in MS (Correale et al. 2017), as it represents a chronic inflammatory demyelinating disease, mediated by Th1, Th17, and B cell activities, that need continuous access to AAs to maintain basal metabolism. RRMS patients showed different AA

trends passing from T0 to T24. Circulating branched-chain AAs, valine and isoleucine, levels gradually decreased in RRMS patients compared with controls. By contrast, plasma leucine levels increased at T6 and remained stable until the end of the FINGO treatment (two years). Therefore, reduced plasma valine and isoleucine levels could indicate a weakened T cell activity and a recovery of immune homeostasis (Fitzgerald et al. 2021).

The reduction of the inflammation response is also evidenced by glycine plasma levels which gradually increase at T6 while reaching a steady state level during the rest of the two years of therapy. Glycine is the major inhibitory neurotransmitter in the brain possessing anti-inflammatory properties which are represented by a systemic modulation of immune cell functions (Carmans et al. 2010). The increased glycine concentration could interact indirectly with glutamate to maintain neural homeostasis (Ďurfinová et al. 2018) by influencing the release of inhibitory amino acids from neurons and astrocytes. Glutamate is a critical mediator of brain function, and its excess, which is associated with the overstimulation of its receptors, may cause excitotoxic injury of neurons and glial cells (Grooms et al. 2003, Pitt et al. 2003). This is particularly important for MS patients, as demonstrated in the study by Sarchielli et al., where either RRMS or secondary progressive MS patients presented increased glutamate concentration in CSF during relapse and in a stable clinical phase (Serchielli et al. 2003). In line with these results, we found a linear and significant decrease in glutamate levels in RRMS patients treated with FINGO which may indicate protection from CNS damage. In agreement with this hypothesis, Noda and Serpero recently demonstrated that patients treated with FINGO had a down-regulation of microglial production of proinflammatory cytokines known to induce hyperactivity of glutamatergic transmission (Noda et al. 2013, Serpero et al. 2013, Centonze et al. 2010, Groves et al. 2013, Pieragostino et al. 2015, Villoslada et al. 2017) , such as tumor necrosis factor-alpha, interleukin-1, and interferon-gamma.

Pyroglutamic acid is a precursor of glutamate which has resulted significantly increased in RRMS patients after FINGO treatment. It is formed through the cleavage of glutathione mediated by the 5-

oxoprolinase in the γ-glutamyl cycle, in which glutathione is decomposed into γ-glutamyl amino acid and again converted to pyroglutamic acid by γ-glutamyl cyclotransferase (Bachhawat et al. 2018). Therefore, the increased plasma levels of pyroglutamic acid in RRMS patients treated with FINGO may indicate the drug-promoted restoration of sera glutathione levels which, in turn, may counteract the oxidative stress typically associated with MS disease.

Several studies have demonstrated that arginine metabolism is frequently altered in MS, in both human and animal models (Murgia et al. 2020, Stojanovic et al. 2012, Mangalam et al. 2013). The conversion of arginine to citrulline represents an important event in the chemical pathogenesis of the demyelinating disease. Therefore, as plasma arginine concentration significantly increased in FINGO-treated RRMS patients, this may indicate a reduced conversion rate to citrulline. This may result in protection from the citrulline-mediated adverse effects, such as destabilization of the membrane architecture and myelin degradation (Pritzker et al. 2000).

Tryptophan has also been shown to contribute to the disease progression of MS through the activation of KP (Rajda et al. 2015). In our study, tryptophan resulted significantly increased in all the time points analysed compared to the T0, possibly due to the reduced activity of the KP pathway. This finding is in line with our previous study showing lower tryptophan levels in MS patients compared to healthy controls (Lorefice et al. 2019). A mechanistic explanation is that FINGO modulates cytokines (including IFN-γ and TNF-α) which are potent inducers of the first-rate limiting enzyme of the KP, namely indoleamine-2-3 dioxygenase (IDO), thus preventing the conversion in quinolinic acid. This latter is a strong N-methyl-D-aspartate (NMDA) receptor agonist which can over-activate NMDA receptors to increase intracellular Ca2+ levels thus leading to oxidative stress and cell death through glutamatergic excitotoxicity (Moroni et al. 2012, Guillemin et al. 2012). Moreover, KP is also implicated in the regulation of the immune system (Vecsei et al. 2013). In particular, IDO is present in various immune cells and can be induced by interferons and LPS (Mandi et al. 2012). The activation of IDO is an important regulator of immune

activation as it counteracts the proliferation of reactive lymphocytes (Rajda et al. 2015). Thus, two years of FINGO treatment, by reducing plasma tryptophan to control levels, may have protected RRMS patients from oxidative stress, cell death, and immune activity.

Plasma glucose and fructose levels resulted statistically increased in RRMS patients starting from T0 up to T24, where the levels of the control group were reached. Glucose is the primary source of energy in the mammalian brain, where it is used in the form of ATP for neuronal and non-neuronal cell survival and the generation of neurotransmitters. In addition, it also represents a substrate consumed by immunity cells to sustain the inflammatory responses (Buttgereit et al. 2000). A situation of chronic immune system activation can lead to overcoming the physiological bioenergetics metabolism consuming a considerable amount of energy (up to 2,000-kJ/day and more) (Straub et al. 2010). Therefore, lower glucose concentrations in RRMS patients at T0 can be associated with its consumption for greater production of proinflammatory cytokines, whereas its increase may indicate a decreased inflammation response due to FINGO treatment.

Several studies have suggested a positive correlation between 2-hydroxybutyrate, arising from lipid oxidation and the pathogenesis of MS due to oxidative stress. Accordingly, the high levels of 2-hydroxybutyrate found in RRMS patients at T0 were considered markers of increased oxidative stress. Opposite, RRMS patients treated with FINGO for two years showed a significant reduction of plasma 2-hydroxybutyrate, indirectly suggesting a reduction of oxidative stress (Kim et al. 2017).

Finally, plasma levels of the metabolite creatine and creatine phosphate did not change during the treatment, possibly indicating muscle weakness, a typical feature in MS patients.

In summary, it is possible to state that although a characteristic profile of the patients affected by RRMS is identifiable at T0, nevertheless, this metabolic phenotype significantly evolves under pharmacological treatment, up to resemble that of the healthy control group[11].

Among RRMS patients some of them responded to FINGO treatment at T24 (R) while others did not respond (NR), according to the NEDA 3 definitions, as extensively discussed in the material

and methods section. Thus, in the second phase of this aim, we have evaluated if specific metabolomic characteristics present at baseline (T0) could predict the therapeutic response to FINGO treatment. Although the preliminary data obtained needs to be further verified in a greater number of samples, they have shown that while the R group was characterized by increased plasma levels of lactate and lysine, the NR group showed high glucose levels. As lactate is the final product of anaerobic glycolysis, its increased basal levels in R patients may suggest the presence of an energy impairment. Conversely, NR patients were characterized by high plasma levels of glucose which could indicate an impairment of glucose metabolism. Moreover, an explorative analysis was conducted at T0 between RRMS patients showing cardiac-side effects after pharmacological treatment (R) and patients who did not show any cardiac-side effects (NR). Unfortunately, the mathematical model used turned out to be not statistically significant, probably due to the small size of the cardiac-side effect group, preventing us to speculate about the possible predictive metabolic biomarkers related to these adverse events.

5. Aim 3: Investigation of the protective role of pregnancy on MS progression by a metabolic point of view.

Materials and Methods

Patients

A total of 155 blood samples were collected at the Multiple Sclerosis Centre of the Binaghi Hospital, Cagliari, from patients affected by MS (according to 2017 revisions of the McDonald criteria). Samples of women affected by MS were collected before and during pregnancy (fertile n=68, pregnancy n=49 respectively) and during the puerperium (woman who has just given birth n=38). Samples of demographically and ethnically matched healthy women were also collected at the Department of Obstetrics and Gynecology of the Policlinico Hospital of Cagliari in the same physiological conditions (before and during pregnancy and during the puerperium). Demographic data are reported in Table 12.

Table 12. Demographic data of the enrolled women.

	Fertile			Pregnancy			Puerpere		
	n	Age ± SD (range)	EDSS (mean)	n	Age ± SD (range)	EDSS (mean)	n	Age ± SD (range)	EDSS (mean)
Healthy	28	31 ±6 (21-43)	-	26	34 ± 6 (16-39)	-	14	33 ± 5 (20-41)	-
MS	68	33 ± 7 (19-46)	1.6	49	34 ± 4 (24-40)	1.6	38	33 ±5 (25-42)	1.4
<u>Therapies</u>									
Interferon β	3								
Glatiramer acetate	3								
Natilizumab	5			4					

Differences between age was not significant.

The institutional ethics committee approved the study, and written informed consent was obtained from each participant.

Sample preparation and data analysis

Sample were prepared and acquired as described in the studies above as the extraction of the hydrophilic phase for serum and plasma is exactly the same. Briefly, serum was extracted by using the Folch method and the hydrophilic phase was concentrated, re-suspended in D_2O and analyzed with the NMR Varian UNITY INOVA 500 spectrometer. Spectra were manually phased and baseline corrected. The chemical shifts were referred to the internal standard, TSP, using the MestReNova software.

Data processing and multivariate analysis were mainly performed starting with the division of each spectrum into consecutive "bins" 0.04 ppm wide. The spectral area under investigation was the region between 0.6 and 8.6 ppm. To remove variations in the presaturation of the residual water resonance and spectral regions of noise, the regions between 4.64 and 5.2 ppm and between 5.32 and 6.6 ppm were excluded. The integrated area within each bin was normalized to the total area=100, in percent values, to minimize the effects of the different concentrations of plasma samples. This generated a final dataset consisting of a 182X223 matrix (variables x patients).

The procedure followed to perform the statistical analysis was similar to the previous study. Briefly, the matrix generated underwent multivariate statistical analysis through the SIMCA-P software (v16.0; Umetrics, Malmö, Sweden). To emphasize all metabolite signals and reduce the spectral noise, the variables were Pareto-scaled. Initially, data analyses were conducted using principal components analysis (PCA), to identify potential outliers and then PLS-DA and OPLS-DA were subsequently applied to maximize the discrimination between samples assigned to different classes and highlighted the variables responsible of the separation. The variance and the predictive ability (R^2X, R^2Y, Q_2) were evaluated for each model as well as the CV-ANOVA ($p<0.05$) and the permutation test (n=400) to validate the models. The most significant variables were extracted by the loading plot and S-plot from each model and identified and quantified using the Chenomx NMR Suite 7.1 which exploits a custom library for unidentified resonances to carry them through the

analysis for relative concentration comparisons. Concentrations of the discriminant metabolites were tested with the univariate statistical analysis, in particular, U-Mann Whitney test was performed to test the significance between mean values of the metabolites between different classes of patients.

Finally, the pathways analysis was conducted with the software MetaboAnalyst 5.0 to explore the most important altered nets involved in the different women life phases based on the presence of the MS.

Results

We performed the analysis of the matrix containing all samples comparing the different stages of the experimental design as follows:

- Comparison of healthy women: fertile *vs*. pregnant, pregnant *vs*. puerperium, fertile *vs*. puerperium.
- Comparison of MS women: fertile *vs*. pregnant, pregnant *vs*. puerperium, fertile *vs*. puerperium.
- Comparison of healthy *vs*. MS: fertile period, pregnancy period, and puerperium period.

A graphic representation of the sequence of the analysis is summarized in figure 25.

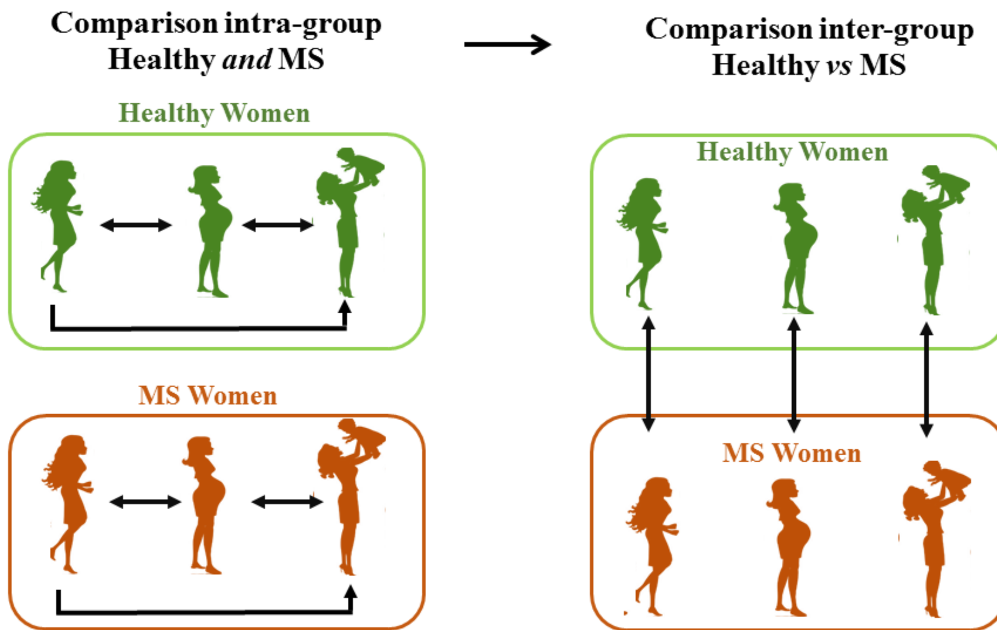

Figure 25. Summary of the analysis performed in this project.

Firstly, to find potential strong outliers which could be confusing for the analysis we applied an unsupervised PCA model (figure 26). Data obtained showed that no strong outliers were present, so all samples were considered for the subsequent analysis.

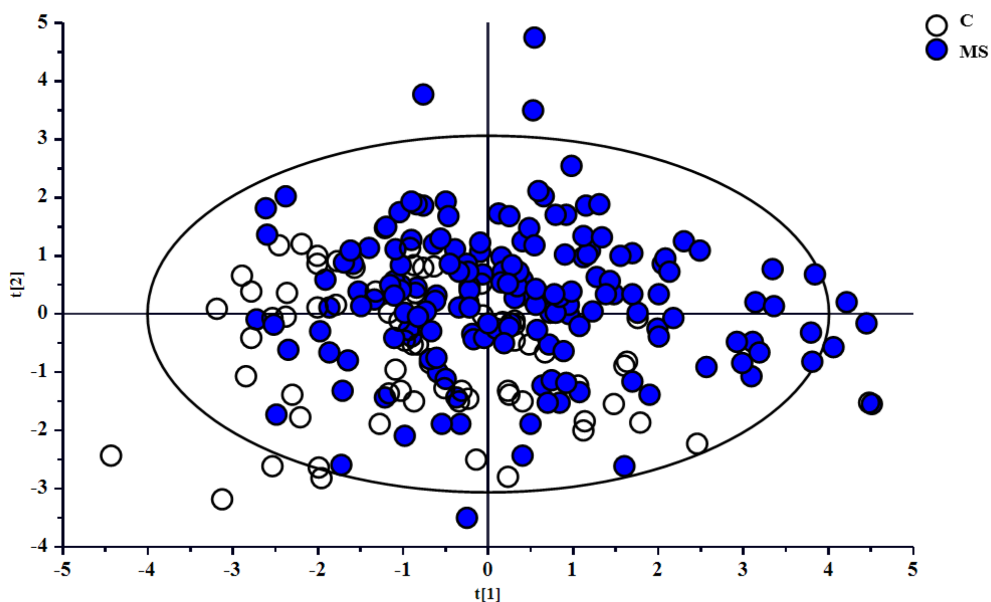

Figure 26. Principal Component Analysis of the samples belonging to the two classes of patients, Healthy controls and MS patients.

As some patients were under therapy during the sample collection, it was evaluated if this factor could affect the analysis of the metabolic profile. The application of a PCA model marking with different colors the patients with therapies showed that these samples were perfectly clustered with the others (figure 27).

Therapy effect

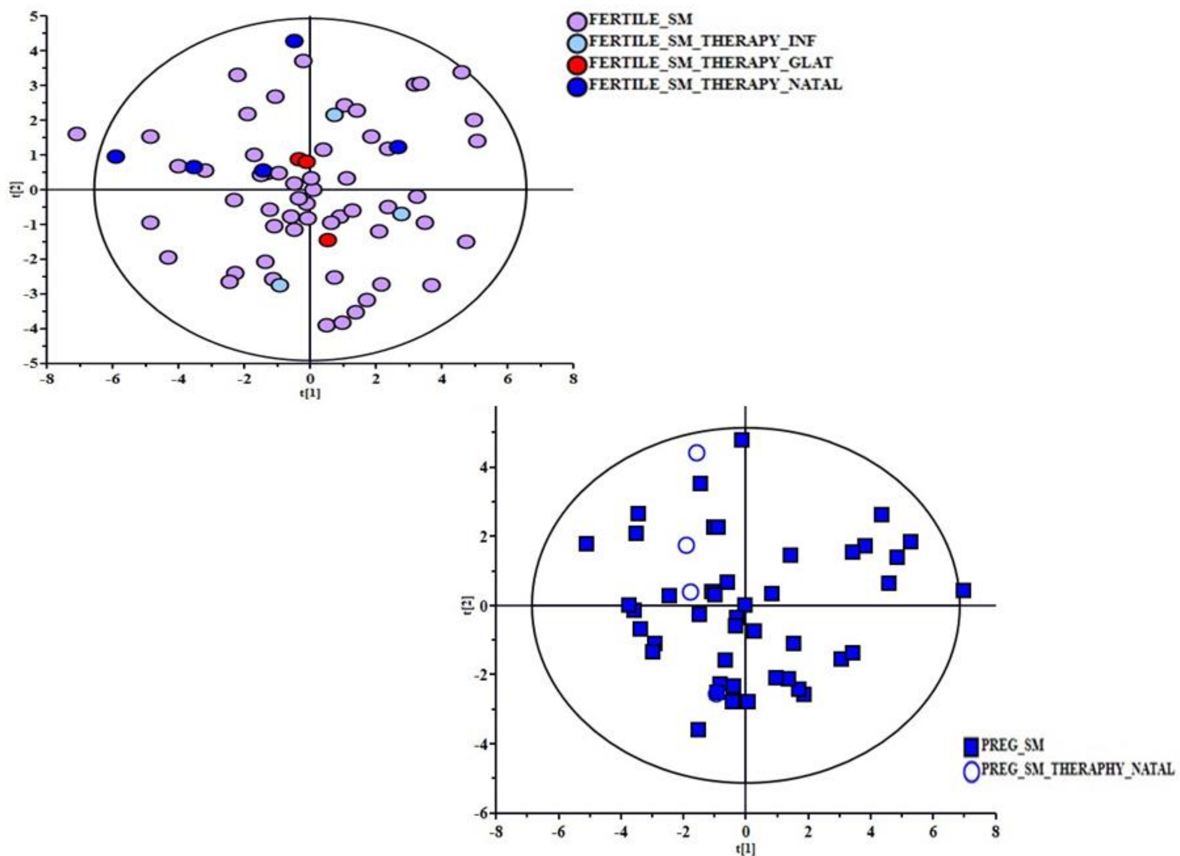

Figure 27. PCA model of the patients enrolled in the study. Colours were assigned based on the different therapy (Interferon beta, Glatiramer acetate, Natalizumab). A) Indicate women at fertile age, B) women during pregnancy.

Next, the analysis using the supervised models was started. Firstly, the analysis was performed by classifying separately the control and MS women according to their different life phases: fertile, pregnancy and puerperium. As shown in figure 28 a clear separation was found both in the control (statistical parameters were R^2X=0,5; R^2Y=0,7; Q^2=0,4; p<0,00001, A) and MS classes (statistical parameters were R^2X=0,5; R^2Y=0,5; Q^2=0,3; *p*<0,00001, B).

SUPERVISED MODELS

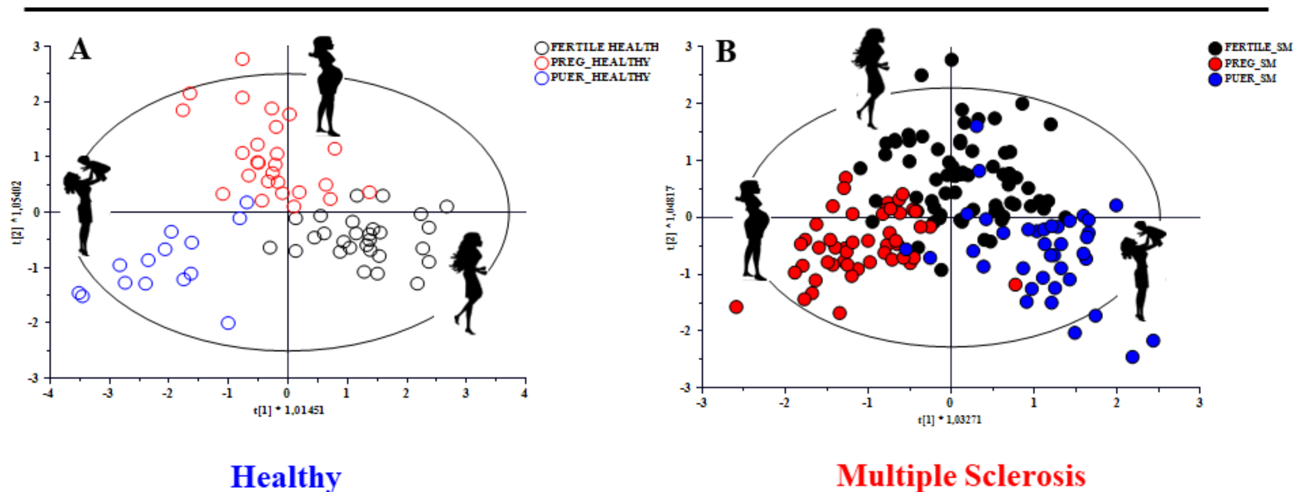

Figure 28. Supervised models of the classes of the controls and MS women. A) Healthy subjects were divided based on the belonging to fertile age (black empty dots), pregnancy (red empty dots), and puerperium (blue empty dots). B) MS subjects were divided based on the belonging to fertile age (black full dots), pregnancy (red full dots), and puerperium (blue full dots).

Moreover, to identify the metabolites responsible for the separation of the different classes (fertile, pregnancy, puerperium) for both controls and MS women, we performed single comparisons. The data obtained for healthy and MS women are shown in figures 29 and 30, respectively. All the models showed a Q^2 between 0.4 and 0.5 and $p<0.0001$ and were validated with the respective permutation test.

Healthy women

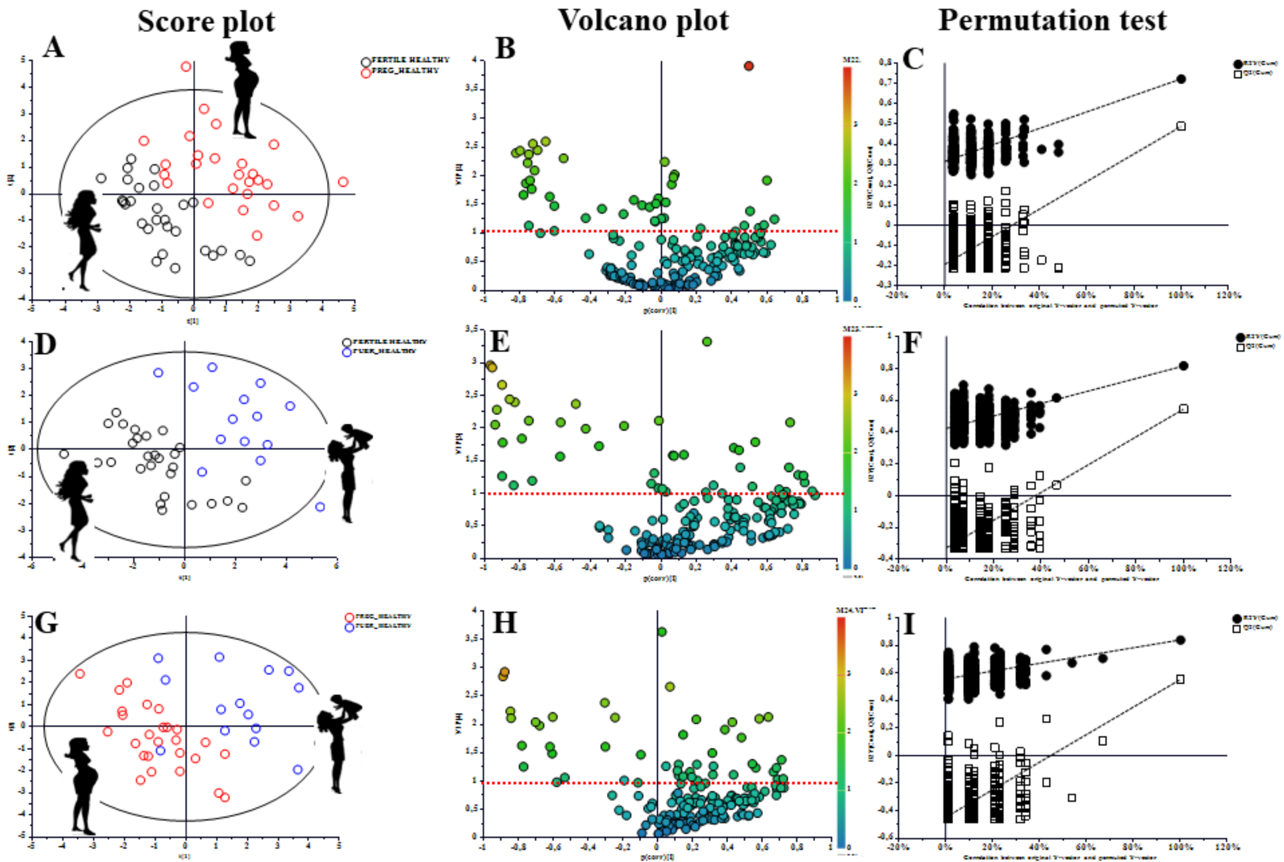

Figure 29. Single comparisons of the healthy women. A) Score plot of the comparison between fertile (black empty dots) and pregnant women (red empty dots). B) volcano plot of the comparison between fertile and pregnant women. C) permutation test of the comparison between fertile and pregnant women. D) Score plot of the comparison between fertile (black empty dots) and puerperium women (blue empty dots). E) Volcano plot of the comparison between fertile and puerperium women. F) Permutation test of the comparison between fertile and puerperium women. G) Score plot of the comparison between pregnancy (red empty dots) and puerperium women (blue empty dots). H) Volcano plot of the comparison between pregnancy and puerperium women. I) Permutation test of the comparison between pregnancy and puerperium women.

MS Women

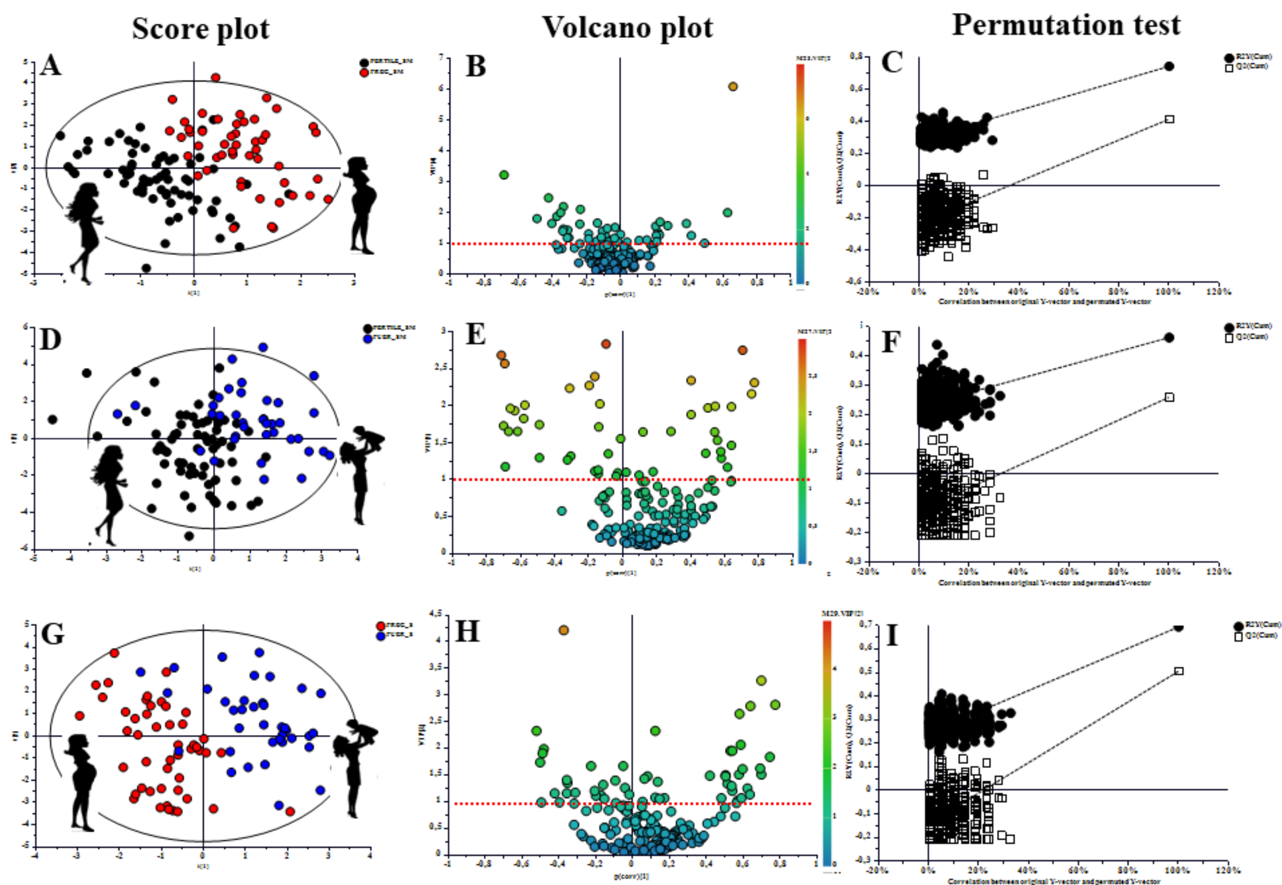

Figure 30. Single comparisons of the healthy women. A) Score plot of the comparison between fertile (black full dots) and pregnant women (red full dots). B) volcano plot of the comparison between fertile and pregnant women. C) permutation test of the comparison between fertile and pregnant women. D) Score plot of the comparison between fertile (black full dots) and puerperium women (blue full dots). E) volcano plot of the comparison between fertile and puerperium women. F) permutation test of the comparison between fertile and puerperium women. G) Score plot of the comparison between pregnancy (red full dots) and puerperium women (blue full dots). H) volcano plot of the comparison between pregnancy and puerperium women. I) permutation test of the comparison between pregnancy and puerperium women.

By analyzing the volcano plot relative to each model, it was possible to find several metabolites which changed their concentration based on the different life phases. Unexpectedly, a partially shared altered metabolic profile was found between healthy and MS women during the three phases analyzed. This, indicate that pregnancy and puerperium strongly affected the metabolism

overcoming the differences due to the presence of the disease. The graphs of the metabolites which resulted in significantly changed in at least one comparison are shown in figure 31 and 32. Most of them presented the same trend in both classes, while someone showed an opposite trend or a class specificity. The summary of the trend is reported in Table 13.

HEALTHY

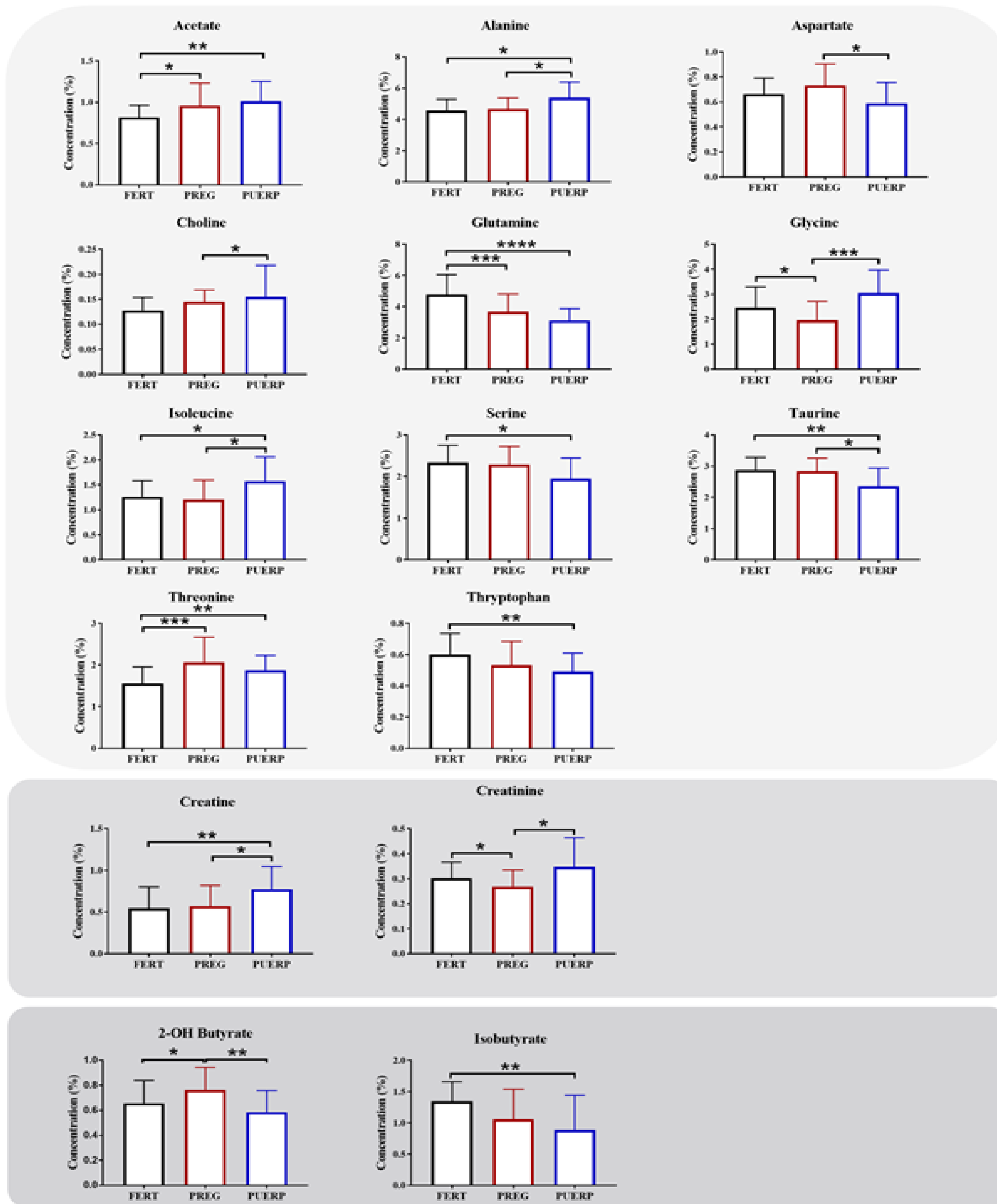

Figure 31 Graphs of the metabolites which resulted significantly changed in at least one comparison in the healthy class.

HEALTHY

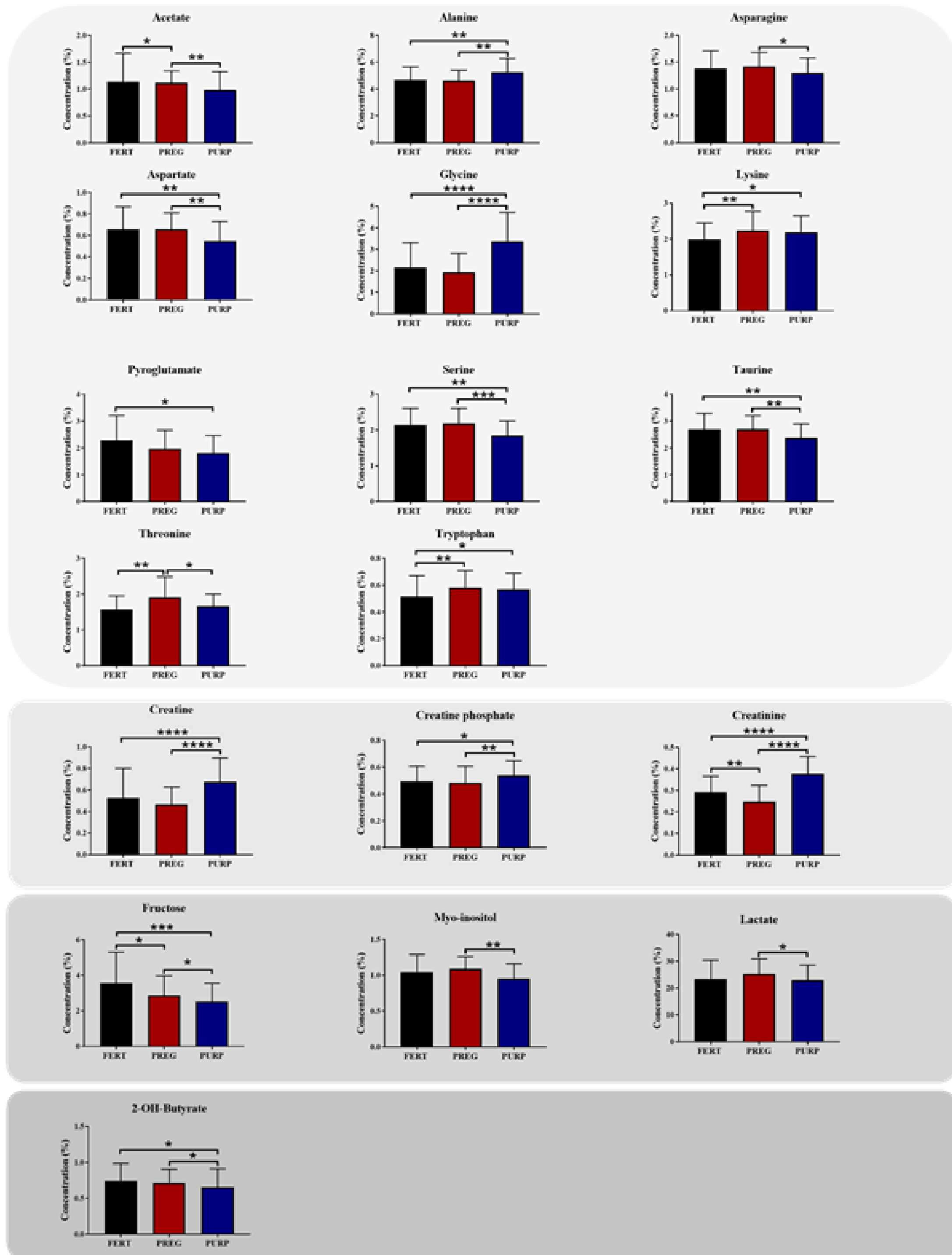

Figure 32 Graphs of the metabolites which resulted significantly changed in at least one comparison in the MS class.

Table 13. Summary of the trend of the metabolites which significantly changed their concentration in at least one comparison in each class of women.

Same trend	Opposite trend	Specific for Healthy women	Specific for MS women
Alanine	Acetate	Choline	Asparagine
Aspartate	Tryptophan	Glutamine	Pyroglutamic
Glycine	2-OH-butyrate	Isoleucine	Fructose
Serine		Isobutyric acid	Myo-Inositol
Taurine			Lactate
Threonine			
Creatine			
Creatinine			

Subsequently, the discriminant metabolites were used to perform the pathway analysis which is important to underline alterations correlated with the different biological phases of the women. The results of the analysis demonstrated a shared alteration between healthy and MS women in the same most important pathways, namely oxidative stress (urea cycle, ammonia recycling and glutathione metabolism), energetic pathways (amino sugar metabolism, phenylacetate metabolism, glucose alanine cycle), amino acid metabolism (mainly glutamate metabolism, aspartate metabolism, glycine and serine metabolism, alanine metabolism, arginine and proline metabolism, taurine and hypotaurine metabolism, methionine and tryptophan metabolism) and biosynthetic activity (purine and pyrimidine metabolism, phosphatidylcholines biosynthesis, etc. Figure 33).

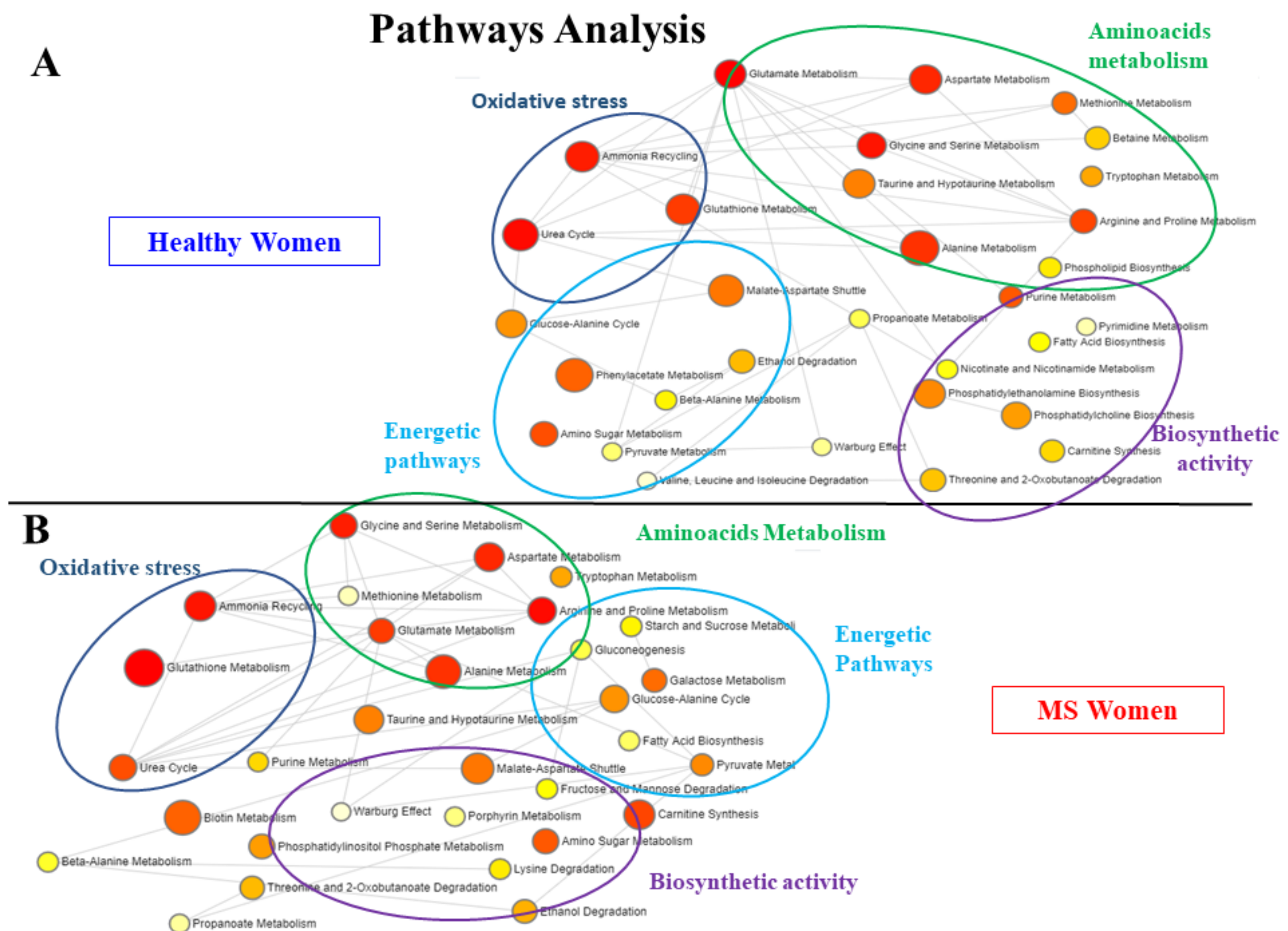

Figure 33. Pathways analysis of the classes under study: healthy women (A), and MS women (B). A shared panel of altered pathways were found.

Finally, we performed the supervised analysis to compare the different life phases, fertile age, pregnancy and puerperium, between the healthy and MS women. Interestingly, only the comparison at the fertile age evidenced a significant result (statistical parameters were R^2X=0,4; R^2Y=0,4; Q^2=0,3; p<0,00001, figure 34). More in detail, the most discriminant metabolites showing a significant decrease in the MS patients at fertile age were creatine phosphate, glutamine, isobutyrate, leucine, lysine, phenylalanine, tryptophan, tyrosine, while acetate, lactate, pyroglutamate, pyruvate appeared to be increased.

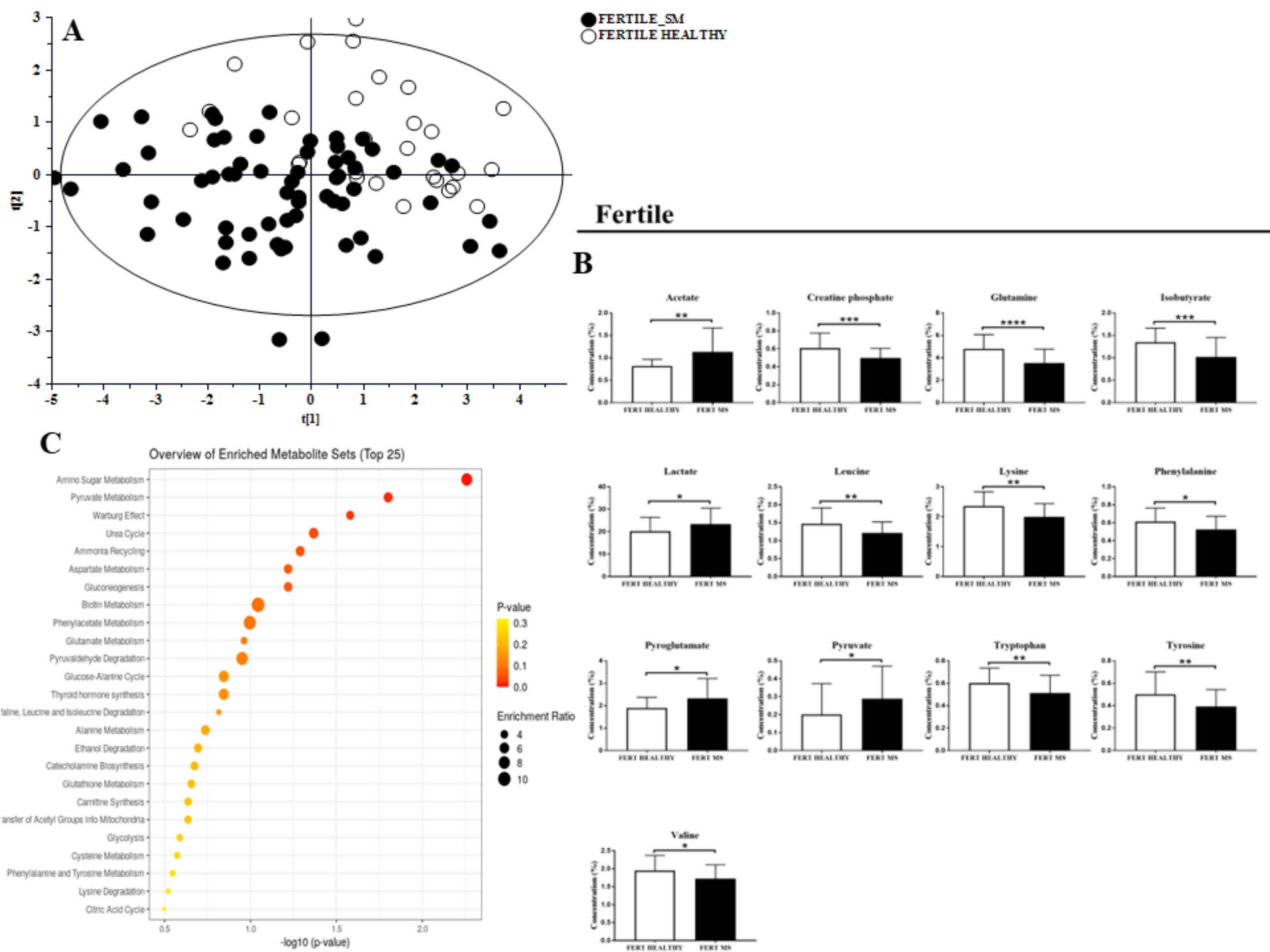

Figure 34. Analysis resulted from the comparison between healthy women and women affected by MS at the fertile age. A) PLS-DA model, white circles represent controls while black circles represent pathological subjects. B) discriminant metabolites resulting from the supervised analysis, white bars are controls while black bars are MS patients. C) pathways analysis showing that energetic homeostasis, oxidative stress and amino acids metabolism are the most altered.

On the other hand, the models of the samples collected during the pregnancy and puerperium did not show any significant separation despite the presence of the disease (pregnancy and puerperium, figure 35).

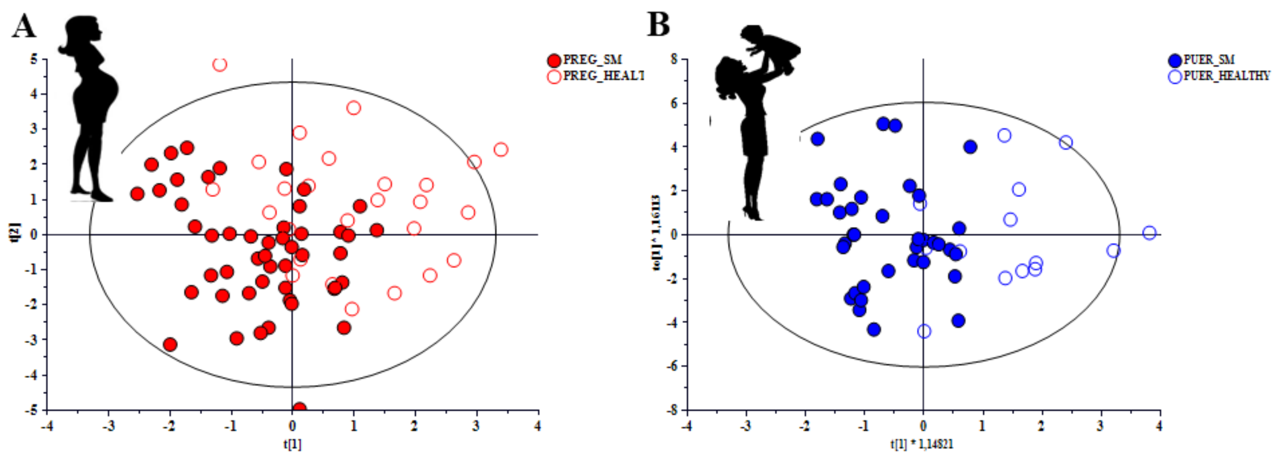

Figure 35. Analysis resulted from the comparison between healthy women and women affected by MS during pregnancy (A) and pueperium (B).

Discussion

Gestation represents an immune-tolerant condition, as the maternal immune system must adapt to the allogeneic fetal tissues (Sicotte et al. 2002, Avila et al. 2018). Cytokine release by the fetus-placental unity modulates maternal cellular immunity promoting a strong Th2 response aimed at preventing the risk of miscarriage (Yoshinaga et al. 2008, Soldan et al. 2003). As a result, during pregnancy, there is an increase in Th2-mediated activity which is associated to the reduction of Th1/Th17-mediated activity (Papenfuss et al. 2011). The immune alterations occurring during pregnancy induce a protective immune shift in MS patients (Voskuhl et al. 2012). Therefore, several studies have shown a 70% reduction of relapses in MS women in the third trimester of

pregnancy when compared to the clinical situation before pregnancy (Soldan et al. 2003, Sicotte et al. 2002, Voskuhl et al. 2016). This represents a remarkable difference considering that current MS therapies induce a 40-70% relapse reduction (Ysrraelit et al.2019).

During pregnancy many hormones present altered levels, such as estriol and progesterone (Gold et al. 2009), potentially playing an immune-stabilizing role. Progesterone and estrogen levels progressively increase during pregnancy, peaking in the third trimester, and then rapidly declining after delivery. Thus, their temporal profile is compatible with the protection from MS relapses observed during pregnancy. It's generally assumed that the estrogens exert their protective role during pregnancy by inducing anti-inflammatory cytokines production and regulatory T (Treg) cell proliferation (McCombe et al. 2013). In line, during pregnancy the circulating levels of Treg cells can increase, either in the periphery or in the placenta, to suppress the allogenic immune response against the fetus. On the other hand, in the post-partum period, which is characterized by hormonal changes and increased relapse rate, an immunological shift toward Th1 responses and estrangement from Th2 responses were observed. A pathophysiological rebound in the first 3-6 months after delivery has been reported by several studies. According to PRIMS study, relapse frequency increased from a value of 0.2 in later pregnancy to a value of 1.2 in the first three months after delivery (Vukusic et al. 2004). This increased disease activity after childbirth seems to depend on the sudden reduction of estrogens occurring post-partum, which affects the immunosuppressive condition characterizing pregnancy, and the increased pro-inflammatory cytokine production occurring in the final phases of pregnancy (Airas et al. 2015).

The key role of sex hormones in the progression of MS it's something undisputed, and in this study, we have been performing the measure of 6 molecules belonging to that class and other neurosteroids (testosterone, DHEA, pregnenolone, allo-pregnenolone, progesterone, estriol, the analysis is still ongoing) to correlated their level with the metabolic profile during the different

reproductive phases of the enrolled MS women and try to explain mechanisms related to the immune-tolerance process still unclear.

To date, only another study in 2018 applied a LC-MS/MS targeted analysis for the determination of serum metabolites, such as steroids, estrogens, sphingolipids, ceramides, AAs, ACCs, SA, nucleosides, and LPCs, on 12 women affected by MS monitored during three trimesters of pregnancy and in the postpartum period. Although the metabolic panel investigated in that study was different from ours, a complex fluctuation of the entire metabolic pattern strongly linked with hormonal variation was suggested, confirming the hypothesis that the study of the correlation between metabolites and hormones is mandatory.

Physiologically, pregnancy can be considered a powerful variable in a woman's life, from several points of view. It causes multiple, systematic, anatomical, and physiologic adjustments that affect the body's metabolism. The physiological modifications depend on several factors, such as environment, physical status, lifestyle behaviour, pre-pregnancy nutrition, and maternal-foetal genetic compositions. The goal of hormonal and metabolic modification during pregnancy is to maintain the constant nutritional needs of the foetus to ensure its regular growth. As a result, metabolism changes from anabolic to catabolic status to promote fetal growth, maturation, and development (Lain et al. 2007, Zeng et al. 2017).

Our metabolomics analysis evidenced that, when the intra-group comparisons of the different "reproductive periods" were considered separately in the class of healthy and MS women, the main altered pathways were similar in both groups, despite the presence of MS (which represents a strong variable in a patient). This concept is reinforced by the fact that there were no significant differences between samples of healthy subjects and MS patients when the pregnancy and puerperium periods were compared. However, a clear separation, in line with the presence of the disease, emerged from the comparison between the serum samples of subjects (controls and pathological) collected at the fertile age. This latter model allowed to define a panel of metabolites

in the MS class which have altered levels compared to the controls: in line with our previous results (Cocco et al. 2016), amino acids were the most altered bio-molecules and, among them, tryptophan, glutamine and lysine showed a p-value<0.001.

More in detail, when we performed the intra-group metabolomics analysis, we found that most of the metabolites changed with the same trend in both controls and MS women, indicating commonly altered pathways which suggested pregnancy as a stronger variable than the presence of MS. These common pathways were involved in oxidative stress (urea cycle, ammonia recycling and glutathione metabolism), energy supply (amino sugar metabolism, phenylacetate metabolism, glucose alanine cycle), amino acids metabolism (mainly glutamate metabolism, aspartate metabolism, glycine and serine metabolism, alanine metabolism, arginine and proline metabolism, taurine and hypotaurine metabolism, methionine and tryptophan metabolism), and biosynthetic activity (purine and pyrimidine metabolism, phosphatidylcholines biosynthesis) and are representative of the physiological alterations occurring during the gestation period.

Increasingly evidence underlines the importance of oxidative stress during pregnancy. The term "oxidative stress" denotes the imbalance between ROS production and the capacity of antioxidant mechanisms to neutralize them (Pizzino et al. 2017). The numerous changes occurring during pregnancy support the "physiological" production of ROS, especially in the second half of pregnancy (Tobola-Wrobel et al. 2020). This is mainly due to the basic metabolism and oxygen "consumption" increases as well as to the use of fatty acids as a primary energy source for most maternal and placental tissues (Duhig et al. 2016). In addition, the placenta is the main source of ROS and their production increases with pregnancy, in association with an increase in placental mass. Nitric oxide is also synthesized by macrophages mainly in the placenta. The presence of oxidative stress is also linked with the nitrogen metabolism related to amino acid oxidation which increases during pregnancy by more or less 40% because of the enhanced protein demand from the mother, fetus, and placenta (Kalhan et al. 2000). Amino acids are essential factors for foetal

development and growth and changes in their metabolism during pregnancy are strongly linked with the boost of biosynthetic activity as they are the precursors for the biosynthesis of many macromolecules (e.g. proteins and nucleotides), have signalling functions, and are involved in adenosine triphosphate (ATP) production (Wu et al. 2013).

The amplification of the biosynthetic activity reasonably leads to an increase in maternal basal metabolic rate (King et al. 1994), augmenting both the resting and total energy consumption to support foetal development and growth (Abeysekera et al. 2016). Both foetal and placental development cause maternal energy intake and expenditure to increase from approximately 375 KJ (89 Kcal) in the first trimester, to 1950 KJ (466 Kcal) in the third trimester (Butte at al. 2005, Armistead et al. 2020). Moreover, there is increased fat storage through an "anabolic" state in the first period which shifts toward a "catabolic" state, characterized by increased lipolysis and the mobilization of fat stores, in the third trimester (Butte et al. 2000). Alteration in the energetic pathways was also related to glucose homeostasis. Indeed, foetal glucose demands increase around week 26 of gestation, requiring increased maternal basal endogenous glucose production via hepatic gluconeogenesis (Bell et al. 1997).

As stated before, all these aspects support our results and represent typical features in a physiological pregnancy, and so are common aspects of both healthy and MS women. Looking at the pathways significantly altered only in MS patients, we found an altered inositols metabolism and increased oxidative stress environment already known as common features of MS. Indeed, alterations in inositol's metabolism in MS patients have been also observed in our previous study. Among inositols, *myo*-inositol, which we found increased in MS women during pregnancy, is one of the components of myelin and plasma membrane and, in the form of inositol phosphates, plays a central role as a second messenger in cells. An imbalance of inositol metabolism identified by the accumulation of *myo*-inositol in the CSF (Reinke et al. 2014) and in normal-appearing white matter was already reported in MS patients by Fernando et al (Fernando et al. 2004). Moreover,

myo-inositol is thought to be a marker of astrocytic activation and proliferation (Sajja et al. 2009) and its reduction is likely to reflect astrocytic necrosis (Ciccarelli et al. 2013). This result supports our finding of a decreased concentration in the peripheral blood of MS patients.

Oxidative stress is commonly implicated in the progress of brain damage, and ROS contribute to numerous mechanisms underlying the pathogenesis of MS lesions (Van der Goes et al. 2001). Among the different damaging mechanisms, high amounts of ROS produced by infiltrated leukocytes induce myelin phagocytosis and breakdown by macrophages (Van der Goes et al. 2001) (oligodendroglial damage) (Van Meeteren et al. 2004), and neuronal and axonal injury (Hendriks et al. 2005). The inflammatory environment in demyelinating lesions causes the generation of oxygen and nitrogen free radicals and proinflammatory cytokines that in turn exacerbates the inflammatory response (Calabrese et al. 2002).

6. Conclusion and future directions

The application of the metabolomics approach has allowed the exploration of pathophysiological features in diseases through rigorous and standardized experiments useful to identify potential relevant metabolic biomarkers. To date, metabolomics represents an innovative approach to discover putative biomolecules that, in a specific pathological contest, can open new avenues for the deep investigation of target mechanisms. . .

In this study metabolomics approach improved our knowledge regarding the pathogenesis of MS, may support its diagnosis and classification, identify effective treatments and define the response to therapy, as we recently showed (Lorefice et al. 2019).

MS is characterize by complex biological mechanisms and diverse phenotypes contribute to its natural history. It is hard that a single metabolite can discriminate a specific disease state or predict treatment response, both in terms of biomarkers discovery and explanation of pathophysiological mechanisms. So, it is probably more productive to search for patterns of multiple metabolites, which may support an MS diagnosis, establish a prognosis, reveal a transition towards the progressive form and monitor treatment efficacy. In this scenario, the association with clinical and radiological measures is desirable because integrating different analytical approaches with clinical characteristics, MRI variables, proteins and metabolite concentrations, represents the best complete method to distinguish MS subtypes better than any single measure. Insights into pathophysiological mechanisms coming from the combination of neuroimaging-based diagnostics and omics could represent an important avenue of research.

On these bases, the aims of this study and the main results obtained have been the following:

AIM 1: <u>Classification of patients affected by different forms of MS</u>.

A different metabolic profile in RRMS or PPMS patients has been found. These differences could be extremely important to explain the specific pathophysiological mechanism behind the two different MS forms (e.g. neuroinflammation *vs* neurodegenerative).

Although we have found a weak correlation between the age of the patients and the concentration of the selected metabolites, age could be considered a confounder factor, and its contribution should not be overlooked especially because, in most cases, it corresponds to more years of illness. A future goal could be the investigation of such correlations in both serum and CSF to find potential markers of "disease aging" in patient affected by MS. Moreover, a comparison between CSF and blood results across MS subtypes may represents a more integrative and informative analysis (especially since the biofluids were obtained from the same patients).

A weak point of the study is represents by the lack of a linear models correcting for sex might which could influence the results, especially considering the well-known gender difference in metabolic phenotype and the higher incidence of MS in females. Further investigations in larger patient cohorts are needed to confirm our preliminary results and to explore the metabolomics pattern related to MS and its evolution. Moreover, the evaluation of a control group, (no recruited due to ethical reasons, especially concerning CSF collection) would have been useful.

AIM 2: <u>Monitoring the response to the therapy</u>

Treatment of MS patients with FINGO influences aminoacidic and energy metabolisms, and reduces oxidative stress and the activity of the immune system, both typical features of the disease.

Some baseline metabolites in MS patients were indicative of a better response to treatment, suggesting their possible use as surrogate biomarkers to predict the FINGO response.

Tryptophan plays a pivotal role in the definition of the metabolic profile of MS patients compared to healthy subjects. Thus, the possibility of deeply investigating the tryptophan metabolism could

represent a potential future target to improve the management of MS patients, especially in terms of response to the therapy.

Considering the complexity of MS management, especially from the pharmacological point of view, ^{1}H-NMR spectroscopy-based metabolomic analysis of blood seems to be a promising, and non-invasive approach to predict the response to MS therapies, with possibly important implications for future personalized therapeutic decision-making processes. However, the analysis of the same cohort of patients with a Mass Spectrometry platform could be extremely important to confirm the results and to measure a larger panel of metabolites which may give a better view of the picture. It will represent the next step of this study. Moreover, a correlation between the metabolic profile and clinical parameters such as EDSS and disease activity represents a necessary point to be developed in the future. Also in this case, a weak point of the study is represents by the lack of a linear models correcting for sex might which could influence the results, especially considering the well-known gender difference in metabolic phenotype and the higher incidence of MS in females.

AIM 3: <u>Investigating the protective role of pregnancy on the MS progression by metabolomics</u>

The known pregnancy's protective role can also be evinced from the metabolic point of view.

Most of the metabolites changed their concentration with the same trend in healthy and MS women during pregnancy, indicating that the presence of the pregnancy is predominant in the MS. Indeed, a clear metabolic separation was evident when we compared the samples of patients affected by MS and controls during the fertile period, but such separation disappeared when the comparisons were performed during the pregnancy and puerperium periods.

The altered pathways in healthy and MS pregnant women mainly reflected physiological alteration occurring during pregnancy.

During the fertile age, increased levels of oxidative stress and alteration in inositol’s metabolisms are specific characteristics of MS patients.

These results are shedding light on the role of sex hormones during pregnancy, and their correlation with metabolic alteration is still part of our project, which is ongoing. Indeed, the evaluation of the hormone balance as well as the typing of lymphocyte subpopulations involved, are essential pieces of a complex puzzle that is the immune-tolerance mechanism which could represent the key to future therapeutic approaches. Also, in this case, the analysis of the same cohort of patients with a MS platform could be extremely important to confirm the results and to measure a large panel of metabolites which can give us a complete view of the picture.

In addition, as lipids can play a role as potential biomarkers of inflammation and disease progression, the lipidomic profile is under consideration. For this purpose, we have started a collaboration with the Rowett Institute (Aberdeen, UK) where there is strong expertise in lipidomic characterization, but the analysis is still ongoing!

Final Conclusion

Considering the three studies, we found a common altered pathways which could be considered hallmarks in the definition of the metabolic fingerprint of the Multiple Sclerosis. Indeed, investigating different aspects of the MS (different forms of MS, response to the therapy and the protective role of pregnancy) some pathways seemed to be redundant confirming their strong values in the description of the pathophysiology of this complex disease. These pathways are involved in the presence of oxidative stress, energetic homeostasis and the tryptophan/kynurenine metabolism.

These pathways may represent a target for future deep investigations in terms of enzymatic functions, correlations with panels of pro-inflammatory cytokines. Moreover, as the tryptophan metabolism is implicated in the immune response, it could be correlated with the changes in the lymphocytes sub-population as well as the hormonal changes which undoubtedly represent a key feature in the management of the MS.

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