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MEDICAL SCHOOL
Department of Health Sciences
The University of Eastern Piedmont "Amedeo Avogadro" Novara, Italy

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THESIS

Serum and CSF Neurofilaments as important diagnostic and prognostic Biomarkers for Multiple Sclerosis patients

Chief Supervisor: Prof. Umberto DIANZANI
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UNIVERSITÀ DEL PIEMONTE ORIENTALE

Contents

Chapter 1 Introduction

1.1) Background

Multiple sclerosis (MS) is the commonest chronic iNFLammatory and demyelinating disease of autoimmune to affect young adults. As reported by {Dobson et al.2019}.MS incidence is increasing worldwide.

Till the moment, the etiology of MS and mechanisms behind incidence increase remain mysterious, Multifactorial complex interactions Between genetic and environmental agents have been demonstrated to play a significant role in MS development, and the progression of MS contributed to some factors like Smoking, vitamin D deficiency, EBV virus infection, and childhood obesity. It is a chronic iNFLammatory autoimmune disease, a neurodegenerative demyelinating disease that infects the central nervous system (CNS) leading to damage of myelin and axonal loss as a result of focal lymphocytic infiltration {Dobson et al.2019}.

Charcot JM (1880) was first to describe MS and the one who named it "sclérose en plaques" disseminates {Murray TJ. 2004}.

MS is a multifocal central nervous system (CNS) disorder leading to axonal damage and can be classified according to clinical courses into 4 types as follow

relapsing-remitting multiple sclerosis (**RRMS**), secondary progressive multiple sclerosis (**SPSM**), primary progressive multiple sclerosis (**PPMS**), and progressive-relapsing multiple sclerosis (**RPMS**) {Kamińska J, et al. 2017}.

On the other hand, MS can be classified according to signs and severity into two types (a) benign MS or (b) malignant MS {Kamińska J, et al. 2017}.MS McDonald's diagnostic criteria determine the severity of the disease by linking clinical manifestation with characteristic lesions demonstrated by magnetic resonance imaging (MRI), and cerebrospinal fluid (CSF) analysis {Kamińska J, et al. 2017}. it has been confirmed that RRMS is the most prevalent MS phenotype characterized by episodes of symptoms exacerbations

1.2) Epidemiology

Epidemiology of MS: Study of the number of MS patients globally, Variation between groups according to demographic data as regions, gender, and age, two common epidemiological terms are prevalence and incidence.

The global prevalence of MS has risen since 2013, and the estimated prevalence in 2020 increased by about 30 % compared to the same estimate done in 2013 for 2.8 million worldwide. the mean age of diagnosis is 32 years. Females are twice as likely to live with MS as males {Walton Clare, et al. 2020}.

For the Italian prevalence, an estimated total number of patients is more than 109,000 according to the Italian population in 2015 with an average of 176/100,000 in the mainland and Sicily, with a high prevalence found to be Sardinia region with an average of 299/100,000 {Battaglia MA 2017}.

MS prevalence in the Tuscany region increased from 189.2 in 2014 to 208.7 per 100,000 in 2017 {Bezzini D, et al. 2019}, while another study estimates only 90 MS cases per 100,000 people in the Campania region explaining that lower prevalence based on conservative a novel case-finding algorithm and direct measuring on population {Moccia M, et al 2020}.

The number of affected children and teenagers under age 18 is about 30,000 children and teenagers with a prevalence of 1.5% of the total number of people with MS in the countries reporting pediatric prevalence data which confirms the increase in the incidence between children and young teenagers than before. {The Multiple Sclerosis International Federation, Atlas of MS, 3rd Edition September 2020}.

Within the same region, the number of females living with MS to the number is usually higher than the number of affected males, which usually at least double the number of affected males represented by a prevalence percentage of 69% of women to 31% of men. {The Multiple Sclerosis International Federation, Atlas of MS, 3rd Edition September 2020}. The gender prevalence ratio varies for different regions, for example, in Europe 2 :1, some countries have a high ratio variation between females to males affected with MS ranging between 3:1 and 4:1 (female: male), with variation of prevalence is present within different countries and within regions in the same country, there are high-prevalence countries and low-prevalence countries as Germany 303 per 100,000 and San Marino 288 per 100,000 {The Multiple Sclerosis International Federation, Atlas of MS, 3rd Edition September 2020}.

The average prevalence of MS patients per 100000 in Europe is 133, followed by America 112, and the low prevalence found in Africa and western Pacific equals 5 per 100000 {The Multiple Sclerosis International Federation, Atlas of MS, 3rd Edition September 2020}. See **Fig (I)**

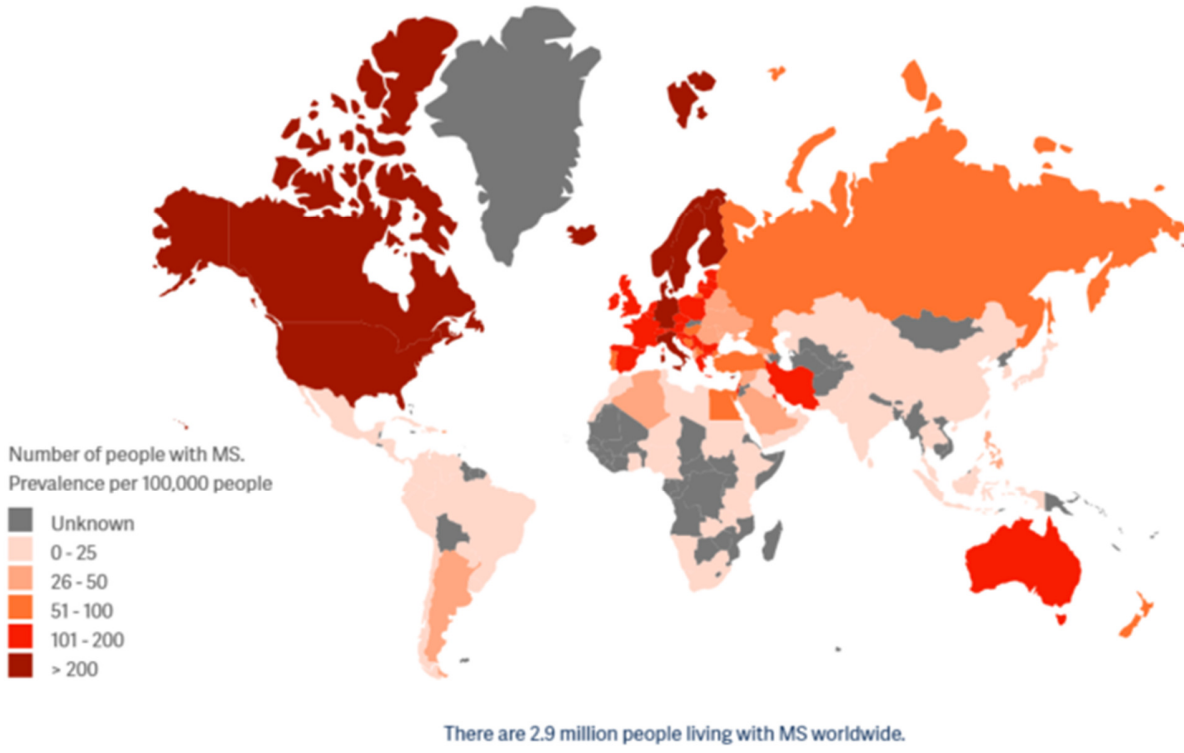


Fig (I), source {The Multiple Sclerosis International Federation, Atlas of MS, 3rd Edition September 2020}

1.3) Clinical features and subtype

MS is categorized into several subtypes based on the pattern of disease progression and the presence or absence of relapses, in 1996 the US National Multiple Sclerosis Society advisory committees (NMSS) classified MS into 4 subtypes based on the clinical course and the phenotypes described by the MS experts as follow Clinically isolated syndrome (CIS), relapsing-remitting multiple sclerosis (RRMS), secondary progressive multiple sclerosis (SPSM), primary progressive multiple sclerosis (PPMS), {US National Multiple Sclerosis Society advisory committees (NMSS) classifications of MS 1996}.

The old classification missing the imaging and biological markers which potentially offer objective criteria for distinguishing disease phenotypes so, it is highly recommend to consider disease progression and the disease activity according to the relapse rate and the image findings {Lublin FD, et al. 2014}

The advisory committee recommended at later time deletion of the term relapse progressive (RPMS) from the classification because it is an obvious term that overlaps with other subtypes {Lublin FD, et al. 2014}

The committee also recommended the replacement of the term chronic progressive with more specific terms of the disease terms SP and PP which refer to the benign and malignant forms of the disease {Lublin FD, et al. 2014}

RRMS relapse remitting multiple sclerosis is the most common course of the disease characterized by attacks of the disease accompanied by new symptoms or the increase of current symptoms, the attacks are called Relapse periods or the active time, followed by a remitting period of nonactivity called relapse time where no symptoms appear which is. So, it includes 4 different stages worsening relapses or non-worsening relapses also Active remission, and non-active remission. Lublin et al., 2014

I. Relapsing-remitting MS (RRMS)

is a clinical pattern with periodic relapses followed by remissions which affect 80% of MS patients The reciprocal patterns may last for decades. {Steinman L, 2014}.

II. Primary progressive multiple sclerosis (PPMS)

The course that characterized by gradual deterioration from the initial relapsing onset independent of relapse occurrence or absence, 15 to 20% of patients present with a gradual deterioration from the onset, with an absence of relapses. {Tafti D et al. 2022}, also the progressive multiple sclerosis PMS is a stage of the disease where the disability increases over time and is reflected into clinical symptoms {Ontaneda et.al 2015}, the atrophy mostly occurs as a result of non-localized degeneration that affects widely both the white matter WM and grey matter GM 55 {Lassmann H et al. 2012} the progressive course of the disease initiated as primary progressive form PPMS in 10% of the total MS counts, furthermore, a large proportion of RRMS patients which may progress to the secondary form of multiple sclerosis SPMS {Ontaneda et al. 2015}.

III. Secondary-progressive MS (SPMS)

The relapse remitting MS can progress to the more symptomatic course of the disease called secondary progressive multiple sclerosis (SPMS) which usually take a long time as far as 10 years but not all cases progress to develop SPMS. the phase characterized by steady progression and aggressiveness compared to RRMS, the periods of remission decreased or disappeared with an increase of symptoms severity during the remission periods., most of untreated RRMS patients ultimately progress to SPMS, the suggested time according to research data the median time for progression from RRMS to SPMS usually takes 19 years after RR onset {Rovaris M et al. 2006}. See Fig (3)

The are many risk factors that accelerate the time of progression from RR to SPMS multiple sclerosis as old age at MS onset, longer disease exposure also male gender is considered as risk factor {Cree BAC et al. 2021}

spinal cord symptoms, and the incomplete recovery of the RR onset is contributed to the acceleration of the progression {Rovaris M et al. 2006}. In most cases, MS starts with the RR course that progresses to worsen disability course SP secondary progressive, the two courses have a different response to the treatment. {Cree BAC et al. 2021}

The secondary progressive course onset main feature is that neuronal damage occurs gradually after the initial course, SPMS may include some relapses {Tafti D et al. 2022}.

IV. Clinically isolated syndrome (CIS)

The earliest clinical phase of MS can be diagnosed by Diagnostic criteria from the International Panel of McDonald's including the spread of disease by the MRI scan time and area specific for the onset {Efendi H et al. 2015}. (CIS) onset caused by a single attack to the CNS that mostly contributed to young adults includes an occurrence of acute or subacute onset with rapid progression till reaches the peak within 2-3 weeks, the acute CIS onset occurs mostly in 85% of young adults, manifestation of the disease may be mono-focal or multifocal affecting the optical nerve, brain stream, spinal cord cerebellum, or cerebral hemispheres. CIS lesions appear silent on MRI without clinical features or availability of diagnostic examination, this increases the risk of developing MS is high. {Efendi H et al. 2015}. The CIS onset is lasting for at least 24 h without

fever or infection or brain injury encephalopathy {Efendi H et al. 2015}. See **Fig II** explaining the possible transitions During MS progression {Hou, Y. et al 2018}

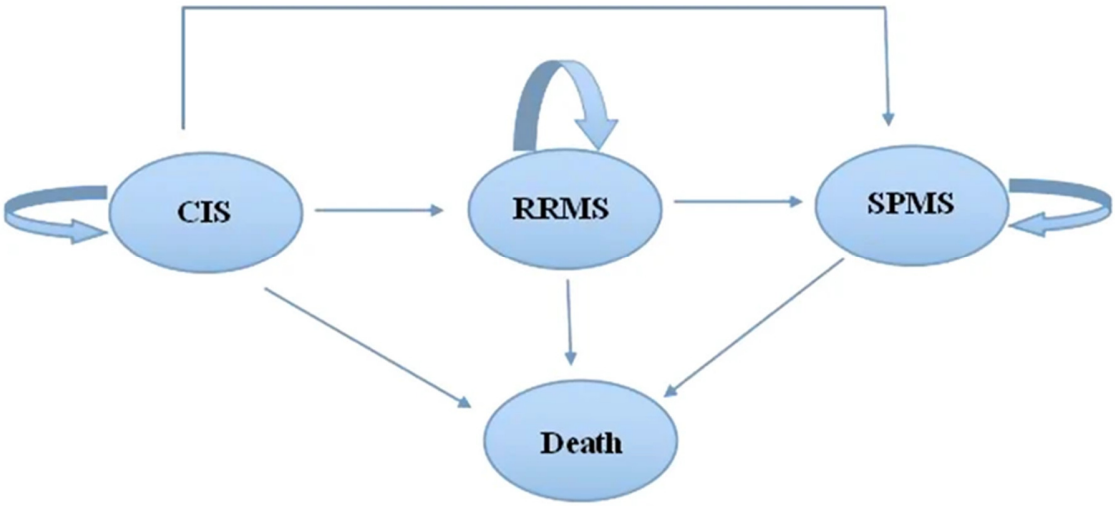


Fig II The possible transitions from CIS during disease progression. CIS: clinically isolated syndrome; RRMS: relapsing-remitting multiple sclerosis; SPMS: secondary progressive multiple {Hou, Y. et al 2018} source https://www.nature.com/articles/s41598-018-29206-y

1.4) Pathophysiology

For centuries MS was classified as 'paraplegia' a condition with progressive neurological deterioration {Murray TJ 2009}.

Dr Robert Carswell introduced first illustration of disease in 1838, the historic descriptions of multiple sclerosis by Rindfleisch and Charcot are well documented in medical literature {Efendi H et al. 2015}. More than 150 years ago, Swiss pathologist Rindfleisch 1863 is the first scientist to recognize that focal MS plaques are centered by small blood vessels and he suggested that the main feature of the lesion requires alteration of these blood vessels and accumulation of round cells {Lassmann H et al. 2005}. Rindfleisch Added another significant observation that nerve fibers lose their myelin and become visible as naked axons in connective tissue {Lassmann H et al. 2005}.

In (1868) a French neurologist Jean-Martin Charcot provided the first succinct disease concept, illustrating a detailed pathological description of MS as structural features of the MS lesions and clinical impairment symptoms observed in the patients {Lassmann H et al. 2005}. he observed that MS pathophysiology is restricted to the primary CNS with main changes seen in MS patients being

Microscopically and macroscopically alterations resulting in two mechanisms of injury to the CNS. Firstly, INFLammation occurs followed by the Formation of plaques and damage of blood-brain barrier blood-brain (BBB) barrier resembling acroscopic changes to the BBB. The second change resembles the microscopic neurodegeneration affecting axons, neurons, and synapses {Tafti D et al. 2022}, he also was the first to give the name of MS after his observations on post-mortem autopsies samples but also provided a detailed description of the microscopic pathology of multiple sclerosis (MS) in the 19th century with his concise disease concept of MS, illustrating the structural features and locations of lesions, {Lassmann H 2005 et al.}, he observed sclerotic changes in brain white matter of glial scars like sclerotic tissue {Herthum H et al.2022}. Charcot described the macroscopic lesions observed in multiple sclerosis and he assured that these lesions can be seen in different loci spine, the medulla, cerebellum, or brain {Zalc B, et al. 2018}.

Furthermore, excellent description by Charcot for what he observed in the center of the lesions, he found demyelinated axons (axons that have lost their protective myelin coating) and refracted axons, he related these findings to axonal degradation {Zalc B, et al. 2018}. Charcot's work helped to establish the link between demyelination and axonal damage in MS which is a hallmark of MS, he also pointed out the importance of the neuroglia in the alterations of the nervous system and stated that neuroglia composed of star-shaped cells, poor of protoplasm with highly thin branches {Zalc B, et al. 2018}, a more deep knowledge about the disease added by Charcot classifying the lesion into zones, he divided sclerotic lesions microscopically into three zones, the periphery zone, the transition zone, and the center of the lesion {Zalc B, et al. 2018}. Charcot also observed the presence of greasy droplets of myelin debris Charcot, he related the presence of these droplets to the destruction of axons. {Zalc B, et al. 2018}, he explained the cause of axon demyelination and axonal destruction releasing the myelin debris to the iNFLammatory response of the immune system that leads to infiltration of immune cells crossing the BBB Blood-brain barrier of CNS. Charcot put the concept of MS pathophysiology and illustrated the hallmarks of MS pathologic features as iNFLammation, demyelination, and relative preservation of axons. {Kornek B, Lassmann H 1999}. The characteristic features of MS are seen in the white matter of the CNS including the formation of large plaque lesions linked to the degradation of axons, reactive astrogliosis and oligodendrocytes. The specific types of immune cells that make

up the iNFLammation can vary among patients and between different stages of the condition but typically include T-cells and macrophages {Kipp M, et al. 2017}.t-Tau and p-Tau can be released in the extracellular milieu and CSF after neuronal damage.

pathophysiology of multiple sclerosis (MS), which involve several interconnected and co-existing stages, {Krieger S, et al 2014} as shown in **Fig (III)**
Acute relapses denote new iNFLammatory activity in the central nervous system (CNS), provoked by autoreactive T-cells initiating an attack on myelin-producing oligodendrocytes (top left panel). The loss of oligodendrocytes and impaired remyelination (top middle panel) may contribute to the accumulation of disability associated with relapses. INFLammatory lesions are also linked to axonal transection and loss, which can manifest early in the disease course and exacerbate over time (top right panel) {Krieger S, et al 2014}

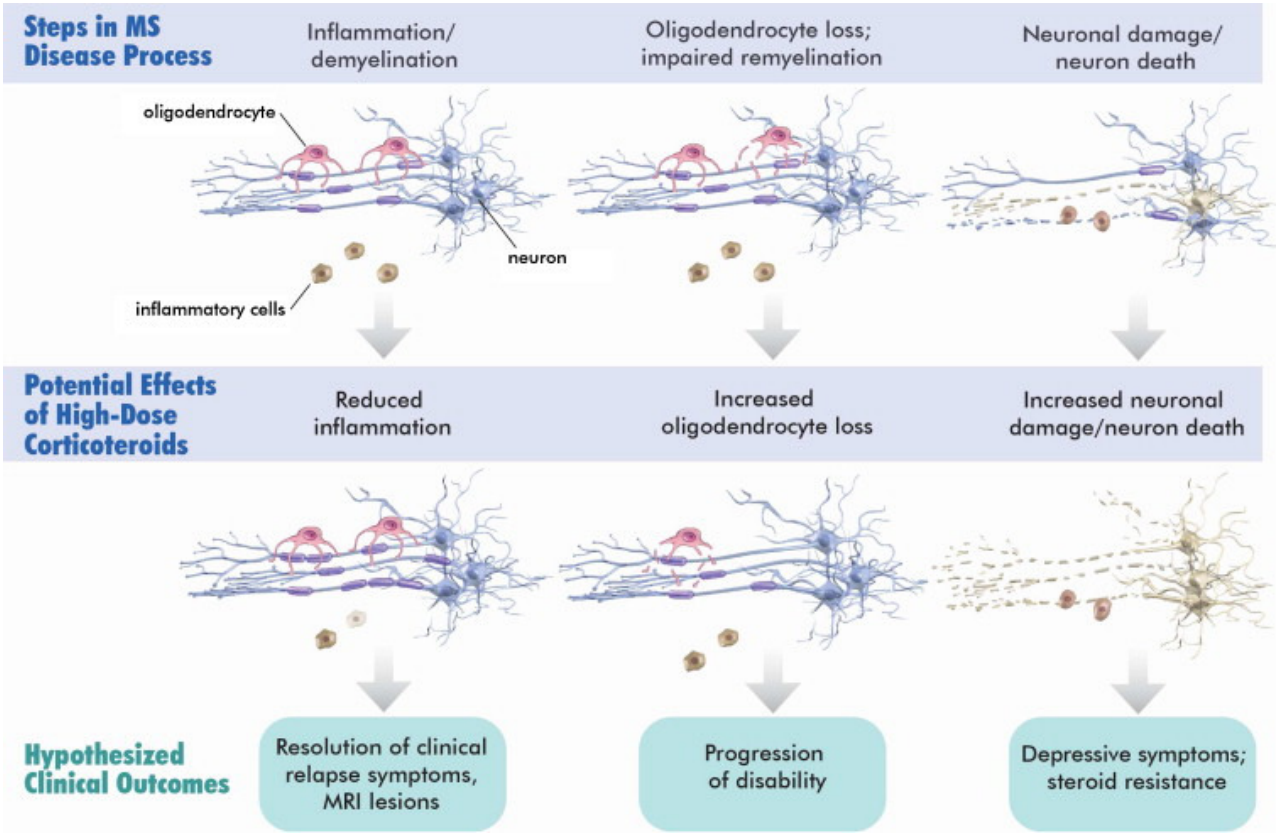


Fig (III) source: {Krieger S, et al 2014} https://doi.org/10.1016/j.clineuro.2013.12.021

1.5) Histology and Immunopathology of MS

MS early-stage initiation is still debated whether it is caused by iNFLammation or by neurodegeneration in absence of iNFLammation that responds to iNFLammation at a later stage causing amplification and modification of lesions.

The first hypothesis is supported by Barnett MH, MS lesion is formed in lack of iNFLammation by T-cells and B-cells destroying oligodendrocytes, following that in later stages, T-cells and B-cells can accumulate in lesions modifying the lesion {Barnett MH et al. 2004}.

In early stages lesions represented by areas of microglial activation with mild axonal damage associated with mild T-cells infiltration confined to the perivascular space in absence of demyelination {Marik C, et al 2007}. In early-stage lesions there is an innate immunity response to a loss of oligodendrocytes and degeneration of myelin by macrophages to advanced stages we can observe the adaptive immunity response to iNFLammation by the presence of CD8+ T cells, CD4+ cells, B cells, and monocytes in a second wave of iNFLammation caused by chemokines formation in response to tissue injury {Henderson AP,2009}.

The hallmark of MS as described more than 150 years ago by Charcot

Are iNFLammation, demyelination, and axonal loss.MS lesions are most commonly associated with white matter, they can also occur in the grey matter of the brain. Grey matter lesions in MS have been recognized as an important aspect of the disease and can be detected with advanced imaging techniques {Geurts JJ et al 2008}.

Revised diagnostic criteria for multiple sclerosis (MS) have been introduced by the International Panel on MS Diagnosis, with the emphasis still placed on the objective identification of lesion dissemination in both temporal and spatial domains {McDonald WI, et al. 2001}.

The diagnosis of multiple sclerosis according to the 2017 McDonald criteria involves a comprehensive assessment of clinical, imaging, and laboratory data. {Thompson, A. J 2008}.

The McDonald's diagnostic criteria for multiple sclerosis (MS) is a set of guidelines developed by an international panel of experts to improve the accuracy and consistency of MS diagnosis. {National Multiple Sclerosis Society

(2022). Diagnosis of MS. Retrieved from}. It was first introduced in 2001 and has been revised several times since then {Thompson et al. 2018}.

The criteria aim to provide a uniform and fact-based approach to the diagnosis of MS {Polman et al. 2005}.

The McDonald criteria rely on the presence of clinical and radiological evidence of MS, as well as the exclusion of alternative diagnoses. According to the criteria, the diagnosis of MS can be made in the following circumstances: including dissemination in space and time

Two or more clinical episodes of neurological symptoms that are consistent with MS {Thompson et.al. 2018}.

A single clinical episode that is accompanied by MRI evidence of new lesions characteristic of MS, Dissemination in space refers to the presence of lesions in different locations within the central nervous system (CNS) and dissemination in time refers to the occurrence of new lesions over time {Swanton et.al. 2007}

MRI evidence of lesions characteristic of MS that are disseminated in time and space {Montalban X, Tintoré et.al. 2010}.

the McDonald's criteria also take into account the presence of oligoclonal bands in the cerebrospinal fluid (CSF) and the results of other diagnostic tests Furthermore, McDonald's criteria are more sensitive than previous diagnostic criteria, including the Poser criteria {Thompson et al. 2018}.

The McDonald criteria have been widely adopted by neurologists and are now the standard for MS diagnosis in clinical practice {Montalban X at al. 2010}.

The McDonald criteria have been shown to have a high sensitivity and specificity for MS diagnosis, with a 90% accuracy rate when compared to pathological diagnosis. The McDonald criteria have been widely adopted by neurologists and are now the standard for MS diagnosis in clinical practice.

1.6) Role of CSF in the diagnosis of MS

Cerebrospinal fluid (CSF) plays a crucial role in the diagnosis of multiple sclerosis (MS), The presence of specific CSF biomarkers, such as oligoclonal bands (OCBs), IgG index, and intrathecal synthesis of immunoglobulins, provides valuable diagnostic information and helps distinguish MS from other neurological disorders. OCBs, which represent a clonal expansion of B-cells in

the CNS, are present in up to 90% of MS patients and are considered a hallmark of the disease. The detection of OCBs in CSF, along with a normal blood-brain barrier (BBB), is highly specific for MS and is included in the McDonald criteria for diagnosis of MS {Thompson et al. 2017}.

In addition, the IgG index, which measures the ratio of IgG in CSF to serum, is often elevated in MS patients, indicating an intrathecal synthesis of IgG. This finding further supports the diagnosis of MS and helps differentiate it from other neurological conditions {Reiber, 2001}.

Finally, the measurement of intrathecal synthesis of immunoglobulins, including IgG, IgA, and IgM, provides additional diagnostic information and helps classify MS patients into different subtypes based on the degree of intrathecal Ig synthesis (3). {Andersson et al. 1994}.

1.7) Biomarkers

Biomarkers are anatomic, physiologic, biochemical, or molecular parameters associated with the presence and severity of specific disease states. Biomarkers are measurable by a variety of methods including physical examination, laboratory assays, and medical imaging {O'Connor KC et al. 2006}. The National Institutes of Health (NIH) established a definition for biomarkers in 1998, stating that they are "an objectively measured characteristic that serves as an indicator of normal biological processes, pathogenic processes, or therapeutic responses to pharmacologic intervention

Biomarkers are objectively measurable characteristics that can indicate normal biological processes, pathogenic processes, or pharmacological responses to treatment. They can be classified into different types, such as Type 0 biomarkers which reflect the natural progression of a disease, and Type I biomarkers which capture the effects of treatment according to its mechanism of action." {Biomarkers Definitions Working Group. 2001}.

disease states. Biomarkers are measurable by a variety of methods including physical examination, laboratory assays, and medical imaging

Several biomarkers have been identified in both blood and cerebrospinal fluid (CSF) that can provide significant pathological insights into the mechanisms of multiple sclerosis (MS). These biomarkers are capable of detecting various forms

of damage, including axonal and neuronal damage, glial dysfunction, demyelination, and CNS iNFLammation {Yang Jet al. 2022}.

CSF biomarkers are considered to be more indicative of CNS iNFLammation compared to serum or urine samples, as they are located closer to the iNFLammatory lesions and are less likely to be degraded by the liver or excreted by the kidneys. This proximity to the iNFLammatory lesions in the CNS may provide a more accurate reflection of the relevant iNFLammatory processes. Additionally, collecting CSF can prevent the biological degradation of excreted markers by the liver or renal excretion {Bielekova B, 2004}. CSF samples are collected by lumbar puncture and have high invasiveness than serum samples, for that reason CSF samples are more suitable for clinical diagnosis but not valuable for medical research {Teunissen CE, et al.2013}.

Biomarkers of Axonal Damage

1.7.1) Neurofilaments

Neurofilaments are a type of cytoskeletal proteins that are discharged from damaged axons and can be detected in both the cerebrospinal fluid (CSF) and blood. Researcher have shown that elevated levels of cNFL are associated with increased levels of CD4+ T lymphocytes, which are believed to play a role in the iNFLammatory processes observed in multiple sclerosis (MS){Sospedra M,2005}.

The correlation between CSF NFL (cNFL) and serum NFL (sNFL) levels showed that the levels of NFL in CSF were 42 times higher than in serum {Disanto, 2017}.

Elevated levels of sNFL have been detected in EBV-infected patients, {Bjornevik, et al. 2022}.

The use of NFL levels as a biomarker for MS relapse lacks specificity since elevated NFL levels can be indicative of infections and several neurodegenerative conditions {Wang, et al. 2012}. Assessing NFL levels can effectively reflect the degree of neuroaxonal injury, particularly in the initial phases of the disease. Numerous studies have provided evidence that NFL levels in both cerebrospinal fluid (CSF) and serum can serve as dependable indicators of MS diagnosis and therapy monitoring {Ferreira-Atuesta C, et al. 2021}.

1.7.2) Neurofilament Light (NFL)

Neurofilaments Nf-light (NFL) are neuron skeleton proteins that consist of 3 subunits (light, medium & heavy chains) and they are released after axon damage to extracellular space, so can be detected in blood and CSF. Release of (NFL) for long time after acute neuronal damage may be an indicator for (BBB) blood brain barrier (BBB) damage, these proteins getting promise interest as useful biomarker of axonal damage and diagnosis of neurodegenerative diseases because their expression is restricted to neurons, furthermore they can be used for predicting neuronal disease progression either chronic or acute phase. NFL are restrictedly expressed in neurons that's why are highly specific for neuronal cell damage.

Chapter (2) Aims of thesis, Hypothesis & expected results

In this work of thesis, we aimed to correlate the diagnostic and prognostic power of the NFL biomarker in serum and CSF obtained by relapsing-remitting naïve MS patients. Furthermore, our objective was to set up a reliable protocol to evaluate the levels of serum and CSF NFL by alternative methods Ella™ and Lumipulse™ to gold standard SIMOA™ investigating its clinical significance.

Summarized points of objectives of the study

A) measure the levels of CSF & serum NFL biomarker of neurodegeneration in clinically diagnosed relapsing-remitting (RR) naïve MS patients and extrapolates the correlation between NFL levels and the pathologic features, in particular Light chain neurofilament (NF-L).

B) comparing of the NFL levels in CSF and matched serum samples to assess their sensitivity to pathologic and clinical features and their power as diagnostic biomarker of MS, furthermore comparing the diagnostic and prognostic power of Matched CSF & Serum samples.

C) comparing the quantitative values of neurofilaments NFLs Biomarker obtained by different immunoassay platforms (Simoa vs Simple Plex ELLA and Fujirebio).

E) demonstrate the correlation between NFL levels in CSF and matched serum samples in the MS patients

Hypothesis, the measured serum NFL levels have an association with the clinical features of early stages of MS and confirm their value as a predictive biomarker of the progression of the disease

Expected results may validate previous data on the predictive values of NFL levels for early disability and clinical progression in MS and investigate the meaning of a modulation on serum NFL levels.

Chapter (3) Methodology

Patient population: 71 newly diagnosed relapsing-remitting (RR) MS patients were recruited at the Multiple Sclerosis Center, SCDU Neurology of Novara. They were enrolled at the time of the diagnosis and serum and CSF samples were withdrawn before steroid or diseaae-modifying treatment initiation.

Samples selection: among the 71 CSF and matched serum samples of naïve RRMS patients stored in the biobank of Clinical Biochemistry Laboratory, Department of Health Sciences, AOU Maggiore della Carità of Novara, 60 serum and CSF matched samples were selected based on the total amount of samples, complete clinical features and the follow-up duration of patients.

Biological assays: NFL levels were determined, as described below, on the selected 60 CSF and matched serum samples by three approaches: 1) the gold standard Quanterix's Simoa® Technology; 2) a benchtop automated ELISA" Enzyme linked immunosorbent Assay" Platform a Simple Plex ELLA™ (Bio Techne); 3) an highly sensitive chemiluminescence method on the automated platform LUMIPULSE™® G600II (FUJIREBIO).

2.1) Study design

Retrospective and prospective study of confirmed patients of relapsing-remitting multiple sclerosis (RRMS) patients whose samples stored in the Laboratory biobank of Multiple Sclerosis Center (SCDU Neurology) of the AOU Maggiore della Carità of Novara (University Hospital Major of Charity).

Participating centers:

a) Multiple Sclerosis Center, SCDU Neurology, AOU Maggiore della Carità (University Hospital Major of Charity) of Novara.
b) Clinical Biochemistry Laboratory, Department of Health Sciences, AOU Maggiore della Carità (University Hospital Major of Charity) of Novara.
c) Laboratory of Immunology, Department of Health Sciences, University of Eastern Piedmont
d) Immunology laboratory at the San Giuliano Hospital.

Study type: Observational
Study Population: Patients hospitalized in the ordinary and Day Hospital regimen in the neurological field undergoing lumbar puncture in the routine diagnostic procedure
Inclusion criteria: Diagnosed with relapsing-remitting multiple sclerosis
Exclusion criteria: None
Duration Of study: 1.5 Years

2.1.1) Ethical considerations & approvals

The study was designed and was conducted in accordance with international and national ethical standards on biomedical research with humans, samples collected for neurology unit SCDU and study doesn't require additional samples but using samples already collected for SCDU unit for routinely clinical diagnosis of suspected MS patients and frozen in Biobank.

Type of consent: informed consent
All the subjects enrolled in the study signed informed consent form before the start of collection and storage of biological samples for both liquor and serum (local Ethics Committee approval – Comitato Etico Interaziendale "AOU Maggiore della Carità" di Novara CE060/2022 and CE 260/2022).

2.1.2) Statistical considerations

i. Continuous variables of data will be reported as median of the first and third quartiles and Wilcoxon-Kruskal-Wallis used for comparison
ii. A categorical variable "Qualitative value "will be reported as percentage (absolute values) Pearson's chi-square test used for comparison
iii. Multiple linear regression (MLR) will be applied on the significant values to predict the outcome and determine predictive values and plot relation between the one single variable and multiple independent variables.
iv. Confidence level used to estimate significance of biomarkers concentration values is 95%, so p-value, or probability value (p-value) of 0.05 is our significance limit.
v. Confidence interval will be calculated around the mean of biomarkers concentrations at confidence level 95%.

Samples & data processing

No blindness required for observational study, but the biological samples (both liquor and CSF) will be pseudonymized by assigning anonymous alphanumeric codes which is known and in the possession of the responsible research

investigator. personal data collected and processed under the regulation of lawfulness, fairness and transparency in accordance to the Decree n.101/18 of 10 August 2018 and maintaining key rules as follow:

i. Purpose limitation: only personal data collected for defined purposes
ii. Data minimization: necessary data only for the purpose of study will be collected and including data correction if necessary
iii. Storage limitation: Data will be stored for limited time not exceeding the time required for data analysis and technically safeguarded against unauthorized access or usage.
iv. Clinical and demographic data will be collected from the patient's historical records in parallel with acquisition of informed consent and reserved in excel file.

Samples types: Matched serum and CSF.

Samples Storage: stored in aliquots of about 500 µL at -80 ° C

Sample size: The study is consider a retrospective and prospective collection of 71 serum and CSF samples, routinely obtained during the diagnostic procedures for MS.

Method of collections: Peripheral venous blood collection for serum samples and lumbar puncture for CSF samples.

Tests done routinely for the collected samples:

a) TLC total leucocyte count and the differential WBCs count at CORE2 sector of the Analysis Laboratory.

b) biochemical tests: CSF glucose (glycorrhachia), total proteins, LDH and chloride at CORE1 sector of the Analysis Laboratory

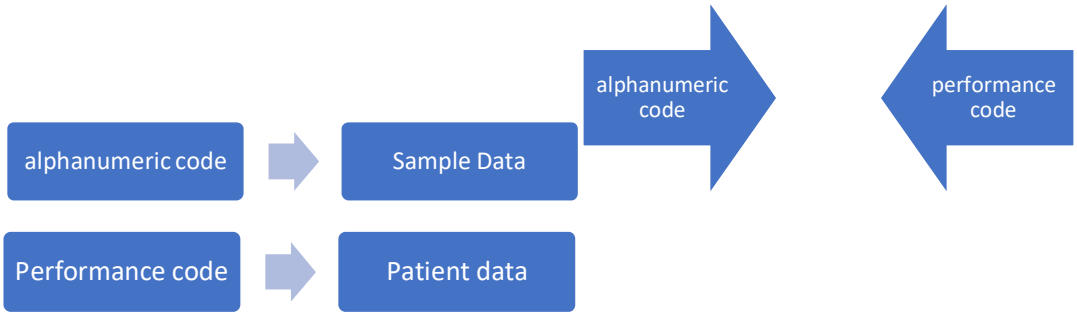
c) Nephelometric analysis of albumin, immunoglobulin G (IgG) total, kappa free light chains (KFLC) and lambda free light chains (LFLC) at Protein Diagnostics Sector of the San Giuliano Hospital, Novara.

d) Albumin quotient: CSF/serum quotient of albumin, known as QAlb

is good biomarker to estimate permeability and function of blood–brain barrier (BBB).

e) Samples (CSF & Serum) will be divided into 3 aliquots with labelled alphanumeric code and stored at -80 ° C in the protein diagnostic center of San

Giuliano hospital, Novara, data of the samples will be stored with alphanumeric code which is tight to the performance code of the samples.



f) link indexes: CSF IgG index (IgG CSF/IgG serum), albumin index, kappa index kappa index which obtained (CSF/serum KFLC) divided by divided by the CSF/serum albumin ratio).

Demographic data of patients assigned shown in table (I), shows assigned number of 25 males Number with 46 females
Mean age: Approximately 39.22 years

Table (I)

No	Date of birth	Gender	Age	Date of acceptance
1	12/1/1962	F	56	31/07/18
2	12/12/1969	F	49	14/05/19
3	8/20/1971	F	48	20/06/19
4	11/1/1983	M	36	02/08/19
5	10/8/1961	F	58	05/08/19
6	9/20/2003	F	15	09/08/19
7	8/23/1991	F	28	16/09/19
8	3/14/1990	F	29	10/01/20
9	1/10/1979	F	41	13/02/20
10	9/26/1990	M	30	24/02/20
11	1/9/1992	F	29	24/04/20
12	4/1/1979	F	41	20/05/20
13	2/9/1992	F	28	21/05/20
14	6/13/1970	M	49	30/12/19
15	11/25/1965	M	54	13/08/20
16	3/5/1993	M	27	24/08/20
17	6/17/2004	F	16	14/09/20
18	4/4/1999	F	21	28/09/20

19	5/28/1977	M	43	05/10/20
20	1/19/2001	M	19	14/10/20
21	3/1/1990	M	29	13/12/19
22	9/9/1989	m	31	23/10/20
23	10/6/3978	F	52	26/10/20
24	12/2/2000	F	20	13/11/20
25	5/1/1980	f	40	23/11/20
26	7/22/1977	F	43	27/07/20
27	3/21/1992	M	28	21/12/20
28	10/14/1993	M	27	21/01/21
29	1/3/1994	F	27	26/01/21
30	6/1/1980	F	40	26/03/21
31	4/8/1969	F	52	14/04/21
32	2/13/1987	M	34	19/04/21
33	7/26/1990	M	30	20/04/21
34	10/16/1997	F	23	22/04/21
35	8/20/1974	F	46	23/04/21
36	1/12/1956	F	65	03/05/21
37	4/26/1980	F	41	10/05/21
38	4/21/1989	F	31	12/03/21
39	10/16/1993	M	27	17/05/21
40	11/6/1989	F	31	20/05/21
41	10/21/1973	F	47	25/05/21
42	10/3/1976	F	44	16/06/21
43	2/26/1975	F	46	17/03/21
44	1/5/1981	F	40	28/06/21
45	2/21/1974	M	47	29/06/21
46	12/29/1974	F	46	07/07/21
47	11/10/1991	F	29	07/07/21
48	1/23/1993	M	28	12/07/21
49	12/17/1997	M	23	15/07/21
50	12/30/1972	F	48	16/07/21
51	2/18/1988	F	33	19/03/21
52	6/5/1973	M	48	29/07/21
53	5/31/1965	F	56	09/08/21
54	2/23/1992	M	29	24/03/21
55	7/3/1980	M	41	13/08/21
56	2/19/1984	M	37	23/09/21
57	2/29/1968	F	53	08/10/21
58	6/6/1978	F	43	18/10/21
59	12/3/1984	M	36	28/10/21
60	7/23/1985	F	36	05/11/21
61	6/21/1980	M	41	24/11/21
62	4/9/1978	F	43	18/12/21
63	7/5/1987	M	34	27/01/22
64	4/4/1961	F	60	04/02/22
65	6/21/2006	F	15	11/02/22
66	3/15/1981	F	40	22/02/22
67	6/13/1986	F	35	17/03/22
68	3/30/1986	F	32	07/06/17
69	7/26/1988	F	31	12/12/19

70	3/27/1994	F	25	19/03/19
71	9/12/1985	F	34	11/02/19

2.2) Devices and techniques

The measurements of NFL were performed with three platforms as follow:

I. LUMIPULSE™® G600II (Fujirebio): Automated immunoassay platform that use the Chemiluminescent enzyme immunoassay technology Known as (CLEIA) for detecting free and bound immune complex antibodies. The system capable of performing 60 tests per hour

II. Simple Plex ELLA™ (Bio Techne analyzer): A benchtop automated ELISA" Enzyme linked immunosorbent Assay" Platform for Consistent Biomarker Detection using sandwich ELISA immunoassay for detecting specific antigen using two loaded antibodies capture antibody (cAb) and detection antibody (dAb) the system uses disposable microfluidic Simple Plex assay cartridge that contain all reagent required and the matched antibodies, the cartridge contain multiple fluidic channels each contain multiple glass nanoreactors (GNRs) coated with capture antibody, only required diluted sample and puffer loading before running the test. Calibration is easier and simultaneous with preloaded factory-calibrated standard curves no need for preparing calibration curve , the cartridges are provided in different designs to for ease of use , a single cartridge provide fast ability for running 72 test for one single sample in tri-replicate for each test , while the Multianalyte provide ability to run 4 ELISA tests for each sample with a total sample load up to 32 samples , and also Multiplex cartridge available for running up to 8 ELISA tests per sample with maximum load of 32 samples per cartridge

III. Quanterix's SIMOA® Technology enables the measurement of biomarkers at low levels than before, Quanterix's highly sensitive biomarker detection is driving breakthroughs previously unattainable due to its unparalleled sensitivity and adaptability. SIMOA® technology resemble the gold standard for early biomarker detection in blood, serum, or plasma, with the capability to quantify proteins at levels far below the Level of Quantification (LoQ).

Chapter (4) Results:

The results are explained in two Sections, A & B. In the section A is described the technical comparison of the dosage of NFL levels between the gold-standard SIMOA™ and two other new commercial platforms consisting in Ella™ (Bio-Techne) and Lumipulse™ (Fujirebio). In the section B is highlighted the correlation between the levels of NFL in serum and CFS of relapsing-remitting MS naïve patients and important clinical features.

Section (A)

Median CSF NFL levels were, 1590.5 pg/ml with Ella™, 1105.0 pg/ml with Lumipulse™, 861.6 pg/ml with SIMOA™, while Median Serum NFL levels were 29.3 pg/ml with Ella™, 14.7 pg/ml with Lumipulse™, 16.3 pg/ml with SIMOA™ As shown in (**Fig. 1.a, Fig 1.b and Table 1**).

Fig (1.a)

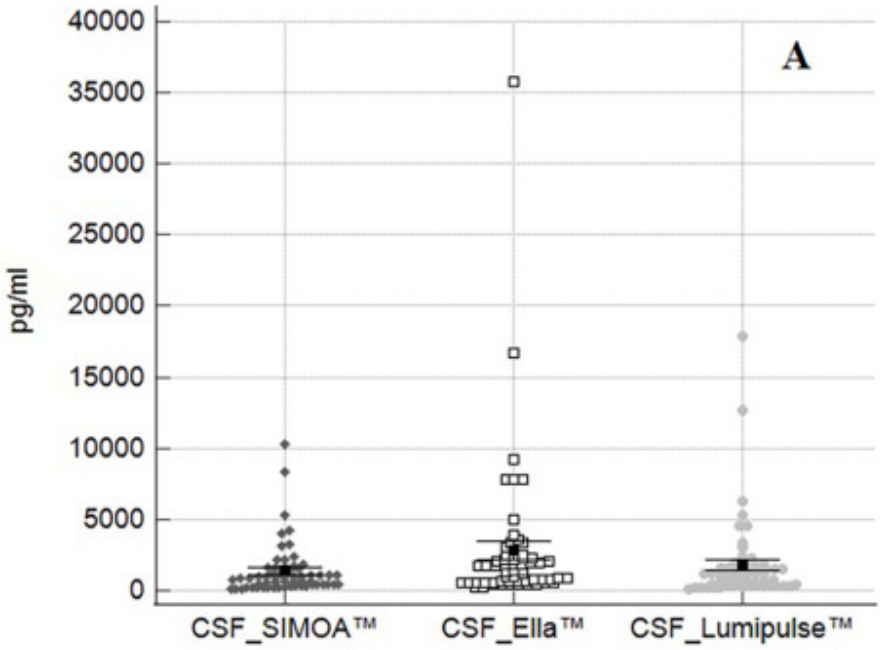


Fig (1.b)

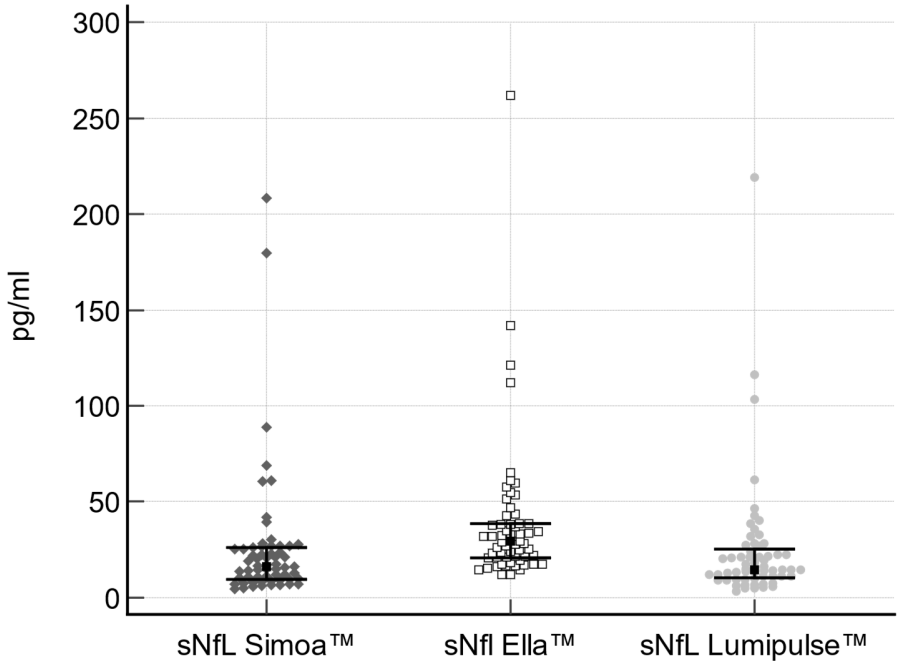


Table (1)

CSF Platform	**Median (pg/mL)**	**nterquartile range (pg/mL**
SIMOA™	861.6	375.3–1533.4
Ella™	1590.5	596.0–2491.0
Lumipulse™	1105.0	363.0–1692.5
Serum Platform	**Median (pg/mL)**	**Interquartile range (pg/mL)**
SIMOA™	16.3	9.6–26.3
Ella™	29.3	20.8–38.7
Lumipulse™	14.7	20.8–38.7

It is observed that Ella™ recorded higher values for both aliquots CSF & Serum compared to other two assays Lumipulse™ and SIMOA™. Nevertheless, we observed strong correlation between values of biological fluids obtained by two

assays and SIMOA™ as shown in Table (2). Using spearman test, (r >0.9) (p <0.0001) we observed stronger correlation for CSF NFL than serum NFL in case we use Ella™ or Lumipulse™ as follow, CSF correlation by spearman 0.98 (0.97–0.99); (p<0.001), and for serum NFL correlation by spearman 0.89 (0.89–0.94) (p<0.001).

Table (2)

Correlation according to Spearman between assays (p<0.0001 for all comparisons) **SIMOA™**		
	SIMOA™ VS Ella™ (95% CI)	**SIMOA™ VS Lumipulse™ (95% CI)**
Serum NFL	**0.93 (0.89-0.96)**	**0.92 (0.86-0.95)**
CSF NFL	**0.93 (0.89-0.96)**	**0.95 (0.91-0.97)**

Afterwards, we compared the three assays with Passing-Bablok regression (Table 3). The analysis of CSF NFL revealed an insignificant intercept and a minimal significant slope when comparing both Ella™ and Lumipulse™ to SIMOA™. There was a proportional error between the two methods with higher value observed for Ella™. On the other side serum NFL didn't reveal any differences between SIMOA™ and Lumipulse™, thereby affirming a very high agreement between methods.

Table (3)
Passing-Bablok regression between SIMOA™, Ella™, and Lumipulse™.

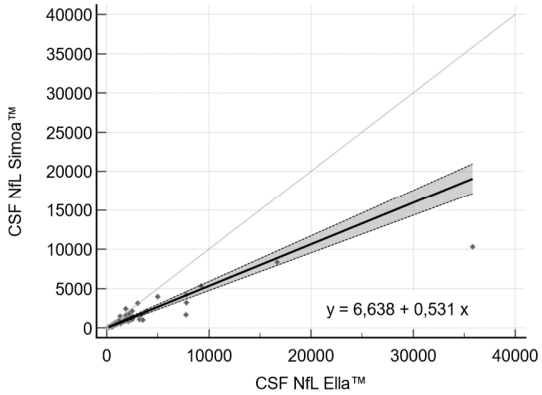
Table (3)

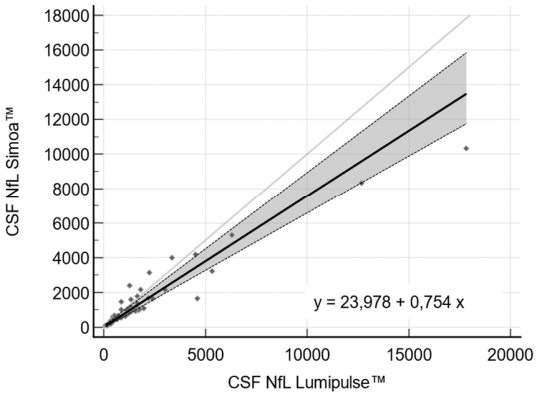
Comparison between assays	Passing- Bablok		
	Intercept (95% CI)	Slope (95% CI)	Linear model validity (p)*
CSF			
SIMOA™/ Ella™	6.6 (-67.6 to 61.5)	0.5 (0.5 to 0.6)	0.22
SIMOA™/ Lumipulse™	24.0 (-37.9 to 86.0)	0.8 (0.7 to 0.9)	0.56
Lumipulse™/ Ella™	-35.5 (-56.1 to -18.8)	0.7 (0.7 to 0.7)	0.56
Serum			
SIMOA™/ Ella™	-5.8(-7.4 to -3.5)	0.8 (0.7 to 0.9)	0.95
SIMOA™/ Lumipulse™	0.04(-2.1 to 1.4)	1.0 (0.9 to 1.2)	0.37
Lumipulse™/ Ella™	-6.3(-8.8 to -3.1)	0.8 (0.7 to 0.9)	0.56

Custom test for linearity confirms the applicability of the Passing- Bablok method if $p>0.05$ the hypothesis of similarity of test is accepted.

The scatter diagram and regression lines are in Fig. 2(a,b). The Bland-Altman comparison of multiple methods confirmed the agreement between the two assays and SIMOA™, used as the reference method (Table 4). The analysis also showed lower biases for CSF and serum NFL detected with Lumipulse™

Fig (2.a) Passing–Bablok regression analysis of CSF NFL values measured by SIMOA™, Ella™, and Lumipulse™ in naïve MS patients. The lines represent the 95% limits of agreement





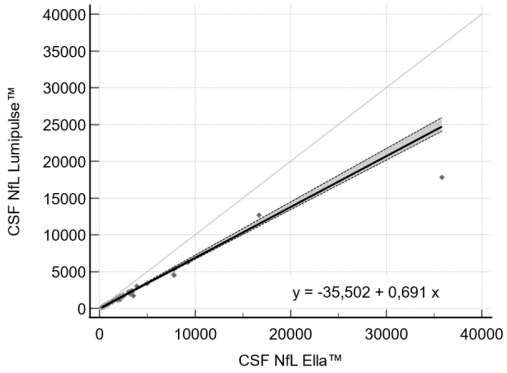
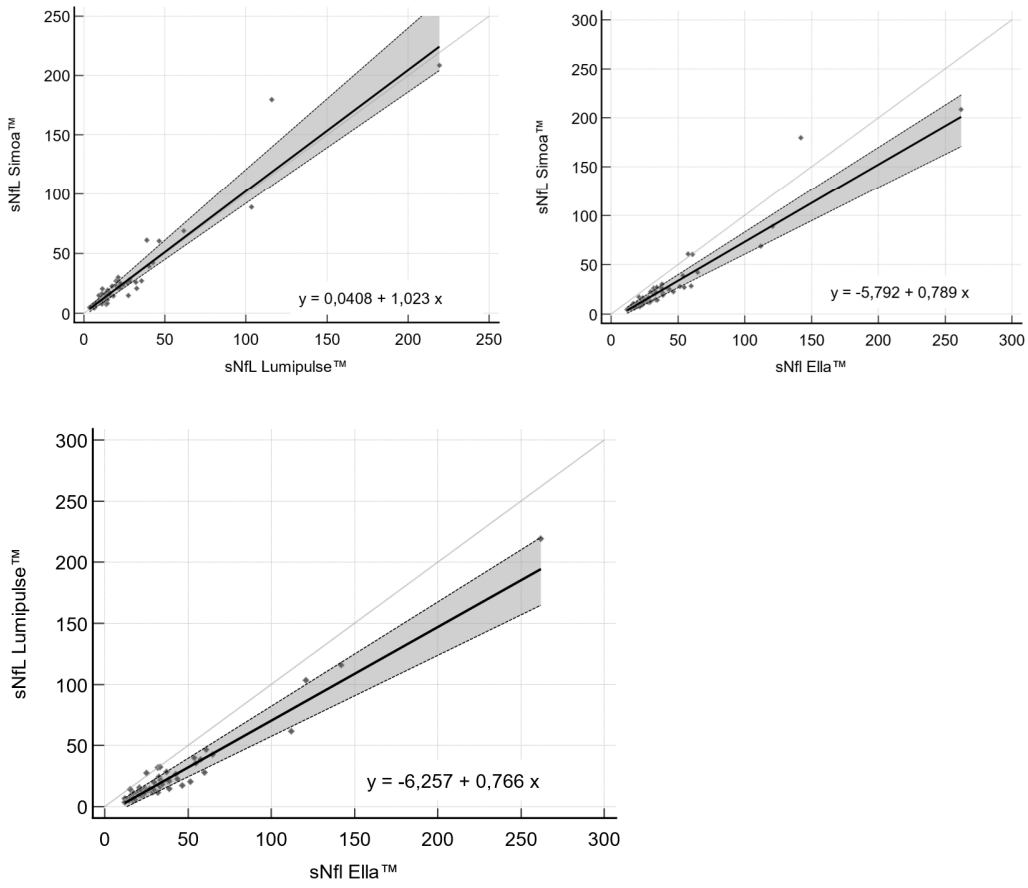


Fig (2.b) Passing–Bablok regression analysis of serum NFL values measured by SIMOA™, Ella™, and Lumipulse™ in naïve MS patients. The lines represent the 95% limits of agreement



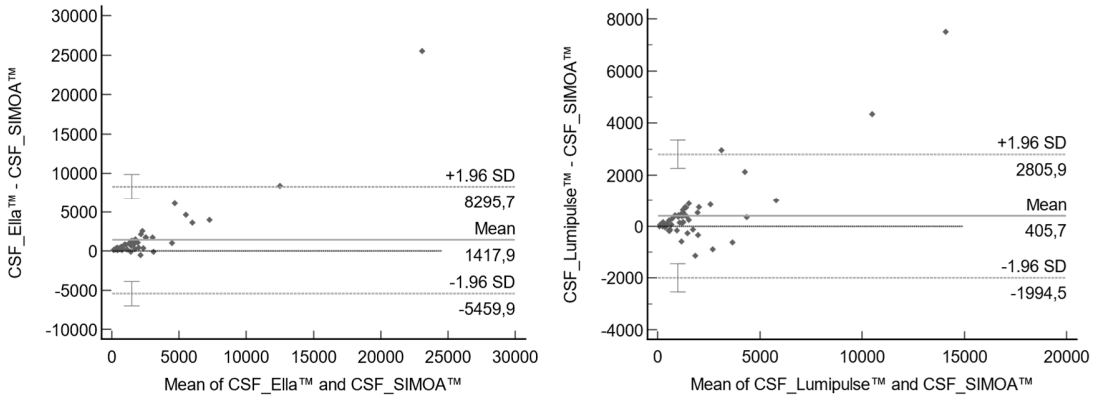
The Bland-Altman comparison of multiple methods validated the consistency between the two assays and SIMOA™, utilized as the reference technique (Table 4). Additionally, the examination indicated lower biases for CSF and serum NFL identified with Lumipulse™ (compared to Ella™) in comparison to SIMOA™. Plots illustrating these findings are presented in Fig (3.a) for CSF and Fig (3.b) for Serum.

Table (4)

Comparison between methods	**Bland-Altman for multiple methods** (SIMOA™ as reference method)			
	Bias (pg/ml) (95% CI)	**Lower limit (pg/ml) (95% CI)**	**Upper limit (pg/ml) (95% CI)**	**% difference (95% CI)**
CSF				
SIMOA™/ Ella™	1417.9 (511.4 to 2324.4)	-5459.9 (-7017.8 to -3902.1)	8295.7 (6737.8 to 9853.5)	58.6 (50.7 to)
SIMOA™/ Lumipulse™	405.7 (89.3 to 722.0)	-1994 (-2538.2 to -1450.9)	2805.9 (2262.2 to 3349.5)	-18.2 (-25.9 to -10.5)
Lumipulse™/ Ella™	1012.2 (397.0 to 1627.5)	-3655.8 (-4713.1 to -2598.4)	5680.2 (4622.9 to 6737.5)	42.5 (39.9 to 45.2)
Serum				
SIMOA™/ Ella™	12.9 (9.9 to 15.9)	-9.6 (-14.6 to -4.5)	35.4 (30.3 to 40.5)	55.5 (48.7 to 62.4)
SIMOA™/ Lumipulse™	-1.5 (-4.1 to 1.2)	-21.3 (-25.8 to -16.8)	18.4 (13.9 to 22.9)	-3.2 (-9.9 to 3.)
Lumipulse™/ Ella™	14.4 (12.0 to 16.7)	3.6 (-7.7 to 0.4)	32.4 (28.3 to 36.4)	58.2 (50.8 to 65.5)

Figure (3.a) for CSF values

Bland-Altman plots with differences between the two methods against the averages of the two methods, black dotted line represents the line of equality



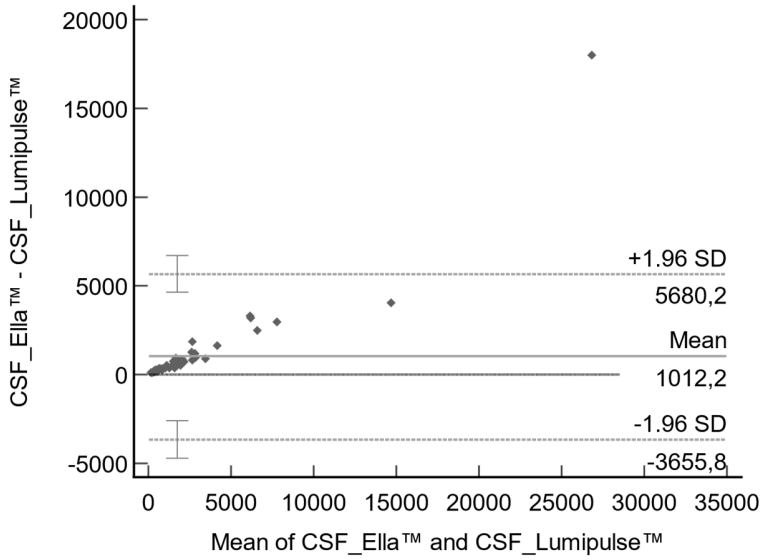
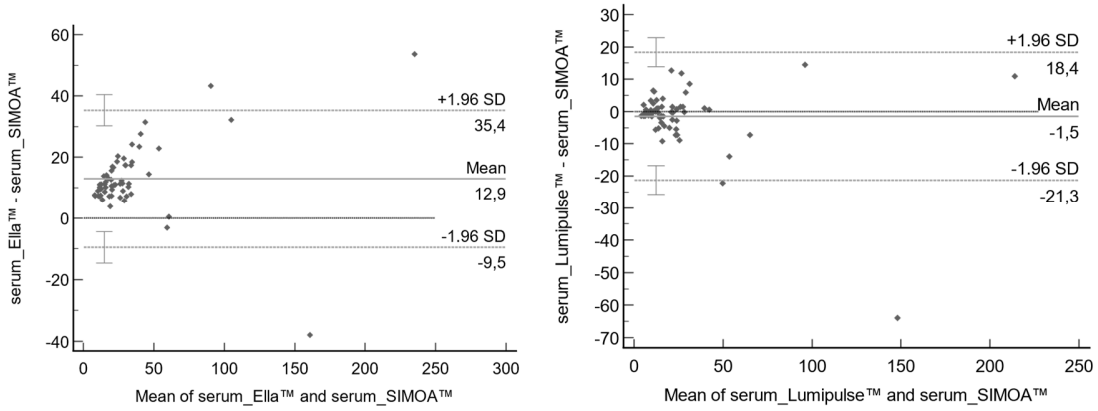
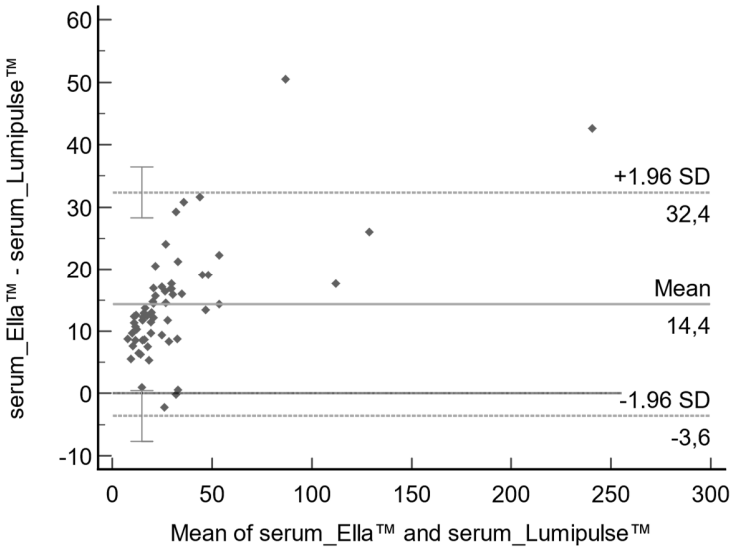


Figure (3.b) for CSF values

Bland-Altman plots with differences between the two methods against the averages of the two methods, black dotted line represents the line of equality





Section (B)

Clinical data were collected from 51 patients who underwent follow-up for RRMS at AOU Maggiore della Carità` University Hospital, University of Piemonte Orientale, Novara, Italy, over a period of 1.5 years after diagnosis. During this time, the first-time EDSS (Expanded Disability Status Scale) was calculated. The patients' data of EDSS at diagnosis and their last visit were utilized to evaluate and validate the reported results regarding the importance of CSF NFL as a diagnostic and prognostic biomarker for RRMS, with a similar evaluation conducted for serum NFL. Additionally, the MSSS (Multiple Sclerosis Severity Score) underwent a regression test with serum NFL levels obtained by Ella to validate the prognostic value of sNFL for suspected patients.

Data of patient shown on table (i)and the result as shown in the figures below

Table (i)

N	cNFL ELLA [155-1757 pg/mL]	sNFL ELLA [6,23-22,2 pg/mL]	EDSS	EDSS Last visit	Date of Birth
1	1733	34.2	1	2	1968
2	3398	59.8	3.5	2.5	1977
3	837	23.4	1	2	1973
4	4973	53.6	1	1	1984
5	807	21	1.5	2.5	1978
6	2315	46.7	1	1.5	1962
7	863	22.1	3.5	2	1980
8	463	16	1	1.5	1980
9	1870	29.5	1	1	1980
10	1369	37	1.5	0	1997
11	2113	21.7	1	2	1989

12	2190	28	3	3	1974
13	1759	43.7	2.5	3.5	1990
14	565	20.6	1.5	1	1979
15	993	16.8	0	2.5	2004
16	407	29.1	1	1.5	1991
17	564	23.7	1	1.5	1978
18	2487	42.7	1	2	1993
19	466	16	2.5	2.5	1989
20	380	19.6	2.5	0	1987
21	1326	26.2	1	1.5	1974
22	2515	34.6	1	1.5	1992
23	1956	15.3	2.5	1.5	1990
24	2285	25.2	1.5	1.5	1977
25	3082	38.6	1.5	1	1994
26	2189	64.9	2	3.5	1992
27	539	31.9	1	1	1987
28	726	14.8	1.5	2.5	1990
29	594	12.1	2	2.5	1990
30	2064	38.7	0	1	1993
31	451	21	1.5	2.5	1965
32	7812	112	2.5	2	1973
33	271	17.2	1	1	1989
34	7812	37.9	1.5	2	1992
35	288	14.3	1.5	1	2001
36	407	14.3	0	1	1976
37	766	21.4	1.5	1	1988
38	3884	60.8	2	4	1986
39	553	17.4	1	4	1975

40	1975	29.6	1.5	2	1978
41	963	24.2	3.5	5.5	1961
42	2495	32.5	1	1	1984
43	7752	54.6	3	6.5	1970
44	3557	33.5	1.5	1	1969
45	599	16.6	1	1	1993
46	9240	121	3.5	3.5	1980
47	1222	33.3	1.5	0	1980
48	1873	38.3	1.5	2.5	1979
49	1749	33.2	1	2	2000
50	634	16.5	0	0	1993
51	201	14.7	1	0	1992

Correlation results between NFL serum levels by Ella™ and the EDSS at two points from first diagnosis to last visit is explained in figures as follow, same procedure done for CSF NFL obtained by Ella™

Table (i.a)

Regression Statistics sNFL ELLA™ / EDSS				
Multiple R	0.400061287			
R Square	0.160049033			
Adjusted R Square	0.142907177			
Standard Error	20.00057636			
Observations	51			
	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	17.89241159	5.619906822	3.1837559	0.002527
EDSS	9.672445972	3.16547372	3.0556077	0.003629

The **Table (i.a),** shows the regression test between EDSS score and serum NFL measured by ELLA™, we observed moderate correlation between the two variables (sNFL levels and EDSS scores) indicated by coefficient of correlation

Multiple R 0.4, the intercept 17.89 represent the level of sNFL when EDSS score equal zero, the p-value of 0.0036 is less than 0.05 ($p < 0.05$) this represents positive correlation between sNFL measured by Ella™ and the EDSS

Table (i.b)

Regression Statistics sNFL ELLA™/EDSS last visit	
Multiple R	0.303150906
R Square	0.091900472
Adjusted R Square	0.073367829
Standard Error	20.7961178
Observations	51

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	23.25685003	5.173982467	4.494961	4.2634E-05
EDSS last visit	5.033167342	2.260222447	2.226846	0.03058514

Table (i.b.) shows the regression test between sNFL ELLA™ and EDSS score measured at last visit for each patient. We aimed to test the stability of correlation of sNFL by progress of disease and at different time interval. The p-value is 0.03058514 ($p < 0.05$) and this indicates that the test is statistically significant and there is strong correlation between the sNFL if measured by ELLA™ and EDSS at last visit.

Table (i.c)

Regression Statistics cNFL ELLA™ and EDSS Last visit	
Multiple R	0.363881023
R Square	0.132409399
Adjusted R Square	0.114703468
Standard Error	1978.714491
Observations	51

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	893.7300521	492.2954458	1.8154343	0.07557928
EDSS Last visit	588.101216	215.0562404	2.7346392	0.00866832

Table (i.c) shows the regression test between cNFL ELLA™ and EDSS score measured at last visit for each patient. We looked to test the stability of sNFL to correlate with progression of the disease different phases. Although intercept P-value doesn't provide strong correlation when the EDSS score equal zero, the p-value of EDSS 0.00866832 ($p < 0.05$) and this indicates that the test is statistically significant and there is strong correlation between the cNFL if measured by ELLA™ and EDSS at last visit.

Table (i.d)

Regression Statistics ELLA™ and the EDSS	
Multiple R	0.376583009
R Square	0.141814762
Adjusted R Square	0.124300778
Standard Error	1967.959855
Observations	51

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	642.303168	552.9716151	1.161548	0.251046
EDSS	886.2998527	311.4672842	2.845563	0.006455

Table (i.d) represent the regression test to validate a correlation between CSF neurofilament assayed by ELLA™ and the EDSS score, the p-Value if intercept 0.251046 doesn't indicate correlation between the two variables when EDSS is zero, however we got strong correlation between the two-variable represented by the p-Value of EDSS 0.006.

Chapter (5) Discussion

In our study, we selected 71 pseudonymized samples from those stored in the biobank of Clinical Biochemistry Laboratory, Department of Health Sciences,

AOU Maggiore della Carità of Novara), with the collaboration with the Multiple Sclerosis Center, SCDU Neurology of the Hospital for clinical evaluations of patients. However, due to a shortage of sufficient aliquots of invasive CSF samples and the difficulty in repeating lumbar punctures, only 60 samples were used for parallel technical comparisons. We conducted comparisons of CSF and serum NFL levels measured using the gold standard SIMOA™, and two other new commercial platforms, Lumipulse™ and Ella™.

Firstly, we verified that neurofilaments Light chain CSF values surpass those in the serum which is very apparent when utilizing SIMOA™ and Lumipulse™. Overall, concentrations of NFL in serum and CSF detected with Lumipulse™ and Ella™ showed a stronger correlation with those detected by the gold standard SIMOA™. However, Lumipulse™ and Ella™ values tended to overestimate the levels measured with SIMOA™.

The results obtained from Lumipulse™ (bias +405.7 pg/ml) were closer to the gold standard compared to those from Ella™ (bias +1417.9 pg/ml).

We found similar bias in case of Serum NFL, were values measured with Ella™ were also notably higher than those obtained with SIMOA™ (bias +12.9 pg/ml), while the levels measured with Lumipulse™ aligned more closely (bias -1.5 pg/ml) with the gold standard. These discrepancies are more pronounced at higher NFL concentrations, both in CSF and serum. We confirmed reported results for Lumipulse™ and Ella™, differences tend to be more apparent at high NFL values, (Notzel ¨ et al., 2022). Gauthier et al. (2021) suggested that these differences may be related to different calibrators used, in fact SIMOA™ uses recombinant human NFL while Ella™ uses Bovine derived calibrator.

Few authors before showed two-by-two differences between platforms (Notzel et al., 2022; Gauthier et al, 2021; Truffi et al., 2022), but, to date, our study represents the first of its kind, as no previous comparisons have been conducted between the three assays for quantifying NFL levels in serum and CSF.

A previous study on 42 undertreatment showed similar bias between Ella™ and SIMOA™ (Notzel et al., 2022). We confirmed previous reported correlation between SIMOA™ and Ella™ plasma NFL {Truffi et al., 2022}. Our study holds a superior position due to its comprehensive examination, particularly in incorporating Lumipulse™ alongside the comparison between Ella™ and SIMOA™. This inclusion expands the scope of analysis beyond previous studies

and provides a more thorough understanding of NFL level quantification. Therefore, clinicians must exercise caution when interpreting data and determining them as pathological, especially if the analysis was conducted using alternative methods. Each platform possesses its own set of strengths and weaknesses. SIMOA™, for instance, offers the lowest limit of quantification (LOQ) rendering it particularly appropriate for patients anticipated to exhibit very low NFL concentrations {Notzel ¨ et al., 2022}. We have the alternative choices between the cost-effective Ella, which is not as flexible due to its single-use cartridges, and the more flexible SIMOA, despite its higher cost {Gauthier et al., 2021}; {Notzel ¨ et al., 2022}. Our study stands out as superior by adding flexibility through confirming the correlation of Lumipulse™ results to the gold standard.

The importance of CSF NFL as Biomarkers for RRMS patients is on rise and it is recognized for prognosis and treatment, monitoring also flexible tool for disease activity follow-up alongside with prognosis of progression from RRMS to SPMS {Igal Rosenstein et .al 2022}.
In our study we confirmed the correlation between serum NFL and RRMS EDSS at different time intervals at beginning of diagnosis also last visit of follow-up for around 1.5 years, as most previous study discusses the relation between CSF NFL which is invasive and require lumbar puncture. The serum NFL is crucial for early diagnosis and flexible tool for follow-up. correlation
Between the NFL biomarker values measured by Ella™ and the clinical data
Since we already verified the correlation between the two alternative assays Ella™ and Lumipulse™ to gold standard SIMOA™ in first part of our study
We selected on alternative technique Ella™ for our clinical correlation. We verified the strong correlation between Neurofilament values either in serum or Or in CSF and Important clinical parameter for progression of MS disease,
Expanded Disability Status Scale (EDSS), we affirmed the correlation that gives or flexibility for clinics to conduct analysis either by CSF or by serum ,with alternative methods also to SIMOA™.

Our study was conducted under the supervision of **Prof. Umberto Dianzani** [a], and technical part of our study was published on open access journal by Vecchio and colleagues (Vecchio D[b] et al. Serum and cerebrospinal fluid neurofilament light chains measured by SIMOA™, Ella™, and Lumipulse™ in multiple

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Conclusion

NFL has emerged as an established biomarker for monitoring MS activity over time (Notzel ¨ et al., 2022; Siller et al., 2019). Baseline values at diagnosis are essential for comparison with subsequent measures to track the disease course. Although CSF values were consistently surpassed serum values across all assays, on the other side CSF is practically constrained for monitoring because of necessity of repeated lumbar puncture procedures over time. Conversely, serum NFL measurements are less invasive puncture and could facilitate the integration of this assay into clinical routine helping early diagnosis of MS.

While all available techniques are effective in detecting serum NFL, the three techniques are detecting serum NFL effectively with minor difference that have to be considered clinically, in our cohort, we report the best agreement between SIMOA™ and Lumipulse™, particularly for serum values.

sNFL beside cNFL are vital biomarkers for early diagnosis and prognosis of RRMS, additionaly sNFL offers more flexibility because non-invasive sampling procedure and ease of collecting.

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