University of Piemonte Orientale

Department of Translational Medicine

Doctor of Philosophy in Medical Sciences and Biotechnology

Cycle XXXII

Project:

Biomarkers in the diagnosis and prognosis of multiple sclerosis

Thesis:

Kappa free light chains as a biomarker in multiple sclerosis

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Introduction.

Multiple sclerosis and the need of biomarkers.

Multiple sclerosis (MS) is a chronic autoimmune and inflammatory disease of the central nervous system (CNS), affecting more than 2 million people worldwide and being a leading cause of disability in young adults (Reich et al. 2018).

MS diagnosis is based on neurological symptoms and signs, alongside evidence of dissemination of CNS lesions in space and time according to the 2017 McDonald criteria (Thompson et al. 2018). Magnetic resonance imaging (MRI) is often sufficient to confirm the diagnosis when characteristic lesions accompany a typical clinical syndrome. Although, "no better explanation" is required to exclude misdiagnosis, and in some cases, supportive information is obtained from cerebrospinal fluid (CSF) analysis and neurophysiological testing (Brownlee et al. 2017).

MS is characterized by the heterogeneity in symptoms, disease course, and outcomes. Typical presenting syndromes vary from, but are not limited to, monocular visual loss due to optic neuritis, limb weakness or sensory loss due to spinal cord lesions, double vision due to brainstem dysfunction, or ataxia due to a cerebellar lesion. Onset symptoms progressively subside completely or not. Sometimes, a first clinical event highly suggestive of demyelinating CNS disease could not meet the criteria of dissemination in time for the diagnosis of MS: this situation is defined clinical isolated syndrome (CIS) and up to 80% convert to MS in 20 years (Miller et al. 2012). A more undetermined condition is the radiologically isolated syndrome (RIS), where incidental imaging suggests demyelination in the absence of clinical signs or symptoms. RIS may raise the suspicion of MS, and there is a 34% risk of first acute or progressive clinical event within 5 years (Okuda et al. 2014). However, after a first clinical event, MS clinical course is characterized by alternating periods of neurological dysfunction, named relapses, lasting at least 24 hours in the absence of infection, and periods of relative clinical stability, named remissions. The frequency of relapses is highly variable but generally does not exceed 1.5 per year. Relapses result in residual deficits in almost half of episodes, leading

to stepwise accrual of impairment. This relapsing remitting (RR) phenotype affects about the 85% of MS patients (Lublin et al. 2014). The majority of untreated RRMS patients do eventually evolve into a secondary progressive (SP) phase about 20 years after the onset. Phenotypically, SPMS consists of periods of progression with possible superimposed relapses or periods of relatively stable disability. On an individual level, it is difficult to determine when exactly the transition to SP starts. Few predictors of earlier progression are higher age at RRMS onset, male gender, spinal cord symptoms and incomplete relapse recovery (Rovaris et al. 2006). Lastly, about 10%–20% of MS patients present a primary progressive (PP) course, characterized by ongoing progression from the disease onset (Ransohoff et al. 2015). In the past years, these clinical phenotypes resulted limited in the individual description of disease course, and some information of "activity and progression over time" were added to enhance the characterization of an ongoing disease. Actually, there are no definitive biomarkers to determine these two entities that remain clinically and radiologically defined. In fact, disease activity is decided upon the presence of clinical or radiological relapses, being this latter at least a gad-enhancing lesion or new/enlarging T2 lesions. On the other hand, progression is estimated by sustained worsening over time, yearly assessed according to the Expanded Disability Status Scale (EDSS) score (Lublin et al. 2014). Still, much has to be discovered in detecting activity and progression toward a more individualized patient care.

Clinical management is challenging since disease onset because of the uncertain long-term prognosis. Studies on MS natural history indicated several clinical factors as predictors of disability (Ebers. 2001) and clearly that early treatment reduced disability accumulation (Cerqueira et al. 2018). The initiation of a disease-modifying therapy (DMT) early improves MS prognosis and the occurrence of neurological damage. In fact, DMT reduces the relapse rate, the appearance of MRI activity, and slows the course of disability progression. It is widely believed that disability results from a series of successive exacerbations, each adding to a growing accumulation of deficits. The impact of treatment may decrease as the disease unfolds in line with the natural history of MS (Tremlett et al. 2004). There is a highly significant association between relapses in the first two years and shortened time to walking aids (Ebers. 2001). Nevertheless, brain atrophy, which accompanies axonal damage and loss, can be observed early in MS disease course, even in patients with CIS. Neurodegeneration continues to progress in all MS patients. Delays in the

diagnosis and treatment relate to severe and irreversible neurological disability (Scalfari et al. 2010). Here comes the need for biomarkers in MS diagnosis, monitoring and prognosis. Early diagnosis and treatment MS can alter disease course and slow disability progression. The search for a reliable biomarker to predict disease progression and monitor response to therapy remains a challenge. Besides, the concept of "no evidence of disease activity" (NEDA) has been introduced as the main therapeutic goal in patients with MS: the more stable the disease is the better in an ongoing process that is requiring more precise outcome measures. Yet, NEDA definition itself is evolving over time: NEDA-3 was defined as no relapses, no disability progression and no MRI activity; NEDA-4 as NEDA-3 plus brain volume loss; and finally, the use of biomarkers is debated as NEDA-5, being the CSF neurofilament light chains the candidate to be included as a measure of neurodegeneration (Giovannoni et al. 2015, Håkansson et al. 2018).

Nonetheless, individual outcomes are still debated as well as a lack of biomarkers that would distinguish MS phenotypes and prognosticate the disease course on an individual patient's level. There is a pressing need, using large datasets of clinically and radiologically well-characterized patients, to explore the potentials of biological markers in supporting (and refining) MS courses since the early phases.

Out of the purpose of this thesis, several instrumental markers have been used to characterize MS diagnosis/prognosis. While T2 and gadolinium-enhancing MRI lesions are common measures of disease activity, the assessment of brain volume loss is ongoing both "directly" with imaging techniques and "indirectly" with optical coherence tomography for with retinal nerve fiber thickness. Further information about their roles as potential markers remains a research priority. There is also a potential value of electrophysiology studies to define MS phenotypes. However, there is considerable interlaboratory variability in those measures. Molecular biomarkers are easily quantifiable and can be complement of MRI and clinical characteristics.

The value of a biomarker is in its ability to predict or be a surrogate for a clinical state of a patient. A biomarker is defined as a characteristic that can be objectively measured, and serves as an indicator of normal biological processes, pathological processes or reactions to therapy. Ideally, if the disease worsens or improves, the concentration of the biomarker should increase or decrease accordingly (Biomarkers Definitions Working Group. 2011). Furthermore, ideal biomarkers are safe for the patient, and easy to

detect with non-invasive methods. The measure should be highly accurate and reproducible, fast and cost-effective (Teunissen et al. 2014). In MS, blood and CSF are particularly suitable. Although the importance of biomarkers has been increasingly recognized, their validation is a lengthy process, so that only a few biomarkers have so far been routinely used in clinical practice. We are focus on biomarkers that are reliable tools for diagnosis and monitoring patients with MS in clinical practice using serum and CSF.

Expected cerebrospinal fluid changes in multiple sclerosis.

For both anatomical and physiological reasons, CSF represents the main source of potential biomarkers for MS among body fluids. The hallmark of CSF changes in MS is the increased production of intrathecal immunoglobulins (Ig) (Link, 1967). The qualitative evaluation of increased intrathecal IgG synthesis is reported as the presence of oligoclonal bands (OB). Two or more OB detected by separation of CSF proteins, not demonstrable in corresponding serum, reflect a local B-cell response present in more than 95% of patients with MS. To date, several studies have attempted to identify the target of the OB, and there has been no definite association with any consistent antigen in MS (Freedman et al. 2005). Once present, CSF OB persists in the individual patient irrespective of MS course. With nearly 86% specificity and more than 95% sensitivity, examination of CSF for OB is strongly recommended to support the diagnosis of RRMS (Link, 2006), and the presence of CSF OB is one of the required criteria for PPMS. Furthermore, to improve early diagnosis, the 2017 McDonald criteria introduced the use of CSF analysis to anticipate MS diagnosis in CIS. In fact, in patients with a typical CIS and clinical or MRI demonstration of dissemination in space, the presence of OB allows MS diagnosis (Thompson et al. 2018). This has been a great example of the re-introduction of a well-established biomarker in MS.

Standardized “gold standard” methodology for detecting is isoelectric focusing (IEF) on agarose gels followed by immunoblotting (Freedman et at. 2005). As a qualitative assessment, CSF OB detection is actually considered a more reliable test than any quantitative assessments of intrathecal synthesis. Using IEF requires a certain level of technical expertise: each gel run requires the presence of controls. Similarly,

the result interpretation necessitates some experience. For this reason, quantitative analysis could give complementary information. The 2017 criteria mention “other tests, such as the IgG index” indicating intrathecal antibody synthesis recommending that positive results on these other tests should be “interpreted with caution” when testing for OB (Thompson et al. 2018).

One of the main purposes of our work was to evaluate the use of complementary quantitative tests to detect intrathecal synthesis in MS.

The most known quantitative evaluation of intrathecal synthesis is the IgG or Link index (Link, 1967). It is calculated as the ratio between IgG quotient (the ratio of IgG in CSF to serum) and albumin quotient. This latter value considers the integrity of the blood-CSF barrier, and is calculated as ratio of albumin in CSF to serum. This is crucial, since the increased concentration of a substance in CSF can be the result of either intrathecal synthesis or increased permeability of the blood-CSF barrier. Regarding the IgG index, a value greater than 0.70 is universally considered suggestive of pathological intrathecal synthesis. With a cut-off value of 0.7, the positive predictive value is 60% for the diagnosis of MS (Mayringer et al. 2005).

Not only Ig are produced in their assembly. Ig are formed by two heavy chains, which determine their class (IgG, IgM, IgE, IgD and IgA) and by two free light chains (FLC), either kappa or lambda. FLC are produced in excess of Ig in the CSF of MS patients from intrathecal humoral activity of plasma cells. In MS they result in particular of kappa subtype (KFLC). Similarly to the Link index, we could calculated a kappa index as the ratio between the CSF/serum KFLC and albumin quotients. During the last years, the kappa index has been explored as a diagnostic biomarker for MS, despite the lack of an unequivocal cut-off value. It gained increasing interest as a possibly more sensitive, less costly and less time-consuming, quantitative marker of intrathecal immunoglobulin synthesis compared to OCB detection. The intrathecal KFLC synthesis can be calculated using different metrics: the linear kappa index (CSF/serum KFLC quotient divided by CSF/serum albumin quotient), the KFLC intrathecal fraction (KFLC IF, which takes into account the non-linear relationship of the blood-to-CSF transfer between albumin and KFLC), or other mathematical models to calculate indexes named Tourtellotte’s or Reiber’s (Reiber et al. 2019, Kaplan et al. 2013).

Conversely, lambda FLC has not resulted as a reliable marker for MS intrathecal synthesis (Desplat-Jégo at al. 2005).

Despite Ig and FLC can be detected by ELISA, Western blotting or nephelometry, this latter technique is the most used (Freedman et al. 2006).

Our analysis concentrated on the role of kappa index in MS diagnosis.

Prognostic value of intrathecal synthesis.

Since CSF analysis has then entered back in the diagnostic work-up again, the presence of intrathecal synthesis has been discussed as a useful tool in MS prognosis.

OB are the hallmarks of definite MS, but some conditions suggesting for demyelination could not fulfil the 2017 McDonald criteria are not fulfilled for MS despite CSF intrathecal synthesis (Thompson et al. 2018). In those case OB are a prognostic marker. Approximately half of the individuals with RIS experiences a first clinical event within 10 years, and OB are a risk factor for clinical manifestations (Lebrun-Frenay et al. 2020, Boyko at al. 2020). In addition, OB also have a predictive role for conversion to MS in those cases remaining CIS according to the 2017 McDonald criteria. Indeed, most publications on the prognostic role of OB considered CIS according to the previous McDonald criteria 2010 (Polman et al. 2010), and brought to the new 2017 version.

Some data have been presented on the prognostic role of intrathecal IgG synthesis in disease course. For example, a low number or no OB at diagnosis resulted predictive for a better prognosis (Avasarala et al. 2001), and patients with no OB had milder disability and less progression in SPMS (Rojas et al.2012). Not conclusive results have been published for IgM (Frau et al. 2018). Looking at quantitative measures, the IgG index has been also related to a major disability progression with greater values in SPMS (Izquierdo et al. 2002). Moreover, a recent retrospective study involving 149 MS and CIS patients related CSF and MRI activity: the IgG index resulted highly correlated with new cerebral lesions as independent predictor.

Data on KFCL are not available so far (Klein et al. 2019), and in our project we searched for any prognostic role for KFLC in early MS.

Aims.

To evaluate the role of KFLC towards MS diagnosis in a large cohort of Italian patients.

To analyse whether the same markers are related to MS prognosis.

Chapter 1: kappa free light chains in multiple sclerosis diagnosis.

Kappa index and isoelectrofocusing in cerebrospinal fluid analysis.

This section is a modified version of an article by Crespi et al. 2017

Introduction.

CSF analysis is a key diagnostic approach in several inflammatory neurological disorders, and the gold standard in suspected MS patients is CSF protein IEF and immunoblotting to detect OB. This procedure is predominantly manual, and the results need trained personnel to be interpreted properly. On the other hand, during Ig assembly, B-lymphocytes and plasma cells synthesize not only Ig but also large amounts of kappa and lambda FLC. In the presence of intrathecal synthesis, an increased CSF/serum Ig or FLC quotients could be evidenced. Since an altered barrier permeability may cause a transfer from plasma to CSF, giving a false increase of the Ig or FLC quotients, they could be corrected by albumin quotient to calculate an index (Reiber et al. 2009, Mayringer et al. 2005). When considering FLC, these measures are called kappa or lambda indexes (Kaplan et al. 2013, Arneth et al. 2009). Few data were available on the combined or sequential use of the indexes and OB detection in the MS work-up. Our aim was to focus on the possible use of both parameters, considering performances and costs, to ameliorate the diagnostic approach of central nervous system inflammatory disorders.

Patients.

Our exploratory cohort included 150 patients (74 females) who underwent CSF study for OB detection. Mean age was 51 years (standard deviation or SD ± 19years). Diagnosis was prospectively collected by a blinded neurologist and compared to the initial suspicion for which the CSF request was sent for. Of the entire cohort 48 (32%) patients were diagnosed with MS according McDonald criteria 2010 (Polman et al. 2010), 32 (21%) of other neurological inflammatory diseases (ID: 12 inflammatory neuropathies, 3 acute

demyelinating encephalomyelitis, 9 systemic autoimmune disorders with CNS involvement, 8 others), 62 (41%) of not inflammatory neurological diseases (NID: 17 amyotrophic lateral sclerosis, 11 dementia, 12 non inflammatory neuropathies, 8 tumors, 14 others), and 8 (5%) had no evidence of neurological diseases. Patients signed an informed consent form for both diagnostic and research purposes.

Studies on FLC were approved by the ethical committee of University Hospital of Novara (reference no. CE1804).

Methods.

The OB detection was achieved by isoelectric focusing and immunofixation (Hydragel 9 CSF Isofocusing; Sebia, Bagno a Ripoli, FI, Italy) on an agarose electrophoresis system (Sebia Hydrasys). The gels were evaluated for the presence of OB by two independent operators. They were classified according the Consensus Report of the Committee of the European Concerted Action for Multiple Sclerosis (Andersson et al. 1994). The results were classified with the attribution of one of the five patterns according to Freedman (Freedman et al. 2005), as follows: (I) normal CSF; (II) CSF-restricted oligoclonal bands: local synthesis; (III) CSF-restricted oligoclonal bands with additional, identical bands in CSF and serum: local synthesis; (IV) identical oligoclonal bands in CSF and serum: not local synthesis; (V) monoclonal bands in CSF and serum: not local synthesis.

Serum and CSF albumin concentration was measured by nephelometric assays using N Antiserum to human albumin and a BN II System (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). Lower limit of the reference curve was 0.017 mg/ml and the total coefficient of variability (CV) of 3.6% and 2.6% for serum and CSF, respectively.

Serum and CSF IgG concentrations were measured by nephelometric assays using N Antiserum to human immunoglobulins and a BN II System (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). Lower limit of the reference curve was 0.0034 mg/ml and the total CV of 2.1% for both serum and CSF.

Kappa and lambda FLC (KFLC and LFLC) were measured with the N Latex FLC kappa/lambda Kit and BN II System (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) based on monoclonal

antibodies that recognize hidden epitopes in intact Ig. Lower limit of the reference curve was 0.000035 mg/ml and 0.000097 mg/ml for KFLC and LFLC. The total CV was 2.9% and 3.4% for serum and CSF KFLC, respectively and 3.5% and 3.6% for serum and CSF KFLC, respectively.

Calibrators and controls were obtained from the manufacturer and consisted of stabilized human sera containing polyclonal KFLC, diluted to the appropriate concentrations. All sample were immediately processed.

Indexes were calculated as follows: IgG or Link index: (CSF IgG/serum IgG)/(CSF albumin/serum albumin), normal ranges ≤ 0.7; kappa index:(CSF KFLC/serum KFLC)/(CSF albumin/serum albumin), normal ranges determined in this study as ≤ 5.0.

Statistics.

Sensitivity (%), Specificity (%), Likelihood ratio for positive test, Likelihood ratio for negative test, Positive Predictive Value (%), Negative Predictive Value (%), Efficiency (%), Pre test probability (prevalence) (%), Pre test odds, Post test odds, Post test probability, and Number Needed to Diagnosis (NID) were measured using the Bayesian calculator developed by SIPMEL (Società Italiana di Patologia Clinica e Medicina di Laboratorio: http://www.sipmel.it/it/).

Statistical analyses were performed using STATISTICA. The Mann-Whitney-U or Kruskal-Wallis test was used for group comparisons of non-parametric values. Qualitative variables were compared with Chi-squared test. Spearman's coefficient was used for correlation analysis. Receiver Operating Characteristic (ROC) curve analysis was performed using XLSTAT statistical package.

Results.

CSF KFLC were significantly higher in MS versus other patients ($p < 0.001$), and no differences were observed in serum KFLC, serum and CSF LFLC, serum and CSF IgG levels (Table 1). These data confirmed an increased concentration of KFLC as a hallmark of MS, as previously suggested (Kaplan et al. 2013). We subsequently compared all the biochemical markers among each group (ID and NID) and MS.

CSF KFLC in MS patients were higher than ID (0.058) and NID (p<0.001) patients (Tables 2-3). Regarding kappa index, MS patients differed significantly from other diseases (p<0.001 among all comparisons).

Table 1. Serum and CSF biochemical markers in MS (N = 48) versus not-MS (N = 102) patients.

	MS	Not-MS	p-value
Serum KFLC: mean±SD (mg/dl)	1.57±0.76	2.22±3.12	0.172
Serum LFLC: mean±SD (mg/dl)	1.82±0.85	3.00±5.15	0.138
CSF KFLC: mean±SD (mg/dl)	0.52±0.57	0.16±0.46	<0.001
CSF LFLC: mean±SD (mg/dl)	0.17±0.24	0.16±0.53	0.924
Serum IgG: mean±SD (mg/dl)	1107.46±179.43	1032.83±298.35	0.149
CSF IgG: mean±SD (mg/dl)	4.94±3.48	6.40±12.72	0.424
Kappa index: mean±SD	74.08±92.12	6.05±13.15	<0.001

Table 2. Serum and CSF biochemical markers in MS (N = 48) versus ID (N = 32) patients.

	MS	ID	p-value
Serum KFLC: mean±SD (mg/dl)	1.57±0.76	2.30±2.73	0.111
Serum LFLC: mean±SD (mg/dl)	1.82±0.85	2.91±4.93	0.173
CSF KFLC: mean±SD (mg/dl)	0.52±0.57	0.25±0.56	0.058
CSF LFLC: mean±SD (mg/dl)	0.17±0.24	0.29±0.78	0.356
Serum IgG: mean±SD (mg/dl)	1107.46±179.43	1044.00±347.48	0.326
CSF IgG: mean±SD (mg/dl)	4.94±3.48	11.05±21.32	0.076
Kappa index: mean±SD	77.54±93.00	10.03±19.58	<0.001

Table 3. Serum and CSF biochemical markers in MS (N = 48) versus NID (N =62) patients.

	MS	NID	p-value
Serum KFLC: mean±SD (mg/dl)	1.57±0.76	1.83±0.92	0.139
Serum LFLC: mean±SD (mg/dl)	1.82±0.85	2.43±1.53	0.024
CSF KFLC: mean±SD (mg/dl)	0.52±0.57	0.12±0.43	<0.001
CSF LFLC: mean±SD (mg/dl)	0.17±0.24	0.12±0.39	0.525
Serum IgG: mean±SD (mg/dl)	1107.46±179.43	1027.81±276.40	0.109
CSF IgG: mean±SD (mg/dl)	4.94±3.48	4.42±5.24	0.581
Kappa index: mean±SD	77.54±93.00	4.57±9.04	<0.001

As a second step we performed the identification of a cut-off of 5 for kappa index in the ROC analysis (AUC = 0.948) in our cohort. Then we compared kappa index to OB determined by IEF, that resulted similarly efficient (Table 4).

Table 4. Test performances of kappa index versus OB by IEF.

	Kappa index	OB
Sensitivity (%)	100	97.9
Specificity (%)	86.3	85.3
Likelihood ratio for positive test	7.3	6.7
Likelihood ratio for negative test	0	0.02
Positive Predictive Value (%)	77.4	75.8

Negative Predictive Value (%)	100	98.8
Efficiency (%)	90.7	89.3
Pre test probability (prevalence) (%)	32.0	32.0
Pre test odds	0.5	0.5
Post test odds	3.4	3.1
Post test probability	77.4	75.8
Number Needed to Diagnosis (NID)	1.16	1.20

Finally, we addressed laboratory costs of MS diagnosis. We proposed 4 scenarios: A) exclusively IEF in all patients: the estimated cost for 150 patients amounted to 6072 euros. B) only kappa index: cost decreased to 2277 euros (-62%). C) both IEF and kappa index contemporarily in all patients: raised costs to 8349 (+37.5%). D) sequential approach with kappa index as first test, followed by IEF as confirmatory test in patients with elevated index: this would lead to an overall cost of 4790 euros (-21.1% compared to A).

Discussion and conclusions.

We evidenced that KFLC are easily determined with high performances and low costs. Kappa index could therefore become a first-line test in MS work-up. Even though IEF for OB has been considered the gold standard, elevated KFLC and kappa index could quantify precisely a certain level of intrathecal synthesis (Zeman et al. 2015). When we planned this study, it was not yet defined whether the use of this index should be preferred to OB detection or performed in combination with IEF to increase both sensitivity and specificity (Senel et al. 2014). Moreover, no analysis on the costs of this procedure was performed. In this study, we primarily looked at the great performance of kappa index as a first-line biochemical approach in MS diagnosis, not excluding IEF, but introducing a potential sequential approach. We propose to use kappa index as a screening test, followed, when abnormal, by OB detection. Nonetheless, from the laboratory point of view, kappa index can be evaluated on automated analytical platforms and the assay gives quantitative results with relatively low inter- and intra-assay variability. In addition, it does not need trained

and independent technicians for performing as compared to OB detection, results are automatically generated and easily interpreted. The sequent use of OB detection for confirming positive results is supported by the established role of this technique in MS diagnostic algorithm. Then the contemporary positivity provided by two different biochemical methods, which are based on completely different principles, could definitively reinforce MS diagnosis. Moreover, our financial evaluation was extremely encouraging. The sequential use of kappa index and IEF if needed was definitely less expensive.

Another important implication was the improvement of diagnostic specificity. As pointed out by Bourahoui et al., OB can be detected in several inflammatory and infective CNS disorders, such as HIV infection, Lyme disease or Sjogern's syndrome. A high number of bands (10 or more) were more frequently found in MS rather than in other ID (Bourahoui et al. 2004). We evidenced that kappa index is significantly higher in MS in comparison to ID, thus suggesting a potential role in further discriminating between these conditions. We found a wide range for kappa index values in MS, from 5.1 to more than 300: this finding could reflect different clinical MS forms or sequential steps in the evolution of the immune response and hence the progression of the disease.

Kappa index versus Link index.

This section is a modified version of an article by Crespi et al. 2019

Introduction.

The gold standard for CSF analysis is IEF to detect CSF specific IgG as OB. Intrathecal synthesis determined with OB detection has been compared historically to elevated IgG (or Link) index. This biomarker is calculated as ratio of CSF/serum IgG to CSF/serum albumin (Link. 1976). Recently, measures of KFLC by nephelometry have been proposed as an alternative method not only to IgG index, but also to OB detection by IEF (Bayart et al. 2018). A sequential method in CSF analysis, using an index and not

excluding IEF, has been considered reasonable (Schwenkenbecher et al. 2018). Our aims were to confirm the role of kappa index in the CSF screening for MS and to compare its performances to that IgG Index and OB in a large cohort of Italian patients.

Patients.

We consecutively enrolled 385 patients who underwent lumbar puncture in their diagnostic work-up for CSF biochemistry and IEF (according to the requesting neurologist), and included those 150 patients described in the previous paragraph. Mean age was 48 years (±SD 18 years). Diagnosis was prospectively collected by a blind neurologist, and compared with the initial clinical suspicion: 127 patients (33%) were diagnosed of MS according to the 2017 McDonald criteria (Thompson et al. 2018); 117 (30%) of other neurological ID (inflammatory neuropathies, acute demyelinating encephalomyelitis, systemic autoimmune disorders with CNS involvement), 141 (37%) of NID (amyotrophic lateral sclerosis, dementia, non-inflammatory neuropathies, tumors). Patients signed an informed consent form for both diagnostic and research purposes at the time of the lumbar puncture.

Methods/Statistics.

See previous paragraphs. Statistical analyses were performed using VassarStats (http://vassarstats.net/). T test for two independent samples was used for group comparison, calculated for either equal and/or unequal sample variances. The difference between three or more samples was calculated using one-way ANOVA. Qualitative variables were compared with Chi-Squared test. Spearman's coefficient was used for correlation analysis. ROC curve analysis was performed using XLSTAT statistical package.

Results.

The 127 MS patients had a median kappa index of 72.9 (± SD 87.9) that resulted significantly higher than other 258 patients who had a median value of 12.7 (± 48.9) ($p<0.0001$) including both ID (23.5 ±70.7; $p<0.0001$) and NID (3.8 ± 8.4; $p<0.0001$). Kappa index resulted higher, than the cut-off of 5, in 96.1% MS, 19.4% not-MS, 33.3% ID, and 7.8% NID. IgG index resulted more elevated in MS (0.86 ± 0.5) then other patients (0.50 ± 0.23; $p<0.0001$), and above the cut-off value of 0.7 in 59.1% MS, 15.1% non-MS, 17.9%

ID, and 12.8% NID. OB type 2 and 3 were found in 123 (96.8%) MS patients and in 44 (17%) with other neurological disorders (35 ID and 9 NID). Data are reported in Table 1.

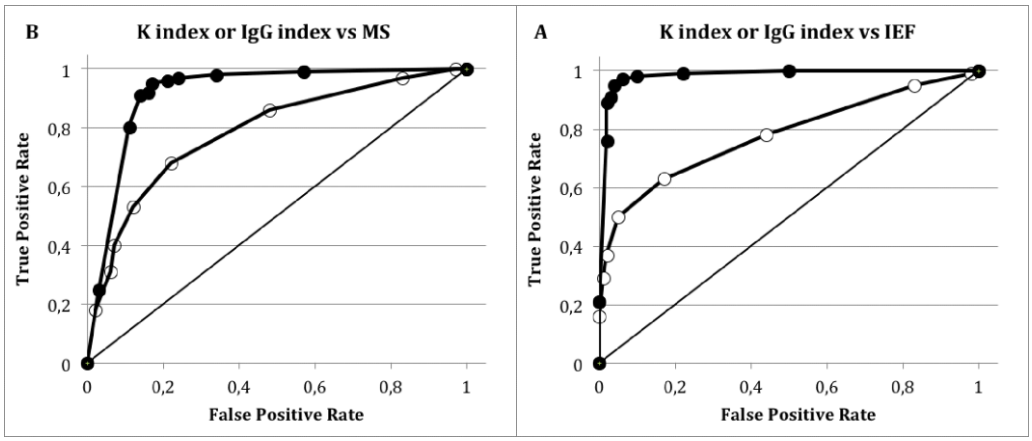
Table 1: Markers of intrathecal synthesis among patients with multiple sclerosis (MS: N=127), inflammatory diseases (ID: N=117) and non-inflammatory diseases (NID: N=141).

	MS	**Not-MS**	**ID**	**NID**
IgG index >0.7: N (%)	75 (59.1%)	39 (15.1%)	21 (17.9%)	18 (12.8%)
Kappa index >5: N (%)	122 (96.1%)	50 (19.4%)	39 (33.3%)	11 (7.8%)
OB: N (%)	123 (96.8%)	44 (17%)	35 (29.9%)	9 (6.4%)

When dividing not-MS patients into ID and NID: kappa index differed among the three groups (ANOVA: $p<0.001$), whereas IgG index was not able to differentiate ID from NID. We also confirmed a correlation between IgG and kappa indexes in all patients (N=385: r=0.75, r2=0.55, $p<0.0001$), and in each group.

To compare the kappa and IgG indexes ability to predict intrathecal synthesis, we analysed their correlation with OB and with MS diagnosis by ROC analysis. Kappa index showed a better AUC (0.981) than IgG index (0.778) to predict OB (Figure 1A). Similar results were in diagnosing MS: AUC for kappa index was 0.949 versus AUC for IgG index was 0.789 (Figure 1B). In panel A, ROC analysis was performed for OB (type 2 and 3 by IEF) versus absence of OB (type 1, 4 and 5). AUC was 0.778 for IgG (open symbols) and 0.981 for kappa index (closed symbols). In panel B, ROC analysis was performed for MS patients versus not-MS patients AUC was 0.789 for IgG (open symbols) and 0.949 for kappa index (closed symbols).

Figure 1. ROC analysis for kappa and IgG indexes in predicting OB detection and diagnosing MS.



We then looked at the diagnostic parameters of both indexes in predicting OB and in diagnosing MS. First in detecting OB, kappa index showed a significantly better sensitivity, likelihood ratio for a positive test, positive and negative predictive values and efficacy than IgG index. By contrast, the specificity was similar for both indexes. Secondly, kappa index had higher sensitivity, negative predictive value and efficiency in diagnosing MS (Table 2).

Table 2: Diagnostic performances of IgG and kappa indexes for OB detection by IEF and MS diagnosis. The analysis was performed for OB (type 2 and 3 by IEF) versus absence of OB (type 1, 4 and 5 by IEF); data are reported with their 95 % confidence intervals.

	OB detection		**MS diagnosis**	
	IgG index	**Kappa index**	**IgG index**	**Kappa index**
Sensitivity (%)	48.0 (41.2 – 54.9)	96.5 (93.0 – 98.3)	49.6 (42.5 – 56.7)	96.1 (90.8 – 98.5)
Specificity (%)	93.8 (90.3 – 96.1)	89.8 (85.7 – 92.9)	88.5 (84.2 – 92.7)	77.9 (72.0 – 83.7)
Likelihood Ratio for a positive test	7.8 (4.8 – 12.6)	9.48 (6.7 – 13.5)	4.3 (3.1 – 7.0)	4.4 (3.1 – 5.9)
Likelihood Ratio for a negative test	0.6 (0.5 – 0.6)	0.04 (0.02 – 0.08)	0.6 (0.5 – 0.7)	0.05 (0.02 – 0.11)
Positive Predictive Value (%)	85.1 (77.4 – 90.5)	87.4 (82.5 – 91.2)	67.7 (58.2 – 76.9)	68.2 (60.9 – 75.4)
Negative Predictive Value (%)	71.1 (66.2 – 75.5)	97.2 (94.4 – 98.7)	78.4 (73.3 – 82.9)	97.6 (94.5 – 98.8)
Efficiency (%)	74.4 (70.3 – 78.1)	92.7 (89.9 – 94.7)	75.8 (70.1 – 80.7)	83.9 (80.7 – 87.5)
Pre test Probability (prevalence) (%)	42.3 (37.9 – 46.8)	42.3 (37.9 – 48.8)	32.6 (28.0 – 37.2)	33.0 (28.5 – 38.3)
Pre Test odds	0.7 (0.6 – 0.9)	0.7 (0.6 – 0.9)	0.5 (0.4 – 0.6)	0.5 (0.4 – 0.6)
Post Test odds	5.7 (2.9 – 11.1)	6.9 (4.1 – 11.9)	2.1 (1.2 – 3.4)	2.1 (1.5 – 3.7)
Post test Probability (%)	85.1 (74.6 – 91.7)	87.4 (80.3 – 92.2)	67.7 (57.9 – 76.8)	68.2 (58.0 - 67.3)
Number Needed to Diagnose (NND)	2.4 (3.2 – 2.0.)	1.2 (1.3 – 1.1)	2.6 (3.75 – 2.1)	1.45 (1.5– 1.2)

Discussion.

This study extends our previous analysis on a larger cohort of patients and compares the diagnostic performances of kappa and IgG indexes in detecting intrathecal synthesis and diagnosing MS. It is widely accepted that intrathecal synthesis of IgG is mirrored by the detection of OB bands in CSF analysed by IEF followed by immunoblotting. These qualitative findings are generally associated with an IgG index above 0.7 and an elevated kappa index (above 5 with our laboratory setting). Since IEF for OB detection is the gold standard approach in MS diagnosis, we compared the "ability" of IgG and kappa indexes both in detecting OB and diagnosing MS. Despite the two markers correlated both in MS and non-MS patients, they showed different diagnostic performances according to ROC analyses. In fact, kappa index presented better AUC not only in predicting OB but also in diagnosing MS. Consequently, we evaluated the performances of a sequential test to detect intrathecal synthesis in MS using a quantitative marker followed by IEF when elevated. Kappa index showed higher sensitivities in predicting OB and diagnosing MS. Kappa index identified the 96.1% of the MS patients whereas IgG index only the 59.1%. Specificity was comparable for the two indexes. We concluded that kappa index resulted most efficient than IgG (Link) index as a quantitative test for intrathecal synthesis.

Conclusions.

These results confirm our previous proposal to use kappa index as a highly sensitive and easy-detectable first-line marker in CSF analysis for intrathecal synthesis. This first approach could be followed by IEF if elevated. IgG index showed lower diagnostic performances to be chosen for this role. The "sequential testing" was an optimal procedure with accurate performance in MS diagnosis. Of note we have been using this sequential testing in our clinical practice since then and according to other recent evidences (Emersic et al. 2019).

Different approaches to calculate kappa free light chain intrathecal fraction.

This section is derived from a modified version of two articles by Crespi et al. 2019 and Vecchio et al. 2020

Introduction.

Some approaches have been studied to calculate the intrathecal fraction of KFLC including different cut-offs of the index, Reiber's diagram, Presslauer's exponential curve, and Senel's linear curve. The calculation of IgG and kappa indexes is based on an equivalent linear relationship between albumin and IgG or KFLC. Consequently, this method is irrespective of the difference in size of the molecules whereas albumin is 69 KDa, IgG 155 KDa, and KFLC 22 KDa. On the contrary, the Reiber's formula employs a hyperbolic relationship to correct for the size (Reiber et al. 2019). In fact, among the different methods to calculate an intrathecal KFLC production, Reiber´s KFLC diagram was recently addressed as the one showing the greater sensitivity in previous studies (Schwenkenbecher et al. 2019). According to these observations, we repeated our analyses and compared the performances of our kappa index cut-off to other methods.

Patients.

See previous paragraphs. This analysis included the above enrolled 385 patients.

Methods.

Intrathecally synthesized KFLC can also be calculated by a non-linear function called "$KFLC_{Lim}$" by Presslauer et al. (2008). This measure relates each KFLC ratio (CSF/serum KFLC) to its corresponding albumin quotient (QAlb) -dependent upper normal limit by the following formula: $KFLC_{Lim}$= 0.9358 x $QAlb^{0.6687}$ = $(3.27 \times (QAlb + 33) - 8.2) \times 103$.

Changes of KFLC concentration in CSF, called "$KFLC_{Loc}$", ware calculated as the difference between $KFLC_{ratio}$ (CSF KFLC/serum KFLC) and $KFLC_{Lim}$, finally corrected for absolute KFLC serum concentration by the following formula: $KFLC_{Loc}$ = ($KFLC_{ratio}$ – $KFLC_{Lim}$) x serum KFLC. Finally, the

relative KFLC intrathecal fraction "$KFLC_{IF}$" or Presslauer exponential curve was displayed as a percentage according to the following formula: $KFLC_{IF}$ = ($KFLC_{Lov}$ / CSF KFLC) x 100.

Finally, Reiber's diagram is defined by the formula: $KFLC_{IF}$ = $KFLC_{Loc}$/ CSF KFLC × 100 or (1 − $KFLC_{Lim}$/$KFLC_{ratio}$) × 100 (Schwenkenbecher et al. 2019).

Statistics.

See previous paragraphs.

Results.

$KFLC_{Loc}$ did not exhibit a better ROC curve neither for OB (AUC for $KFLC_{Loc}$ versus kappa index: 0.978 versus 0.981) nor MS diagnosis (AUC for $KFLC_{Loc}$ versus kappa index: 0.921 versus 0.949). No significant differences were also observed according to the other performance parameters (Table 1).

Table 1: Diagnostic performances of kappa index and $KFLC_{Loc}$ for OB detection by IEF and MS diagnosis.

The analysis was performed for OB (type 2 and 3 by IEF) versus absence of OB (type 1, 4 and 5 by IEF); data are reported with their 95 % confidence intervals.

	OB detection		**MS diagnosis**	
	Kappa index	**$KFLC_{Loc}$**	**Kappa index**	**$KFLC_{Loc}$**
Sensitivity (%)	96.5 (93.0 – 98.3)	95.8 (91.8 – 97.8)	96.1 (90.8 – 98.5)	95.8 (90.5 – 98.2)
Specificity (%)	89.8 (85.7 – 92.9)	93.3 (89.4 – 95.8)	77.9 (72.0 – 83.7)	80.7 (75.5 – 85.0)
Likelihood Ratio for a positive test	9.48 (6.7 – 13.5)	14.2 (8.8 – 22.9)	4.4 (3.1 – 5.9)	5.0 (3.9 – 6.4)
Likelihood Ratio for a negative test	0.04 (0.02 – 0.08)	0.04 (0.02 – 0.09)	0.05 (0.02 – 0.11)	0.05 (0.02 – 0.12)

Positive Predictive Value (%)	87.4 (82.5 – 91.2)	91.9 (87.2 – 94.9)	68.2 (60.9 – 75.4)	69.5 (62.1 – 76.0)
Negative Predictive Value (%)	97.2 (94.4 – 98.7)	96.5 (93.3 – 98.2)	97.6 (94.5 – 98.8)	97.6 (94.6 -98.9)
Efficiency (%)	92.7 (89.9 – 94.7)	94.4 (91.8 – 96.2)	83.9 (80.7 – 87.5)	85.4 (81.5 – 88.6)
Pre test Probability (prevalence) (%)	42.3 (37.9 – 48.8)	44.3 (39.6 – 49.0)	33.0 (28.5 – 38.3)	31.5 (27.0 – 36.3)
Pre Test odds	0.7 (0.6 – 0.9)	0.8 (0.7 – 0.96)	0.5 (0.4 – 0.6)	0.5 (0.4 – 0.6)
Post Test odds	6.9 (4.1 – 11.9)	11.3 (5.8 – 22.0.)	2.1 (1.5 – 3.7)	2.1 (1.5 – 3.6)
Post test Probability (%)	87.4 (80.3 – 92.2)	91.9 (85.3 – 95.7)	68.2 (58.0 - 67.3)	69.5 (58.8 – 78.5)
Number Needed to Diagnose (NND)	1.2 (1.3 – 1.1)	1.1 (1.2 – 1.1)	1.45 (1.5– 1.2)	1.47 (1.5 – 1.2)

In conclusion, we have not been able to demonstrate that the correction of kappa index according to other methods could ameliorate the diagnostic power instead of using our cut-off of 5.

Comparisons to Reiber's diagram are mentioned in the next paragraph.

Kappa index confirmed: better to include the blood-CSF barrier permeability.

This section is a modified version of an article by Vecchio et al. 2020

Introduction.

Recently, the great sensitivity of intrathecal KFLC fraction towards MS diagnosis has been confirmed even using different approaches (Schwenkenbecher et al. 2019). Other parameters with diagnostic role toward MS were: OB detection, IgG (or Link) index and LFLC index. All these markers are corrected for blood-CSF barrier permeability: by comparison to serum for OB and by albumin ratio (serum/CSF albumin) for indexes (Presslauer et al. 2016). In this study we also considered measures for the excess of kappa and lambda FLC only in the CSF, called CSF ratios, that are not albumin and serum-corrected.

Patients.

We finally enrolled 406 consecutive patients who underwent a spinal tap during their diagnostic work-up for a neurological disorder between January 2015 and December 2019. We included in the present analysis 373 patients: 133 (88 females) MS according to McDonald criteria 2017 (Thompson et al. 2018), 93 (50 females) with other neurological ID of the peripheral/CNS, and 147 (72 females) patients with NID. MS patients at diagnosis were classified as: 118 RR, 12 SP and 3 PP. ID included: RIS-CIS (N=18 patients) according to McDonald criteria 2017 (Thompson et al. 2018), isolated myelitis (N=8 patients), acute demyelinating encephalomyelitis (N=3), neuromyelitis optica spectrum disorders (N=5), systemic autoimmune disorders with CNS involvement (N=14), autoimmune encephalitis (N=8), inflammatory neuropathies (N=33 that were classified as acute/chronic inflammatory demyelinating polyneuropathies in 13 cases, antibody-mediated or in systemic autoimmune disorders in 20), acute cerebellitis (N=3), Behcet syndrome (N=1). NID were: amyotrophic lateral sclerosis, dementia, non-inflammatory neuropathies, tumors. We excluded: 5 cerebral lymphomas, 16 CNS infectious diseases, 12 with no evidence of neurological disease at the end of the diagnostic work-up. Mean age of the included subjects was: 39.6 years (± SD 12.9) in MS, 51.1 (±19.7) in ID and 57.2 (±16.7) in NID.

Methods.

We calculated two groups of markers: a) indexes (corrected for blood-CSF barrier permeability) that were IgG, KFLC (both employing our cut-off of 5.0 and Reiber´s KFLC diagram), and LFLC indexes (Reiber et al. 2018). b) CSF ratios (not albumin and serum-corrected): CSF KFLC/LFLC, CSF KFLC/IgG, CSF LFLC/IgG. Thirdly, OB were detected according to standard methods (previously described).

Statistical analysis.

Continuous variables were expressed with mean and SD. Their distributions were checked with Shapiro-Wilk test and resulted not normally distributed. To compare data of multiple groups (MS, ID and NID patients), a non-parametric ANOVA (Kruskal-Wallis analysis) was applied with Bonferroni correction for multiple comparisons (p-values below 0.005 were considered to be significant). Sensitivity was calculated as "true-positive/(true-positive+false-negative)", specificity as "true-negative/(true-negative+false-positive)". Area under curve (AUC), sensitivity and specificity were performed on ROC using a VassarStat software and with a Bayesian calculator made available by The Italian Society of Laboratory Medicine (SIPMEL).

Results.

We included 373 patients for KFLC and OB evaluation, 223 of them were tested also for LFLC.

Table 1. Absolute concentrations of kappa (K) and lambda (L) free light chains (FLC), CSF ratios and indexes were determined in multiple sclerosis (MS), inflammatory neurological diseases other than MS (ID), and non-ID (NID).

Legend: Values are expressed in mean ± SD. "*" means significantly different in MS from ID and NID ($p<0.005$ was considered significant according to Bonferroni correction for multiple comparisons). "#" means significantly different among three groups (MS *versus* ID *versus* NID, $p<0.05$). OB yes/no: "yes" was intended for types II and III

	MS (n =133)	ID (n= 93)	NID (n = 147)	Sensitivity (%)	Specificity (%)
CSF markers (not albumin or serum-corrected): mean ± SD					
KFLC (mg/dl)	0.48±0.56*#	0.20±0.53	0.03 ±0.13	78.9	97.5
LFLC (mg/dl)	0.13±0.16	0.05±056	0.06±0.22	56.3	81.8
IgG (mg/dl)	4.90±3.24	6.64±12.71	3.91±6.28	60.9	62.1
KFLC/LFLC ratio	16.24±40.47	8.31±33.37	0.98±0.53	77.8	77.5
KFLC/IgG ratio	85.44±66.67*#	33.36±57.08	9.20±7.49	86.5	87.9
LFLC/IgG ratio	2.81±2.86*	1.17±0.99	1.17±0.83	51.3	88.1
Markers corrected for blood-CSF barrier permeability					
IgG index: mean ± SD	0.85±0.46*	0.56±0.22	0.50±0.13	70.5	68.8
KFLC index: mean ± SD	70.84±86.70*#	26.38±77.25	3.08±6.21	93.2	85.4
LFLC index: mean ± SD	17.07±23.00*	3.93±6.88	3.76±8.43	80.3	80.3
Reiber´s KFLC diagram: mean ± SD	88.2±18.2*#	34.5±22.10	16.4±22.6	98.1	53.2
OB: yes/no	127/6	28/65	7/140	95.5	85.2

KFLC differentiated MS patients from those with ID and NID ($p<0.005$). In fact, KFLC index and CSF KFLC/IgG ratio were significantly higher in MS than in other neurological conditions. Similarly, MS patients presented increased absolute concentrations of KFLC (mean value was 0.48 mg/dl) compared to both ID (0.20 mg/dl) and NID patients (0.03). The KFLC, despite considering different interpretation approaches, permitted also to distinguish among the three groups (MS *versus* ID *versus* NID). Conversely, LFLC were not relevant in MS diagnosis (223 patients of the 373 included were tested for LFLC). LFLC index and CSF LFLC/IgG ratio resulted greater in MS then in other neurological conditions, but did not differ significantly among the three groups.

KFLC index emerged as the most sensitive marker corrected for blood-CSF barrier permeability in diagnosing MS. Its sensitivity of 93% overtook that of IgG index (70.5%), and was only slightly lower than of OB (95.5%). Accordingly, we confirmed the greater accuracy of OB in MS diagnosis, according to McDonald criteria 2017 (Thompson et al. 2018). Of note, in our study the specificity of OB was similar to that of KFLC index (85%). If comparing different approaches to calculate KFLC intrathecal fraction in our cohort, sensitivity towards MS diagnosis was 98% for Reiber´s KFLC diagram (Reiber et al. 2019), in face of 53% specificity. Thus, concerning MS diagnosis, KFCL index performances resulted more similar to that of OB in our population. Among CSF markers, only KFLC/IgG ratio resulted a sensitive marker of intrathecal IgG synthesis (sensitivity 86.5%).

Conclusions.

Our study confirmed the role of KFLC in the diagnostic work-up for MS. Both KFLC index (corrected for blood-CSF barrier permeability) and KFLC/IgG ratio (evaluating the overproduction of KFLC in CSF only) showed a high sensitivity and decent specificity towards MS diagnosis. Overall, OB remained the gold standard for CSF analysis in MS.

Future perspectives: any influence by demographic features

Some demographic features have been associated to changes in the blood-CSF barrier permeability, that seems to increase with age and being higher in men (Castellazzi et al. 2020; Parrado-Fernandez et al. 2018). Since this measure was included in the formula of kappa index, we evaluated if gender and age could affect our data.

Regarding gender, we first looked if there was any association to the KFLC intrathecal fraction. Secondly, we checked if the albumin quotient, quantifying the blood-CSF barrier permeability, differed between males and females. We included 373 patients (as in previous paragraphs): 133 (88 females) MS, 93 (50 females) with other neurological ID of the peripheral/CNS, and 147 (72 females) patients with NID. Continuous variables (reported as means ±SD) were analysed with a one-way ANOVA, and categorical variables (reported as count and percentage) with Fisher's exact test (for data not normally distributed according to Shapiro-Wilk test).

Mean values of KFLC according to gender are presented in Table 2.

Table 2. Kappa (K) free light chain (FLC) intrathecal fraction and albumin quotient according to gender.

Legend: Values are expressed in mean ± SD.

	Males (n =166)	**Females (n= 207)**	**p-value**
CSF KFLC (mg/dl)	0.20±0.44	0.27±0.50	0.21
KFLC quotient	0.16±0.39	0.20±0.39	0.36
Kappa index	24.89±64.45	39.69±74.60	0.05
Albumin quotient	0.014±0.08	0.006±0.008	0.15

We did not find any significant difference between males and females. Although, when considering those patients with kappa index above 5 (intrathecal synthesis), 108/169 (64%) were females (p=0.003). This result is probably biased by the higher incidence of MS in females (65% of MS cases were women). Moreover, there was no gender difference in the blood-CSF barrier permeability in the overall population (Table 2). Subsequently, we identified those patients with dysfunctions in the blood-CSF barrier. To categorize these patients, we considered normal values of the albumin quotient among ages as follows: < 6.5 for 15–40 years, < 8.0 for 41–60 years and < 9.0 for over 60 years (Castellazzi et al. 2020). One hundred-one (29%) patients presented blood-CSF barrier's dysfunction. About 39% (64/166) male patients had an altered blood-CSF barrier compared to 18% (37/207) females (Chi square: p<0.0001).

Concerning age-dependant changes in CSF proteins (Parrado-Fernandez et al. 2018), we explored differences in the KFLC intrathecal fraction and in the albumin quotient in 367 patients (Table 3). Those

cases below 15 years old were excluded, and others were divided into three subgroups aged: 15–40, 41–60 and above 60 years old (Castellazzi et al. 2020).

Table 3. Kappa (K) free light chain (FLC) intrathecal fraction and albumin quotient according to age.

Legend: Values are expressed in mean ± SD.

	15-40 years (n =166)	**41-60 years (n= 207)**	**>60 years (n=**	**p-value**
CSF KFLC (mg/dl)	0.40±0.64	0.23±0.43	0.08±0.19	<0.001
KFLC quotient	0.32±0.53	0.17±0.33	0.05±0.14	<0.001
Kappa index	56.52±84.93	33.75±66.67	5.59±12.95	<0.001
Albumin quotient	0.006±0.055	0.007±0.009	0.018±0.096	0.16

KFLC intrathecal fraction resulted higher in younger patients, and regardless albumin correction. Yet, this data probably reflects the higher incidence of MS in these subgroups (mostly patients aged 15-40 years old). Looking at age-related changes, the albumin quotient was more elevated in elderly, despite our analysis did not result significant among age subgroups.

In conclusion, we confirmed that dysfunctions in the blood-CSF barrier are more frequent in men, and excluded gender-related differences in the KFLC intrathecal fraction. Data on age-related changes in CSF proteins need further discussion.

Chapter 2: kappa free light chains in multiple sclerosis prognosis.

Radiological and clinical isolated syndrome.

This section is a modified version of an article by Vecchio et al. 2020

Introduction.

Kappa index has been described as a reliable marker of intrathecal IgG synthesis in MS being quantitative biomarker that could be easily measure to select which cases should have CSF IEF. The presence of OB during the early MS phases have been discussed also as a negative prognostic indicator for disease outcome (Avasala et al. 2001. Amato et al. 2001), and robust data have been published on the role of OB in predicting CIS conversion to MS (Kuhle et a. 2015).

We aimed to evaluate whether the markers of intrathecal synthesis in MS that consider KFLC could have prognostic value in radiologically and clinically isolated syndromes (RIS-CIS) to identify which patients were at higher risk of conversion to MS.

Patients.

See previous paragraphs. This analysis included the 18 RIS-CIS patients according to McDonald criteria 2017 (Thompson et al. 2018) from the above-mentioned cohort of 373. We included 3 patients with RIS and 15 with CIS. Mean age of the 18 subjects (11 females) was: 36.3 years (± SD 8.5). CIS presentations included: unilateral optic neuritis (6 patients), focal supratentorial syndrome (4), and partial myelopathy (5). Brain and spinal magnetic resonance (performed at the time of diagnostic work-up) did not fulfilled criteria for dissemination in space in 12 cases, and for dissemination in time in the remaining 6.

Methods.

See previous paragraphs. We included: a) IgG, KFLC and LFLC indexes (corrected for blood-CSF barrier permeability) b) CSF ratios (not albumin and serum-corrected): CSF KFLC/LFLC, CSF KFLC/IgG, CSF LFLC/IgG. c) OB were detected by isoelectrofocusing and immunoblotting.

Statistical analysis.

See previous paragraphs. Differences between patients with RIS-CIS, that converted to MS, and those who did not covert were explored by Mann-Whitney test. The prognostic value of KFLC was determined by comparing converters *versus* non-converters by binary logistic regression analyses. P-values below 0.05 were considered to be significant.

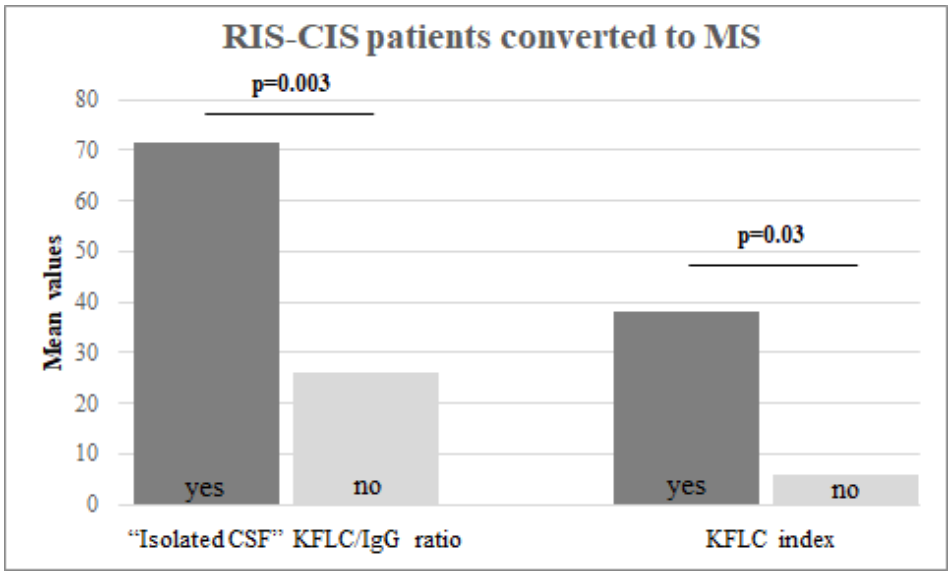
Results.

Six (33%) patients converted to MS during the follow up (that lasted at least one year), developing new lesions over time. Mean follow up of this subgroup of 18 subjects was 3.6 years (± SD 3.6). All the subjects that converted to MS presented OB and significantly higher KFLC then those who remained RIS-CIS (Figure 1). Mean values were: CSF KFLC/IgG ratio 71.5 (± SD 45.9) in RIS-CIS converted to MS versus 26.0 (±28.8) in non-converted (p=0.003); KFLC index 38.2 (±63.5) versus 5.9 (±10.8) (p=0.03). No difference in LFLC was found between RIS-CIS patients with and without conversion to MS. CSF KFLC/IgG ratio resulted more informative in detecting whose patients were at risk of convert to MS. In fact, RIS-CIS patients with elevated CSF KFLC/IgG ratio had a higher risk to convert to MS (hazard ratio, HR=1.05; 95% CI=1.01–1,10; p=0.02). Conversely, regression was not significant for KFLC index (HR=1.07; 95% CI=0.99–1,16; p=0.09).

Gender and age at onset did not differ significantly among RIS-CIS patients who converted or not to MS. Those patients who presented with optic neuritis converted less to MS then other types of onset (RIS, focal supratentorial syndrome, or partial myelopathy) (p=0.07).

Figure 1: KFLC in patients with radiological or clinically isolated syndrome (RIS-CIS) according to conversion to multiple sclerosis (MS) after a minimum follow up of one year.

Legend: Mean values were: CSF KFLC/IgG ratio 71.5 (± SD 45.9) in RIS-CIS converted to MS versus 26.0 (±28.8) in non-converted (p=0.003); kappa index 38.2 (± 63.5) versus 5.9 (±10.8) (p=0.03).



Discussion.

In this study we included a small group of RIS-CIS patients and evaluated conversion to MS in the short term. KFLC through different interpretation approaches resulted higher in those subjects who converted to MS during the follow up, being CSF KFLC/IgG ratio more significant than kappa index. A prognostic value for KFLC have been discussed in few recent studies (Rinker et al. 2006, Voortman et al. 2016). Villart et al. associated high CSF KFLC absolute concentrations (categorized versus less than 0.53 mg/l) to a greater probability of conversion to MS in 78 CIS patients (Villar et al. 2012). A similar prognostic role was confirmed for KFLC index by Makshakov et al. (Makshakov et al. 2015). There is no prognostic data on the excess of KFLC in the CSF (using the ratio that includes CSF IgG as we did). Moreover, in our study, the CSF KFLC/IgG ratio better stratified the risk of conversion to MS if compared to KFLC index. LFLC did not differ among the groups, as previously described (Voortman et al. 2016). Early conversion to MS was less frequent with optic neuritis onset, whereas other clinical/paraclinical parameters failed to identify

converters in our cohort (possibly because of the small sample size). Senel et at. enrolled 77 CIS patients according to Mc Donald 2010 criteria (Polman at al. 2011), of whom 38 converted to MS. They showed that KFLC are predictors for conversion to MS (almost as sensitive as OB) (Senel et al. 2014). In the present study, the application of 2017 McDonald criteria reduced the number of cases that could be classify as RIS-CIS, and definitely, a prolonged follow up with long-term outcomes could improve the prognostic role of KFLC.

Conclusions.

We suggested that CSF KFLC/IgG might be employed to find whose RIS-CIS patients will convert to MS. Of note, not only this marker could be used to search for intrathecal IgG synthesis in suspect MS if serum is not available, but also supported the hypothesis that MS patients have an "excess" of KFLC production limited to the CSF.

Early multiple sclerosis prognosis.

This section is a modified version of an article by Vecchio et al. 2019

Introduction.

Although OB have been related to CIS conversion to MS (Dobson et al. 2013), a role in predicting progression is still debated (Becker et al. 2015) especially if unrelated to clinical and radiological data (Moroso et al. 2015). Several authors considered not only the presence, but also the number of OB (Avasarala et al. 2001) as a marker for MS prognosis. Moreover, not only the presence of IgG but also of IgM OB at the time of diagnosis has been related to a worse outcome (Mandrioli et al. 2008). However, OB remain a mainly qualitative measure of intrathecal synthesis whose result could be also influenced by operator interpretation. Despite OB were associated to poor disease prognosis, little is known on FLC in predicting MS early progression (Rathbone et al, 2018) and could probably not be related to lesion load

(Voortman et al. 2014). Our aim was to evaluate the prognostic value of KFLC in a cohort of Italian MS patients who underwent lumbar puncture in their diagnostic MS work-up.

Patients.

We recruited 100 patients (64 females) who underwent lumbar puncture in their diagnostic MS work-up including who: 1) had a CSF study at the time of their MS work-up including OB and FLC measures. 2) had a diagnosis of CIS or MS according to McDonald 2017. 3) had a minimum follow up of 1 year. Control population included 97 NID excluding lympho-proliferative disorders.

Demographic and clinical variables at diagnosis were recorded: gender, age at onset, clinical course, early MS treatments (within 1 year), the presence of gadolinium-enhancing (Gd+) lesions. Brain MRI was performed on 1.5 T with single dose of gadolinium (Gd) within 3 months from the LP.

Methods.

See previous paragraphs. MS patients were followed up over time to detect their disability according to the expanded disability status score (EDSS) (Kurtzke et al). This score has been corrected by time-measure using the MS severity score (MSSS) (Roxburgh et al).

Statistics.

Data were stored and analysed in SPSS 22.0. Continuous data were presented in mean and standard deviation, while categorical data was presented in median and ranges. Comparisons between groups were made using the Mann–Whitney test for continuous variables. Correlations were performed with Spearman's rank correlation coefficient, and linear regression for significant predictors in the univariate model. P value 0.05 was considered statistically significant.

Results.

Baseline features are summarized in Table 1.

Table 1. General features of 100 patients.

	N (%)	Mean (SD) / Median (range)
Gender: female	64 (64%)	-
Age at onset (years)	-	34.4 (10.7)
Initial MS course:		
radiological isolated syndrome	2 (2%)	-
clinical isolated syndrome	9 (9%)	
relapsing-remitting	84 (84%)	
progressive	5 (5%)	
CSF OB	92 (92%)	-
Number of CSF bands		13 (0-42)
CSF IgG	-	4.7 (3.4)
Kappa index*	-	68.2 (84.4)
Gadolinium-enhancing lesions at baseline (N=99)	32 (32%)	
Disease modifying treatments within 1 year	50 (50%)	
Time to last follow up (years)	-	3.9 (5.0)
EDSS at last follow up	-	4 (0-7)
MSSS at last follow up	-	2.87 (0.05-9.68)

*Of note we considered a cut-off value of 5 ± 0.8 for Kappa index that resulted elevated in 92 cases.

Median age at onset was 34.4 years (SD ±10.7). MS course at diagnosis was: 2 RIS, 9 CIS, 84 RR MS, and 5 progressive MS. Median time from onset to last follow up was 3.9 years (±5.0).

Overall, 92 patient had intrathecal synthesis, 5 had no or indistinct bands, and 3 had similar bands both in serum and CSF; median number of CSF bands was 13 (range 0-42). Mean kappa index 68.2 (SD± 84.4) and CSF IgG 4.7 mg/dl (3.4). Half of our cohort started a DMT within one year after diagnosis (defined as early treatment), at last follow up median EDSS was 4 (range: 0-7) and MSSS 2.87 (0.05-9.68). Kappa index resulted as significant predictor for disability over time according to MSSS since patients with higher CSF KFLC presented a higher risk of poor outcomes in the short term (HR 0.22, 95% IC 0.002-0.011, p=0.007). We also confirmed the prognostic role of age at onset for MS disability, but not for other clinical/paraclinical factors at diagnosis. According to a univariate model, patients with a lower age at onset reached a lower MSSS at their last follow up (rho 0.55, p<0.001). This was also the case of patients with an initial diagnosis of RIS/CIS (median MSSS 1.89, range 0.35-5.87) versus MS (3.34, 0.50-9.68) (rho 0.20, p=0.044). Among CSF markers, lower KFLC index (rho 0.19, p=0.047) and absence of OB (rho 0.36, p<0.001) correlated to a better early-outcome. MSSS did not relate to: CSF IgG, number of CSF bands, Gd+ lesions and early MS treatment. Consequently, we performed a multivariate analysis using MSSS as independent factor and variables that were significant in the univariate model (i.e. age at onset, initial MS diagnosis and kappa index) as predictors. Kappa index and age at onset resulted the strongest predictors for MSSS in the first years of disease course (df 3, p<0.0001). According to our model, MSSS increased of 0.06 for each point of kappa index (p=0.007). Despite PR MS tended to have higher MSSS than RIS/CIS and RR, initial MS diagnosis was not significant, possibly due to the small sample size (Table 2).

Table 2. Prognostic factors at MS diagnosis for early disability according to MSSS at last follow up (N=100).

	Multivariate analysis		
	Beta	**95% CI**	**P value**
Age at onset	0.11	0.07-0.15	<0.0001
Initial MS course:			
radiological/clinical isolated syndrome	reference		
relapsing-remitting	0.77	-0.56-2.10	0.25
progressive	2.15	0.18-4.33	0.03
Kappa index	0.006	0.002-0.011	0.007

We then evaluated how KFLC varied according to several patients' features. Kappa index differed among initial MS diagnosis resulting lower in RIS/CIS (N=11: median kappa index 2.9, 25th-75th percentiles 2.1-45.5) versus MS (N=89: 39.8, 18.1-105.5) (Mann-Whitney, p=0.003), although it was not able to identify progressive MS (median kappa index 16.1, 25th-75th 14.5-96.5 versus 36.6, 17.3-74.8 in RR). Patients who did not start early MS treatments (N=50/100) had lower Kappa index (29.5, 12.6-65.7) than those who commenced DMTs within one year (41.0, 24.3-107.3) (p=0.046). No significant association was found for kappa index and gender (p=0.5), age at onset (p=0.7), Gd+ lesions (N=32/99: p=0.7).

Discussion.

Our data suggest a prognostic role of kappa index for developing early disability in MS: this quantitative marker of intrathecal synthesis showed a direct correlation with MSSS and could be effective in stratifying the risk among those patients who had classically defined OB. In fact, we also confirmed the correlation of disability and OB that remains an established but qualitative and frequent marker to intrathecal synthesis. Notably, the number of bands or CSF IgG did not relate to MSSS in our cohort. We decided to focus on

kappa index since this quantitative measure could be more informative then a categorical binary variable and have a great variability among patients with OB. Thus, we suggest that KFLC could be used both to confirm MS diagnosis and to enrich the prognostic evaluation at diagnosis.

The first potential strength of our results is the use of a biomarker that could be routinely available in the clinical practice. In fact, LP regained a role in MS work-up according to 2017 McDonald criteria (Thompson et al. 2018), and CSF is a potential source of biomarkers at diagnosis. IgG OB have been in fact largely evaluated in relation to CIS/MS prognosis with still unclear conclusions. Moreover, OB are present in about 95% of MS patients being poorly sensible to differentiate patients for disease course at diagnosis. To overcome this limit, Avasarala et al. associated no or low number of OB to a better prognosis in 44 patients according to their EDSS at minimum 10 years (Avasala et al. 2001). We did not confirm the association between the number of bands and disability in the short term. Then, some authors focused on IgG index (Izquierdo et al. 2002) suggesting a correlation to a measure of disability over time and related the progression index (EDSS at last examination divided by the evolution time) to a very high Link index. Similarly, we are proposing that the use of this easy-detectable quantitative marker could overcome the limit of OB execution and interpretation. Kappa index has been proposed as a prognostic marker because of a possible relation between a benign outcome and no CSF humoral immune response. Kappa index displayed a higher variability in our cohort, and its value directly correlated to MSSS at minimum one year. Similar results had been proposed by Rudick at al. in 36 patients: they showed that CSF KFLC were related to disability progression according to EDSS in 36 months (Rudick et al. 1995). Another group confirmed in 57 MS patients that higher CSF KFLC predicted the need of ambulatory aids and MSSS at 10 years (Rinker et al. 200). Results on EDSS progression were not confirmed by Presslauer at al., although this study is not comparable to our since only moderately and very high values of kappa index were included (and categorized using the cut-off of 100) (Presslauer at al. 2014). We analysed the kappa index as a continuous variable including all the results of our MS population.

Looking at short-term outcome, we decided to evaluate early MS treatment as another paraclinical measure of severity at diagnosis. In fact, patients who started early DMTs according to clinical practice, and not influenced by CSF analysis, showed higher kappa index then those who remained untreated over the first

year after MS diagnosis. Early treatment could be considered a surrogate marker to identify those patients who had worse prognostic factors at onset according to clinicians (Comi et al. 2017). Since KFLC could differ among MS patients with OB, and intrathecal synthesis is still unclearly related to disability (Frau et al. 2017) we could speculate that kappa index represents a more sensible marker of humoral immune response that may help in treatment decisions at diagnosis.

In our study we also confirmed the prognostic role of age at onset for MS disability in the short term, but not for other clinical/paraclinical factors at diagnosis. This "age-dependent" disability has already been established (Ramachandran et al. 2014), and allows us to hypothesize that the selected population could be an adequate MS sample.

Our study is limited by the short term follow up. We calculated MSSS as a time-related disability measure to overcome the limits of reaching EDSS milestones in few years. MSSS has been established as an appropriate method for comparing disease progression using a single assessment (Roxburgh et al. 2016). Secondly, we enrolled all the patients presenting to our MS Center, mostly RR cases.

Conclusions.

We suggest a prognostic value of intrathecal synthesis using kappa index in terms of disability over time. This quantitative marker is not a substitute for OB in MS diagnosis, but could help also in differentiating patients with intrathecal synthesis according the risk of severe outcomes.

Future perspectives: any role to predict treatment response and long-term prognosis.

No data have been published on KFLC and MS long-term outcome, and two aspects could be proposed as future perspectives.

First, MS treatments could affect B-cells as well as KFLC production. Could intrathecal synthesis predict the response to specific DMT or be used as marker of responses at least with repeated measure in serum? Few is known also about OB: various immunomodulators and immunosuppressors have not been found to modify them, whereas natalizumab (an highly effective DMT) reduced OB to undetectable levels (von Glehn et al. 2012). Besides, a single study evaluated the effect of steroids in serum, non in CSF, KFLC. They showed a continuous decrease after each administration of methylprednisolone. In contrast, high-dose methylprednisolone did not influence immunoglobulin amount or OB (Kohen et al. 2020). No data are available on other immune therapies and KFLC concentrations over time.

Secondly, long-term disability has not been discussed yet in relation to KFLC. A unique paper suggested KFLC ratio as an independent predictor of second relapse and disability worsening at 79 months in 28 MS patients (Salavisa et al. 2020). We could plan to include other measures of long-term MS outcome in our analysis, and disability could be check over time. A limit arises, fortunately, from MS therapy that is largely changing natural disease history in terms of disability progression.

Moreover, it is difficult to plan to serial CSF analyses, so we could discuss if changes in serum or other fluids, such as tears, could be used to monitor KFLC intrathecal synthesis with the starting of a DMT. This application could be able to define those patients who reply poorly to the current treatments and support a personalized therapeutic strategy.

Overall discussion and Conclusions.

Diagnosis.

Kappa index has been described as a reliable marker of intrathecal IgG synthesis in MS resulting even more accurate than IgG index to discriminate MS from other neurological diseases. Our study confirmed the role of KFLC in the diagnostic work-up for MS. Kappa index (corrected for blood-CSF barrier permeability) showed a high sensitivity and decent specificity towards MS diagnosis. Overall, OB remained the gold standard for CSF analysis in MS. The high sensitivity and specificity associated with the lower costs of kappa index suggested to use this test first, followed by IEF as a confirmative procedure. The sequential use of IEF and kappa index showed high diagnostic efficiency with cost reduction of 43 and 21%, if compared to the contemporary use of both tests, or the unique use of IEF in all patients. If compared to by IEF and immunoblotting, KFLC can be completely automatized, it is operator-independent in interpretation, less time-consuming and less expensive. Enlarging the sample size during these analyses, we confirmed this "sequential testing" as an optimal procedure on a larger sample and applied this method routinely.

Recently, the great sensitivity of intrathecal KFLC fraction has been confirmed even using several approaches among whom Reiber's diagram had a greater sensitivity towards intrathecal Ig synthesis. In our cohort we confirmed the greatest sensitivity (98%) of Reiber´s KFLC diagram toward MS. Although, this measure lacked of specificity in our population, so we confirmed the sequential testing using as first-line test kappa index (with cut-off of 5). Moreover, "false positive" values for kappa index were double-check for the clinical diagnosis at the end of diagnostic work-up and after a follow up of one year.

In conclusion, these data confirmed a "kappa-oriented" immune reaction in MS CSF. To our knowledge, the KFLC overproduction in MS patients has not been clarified yet. Increased concentrations of serum FLC have been described in several autoimmune disorders (and related to disease activity in few) in relation to

the phenomenon of “antigen excess”. Although not explaining the “kappa” prevalence, it remains of diagnostic utility in MS.

Prognosis.

MS course presents a high variability among individuals ranging from a minimal disability over time to a rapid and severe progression (Tutuncu et al. 2013). DMTs with a different risk to benefit ratios impact on disease history, and the possibility of predicting early MS course is crucial. Many clinical and radiological factors at MS diagnosis have been related to MS prognosis, such as gender, age of onset, disability, number of relapses, MRI measures (Swanton et al. 2014. Wattjes et al. 2015). A similar role has been proposed for CSF biomarkers, and our study confirmed that KFLC could be apply in MS prognosis.

First, CSF KFLC/IgG might be employed to find whose RIS-CIS patients will convert to MS.

Secondly, kappa index resulted a significant predictor for disability over time being higher in patients who developed greater MSSS. Accordingly, kappa index was also significantly increased in patients undergoing early versus delayed treatment.

Limits.

A limit of this study was that our patients underwent a unique lumbar puncture. Consequently, we do not have data on any changes of FLC levels over time, despite foregoing reports suggested they remain stable. Unfortunately, we were not able to discuss any change within KFLC with treatments. There are few data how DMT could able to affect local humoral production. It seems that only natalizumab and cladribine affect intrathecal Ig synthesis, ultimately leading to CSF OB disappearance in some cases (Rejdak et al. 2019, Mancuso et al. 2014). It is questionable whether KFLC could change with treatment particularly targeting B-cells (Rudick et al. 1999).

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Publications related to the thesis.

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