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Intrathecal immunoglobulin synthesis: The potential value of an adjunct test

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ABSTRACT

Background: Detection of cerebrospinal fluid (CSF) specific oligoclonal bands (OCB) supports the diagnosis of multiple sclerosis (MS), but the method is technically demanding and gives only qualitative information. Kappa free light chains (KFLC) quantification could represent a convenient alternative. We evaluated the diagnostic accuracy of OCB and KFLC in our cohort to further estimate the gain in diagnostic performance when combining both of them.

Methods: KFLC were measured in paired serum and CSF samples of 80 patients with MS and 50 patients with non-inflammatory neurological disorders. OCB were detected using an in-house alkaline phosphatase assay. Likelihood ratio (LR) was used to explore the benefit of the combined KFLC and OCB test.

Results: Sensitivity of KFLC index (≥5.3) and intrathecal KFLC fraction (≥10%) was 96% and 95% respectively, compared to 91% sensitivity of OCB assay. Specificity was 96% for intrathecal KFLC synthesis and 98% for OCB. Probability of MS in the absence of OCB was further reduced with concurrently normal KFLC index.

Conclusions: Normal KFLC parameters allow confident exclusion of intrathecal inflammation, but probability of MS is greater with positive OCB. Use of KFLC as an adjunct test might be beneficial in specialized MS centers with larger pretest probability.

1. Introduction

The diagnosis of multiple sclerosis (MS) is mainly based on MRI findings, but cerebrospinal fluid (CSF) analysis remains an important diagnostic tool to confirm inflammatory nature of the disease and to exclude alternative conditions [1]. Currently, the most reliable evidence of intrathecal immune response are CSF specific oligoclonal bands (OCB) detected in approximately 90% of MS patients [2–4]. OCB positivity independently increases the risk of conversion to MS in patients with clinically isolated syndrome (CIS) and thus has an added value to the MRI features [5–9]. With the role of CSF examination re-emphasized in the new revisions to the McDonald criteria optimization and standardization of OCB (intrathecal inflammation) detection methods is again to be addressed [10].

The recommended method for OCB determination consists of isoelectric focusing with subsequent immunodetection (either blotting or fixation) [2]. Although good reproducibility of different techniques was reported (semi-automated commercially available and in-house high sensitivity assays), the procedure is time consuming and gives only qualitative information. Furthermore, it is still largely dependent on technical skills and enables subjective interpretation, which is an important source of inter-laboratory variability [2,11,12].

Quantification of immunoglobulin free light chains (FLC) rather than intact antibodies was proposed to overcome these issues [13,14]. As FLC are secreted in excess by plasma cells during the immunoglobulin (Ig) production they also accumulate in the CSF in the presence of intrathecal B cell activity. Several studies demonstrated comparable diagnostic accuracy of kappa FLC (KFLC) and OCB in MS, however different methodologies and KFLC parameters were employed [13–16].

In this study we aimed to compare the diagnostic accuracy of various KFLC parameters and OCB positivity in our MS cohort and to further estimate the potential value of KFLC as an adjunct test for detection of intrathecal inflammation.

Abbreviations: CIS, clinically isolated syndrome; CSF, cerebrospinal fluid; FP, false positives; IEF, isoelectric focusing; (K)FLC, (kappa) free light chains; LR, likelihood ratio; NIND, non-inflammatory neurological disorders; OCB, oligoclonal bands; QAlb, albumin quotient; QKFLC, kappa free light chain quotient; TP, true positives; TPC, total protein concentration; WBC, white blood cells

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2. Methods

2.1. Patient selection

Written informed consent was obtained from the patients and the study protocol was approved by the National Medical Ethics Committee of Slovenia. Patients hospitalized at the Department of Neurology, University Medical Centre, Ljubljana, who underwent diagnostic lumbar puncture between 2014 and 2016 were included in the study. As inclusion criteria, at least 0.5 mL of patient's CSF and results of routine CSF analysis (leukocyte count, total protein concentration, OCB status) had to be available. The diagnosis of MS was established using the 2010 revision of the McDonald criteria [1]. Upon review of patients' records, 80 patients with MS (78 relapsing remitting, 1 primary progressive and 1 secondary progressive MS) were identified and 50 were allocated to the control group of non-inflammatory neurological disorders (NIND). Control group patients presented either with somatization disorder ($n = 16$), primary headache syndrome ($n = 8$), unexplained paresthesia (n = 8), spondylopathy ($n = 7$), mood disorder ($n = 4$), acute vertigo ($n = 2$), arachnoid cyst ($n = 1$), idiopathic intracranial hypertension (n = 1), unexplained hearing loss (n = 1), fibromyalgia (n = 1) or tinnitus (n = 1).

2.2. Routine CSF analysis

Results of leukocyte count, total CSF protein concentration (TPC), CSF/serum albumin quotient (QAlb) and IgG index (intrathecal synthesis) were obtained from respective laboratory (Laboratory for CSF diagnostics or Protein-lipid laboratory, UMC Ljubljana). Total CSF protein concentration was measured by nephelometry, using trichloroacetic acid precipitation method. Albumin and IgG concentrations were measured by immunonephelometry, using antiserum to human albumin or human IgG (N Antiserum to Human Albumin and N Antiserum to Human IgG, both by Siemens Healthcare Diagnostics GmbH, Germany). Following routine analysis, CSF and serum samples were stored at −80 °C up to the day of KFLC determination.

2.3. Oligoclonal band determination

Determination of OCB was performed as previously described [17,18]. Briefly, OCB assay consisted of agarose isoelectric focusing (IEF) and subsequent immunoblotting. Immunodetection by alkaline-phosphatase labelled anti-human IgG antibody was used. This in-house assay demonstrates at least four times higher analytical sensitivity than the standard peroxidase method and has improved diagnostic sensitivity for MS in our clinical setting by 6–8% [17,19]. OCB patterns were interpreted following international recommendations [20].

2.4. KFLC determination

KFLC concentrations in CSF and serum samples were measured with Siemens BN II nephelometer, using N Latex FLC kappa assay (Siemens Healthcare Diagnostics GmbH, Germany) according to the manufacturer's instructions. Latex enhanced assay is based on highly specific monoclonal antibodies to KFLC and has a lower limit of detection below 0.1 mg/L [21,22]. Estimated intralaboratory imprecision (CV %) was below 4.2% for serum and under 5.5% for CSF samples. All samples were run in a blind manner.

2.5. Data analysis

GraphPad Prism 7 (GraphPad Software Inc., CA, USA) was used for statistical analysis and two-tailed p values < 0.05 were considered statistically significant. All data was tested for normality and Mann-Whitney-U test was used for group comparisons, when appropriate. Qualitative variables among groups were compared with χ^2 test. Sensitivities and specificities of different KLFC parameters were assessed by receiver-operating characteristics (ROC) analysis, using maximization of the Youden index to determine optimal cut off values.

3. Theory and calculations

3.1. Calculation of KFLC parameters

Different quantitative measures of intrathecal KFLC production were evaluated. KFLC index was calculated as a quotient of the CSF/serum ratios of KFLC and albumin concentrations. To obtain local secretion of KFLC in CSF compartment each corresponding QAlb-dependent upper normal limit of KLFC was subtracted from CSF/serum KFLC ratio and corrected for absolute KFLC serum concentration, as defined in previous studies [23–25]. Intrathecal KFLC fraction (%) was assessed as a relative fraction of locally synthesized KFLC in the CSF ($KFLC_{LOC}/KFLC_{CSF} \times 100$) [25]. Additionally, CSF KFLC to total protein (TPC) ratio was used as the simplest measure accounting for blood-CSF barrier integrity.

3.2. Comparison of diagnostic performances of individual tests

McNemar's' hypothesis test was used to estimate significance of differences in sensitivity and specificity of OCB and KFLC index, however it only examines the extent of disagreement (discordant observations) rather than all the information included in the sensitivity and specificity [26]. Additional performance measures were therefore considered. First, area under the ROC curve (AUC) as an informative indicator of overall test performance was derived from ROC analysis for continuous variables. For OCB, only one paired value of sensitivity and specificity exists (a binary test with only one possible cut-off), so the corresponding ROC “curve” does not have a concave shape but is made of two line segments connecting origin, the point representing test's sensitivity and (1-specificity) and the point located in the top right corner of the ROC space. AUC for a binary test can be calculated as [27]:

$$(sensitivity + specificty)/2$$

Likelihood ratios (LR) were used as another measure of diagnostic accuracy which is independent of disease prevalence and suitable for dichotomous or continuous data. LR compare the probability of positive (or negative) test results in patients with the probability of the same results in non-diseased:

$$LR^+ = sensitivity/(1 - specificity) \text{ and } LR^- = (1 - sensitivity)/specificity$$

LR have greater clinical applicability and often enable more appropriate interpretation of results [28,29], because they also relate pretest probability to posttest probability in Bayes' theorem:

$$pretest\ disease\ odds \times LR = posttest\ disease\ odds$$

3.3. Diagnostic performance of the combined test

Direct comparison of sensitivity and specificity can be misleading when evaluating coupled testing with one of its component tests as the apparent gain in sensitivity can occur by chance alone or at the cost of significant loss in specificity. Furthermore, the trade-off between sensitivity and specificity does not automatically lead to the trade-off in predictive value of a positive and negative test result [30]. To explore the potential benefit when combining OCB and KFLC index an approach described by Macaskill et al. [30] was used. LR of the combined and conventional single (OCB) test was compared and the trade-off (T) in the expected number of additional true positives and false positives determined by the following formula [30]:

$$T = R \times \theta \times p^+_{-+} - (1 - \theta)p^-_{-+}$$

A trade-off zero ($T = 0$) corresponds to equal performance of the combined and the single test. R is the ratio of the additional false positives we are willing to accept for each new patient detected (a beforehand decision) and θ is the prevalence of the condition in a population to be tested. Probabilities of a positive result with a combined test if the standard OCB test is negative (p_{-+}) were calculated for patients (additional true positives, p^{+}_{-+} and non-diseased (additional false positives, p^{-}_{-+}) based on paired observations in this study (both tests were applied to the same individuals).

3.4. Pretest probability and cost estimation

The prevalence of the condition (θ) among tested depends on the referral filter before the test is applied. Lumbar tap with OCB testing is performed in patients with suspected demyelinating disorder or to confirm inflammatory nature of the disease, so the spectrum of disease in tested is already limited and patients with very mild or severe forms might be excluded (diagnosis confirmed or omitted with previous investigations, e.g. MRI). To estimate pretest probability (θ) in our laboratory patients' records were reviewed or referral neurologist inquired in order to obtain clinical diagnosis for all who underwent OCB testing within the same time period as our study cohort (2014–2016). Costs per each analysis (OCB or KFLC index) were assessed with consideration of direct material expenses, equipment maintenance and personnel costs (increase or decrease in manual work).

4. Results

4.1. Demographic and basic CSF characteristics

Patients from both groups did not differ significantly in gender distribution (Yates' corrected $\chi^2 = 1.288$, $p = 0.256$), however mean age at the time of lumbar puncture was significantly higher in controls. Among basic CSF parameters, only differences in white blood cell (WBC) counts were significant (Table 1). According to the age related reference value 15% of MS and 26% of control group patients had elevated CSF/serum albumin quotients (QAlb; Yates' corrected $\chi^2 = 1.741$, $p = 0.187$), indicating comparable blood-CSF barrier function in both groups.

4.2. KFLC concentrations in CSF and serum

CSF KFLC concentrations as well as CSF/serum KFLC quotients (QKFLC) were significantly elevated in MS patients compared to the control group. In serum, similar concentrations of KFLC were found in both groups (Table 2). For absolute CSF concentration a cut off value of 0.3 mg/L was derived from the ROC curve analysis. When comparing CSF KFLC concentrations among different OCB patterns, significant differences were observed between OCB types indicating intrathecal IgG production (type 2 and 3) and others (type 1 and 4) (Fig. 1). KFLC concentrations in CSF correlated significantly with IgG index ($p < 0.0001$, $R = 0.848$), Reiber's IgG synthesis ($p < 0.0001$, $R = 0.785$) and WBC count ($p < 0.0001$, $R = 0.660$). In control group, CSF KFLC concentrations also correlated with serum KFLC concentrations ($p < 0.0001$, $R = 0.569$), TPC ($p < 0.0001$, $R = 0.584$) and QAlb ($p < 0.0001$, $R = 0.632$).

4.3. Assessment of intrathecal KFLC production

Intrathecal KLFC synthesis (expressed as KFLC index or intrathecal KFLC fraction) and the CSF KFLC/TPC ratio were significantly higher in MS patients compared to patients with non-inflammatory neurological conditions (Table 2). As with CSF KFLC concentrations, significant correlation of complex KFLC measures with IgG index, IgG synthesis according to Reiber and WBC count was observed. KLFC index cut-off ≥5.3 optimized sensitivity and specificity for MS. ROC curve-derived cut-off for CSF KFLC/TPC ratio was 0.07%, whereas intrathecal KFLC fraction > 10% was considered elevated, analogously to Reiber's IgG intrathecal synthesis.

4.4. Diagnostic performance of oligoclonal bands and different KFLC measures

Different indicators of intrathecal B cell activity were evaluated (Table 3, Fig. 2). OCB type 2 or type 3 were detected in 91.3% of MS patients and had 98% specificity. Among many KFLC measures linear function of KFLC index offered the best sensitivity and specificity (both 96%), but the gain in sensitivity was not statistically significant when compared to the gold standard OCB method (McNemar's $p = 0.1336$, with continuity correction). However, in 4 out of 7 OCB negative (type 1 or type 4) MS patients all KFLC measures were elevated above the used cut-offs. In the rest of them intrathecal plasma cell activity could not be detected with any of the CSF analysis used in this study. None of the OCB positive patients had KFLC index < 5.3. Elevated CSF KFLC concentration, KFLC index and intrathecal fraction were found in two control group patients, but only one who presented with lower back pain (WBC count = 4, QAlb < $QAlb^{REF}$) was also OCB positive. The second, OCB negative, presented with unexplained left foot paresis (WBC count = 3, QAlb < $QAlb^{REF}$). AUC which reflects the proportion of correctly identified subjects was deemed informative (Table 3) but was not further compared between KFLC parameters and OCB due to inherent difference in the surface under the ROC curve for a binary or continuously valued test result (*3.2 Theory and calculation*). LR, on the other hand, manages continuous and binary test outcomes equally well. LR for the positive OCB result (LR^{+}) was 45.6, indicating the magnitude of change from initial suspicion of disease to the posttest probability of MS. In our setting with estimated 25% pretest probability this meant that positive OCB result would increase disease odds from 1:3 (pretest odds if one of 4 patients tested is MS patient) to 15:1 ($0.333 \times 45.6 = 15.2$). Thus, 15 out of 16 positive OCB patients would be expected to have MS. With KFLC index ≥5.3 odds of having a disease increased 24-times ($LR^{+} = 24.1$), resulting in 8:1 posttest odds in our setting. On the contrary, normal KFLC index ($LR^{-} = 0.039$) allowed more confident exclusion of MS with a decrease in disease odds to 1:77 ($0.333 \times 0.039 = 0.013$). Probability of MS decreased approximately 10-times in the absence of OCB ($LR^{-} = 0.089$), meaning that one out of 35 OCB negative patients (posttest disease odds 1:34) in our laboratory could still have had MS.

Table 1
Demographic and basic cerebrospinal fluid characteristics.

Patient group	N	Gender, F N (%)	Age (years) Mean ± SD	WBC ($\times 10^6$/L) Median (IQR)	CSF TPC (mg/L) Median (IQR)	QAlb ($\times 10^{-3}$) Median (IQR)
MS	80	54 (68)	38 ± 11	4 (3–8)	397 (315–457)	5.2 (3.7–6.2)
NIND	50	28 (56)	46 ± 15	1 (1–2)	375 (286–453)	5.6 (3.8–7.5)
p value		0.256	0.002	< 0.0001	0.604	0.106

MS: multiple sclerosis, NIND: non-inflammatory neurological disorders, WBC: white blood cells, CSF: cerebrospinal fluid, TPC: total CSF protein, QAlb: cerebrospinal fluid/serum albumin ratio, SD: standard deviation, IQR: interquartile range.

Table 2
KFLC concentrations and calculated measures.

Patient group	CSF KFLC (mg/L) Median *(IQR)*	Serum KFLC (mg/L) Median *(IQR)*	QKFLC ($\times 10^{-3}$) Median *(IQR)*	CSF KFLC/TPC ($\times 10^{-3}$) Median *(IQR)*	KFLC index Median *(IQR)*	KFLC synthesis (%) Median *(IQR)*
MS	1.7 *(0.9–4.6)*	10.3 *(9.0–12.2)*	157.8 *(91.7–407.0)*	4.6 *(2.1–12.7)*	31.6 *(15.5–94.2)*	83.0 *(66.3–94.0)*
NIND	0.1 *(0.1–0.2)*	11.0 *(8.9–13.8)*	10.0 *(6.9–14.9)*	0.3 *(0.2–0.5)*	1.8 *(1.4–2.1)*	0 [a]
p value	< 0.0001	0.129	< 0.0001	< 0.0001	< 0.0001	< 0.0001

MS: multiple sclerosis, NIND: non-inflammatory neurological disorders, CSF: cerebrospinal fluid, KFLC: kappa free light chains, QKFLC: cerebrospinal fluid/serum KFLC ratio, TPC: total CSF protein, IQR: interquartile range. [a] Local KFLC synthesis was 0% in 48 out of 50 NIND patients.

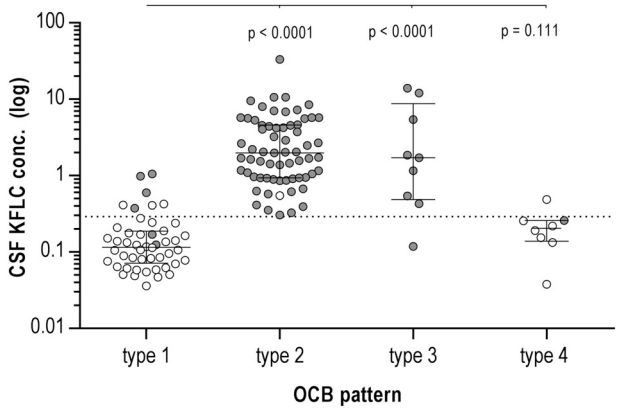

Fig. 1. CSF KFLC concentrations among different oligoclonal band patterns. Four out of 5 classic oligoclonal band patterns were observed: type 1 (no bands in CSF and serum), type 2 (bands in CSF but not in serum), type 3 (bands in CSF and serum, but more/additional bands in CSF) and type 4 (identical bands in CSF and serum, indicative of a systemic not intrathecal immune response). Grey circles represent patients with MS, white patients with non-inflammatory neurological disorders. KFLC concentrations are plotted on a logarithmic scale and an arbitrary cut-off (0.3 mg/L) is depicted by the dotted line. *p*-values for pairwise comparisons with type 1 are given. CSF KFLC concentration was above 0.3 mg/L in 4 out of 7 OCB negative (type 1 and 4) MS patients. One OCB positive (type 3) MS patient had CSF FLC concentration < 0.3 mg/L. CSF: cerebrospinal fluid, KFLC: kappa free light chains, OCB: oligoclonal bands, MS: multiple sclerosis.

4.5. Diagnostic performance of a combined test

Because no simultaneous improvement in positive and negative LR (the greater positive the better and vice versa for negative) was observed when using KFLC index instead of OCB, the benefit of a combined test was investigated. From the viewpoint of diagnostic performance, the use of KFLC index as an adjunct test is reasonable only if "either positive rule" is applied (combined test is positive if either component test is positive), whereby the combined test gains in sensitivity on the account of greater KFLC index sensitivity. Specificity, in this case, is no greater than the lower specificity of the component test (combined test is negative only if both component tests are). Again, a clear choice between a combined and a single component test based on their likelihood ratios can only be made when $LR_{combined}{}^{+} > LR_{single}{}^{+}$ and $LR_{combined}{}^{-} < LR_{single}{}^{-}$. The combined test in our study "adopted" positive and negative LR of KFLC index component test. Accordingly, decreased LR^{-} (from 0.089 with OCB alone to 0.039) favored combined testing whereas a decrease in LR^{+} implied to use OCB alone. Taken together, this indicated a potential value of KFLC index as an adjunct test. A trade-off in the expected number of additional true positives (TP) and false positives (FP) per 100 persons tested (100 T) was then calculated for hypothetical values of R (acceptable ratio of additional FP/TP) as prevalence (θ) varies across a range of plausible values (Fig. 3, Table 4). When $T > 0$ the combined test would be preferred as the number of additional true positives outweighs the number of false positives. $T < 0$ implies there is no benefit of using both tests and at $T = 0$ both, the combined and single test would perform equally. Due to clinicians referral filter the estimated pretest probability (θ) in our laboratory was 20–30% within a 3-year period (grey shaded area, Fig. 3A). A critical ratio (R*) of additional FP to TP that would result in equivalence (T = 0) of the combined and a single test at our prevalence was estimated at 0.9 (95% CI 0.3–2.5) to 1.6 (95% CI 0.6–4.4) (Table 4). Apparently, we could gain from adjunct test in our setting only if we could afford more false positives per each new patient detected than the value of R* is (R > R*). For instance, if we can accept 2 additional false positive results for each additional patient detected ($R = 2$), we could gain 1.6 patients more than false positives per 100 tested at our prevalence. If an estimated pretest probability in a specialized MS center was 50% and the same test performance and tolerance for false positives was assumed (R = 2), the number of additionally detected MS patients could outweigh false positives by 4 (Fig. 3A, Table 4).

4.6. Cost-effectiveness of sequential testing

If OCB testing was done conditional on the result of KFLC test – only as confirmatory analysis for positive test results, the number of OCB assays performed would reduce by approximately two thirds in our

Table 3
Diagnostic sensitivity and specificity of oligoclonal bands and different KFLC measures.

	Sensitivity (%)	Specificity (%)	AUC	LR^{+}	Cut-off used
OCB	91.3 (84.8–100)	98 (92.0–100)	0.9436	45.6	> 2 bands
IgG index	60.3	100	0.9154 *	–	> 0.6
IgG synthesis (Reiber)	38.5	100	0.7628 *	–	> 10%
CSF KFLC concentration	95.0	90.0	0.9751	11.4	> 0.3 mg/L
QKFLC	93.8	96.0	0.9885	23.4	≥ 30×10^{-3}
KFLC index	96.3 (89.4–99.2)	96.0 (86.3–99.5)	0.9929	24.1	≥ 5.3
KFLC intrathecal fraction	95.0	96.0	0.9735	23.8	≥ 10%
CSF KFLC/TPC	95.0	94.0	0.9848	15.8	≥ 0.07%

OCB: oligoclonal bands, CSF: cerebrospinal fluid, KFLC: kappa free light chains, QKFLC: cerebrospinal fluid/serum KFLC quotient, TPC: total CSF protein. AUC: area under the curve. *AUC for our data only (IgG was determined in 78 MS and 42 control group patients).95% confidence interval of the estimated sensitivity and specificity is given in brackets for the gold standard method and the best KFLC measure in this study.

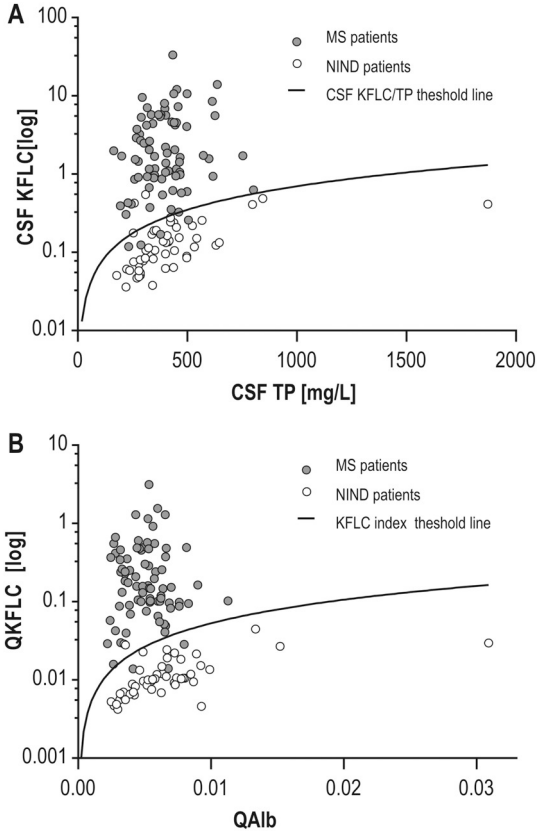

Fig. 2. Similar discriminatory potential of two KFLC measures. Results of MS and NIND patients are plotted on semi-logarithmic graphs with individual threshold lines: (A) CSF KFLC/TPC ratio threshold = 0.07%. CSF TPC is only a crude estimate of blood-CSF barrier integrity, yet diagnostic performance of a simple CSF KFLC/TPC ratio was almost as good as with KLFC index. (B) KFLC index threshold = 5.3. MS: multiple sclerosis. NIND: non-inflammatory neurological disorders. CSF: cerebrospinal fluid, KFLC: kappa free light chains, TPC: total protein concentration. QAlb: cerebrospinal fluid/serum albumin ratio.

laboratory based on our pretest probability estimates. When considering material expenses, decrease in manual work and diagnostic accuracy of both tests sequential testing would reduce our costs by 17%. Because in this case "both positive rule" would be applied (result is positive only when both tests are positive), another highly sensitive and possibly less specific KFLC parameter could be used. A simple KFLC/TPC ratio incorporates only two measurements instead of four needed for calculation of KFLC index (KFLC and albumin in CSF and serum) and has comparable sensitivity as more complex measures. If KFLC/TPC ratio was used for screening, sequential testing would reduce our costs by 48%.

5. Discussion

Similar diagnostic performance of OCB and KFLC was found in our study. Among different KFLC measures, linear function of KFLC index provided the best sensitivity and specificity (both 96%), though gain in sensitivity was not statistically significant compared to the gold standard method. Since statistical analysis depends only on the extent of disagreement between the two methods, likelihood ratio with its inherent trade-off between sensitivity and specificity was rather used for direct comparison of test performances. Furthermore, sensitivity and specificity are less convenient for interpretation of individual test results when we are interested in the odds of a disease given a positive or a negative test result. If LR for the test result is known, posttest odds of a disease will simply be the arithmetic product of LR and clinician's appraisal of pretest odds [28]. Importantly, LR do not vary as a function of disease prevalence, are indifferent to the test outcome format (dichotomous or continuous) an can be used when comparing diagnostic performance of combined test with one of its component tests [28,30]. In our setting, the probability of MS was lower if patient tested positive for KFLC index (≥ 5.3) than if he was OCB positive, but odds of having a disease were smaller if KLFC index < 5.3 versus a negative OCB result. Neither KFLC index nor OCB thus appeared superior for MS upon direct comparison of diagnostic performances.

Our results are in agreement with the findings of previous studies reporting high sensitivity and specificity of KFLC index for MS diagnosis, irrespectively of the method used for KFLC determination [13,23,31]. Assays for (K)FLC quantification that were originally developed for detection and monitoring of patients with plasma cell dyscrasias have been less commonly validated in other diseases and

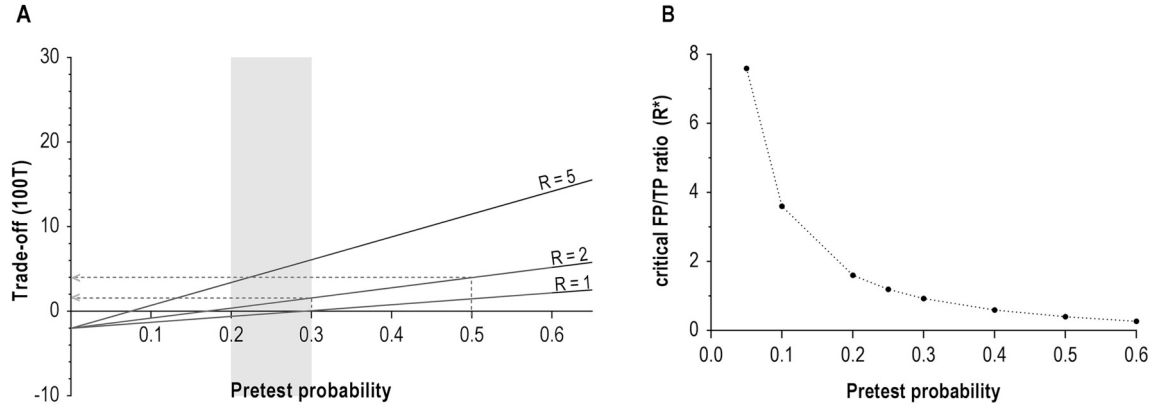

Fig. 3. The expected trade-off per 100 tested (A) and critical value of R* (B) against pretest probability. (A) A trade-off between the combined and a standard (OCB) test will depend on the prevalence among tested, which was estimated at 20–30% in our center (grey shaded area). The point where each line ($R = 1$, $R = 2$, $R = 5$) crosses $100{\cdot}T = 0$, gives the pretest probability at which the combined and single test are equivalent for the given value of R. If $100 \times T > 0$ the combined test would be preferred, while $100 \times T < 0$ implies there is no benefit of coupled testing. The greater the pretest probability, the larger gain we can expect with equal tolerance for false positives. For example, if R = 2, the difference between additional TP and FP per 100 tested would be 1.6 at our prevalence. If a specialized MS center has 50% disease prevalence among tested, the number of additionally detected MS patients could outweigh false positives by 4 (dashed arrows). (B) Critical ratio (R*) of additional FP to TP that would result in equivalence (T = 0) of the combined and a single test. By varying prevalence across the plausible range, we can assess whether the R* takes an acceptable value. *Calculations for $R = 2$ and R^* are provided in*Table 4. FP: false positives, R: the number of additional false positives we are prepared to trade for each new true positive. T: trade-off, TP: true positives.

Table 4
Calculatio`lse positives to true positives.

$100T = 100R \times \theta \times p^{+}_{-+} - 100(1-\theta)p^{-}_{-+}$				$R^{*} = (1-\theta)p^{-}_{-+}/\theta \times p^{+}_{-+}$
	R = 2			$T = 0$
Prevalence	R*100* TP	100*FP	100 T [95% CI]	R* [95% CI]
0.1	1	1.8	**−0.8** [−0.84,-0.76]	**3.6** [1.3, 9.9]
0.2	2	1.6	**0.4** [0.36, 0.44]	**1.6** [0.6, 4.4]
0.3	3	1.4	**1.6** [1.56, 1.64]	**0.9** [0.3, 2.5]
0.4	4	1.2	**2.8** [2.76, 2.84]	**0.6** [0.2, 1.7]
0.5	5	1.0	**4.0** [3.95, 4.05]	**0.4** [0.1, 1.1]
0.6	6	0.8	**5.2** [5.14, 5.26]	**0.3** [0.1, 0.8]
0.7	7	0.6	**6.4** [6.33, 6.47]	**0.2** [0.1, 0.5]

If we allow 2 additional FP per each new TP (we set $R = 2$), we can estimate the trade-off per 100 tested as the prevalence varies. Alternatively, if we set $T = 0$, we can calculate the critical ratio of FP/TP (R*) that would result in equivalence of the coupled and single test and assess whether R* lies in an acceptable range. We can expect to gain from the combined test if we can afford more FP per each TP than the value of R* is. $p_{-+}{}^{+}$:probability of a positive result with a combined test if the standard OCB test is negative when someone is truly MS patient. $p_{-+}{}^{-}$:probability of a positive result on an adjunct test among non-diseased if the standard test is negative; multiplication by $(1-\theta)$ gives additional number of FP, CI:confidence interval, FP: false positives, R: ratio of FP/TP, θ: prevalence, TP: true positives.

clinical settings [32,33]. Because substantial differences in serum FLC were reported between the mono- and polyclonal assay when samples contained monoclonal FLC (including cases where former failed to detect monoclonal gammopathy [34]), International myeloma working group guidelines, for instance, clearly state that recommended decision levels only apply to polyclonal assay [33]. However, sensitivity of monoclonal assays does not seem to be controversial in MS [13–15] and good concordance with similar reference ranges was observed between both nephelometric assays in normal - polyclonal FLC samples [22,35]. In line with that, our threshold for KFLC index derived from ROC analysis (5.3) is very close to the cut-off value recently validated in a multicenter study using polyclonal nephelometric assay [25]. Actually, diagnostic performance of KFLC index in our study would not change if cut-off 5.9 was applied. On the other hand, higher KFLC index cut-off (≥12) was proposed in a study employing the same monoclonal assay as ours, probably on the account of the additional patient group – patients with other inflammatory neurological disorders [13]. Moreover, in studies using enzyme immunosorbent assays [14,15], somewhat lower KFLC concentrations were observed, which demonstrates that method-specific (and preferably laboratory own) reference values should be applied in the absence of internationally recognized reference material.

As might be expected, all KFLC parameters (intrathecal synthesis, KFLC index, KFLC/TP ratio, QKFLC and absolute CSF concentration) were significantly higher in our OCB positive patients (only data for absolute CSF concentrations shown), which supports earlier observation that QKFLC correlates significantly with OCB positivity [14]. Considerably lower serum KFLC were previously reported in MS and CIS patients compared to NIND group [14,25], but not observed in our study. Absolute KFLC concentrations and intrathecal KFLC synthesis (index or intrathecal fraction) were almost equally sensitive in our study, yet most of our patients had normal blood-CSF barrier function (in ~80% QAlb < $QAlb^{REF}$). Specificity improved with either of the measures accounting for barrier integrity. Given that KFLC is unspecific marker of inflammation, elevated CSF concentration (intrathecal synthesis) is expected and has been reported in other inflammatory neurological conditions [14,15]. Similar applies to OCB, although from what we know, quantification of KFLC is not enough to identify the number of antibody producing clones involved in the intrathecal humoral immune response. Indeed, lower specificity of KFLC index (> 6.3) for CIS and MS diagnosis (83% in both) compared to OCB (92%) was found in recent multicenter study [36] and observed also in our MS cohort.

We acknowledge that only KFLC index or local KFLC synthesis should be applied as indicators of intrathecal inflammation. Since elevated KFLC concentrations in the CSF could also be a consequence of barrier dysfunction (decreased CSF flow rate), determination of serum KFLC and reference to albumin quotient is needed for accurate interpretation. Total CSF protein concentration is only a crude estimate of blood-CSF barrier integrity, nevertheless, we showed that a simple CSF KFLC to TPC ratio can reach similar diagnostic sensitivity and specificity as more complex KFLC measures. It might thus be considered as an economical screening test with subsequent OCB detection.

Intrathecal KLFC production (as defined by intrathecal KFLC fraction, KFLC index or CSF KFLC/TP ratio) was detected in almost 10% of all our OCB negative patients, but more importantly, in > 50% of OCB negative MS patients. As shown earlier, intrathecal KFLC fraction enabled detection of inflammation also in 53% of OCB negative CIS patients [25]. Analogously to OCB, prognostic value of KFLC in CIS and MS has already been reported [14,15,37], but more thorough prospective studies are needed (e.g. in OCB negative KFLC positive patients) before KFLC index can be deemed superior. If increased KFLC index (intrathecal fraction) is ever regarded equivalent to OCB positivity for MS diagnosis, possibly lower specificity could pose a concern, especially since the latest 2017 revisions to the McDonald criteria already enable faster diagnosis [38]. Nevertheless, simple and automated quantification with unambiguous interpretation might favor KFLC index in general laboratory settings, not specialized or less experienced in CSF diagnostics. When differential diagnosis is more difficult OCB would probably still be preferred due to slightly better specificity.

Rather than competitive alternative KFLC could be implemented as an adjunct test to aid the interpretation of equivocal OCB (e.g. type 3 vs. type 4) or to guide further CSF analysis. We considered both possible testing designs. Since MS diagnosis would be very unlikely if KFLC parameters are normal, use of KFLC index for screening and OCB as confirmatory analysis seems straightforward. Based on pretest probability of intrathecal inflammation, material expenses and diagnostic accuracy of both tests in our setting sequential testing would reduce our costs by 17% or even by 48% if a simple KFLC/TPC ratio was used as primary test. Cost-effectiveness of this stepwise CSF analysis has been observed before [39]. However, with sequential testing design only OCB positivity is concluded positive test result and we do not benefit of greater KFLC index sensitivity which might be prominent in CIS patients [25]. Assuming it is feasible to do both tests in an individual, we wanted to evaluate the gain of simultaneous testing. Because no clear choice could be made between the combined and a standard OCB test based on their likelihood ratios, a trade-off in the expected number of additional true positives and false positives was calculated. We highlighted that potential value of an adjunct simultaneous test will largely depend on pretest probability of a disease and should thus be assessed for the respective setting. Referral of various neurological conditions to our department was responsible for relatively low pretest probability of

intrathecal inflammation, so the benefit of simultaneous testing was less obvious in our laboratory. However, if pretest odds would raise to 1 (probability 0.5) less false positives would have to be traded for each new patient detected in order to gain from an adjunct test (lower R*). A better trade-off with more carefully selected population (e.g. in specialized centers) might therefore be expected. Clearly, we should determine the maximal acceptable R after considering costs and consequences of new false positive results (e.g. in euros and adverse events of unjustified treatment) versus the benefits of additionally identified patients (e.g. in quality of life years due to faster diagnosis and earlier treatment).

There are limitations of our study which need to be mentioned. Similar to intrathecal IgG production KFLC reflect B cell activation regardless of the underlying cause or pathology. Consequently, diagnostic specificity of KFLC parameters would decrease if comparisons were done with other inflammatory conditions. From our experience OCB positivity is often observed in patients with inflammatory neurological disorders, such as neuroborreliosis or paraneoplastic neurological syndromes - the same would be expected for KFLC. Furthermore, for the assessment of the gain from an adjunct test pretest probability in our setting was estimated within a 3-year period whereas probabilities for test results were calculated based on observations in this study. Our cohort might not be completely representative of all patients who underwent OCB testing within this time because availability of CSF and serum samples was one of our inclusion criteria. A larger prospective study with parallel use of both tests would allow better estimation of the trade-off. Lastly, in CSF studies it is almost impossible to include completely healthy individuals in the control group. Even though no obvious signs of inflammation were found in our control group patients (leukocyte count $< 5 \times 10^6$/L), some of them (e.g. patients with unexplained paresthesias) could still have an early inflammatory condition which would contaminate the results of our study. Control group patients were also not completely age matched to MS patients, however correlations of studied parameters with the age were negligible in our study.

6. Conclusions

Due to fast, relatively inexpensive, automated and rater-independent analysis KFLC quantification has a reasonable potential for implementation in the diagnostic work-up of MS. Even with the use of monoclonal nephelometric assay, different KFLC parameters are as good as OCB in terms of sensitivity and specificity for MS. If neither of the tests proves clearly superior sequential analysis could be cost-effective whereas gain in diagnostic performance might be achieved by simultaneous testing. Since the potential value of an adjunct test will largely depend on pretest probability it is best estimated in a respective setting.

Author contributions

MK and UR designed the experiment and contributed materials/analysis tools. AE and VA performed the experiment and analyzed the data. AE, VA and UR wrote the paper. MK and UR read and approved the final manuscript.

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Conflicts of interest

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