Two-level diagnostic classification using cerebrospinal fluid YKL-40 in Alzheimer's disease

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Abstract

INTRODUCTION: We aimed to assess diagnostic accuracy of cerebrospinal fluid (CSF) YKL-40 in discriminating: (i) Alzheimer's disease (AD), cognitively healthy controls (HC), and frontotemporal dementia (FTD) in a purely clinical analysis (Level I), (ii) patients showing different AD pathologies from HC and FTD in an analysis independent of cognitive impairment severity, following an unbiased descriptive categorization based on CSF core biomarkers (Level II).

METHODS: In a cross-sectional multi-center study, YKL-40 was compared among HC (n=21), mild cognitive impairment (MCI) (n=41), AD (n=35), FTD (n=9) (Level I); among HC (n=21), AD pathology (tau and amyloid-β) negative (n=15), tau-positive (n=15), amyloid-β-positive (n=13), AD pathology-positive (n=33), and FTD (n=9) (Level II).

RESULTS: Level I: YKL-40 discriminated AD from HC and FTD with AUROCs=0.69, 0.71, respectively. Level II: YKL-40 discriminated tau-positive and AD pathology-positive patients from HC, and AD pathology-positive patients from FTD (AUROCs=0.76, 0.72, 0.73, respectively).

DISCUSSION: YKL-40 provides fair performance in distinguishing tau-positive patients from HC.

Key words: Alzheimer's disease, Alzheimer's disease pathophysiology, biomarkers, biomarker-based diagnosis, cerebrospinal fluid, clinical diagnosis, dementia, diagnostic biomarkers, Frontotemporal dementia, mild cognitive impairment, neurodegeneration, neuroinflammation, YKL-40

Abbreviations: Alzheimer's disease (AD); amyloid-β 1 to 42 ($A\beta_{1-42}$); area under the receiver operating characteristic curve (AUROC); A/T/N system: A= Aβ, T= phospho-tau, N= total-tau; cerebrospinal fluid (CSF); cognitively healthy controls (HC); ^{18}F-fluorodeoxyglucose-PET (^{18}F-FDG-PET); False Discovery Rate (FDR); fronto-temporal dementia (FTD); hyperphosphorylated tau (p-tau); Institute of Memory and Alzheimer's Disease (IM2A); International working group-2 (IWG-2); Kruskal-Wallis (KW); leave-one out cross validation (LOO-CV); mild cognitive impairment (MCI); Mental-State Examination (MMSE); National Institute on Aging–Alzheimer's Association (NIA-AA); National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA); pairwise multiple comparison of mean ranks (PMCMR); total tau (t-tau)

1.1 INTRODUCTION

Alzheimer's disease (AD) is a clinically and neuropathologically heterogeneous and multifactorial disorder [1–3] whose primary pathophysiological hallmarks are amyloid plaques and neurofibrillary tangles depositions. However, in a majority of patients AD is combined with other types of pathology [4,5]. Currently, three cerebrospinal fluid (CSF) biomarkers have shown to be able to track the *in vivo* pathophysiological mechanisms of AD both in the prodromal and preclinical phases [6,7]. In particular, (I) the amyloid-β 1 to 42 ($A\beta_{1-42}$) peptide is considered a marker of amyloid brain deposition, (II) total tau (t-tau) protein is thought to be a marker of neuronal injury (not exclusively due to AD) and (III) hyperphosphorylated tau (p-tau) protein is a marker reflecting deposition of neurofibrillary tangles [8]. Although neuroinflammation has been suggested to be a relevant pathophysiological mechanism in AD [1,9], a validated CSF biomarker to monitor neuroinflammation in AD is not available. So far, YKL-40, a glycoprotein belonging to the chitinase-like proteins group, represents a promising inflammatory marker for AD, although its exact pathophysiological role is unclear [10]. YKL-40 is a differentiation marker of macrophages [11–13] and is expressed in microglia and astroglia within the central nervous system [14]. Recently, elevated CSF concentrations of YKL-40 have been reported in AD compared with cognitively healthy controls (HC), also in its prodromal and preclinical phases [15–28].

The aim of this study was to assess the diagnostic accuracy of CSF YKL-40 in distinguishing among groups of cognitively impaired patients. In a first step (Level I), we tested the ability of YKL-40 in discriminating AD dementia patients from HC subjects and frontotemporal dementia cases (FTD), identified according to a purely clinical diagnostic approach. Successively, in a second level of analysis (Level II) [4], we adopted an unbiased descriptive categorization system based on core biomarkers (A/T/N system: A= Aβ pathology, T= tau pathology, N= neurodegeneration) for characterizing AD pathology which was independent of the severity of cognitive impairment. In this context, we determined the diagnostic accuracy of YKL-40 in discriminating patients within the AD pathology spectrum (patients showing both decreased $A\beta_{1-42}$ and increased T-tau or P-tau CSF levels [7], patients which were only tau positive, patients which were only Aβ positive, patients negative to both biomarkers) from HC and FTD cases (Level II).

2.1 METHODS

2.1.1 Population

Clinical and biological data from a convenience sample of 108 individuals (AD= 35, FTD= 9, MCI= 41, and cognitively HC= 23) were retrospectively collected in a multi-centre cross-sectional study involving three independent academic AD research centres and memory clinics. Thirty-five subjects were recruited at the Institute of Memory and Alzheimer's Disease (IM2A) at Pitié-Salpêtrière University Hospital in Paris (France); 57 at the German Centre for Neurodegenerative Diseases (DZNE) in Rostock (Germany); 16 at the Institute of Neuroscience and Physiology at Sahlgrenska University Hospital in Göteborg (Sweden).

The study was conducted according to the provisions of the Declaration of Helsinki. All participants or their representatives gave written informed consent for the use of their clinical

data for research purposes and the local Ethical Committees at the respective universities approved the study.

2.1.2 Patient stratification

2.1.2.1 Level I (purely clinical diagnostic approach)

The first group was composed of 23 cognitively HC. Two individuals from the Göteborg cohort resulted asymptomatic-at-risk of AD [7] or preclinical AD [29] because of high CSF t-tau concentrations and were thus excluded from further analyses. The second group included 41 MCI patients [6]. The third group included 35 AD dementia patients [30]. Finally, the fourth group included 9 FTD patients [31] (**Figure 1**). The clinical diagnosis of AD dementia was performed according to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) consensus criteria [30]. The clinical diagnosis of MCI was made according to the MCI core clinical criteria [6]. The clinical diagnosis of FTD was made following the consensus on clinical diagnostic criteria of 1998 [31]. Cognitively HC were individuals who volunteered for a lumbar puncture; the inclusion criteria were the absence of history of neurological or psychiatric diseases and Mental-State Examination (MMSE) score between 27 and 30.

2.1.2.2 Level II (unbiased categorization based on CSF core AD biomarker profiles)

AD dementia and MCI patient categorization followed an unbiased biomarker-based descriptive classification system recently proposed by Jack and colleagues: the "A/T/N" system [4]. This classification considers 3 binary (i.e. positive or negative) categories: "A" referring to an amyloid biomarker (CSF $A\beta_{1-42}$ or amyloid-PET), "T" to a tau pathology biomarker (CSF p-tau or tau-PET), and "N" to a quantitative or topographic biomarker of neurodegeneration or neuronal injury (CSF t-tau, ^{18}F-fluorodeoxyglucose-PET (^{18}F-FDG-PET), or structural MRI). Since each individual score is displayed as an "A±/T±/N±" arrangement, eight different

categories are possible [4]. The A/T/N classification system is linked to the biomarker classification frameworks i.e. the International working group-2 (IWG-2) criteria [7] and the National Institute on Aging–Alzheimer's Association (NIA-AA) guidelines [5,29,30], and is able to chart both diagnostic classifications. The A/T/N system was utilized in a simplified version which employed only CSF markers and excluded the imaging-related ones (amyloid PET, tau PET, FDG-PET, or structural MR) to define 5 categories (groups) which were independent from severity of cognitive impairment:

Group 1 consisted of cognitively HCs (n= 21), *a priori* defined as both Aβ and tau negative [A-/T-/N-]; group 2 [A-/T-/N-] (n= 15), included 2 AD dementia and 13 MCI patients which were both Aβ and tau negative; group 3 [A-/T±/N+ or A-/T+/N±] (n= 15), encompassed 6 AD dementia and 9 MCI patients which were tau positive but Aβ negative; group 4 [A+/T-/N-] (n=13), contained 5 AD dementia and 8 MCI patients which were Aβ positive only; group 5 [A+/T±/N+ or A+/T+/N±] (n=33) included 22 AD dementia patients in line with the IWG-2 criteria [7] and the NIA-AA guidelines [32], and 11 prodromal AD [33] or MCI due to AD [6] cases, all of which were both Aβ and tau positive; group 6 comprised all FTD cases (n=9) including seven patients which were both $A\beta_{1-42}$ and tau negative, one patient which was $A\beta_{1-42}$ negative and tau positive, and one which was patient $A\beta_{1-42}$ positive and tau negative. According to the IWG-2 criteria this last participant should be defined as a case of FTD and not as a patient with a frontal variant of AD [7]. Of note, since the A/T/N system is not directly applicable to FTD, this last group was analysed exclusively in terms of clinical diagnosis (**Figure 1**).

2.1.3 CSF sampling

All CSF samples were collected in polypropylene tubes, centrifuged (1000 g, 10 minutes, +4°C (sample collected at IM2A laboratory for the Paris cohort), 1500 g, 10 minutes, +4°C (sample collected at DZNE laboratory for the Rostock cohort), 1800 g, 10 minutes, +4°C

(sample collected at Mölndal Clinical Neurochemistry Laboratory for the Göteborg cohort)), and the collected supernatant was stored at –80°C pending biochemical analysis.

2.1.4 Immunoassays for core biomarkers

All the core biomarkers ($A\beta_{1-42}$, t-tau, and p-tau) were measured in the CSF of each subject. For the Paris cohort, CSF analyses were performed at the Laboratory of Biochemistry, Unit of Biochemistry of Neurometabolic diseases, Pitié-Salpêtrière University Hospital of Paris.

For the Rostock cohort, CSF analyses were executed in two different units: the Institute of Clinical Chemistry and Laboratory Medicine, Rostock University Medical Centre, after 06/2012, and the Laboratory of Neurochemistry, Department of Neurology, Göttingen University Medical Centre, before 06/2012.

For the Göteborg cohort, CSF analyses were executed at the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital, Mölndal.

T-tau was measured using a sandwich ELISA (INNOTEST hTAU-Ag, Fujirebio Europe, Gent, Belgium) specifically constructed to measure all tau isoforms irrespective of the phosphorylation status [34]. Tau phosphorylated at threonine 181 ($p\text{-}tau_{181}$) was measured using a sandwich ELISA (INNOTEST Phospho-Tau[181P], Fujirebio Europe, Gent, Belgium) constructed to specifically measure tau protein phosphorylated at the amino acid threonine 181 [35]. $A\beta_{1-42}$ was measured using a sandwich ELISA (INNOTEST β-AMYLOID(1-42), Fujirebio Europe, Gent, Belgium), specifically constructed for the quantitative determination of $A\beta_{1-42}$ [36]. All analysis were performed by board-certified laboratory technicians blinded to clinical information.

CSF biomarkers abnormalities were defined based on reference values currently used in each memory clinic: at IM2A in Paris, $A\beta_{1-42} < 500$ pg/mL, T-tau > 450 pg/mL, $p\text{-}tau_{181} > 60$ pg/mL; at DZNE in Rostock, $A\beta_{1-42} < 567$ pg/mL, T-tau > 512 pg/mL, $p\text{-}tau_{181} > 66$ pg/mL for the CSF samples measured before 06/2012 and $A\beta_{1-42} < 450$ pg/mL, T-tau > 450 pg/mL, $p\text{-}tau_{181} > 62$

pg/mL for the CSF sampels measured after 06/2012; at Mölndal Clinical Neurochemistry Laboratory, $A\beta_{1-42}$ < 550 pg/mL, T-tau > 400 pg/mL, p-tau_{181} > 80 pg/mL.

2.1.5 Immunoassay for YKL-40

All CSF YKL-40 analyses were performed at the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital, Mölndal, Sweden, using a commercial available ELISA kit (R&D Systems, Minneapolis, MN, US), according to manufacturer instructions. The measurements were performed in one round of experiments using one batch of reagents by board-certified laboratory technicians who were blinded to clinical data. Intra-assay coefficients of variation were below 10%. All samples were well within the linear range of the assay.

2.1.6 Statistical Analysis

Associations between sex and diagnostic group were assessed by Fisher's exact test, and the associations between age and diagnostic group was assessed through a nonparametric Kruskal-Wallis (KW) test. Subsequently, as a preprocessing step, all YKL-40 values were adjusted for age, sex and site employing nonparametric regression to enable age-,sex- and site- independent assessment of the diagnostic potential of YKL-40 while foregoing assumptions of normality. We conducted group-wise comparisons of YKL-40 values through nonparametric KW tests followed by pairwise post-hoc comparison (Conover's-test for multiple comparisons) whenever the result of the KW test was statistically significant ($p<0.05$). Results of post-hoc testing were corrected for multiple comparisons using a False Discovery Rate (FDR) procedure ($\alpha=0.05$).

We then evaluated the diagnostic potential of YKL-40 using logistic regression within a leave-one out cross validation (LOO-CV) approach in the following *a priori* comparisons: HC vs. AD and AD vs. FTD (Level I), HC vs. group 3 [A-/T±/N+ or A-/T+/N±], HC vs. group 4 [A+/T-/N-], HC vs. group 5 [A+/T±/N+ or A+/T+/N±] (Level II). In this analysis, the age-, sex-, and site adjusted YKL-40 values were entered as predictors and the diagnostic group was entered as the

dependent variable. After model fitting, we calculated the area under the receiver operating characteristic curve (AUROC) and its associated confidence intervals using a bootstrap procedure (100000 bootstraps) [37] by pooling predictions computed on the test sets from each train-test split in the LOO-CV procedure. The discriminatory ability of YKL-40 to correctly allocate participants to diagnostic groups was classified as follows: excellent (AUROC 0.90-1.00), good (AUROC 0.80-0.89), fair (AUROC 0.70-0.79), poor (AUROC 0.60-0.69), or fail/no discriminatory capacity (AUROC 0.50-0.59) [38].

All statistical analyses were performed in the R statistical environment version 3.2.3 (available at https://www.R-project.org/) under a Linux environment using the nonparametric kernel smoothing methods for mixed data types package (np package) [39], partial ROC (pROC) package [37], and the pairwise multiple comparison of mean ranks (PMCMR) package [38]. Two-tailed P values < 0.05 were considered statistically significant.

3.1 RESULTS

3.1.2 CSF YKL-40 levels in the population categorized according to Level I

Table 1 summarizes the levels of all analytes, combined with the demographic and clinical data of the population classified in line with Level I classification. Cognitively HC were slightly but significantly younger than MCI, AD, and FTD patients. MMSE scores were significantly lower in AD compared with cognitively HC and MCI. Compared with HCs group, CSF YKL-40 levels were significantly increased in AD (P=0.032) and FTD (P=0.049) (**Figure 2A**).

3.1.3 CSF YKL-40 levels in the population categorized according to Level II

Table 2 summarizes the levels of all analytes, combined with the demographic and clinical data of the population classified in line with Level II criteria. Cognitively HC (group 1) and patients belonging to group 2 [A-T-N-] were significantly younger than all the other groups

(Table 1). Compared with group 1 (HC), CSF YKL-40 levels were significantly increased in group 3 [A-/T±/N+ or A-/T+/N±] (*P*= 0.002), and group 5 [A+/T±/N+ or A+/T+/N±] (*P*= 0.002). Group 3 [A-/T±/N+ or A-/T+/N±], and group 5 [A+/T±/N+ or A+/T+/N±] patients presented substantially higher CSF YKL-40 concentrations compared with group 4 [A+/T-/N-] (*P*< 0.001 for both) patients, and compared with cases belonging to the FTD group (*P*=0.006 and *P*=0.007, respectively); group 3 [A-/T±/N+ or A-/T+/N±] patients presented higher CSF YKL-40 concentrations compared to group 2 [A-/T-/N-] patients (*P*=0.033), (**Figure 2B**).

3.1.4 Diagnostic value of CSF YKL-40 in the population at Level I

We found that YKL-40 differentiated HC from AD patients with an AUROC of 0.69 (95% CI, 0.55-0.84) (**Figure 3A**). CSF YKL-40 discriminated AD from FTD patients with an AUROC of 0.71 (95% CI, 0.51-0.91) (**Figure 3B**) .

3.1.5 Discriminative value of CSF YKL-40 in the population at Level II

CSF YKL-40 discriminated cognitively HC from the group 3 [A-/T±/N+ or A-/T+/N±], group 4 [A-/T±/N+ or A-/T+/N±], group 5 [A+/T±/N+ or A+/T+/N±] with AUROCs=0.76, (95% CI, 0.58-0.94), 0.52 (95% CI, 0.29-0.74), and 0.72 (95% CI, 0.58-0.87) (**Figure 4A-C**), respectively. CSF YKL-40 differentiated group 5 [A+/T±/N+ or A+/T+/N±] from the FTD with AUROC= 0.73 (95% CI, 0.54-0.92) (**Figure 4D**).

4.1 DISCUSSION

In Level I, CSF YKL-40 concentrations were significantly increased in clinically diagnosed AD patients compared with HC (**Figure 2A**). Moreover, the corresponding AUROC was poor/borderline fair in discriminating the two groups (**Figure 3A**). These findings partly

confirmed previous studies [16,18,20,25,26,28] and also the data from a recent meta-analysis [14]; in contrast, one study showed no differences between AD and HC [22]. Importantly, AD patients showed higher levels of CSF YKL-40 when compared to FTD; indeed, CSF YKL-40 gives a fair performance in distinguishing between the two groups (**Figure 3B**). In the literature, very few studies evaluated the diagnostic accuracy of CSF YKL-40 in discriminating between AD and FTD patients, and these studies reported conflicting results. In particular, Craig-Shapiro and colleagues reported higher levels of CSF YKL-40 in FTD compared to mild AD [18]; conversely, two other studies found no significant differences between AD and FTD [15,19].

Level II analysis showed that CSF YKL-40 concentrations were significantly increased in patients which were tau-positive only and those with AD pathology compared to HC (group 1) (**Figure 2B**). We found that YKL-40 delivered fair performance in discriminating tau-positive and AD pathology patients from HC (**Figure 4A and 4C**), but not in discriminating Aβ-positive only patients from HC (**Figure 4B**). These results generally agree with currently available studies which point toward the idea that CSF YKL-40 concentrations are more related to tau protein pathology as opposed to Aβ pathology [15,17,19,20,23,24]. Tau-positive patients revealed higher CSF concentrations of YKL-40 compared to patients with non-AD pathology, patients which were Aβ-positive only, and FTD patients. Similarly, AD pathology patients showed higher CSF levels of YKL-40 compared with patients which were Aβ-positive only, FTD patients, and a trend towards higher levels of YKL-40 in comparison to non-AD pathology patients. In particular, the AUROCs related to discriminating between AD pathology patients and FTD was fair (**Figure 4D**), i.e. comparable to what we found in Level I analysis. Several explanations support the fact that FTD patients can display lower CSF YKL-40 levels when compared to AD patients. In particular, FTD patients may have an underlying neurodegenerative process not related to tau protein [40]. This pathological variability possibly reflects the common clinical finding that FTD is a heterogeneous syndrome with different and overlapping phenotypes.

CSF YKL-40 can be considered as a biomarker of a specific pathogenic mechanism, allowing for *in vivo* measurement of neuroinflammatory processes that may be complementary to the core AD CSF biomarkers $A\beta_{1-42}$, T-tau and p-tau. The importance of having an early biomarker of neuroinflammation in AD is intriguing not only for diagnostic purposes (neuroinflammation is probably involved in other, additional neurodegenerative diseases [41]) but also because it can be predictive of response to novel anti-inflammatory drugs. In fact, epidemiological studies indicate that non-steroidal anti-inflammatory drugs (NSAIDs) may lower the risk of AD [42,43], although a number of trials reported negative results [9]. However, anti-inflammatory treatments may not be efficacious when administered during the dementia stage of AD. Notably, the naproxen trial in AD initially reported negative results; conversely, longer-term follow-up results suggested that naproxen may exert a protective role in asymptomatic subjects at baseline, thus reducing the conversion rate to AD [44,45]. The discovery and validation of a reliable inflammatory biomarker in prodromal AD as well as in preclinical phases, with the aim of tracking the response to an anti-inflammatory drug, might therefore represent an innovative step in developing novel therapeutic strategies for AD.

Our study represents the first attempt to apply YKL-40 as a diagnostic CSF biomarker for AD following a new unbiased biomarker-based classification [4]. Some limitations needs to be mentioned. First, in Level II, the categorization of our patients was based on CSF biomarkers only - i.e. the A/T/N system was used without considering neuroimaging markers. Also, this is a cross-sectional study and longitudinal data are not available. In particular, we are not able to differentiate potentially stable MCI patients from MCI patients converting to dementia, or to provide data about the possible different prognosis and rate of cognitive impairment progression. Furthermore, the diagnosis of MCI was made in a clinical setting and extensive and/or homogeneous psychometric data were not available. Additionally, given the relatively low number of patients, we did not test the CSF YKL-40 levels in all possible (eight) categories reported in the classification by Jack and colleagues [4]; we merged MCI patients with those in

the dementia stage of AD on the basis of core biomarkers assessment, without considering the degree of severity of cognitive impairment. However, the clinical distinction between MCI and dementia is not clear and time dependent; in this regard, the IWG-2 criteria consider MCI patients with AD pathology as AD in its prodromal phase [7]. Finally, with the exception of YKL-40, the measurements of the CSF core AD biomarkers, were performed in different laboratories and, while we controlled for center effects in our statistical analysis, additional inter-laboratory variability cannot be completely ruled out.

In conclusion, our study indicated that YKL-40 is poor/borderline fair and, therefore, it might not be able to satisfactorily differentiate AD from cognitively HC based only a purely clinical level of categorization. CSF YKL-40 deliveres a fair performance in discriminating between clinical AD and FTD. Based on core biomarker classification, CSF YKL-40 levels fairly distinguished HC individuals from cognitive impaired patients with both Aβ and tau pathology and cognitively impaired patients with tau pathology only, and both Aβ and tau pathology patients from FTD. In contrast, CSF YKL-40 levels were not useful in distinguishing between HC and cognitively impaired patients who were Aβ-positive only. Overall, CSF YKL-40 does not seem to play a major role in distinguishing clinical AD and AD pathology patients from HC subjects or from FTD cases. However, our results confirm that CSF YKL-40 levels should be considered a biomarker of neuroinflammation potentially related to neurodegenerative processes associated with tau protein.

We believe that, in the future, large longitudinal studies will be able to investigate the AD spectrum by applying the unbiased A/T/N classification system which can be considered an adaptive and flexible "open source" approach, based on a pattern of established biomarkers which however, can be potentially expanded to integrate novel biological biomarkers, genetic and epigenetic factors [46] as well as, possibly, indicators spanning different dimensions of pathology such as MRI-derived grey matter atrophy or functionally relevant burden of white matter damage [47].

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Drs. Blennow and Zetterberg are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg.
Dr Blennow has served as a consultant or at advisory boards for IBL International, Roche Diagnostics, Eli Lilly, Fujirebio Europe, and Novartis.
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