Reproducibility of Alzheimer's Disease Cerebrospinal Fluid-Biomarker Measurements under Clinical Routine Conditions

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Abstract. Analysis of cerebrospinal fluid (CSF) is one of the key tools for the state-of-the-art differential diagnosis of dementias. Dementia due to Alzheimer's disease (AD) is characterized by elevated CSF levels of total Tau (tTau) and phospho-181-Tau (pTau) and low CSF amyloid-β_{42} (Aβ_{42}). Discrepancies in the laboratory analysis of human materials are well known and much effort has been put into harmonization procedures. In this study, we measured CSF biomarkers of more than 100 patients obtained under clinical routine conditions in two different clinical laboratories. The CSF biomarker levels obtained from the two different sites were significantly correlated: $R^2 = 0.7129$ (tTau, $p < 0.001$), 0.7914 (pTau, $p < 0.001$), 0.5078 (Aβ_{42}, $p < 0.001$), 0.5739 (Aβ_{40}, $p < 0.001$), and 0.4308 (A$\beta_{42/40}$, $p < 0.001$). However, the diagnostic classifications of the Aβ_{42}, tTau, and pTau levels of identical subjects into normal versus pathological range made by the two different sites showed substantial discrepancies (31.5%, 29.6%, and 25.0% discordant cases, respectively). Applying A$\beta_{42/40}$, instead of CSF Aβ_{42} alone, lead to a reduction of the discordant cases to 16.8%. Our findings suggest that CSF A$\beta_{42/40}$ can outperform Aβ_{42} as a biomarker for AD neuropathology, not only under well-controlled study conditions but also in real life clinical routine. Thus, we recommend the inclusion of A$\beta_{42/40}$ as a CSF biomarker in the diagnostic procedure.

Keywords: Alzheimer's disease, amyloid-β, biomarker, cerebrospinal fluid, clinical diagnostics

INTRODUCTION

The diagnosis of Alzheimer's disease (AD) is based on different pillars. In addition to medical history, clinical examination, neuropsychological testing, and imaging methods, analysis of the cerebrospinal fluid (CSF) plays an important role in the biomarker-supported differential dementia diagnosis. According to different international [1] and national guidelines (e.g., German "S3-Leitline Demenz" [2]) for the clinical diagnosis of Alzheimer's disease as well as the recommendations of the "National Institute on Aging and the Alzheimer's Association working group" [3], increased CSF levels of total-Tau (tTau) and phospho(181)Tau (pTau), and low CSF Amyloid-β_{42} (Aβ_{42}) can be considered valid AD

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biomarkers and have been recommended to support an accurate diagnosis.

A non-invasive way to measure the amyloid burden in the central nervous system (CNS) display nuclear medicine imaging methods for *in vivo* detection of amyloid plaque density. The ^{18}F-labelled amyloid tracer Florbetaben shows high sensitivity and specificity for the detection of cerebral amyloid plaques in correlation to histopathology [4]. Therefore, amyloid PET imaging might be a valuable addition to common CSF-Aβ diagnosis.

In addition to $A\beta_{42}$, the analysis of the shorter Aβ-variant $A\beta_{40}$ seems to be useful. $A\beta_{40}$ is the most abundant C-terminal Aβ-variant in CSF, followed by $A\beta_{38}$ and $A\beta_{42}$ [5]. In CSF, $A\beta_{40}$ correlates well with the total Aβ concentration [6] and thus can serve as a surrogate-marker for total CSF Aβ. In patients with either unusually low or high overall Aβ, the $A\beta_{42}$ to $A\beta_{40}$ concentration ratio ($A\beta_{42/40}$) can be a helpful tool to normalize the $A\beta_{42}$ levels to $A\beta_{40}$ (as a proxy of total Aβ) and thereby improve the diagnostic accuracy [7–9].

CSF $A\beta_{42/40}$ was reported to correlate more closely with tracer retention on Amyloid-PET imaging than $A\beta_{42}$ alone [10] and appears to be less prone to variances resulting from pre-analytical sample handling [9]. Furthermore, in our hands, $A\beta_{42/40}$ turned out to provide higher inter-assay and between lot reproducibility than CSF $A\beta_{42}$ alone, when assessed with a multiplex chemiluminescence immunoassay (Aβ V-PLEX, Mesoscale) [6].

Several studies and meta-analyses described that the core AD CSF biomarkers tTau, pTau, and $A\beta_{42}$ provide good differentiation between AD and controls as well as mild cognitive impairment (MCI) due to AD and stable MCI. Consequently, their use in clinical practice and clinical research was clearly recommended by these authors [11–13]. However, it appears that the appraisal regarding the clinical use of these biomarkers, in particular in patients with MCI, is quite controversial: For example, in their 2014 Cochrane review, Ritchie et al. did not recommend CSF Aβ levels as an accurate test of AD pathology in patients with MCI [14, 15]. It is important to note that the published biomarker studies presumably have been performed in general according to highly standardized and well controlled operating procedures and with very carefully selected clinical cohorts. Therefore, it is not obvious to what extent these optimized study conditions reflect the situation in everyday clinical routine in memory clinics or at general practitioners (GPs). Although CSF biomark-

ers are recommended for clinical research, McKhann et al. did not support the use of CSF biomarkers for routine diagnostics [3].

To address this question, we have compared parallel CSF biomarker measurements and their interpretations executed on a fee for service basis by two different certified clinical laboratories. To this end, parallel aliquots of 114 CSF samples were sent to two independent laboratories for the simultaneous determination and interpretation of t-Tau, p-Tau, $A\beta_{42}$, and $A\beta_{42/40}$ according to the respective lab-specific standard operating procedures (SOPs) and cut-off points. The measured biomarker levels and corresponding diagnostic classifications obtained from the two different sites for each one of the 114 subjects were compared and the degree of agreement between the different sites in term of the diagnostic interpretations was assessed. An important question to be addressed was whether $A\beta_{42/40}$ would show a higher level of agreement between the two sites than $A\beta_{42}$ alone in term of diagnostic classifications.

A small number of patients from this cohort underwent an additional amyloid-PET diagnostic to measure the central amyloid deposition as a marker of amyloid pathology. We have also compared $A\beta_{42}$ and $A\beta_{42/40}$ classifications from both centers with the amyloid-load as measured by amyloid-PET.

MATERIALS AND METHODS

Study design

Over a period of two years (September 2014 until August 2016), parallel aliquots of CSF samples of 114 patients who underwent a lumbar puncture for dementia differential diagnostic reasons were sent to two different, independent, certified clinical laboratories for the parallel determination of tTau, pTau, $A\beta_{42}$, and $A\beta_{42/40}$. CSF biomarkers were analyzed according to the respective internal laboratory routine procedures and evaluated with regard to the particular diagnostic cut-off points. Lumbar puncture was performed with ether a "Quincke needle" (BD) or "Sprotte cannula" (Pajunk). CSF was collected in polypropylene CSF tubes (Sarstedt). Importantly, this study was not designed as a Round-Robin study. None of the two clinical laboratories had been informed about the parallel measurements and, consequently, no efforts of any kind for standardization between laboratories were made. We have compared the biomarker data and diagnostic classifications

obtained from both different clinical laboratories for each one of the 114 subjects included in this study retrospectively. We chose this kind of study design to reflect clinical everyday conditions. The interpretation of the measured biomarkers was done according to the respective reference values of the two clinical laboratories.

Amyloid-PET

Amyloid PET/CT was performed on a Philips Gemini TF16 PET/CT-scanner (Philips Medical Systems, Cleveland, OH, USA) with a 128×128 matrix with 2 mm slice thickness and acquisition time of 20 min. Low-dose-CT with a 512×512 matrix with 2 mm slice thickness was used for attenuation correction (CTAC-SG algorithm). Reconstruction was performed using the LOR-TF-RAMLA ("BLOB-OS-TF") algorithm. 300 ($\pm$20%) MBq 18F-Florbetaben (Piramal Imaging Ltd, Cambridge, UK) was administered intravenously and PET/CT was performed 90 min post injection. PET/CT data were visually assessed by an experienced nuclear medicine physician who underwent a specific reader training for amyloid imaging. Scans were scored 'negative' when the amyloid load was within the physiological range and 'positive' when the amyloid load was increased.

Study cohort

114 patients from the Department of Psychiatry and Psychotherapy of the University Medical Center Goettingen were included in this study. All patients underwent lumbar puncture for diagnostic reasons within the diagnostic procedure of dementia. Due to the high impact of CSF biomarkers into the clinical diagnosis, we renounced to include the clinical diagnosis. The mean age of this study cohort was 66.46 ± 10.16 years. In total, 59 men (51.75 %) and 55 women (48.25 %) were included (Table 1).

Statistics

All results are presented as mean $\pm$ SD, unless otherwise indicated. Bland-Altman analysis was

Table 1
Cohort description

included patients [number]	114
Male [n] (%)	59 (51.75)
Female [n] (%)	55 (48.25)
Mean age at CSF diagnosis [y] (SD)	66.46 (10.16)

performed to evaluate systematic center differences. A Pearson correlation and a two-tailed paired student *t*-test was calculated to compare the CSF biomarker measurements done on parallel aliquots of the same set of CSF samples by the two different centers. To compare the agreement on interpretation of the results between both centers, the inter-rater reliability was calculated as Cohen's kappa [16].

Ethical commitment

This study was performed according to the Declaration of Helsinki in the current version. The data-collection and analysis was approved by the ethical committee of the University Medical Center Goettingen (ethical vote 4/1/17).

RESULTS

We compared the results from parallel measurements and diagnostic classifications of the CSF levels of tTau, pTau, $A\beta_{42}$, and $A\beta_{42/40}$ obtained from two different certified diagnostic clinical laboratories under everyday clinical conditions. In total, $n = 114$ subjects were included in this study. Bland-Altman method comparisons suggested systematic center differences regarding $A\beta_{42}$ and $A\beta_{42/40}$, but not $A\beta_{40}$, tTau, and pTau (Fig. 1). This can be explained by the use of different assay kits at the two sites for measuring CSF $A\beta_{42}$ (IBL versus Fujirebio).

The CSF levels of tTau, pTau, $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{42/40}$ as well as the corresponding cut-off values are summarized in Table 2 and illustrated in Figs. 2 and 3. The mean concentration of CSF $A\beta_{42}$ was on average 233.4 pg/ml higher in laboratory 1 (1034.0 ± 421.0 pg/ml) compared to laboratory 2 (800.6 ± 398.3 pg/ml) (Fig. 2D). The cut-off point used by laboratory 2 (620 pg/ml) was 170 pg/ml higher than the one used in laboratory 1 (450 pg/ml). Thus, a higher number of pathological $A\beta_{42}$ samples was found in laboratory 2. For $A\beta_{42/40}$, identical cut-off points were used (0.05 $A\beta_{42}/A\beta_{40}$) at the two sites. The mean of the calculated $A\beta_{42/40}$ ratio was 0.028 higher in laboratory 1 (0.086 ± 0.034 $A\beta_{42}/A\beta_{40}$) compared to laboratory 2 (0.058 ± 0.018 $A\beta_{42}/A\beta_{40}$) (Fig. 2E). A substantially higher discrepancy at higher $A\beta_{42/40}$ (indicating neurochemically healthy subjects) could be seen in the Bland-Altman-Analysis (Fig. 1E).

Both tTau and pTau measurements of the two centers were strongly correlated (tTau: $R^2 = 0.7129$, $p < 0.0001$; pTau: $R^2 = 0.7914$, $p < 0.0001$) (Fig. 3A,
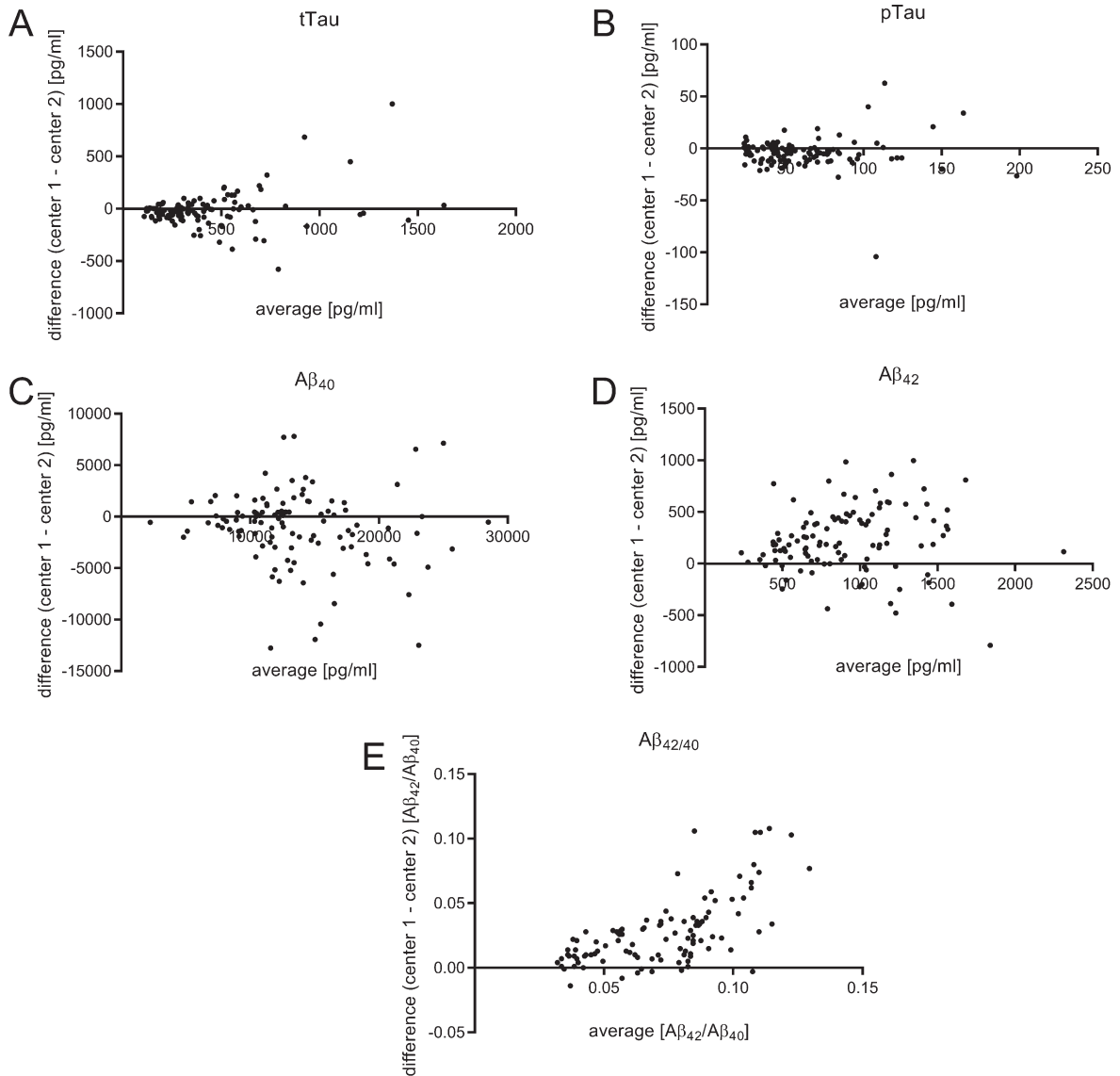
Fig. 1. Bland-Altman plots for each biomarker. The mean value of the two biomarker measurements was plotted on the X-axis. On the Y-axis the difference between both centers (center 1 – center 2) was pictured. This presentation facilitates the direct comparison between two different centers. While no systematic differences were observed regarding the measurements for tTau, pTau, and $A\beta_{40}$, the measured $A\beta_{42}$ levels and, as a result $A\beta_{42/40}$, were generally higher in center 1.

B). However, the diagnostic interpretations (normal CSF level versus pathological level) obtained from the two different laboratories were discordant in 29.6% of the cases with regard to tTau and in 25.0% of the patients with regard to pTau. The $A\beta_{42}$ and $A\beta_{40}$ measurements of the two different laboratories also showed statistically significant correlations: $R^2 = 0.5078$ ($p < 0.0001$) for $A\beta_{42}$ and $R^2 = 0.4308$ ($p < 0.0001$) for $A\beta_{42/40}$, respectively (Fig. 3D, E). CSF $A\beta_{40}$ correlated with $R^2 = 0.5739$

($p < 0.0001$) between both centers (Fig. 3C). Although the cut-off points were different in both centers, this could not explain the different interpretations completely: parallel measured CSF aliquots were significantly different for pTau, $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{42/40}$. Only tTau measurements were similar in both centers (Fig. 2, Table 2).

The diagnostic interpretations of the $A\beta_{42}$ CSF levels (normal versus pathological range) obtained by the two different sites were discrepant in 31.5% of
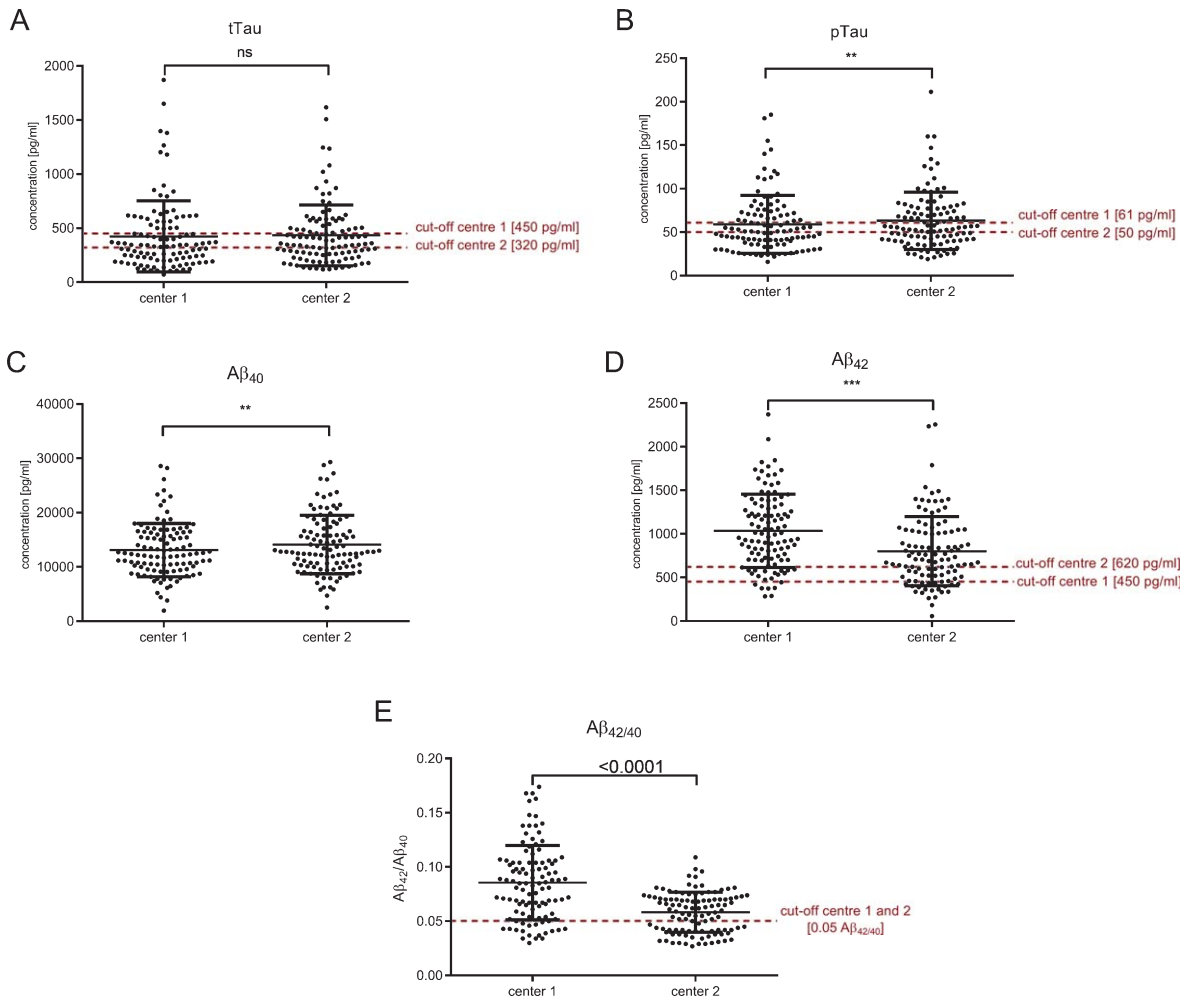
Fig. 2. Measured concentrations of tTau, pTau, $A\beta_{40}$, and $A\beta_{42}$ in both centers presented as scatter blots with the corresponding cut-off points. For $A\beta_{42/40}$, both centers had the same cut-off points (0.05 $A\beta_{42}/A\beta_{40}$). Interestingly, in center 2, two different subgroups, most likely AD patients and controls, can be seen, which are separated by the cut-off of 0.05 (E). In contrast, no such separations can be seen in center 1. Significant differences are indicated as **$p < 0.01$ and ***$p < 0.001$.

the cases. The corresponding Cohen's kappa (κ), as a measure of inter-rater agreement, was 0.170 indicating a poor agreement between the two different clinical laboratories [16]. In contrast, the diagnostic classifications of the subjects according to the $A\beta_{42/40}$ ratio were discordant in only 16.8% of all cases, with $\kappa = 0.589$ indicating a moderate agreement. For tTau and pTau, calculated values of Cohen's kappa were 0.449 and 0.528 (moderate agreement), respectively.

For a subset of the subjects included in this study, additional *in vivo* amyloid PET imaging data was available. Seven out of ten patients showed an increased amyloid-load within the CNS. Table 3 summarizes the degree, to which the diagnostic interpretations of the CSF $A\beta_{42}$ levels and $A\beta_{42/40}$ obtained from the two different sites were in line with the observations from amyloid PET imaging. On average, in only 55% of the cases both, CSF biomarkers and amyloid imaging suggested concordantly either absence or presence of brain amyloid deposition.

DISCUSSION

In this study, we report on the comparison of parallel CSF-biomarker measurements in a clinical cohort
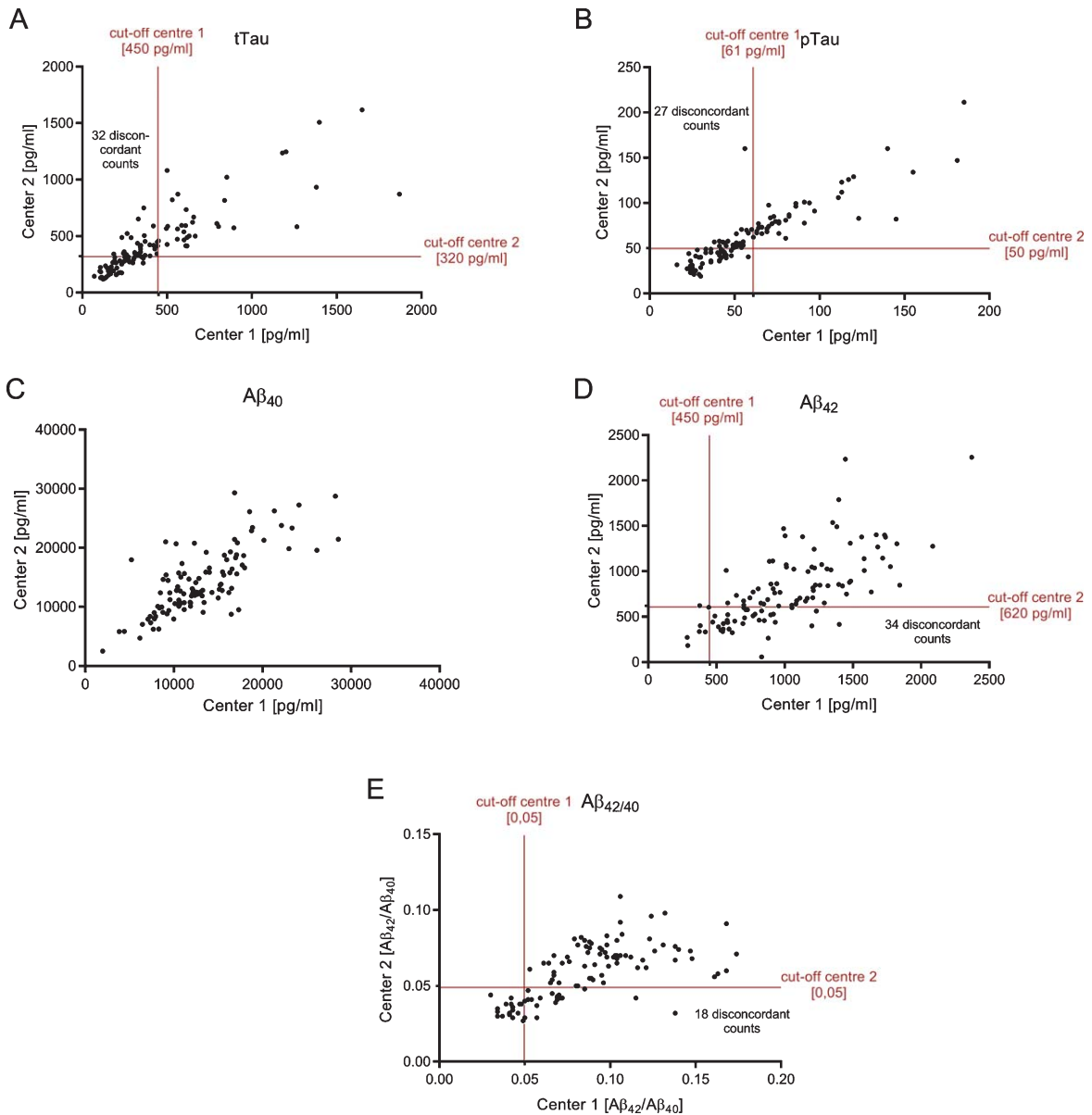
Fig. 3. Correlation between CSF-biomarker measurements in the two different centers. Cut-off points of each center are indicated as red horizontal and vertical lines. Patients with discordantly interpreted biomarkers can be found in the upper left square for tTau (A) and pTau (B), whereas for $A\beta$, discordant biomarkers are mainly in the lower right square (C: $A\beta_{42}$ and D: $A\beta_{42/40}$). All biomarkers correlated between both centers: $R^2 = 0.7129$, $p < 0.001$ (tTau); $R^2 = 0.7914$, $p < 0.001$ (pTau); $R^2 = 0.5078$, $p < 0.001$ ($A\beta_{42}$); $R^2 = 0.5739$, $p < 0.001$ ($A\beta_{40}$); $R^2 = 0.4308$, $p < 0.001$ ($A\beta_{42/40}$).

and their diagnostic interpretations executed under everyday routine conditions in two different clinical laboratories on a fee for service basis. In a sample of more than 100 patients, tTau, pTau, $A\beta_{42}$, and $A\beta_{42/40}$ were measured in parallel in two different laboratories according to the respective SOPs and the measured concentrations were classified according to the respective laboratory-specific cut-off points.

We observed substantial discrepancies between the two centers—with regard to the reported CSF $A\beta_{42}$ concentrations and the $A\beta_{42/40}$ ratios, which can be easily explained by the use of different assay kits for measuring $A\beta_{42}$. More importantly, the diagnostic interpretations, provided by the different centers, were often discordant, not only for CSF $A\beta_{42}$ but also for tTau and pTau. The classifications (normal

Table 2
Measured mean values, cut-off points, correlation, inter-rater reliability and discordance rate

		tTau		pTau		$A\beta_{42}$		$A\beta_{40}$		$A\beta_{42/40}$	
		center 1	center 2	center 1	center 2	center 1	center 2	center 1	center 2	center 1	center 2
mean ± SD	[pg/ml]	423.4 ± 328.5	433.6 ± 280.3	58.96 ± 33.24	63.17 ± 32.98	1034.0 ± 421.0	800.6 ± 398.3	13109 ± 4916	14128 ± 5386	0.086 ± 0.034	0.058 ± 0.018
paired *t*-test	*p* value	0.5471		0.0059		<0.0001		0.0041		<0.0001	
cut-off	[pg/ml]	450.0	320.0	61.00	50.00	450.0	620.0	–	–	0.05	0.05
correlation	Pearson R^2	0.7129		0.7914		0.5078		0.5739		0.4308	
	p value	<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	
Cohen's kappa		0.449		0.528		0.170		–		0.589	
discordance	[%]	29.6		25.0		31.5		–		16.8	

Although $A\beta_{42/40}$ showed the lowest correlation between both centers, $A\beta_{42/40}$ outperformed the discordance rate and inter-rater reliability, compared to $A\beta_{42}$, tTau, and pTau.

Table 3
Concordance of CSF-biomarkers and amyloid load in the CNS

	$A\beta_{42}$		$A\beta_{42/40}$	
	center 1	center 2	center 1	center 2
Patient 1	–	–	+	+
Patient 2	–	+	–	–
Patient 3	–	+	–	+
Patient 4	+	+	+	+
Patient 5	–	+	–	–
Patient 6	+	+	+	+
Patient 7	–	+	–	+
Patient 8	+	–	+	–
Patient 9	+	+	+	+
Patient 10	–	–	–	–
In line with Amyloid-PET	40%	70%	50%	60%
	55%		55%	

If CNS amyloid load, measured by Amyloid-PET, corresponds to the CSF biomarkers $A\beta_{42}$ and $A\beta_{42/40}$, patients are marked with "+", if not, patients are marked with "–". Overall, 55% of all CSF biomarkers were in accordance to the Amyloid-PET.

range versus pathological range) made by the two centers were discrepant in 25.0% of the cases with regard to pTau, in 29.6% of the cases for tTau and in 31.5% of the cases regarding $A\beta_{42}$. Considerable inter-laboratory variability in the immunological measurements of tTau, pTau, and $A\beta$ peptides in CSF has been reported previously, also in carefully conducted multicenter quality survey studies [17, 18]. In particular, the reliable and reproducible determination of CSF $A\beta_{42}$ is known to be technically challenging, and consequently efforts on standardization, including, for example, the development of certified reference materials and reference measurements procedures, have been launched [19].

In the samples studied here, the diagnostic classifications of the $A\beta_{42/40}$ concentration ratios showed substantially less discordance between the different centers than those of the CSF $A\beta_{42}$ levels. This observation further supports $A\beta_{42/40}$ to represent the better neurochemical biomarker of pathological cerebral $A\beta$ accumulation than $A\beta_{42}$ alone [9, 20, 21].

The incorporation of additional diagnostic techniques is particularly important in the case of patients with atypical CSF-constellations or clinical appearance. PET-imaging is expensive and extensive, but might lead to an improved diagnostic accuracy in some individual cases.

In the present cohort, the agreement between CSF biomarkers and Amyloid-PET imaging was moderate. Overall, 55% of the CSF biomarker interpretations were in line with the Amyloid-PET results, whereby the agreement between the different centers

and biomarker modalities ($A\beta_{42}$ versus $A\beta_{42/40}$) were varying. However, there was only a small number of imaging results available. Mattsson et al. showed that the correlation between CSF $A\beta_{42}$ and amyloid load, measured with Florbetapir, depends on the diagnosis and clinical stadium [22]. In cognitively healthy subjects, 21% of all cases showed discordant results between $A\beta_{42}$ and Amyloid-PET with mainly pathologically decreased $A\beta_{42}$ but normal Amyloid-PET results. In patients with AD, only 6% discordant cases were observed. Hence, the authors concluded that decreased $A\beta_{42}$ in CSF without Amyloid-PET abnormalities corresponded to early stages of AD, whereas Amyloid imaging changes to pathological in progressed stages [22]. The high discrepancy between the Amyloid-PET results and the measured CSF-biomarkers we observed here could be due to diagnostically inconclusive patients. In this cohort Amyloid-PET was not routinely performed, thus mainly clinical atypical or unclear patients underwent an additional Amyloid-PET.

As Amyloid-PET might not yet be available at many diagnostic sites, most centers will most probably make the diagnosis of AD supported by CSF-biomarkers. This study clearly reveals the importance of improving the possibilities to interpret CSF markers in subjects in order to reduce false negative and particularly false positive AD diagnoses, an issue of enormous importance to subjects suspected to suffer from AD.

In view of the here observed discordance in the diagnostic interpretation of CSF-biomarker data of up to 31.5% between the two centers, there is a great need for multicenter efforts to define valid cut-off points and to harmonize biomarker data interpretation. Different efforts on biomarker harmonization studies have been done so far. For example, a quality control program has been established, the Alzheimer's Association QC program for CSF biomarkers (http://www.neurochem.gu.se/TheAlzAssQCprogram) [23, 24]. In addition, Bjerke et al. studied certified reference materials for the comparison between different assay techniques, which might be a useful tool for quality controls [25]. Besides, more and more efforts are made on the implementation of fully automated analyzers for CSF measurements [19, 26–28]. Recent data suggest the superiority of these fully automated electrochemiluminescence immunoassay, such as the novel Elecsys β-amyloid (1–42) assay by Roche Diagnostics, over classical ELISAs [29]. Besides Roche Diagnostics, Fujirebio (Lumipulse G600II) and Euroimmun (EUROIMMUN RA Analyzer 15)

also provide fully automated immunoassays. All of these systems are supposed to provide a minimal lot-to-lot variation and thus might lead to a higher agreement between different centers and measurements. Further studies on fully validated systems are still pending to evaluate the benefit of automated immunoassays.

Nevertheless, Mattsson et al. could clearly demonstrate that the CSF biomarker variability is too high to calculate general cut-off values using the classical immunoassay based assays [28]. Experiences with fully automated immunoassays to determine general cut-off values are still insufficient. Therefore the determination of center-specific cut-off points, which need to be adapted within certain periods, is of high importance [30].

With a prevalence of estimated 5.4 million affected AD persons in the USA in 2016 [31], there is a substantial need for reliable CSF diagnostics. Thus, there is a big need for an improved inter-laboratory standardization. Since this study did not focus on the reason for the observed discrepancies (e.g., transport, SOP, methods, reference values), further research on the identification of influencing factors and consecutively optimization is required.

Conclusion

The analysis of neurochemical AD biomarkers in CSF can provide important support for the early and differential diagnosis of dementia. However, biomarker data have to be interpreted with caution and always in the context of the available clinical and diagnostic information. Biomarker data cannot serve as the sole basis of a diagnosis and need to be assessed in the context of medical history, neurological examination, and neuropsychological testing [2, 3, 32]. While high sensitivities and specificities of CSF biomarkers for the discrimination between controls and AD patients have been observed in well controlled biomarker studies [2, 33], this may not be feasible to the same extent in clinical every day routine. Measured CSF-biomarkers and their interpretation should be considered critically and taken as one part of a versatile diagnostic process. We could show that under real life clinical routine conditions the use of CSF $A\beta_{42/40}$ instead of CSF $A\beta_{42}$ can reduce the discrepancies between two different sites in terms of diagnostic biomarker interpretation. As a result, we support the implementation of $A\beta_{42/40}$ in routine clinical diagnosis to improve the diagnostic certainty. Nevertheless, it is important to be aware

of the limitations of the diagnostic performance of biomarker measurements.

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