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Serum and cerebrospinal fluid Neutrophil gelatinase-associated lipocalin (NGAL) levels as biomarkers for the conversion from mild cognitive impairment to Alzheimer's disease dementia

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ABSTRACT

Neutrophil gelatinase-associated lipocalin (NGAL) is an acute phase protein that has been reported as a potential marker for pre-dementia stages of Alzheimer's disease (AD). Longitudinal studies for its association with the conversion of mild cognitive impairment to AD is still lacking. This study included n = 268 study participants with subjective cognitive decline (SCD) (n=82), mild cognitive impairment (MCI) (n=98) and AD dementia (n=88) at baseline and two-year follow-up clinical assessments. Serum and cerebrospinal fluid (CSF)NGAL, CSF amyloid beta$_{1-42}$, total-Tau, and phospho-Tau levels were measured with ELISA analysis. CSF NGAL levels were significantly lower in MCI participants compared to people with SCD at baseline. Lower baseline CSF NGAL levels predicted MCI converters to AD dementia vs. non-converters after 2-years follow-up. A positive correlation between CSF NGAL and amyloid beta$_{1-42}$ was found particularly in MCI participants at baseline. NGAL in CSF holds potential to be used as a predictive marker for the conversion of MCI to AD dementia and may reflect pathophysiological processes of prodromal AD neuropathology.

1. Introduction

A chronic inflammatory response, in addition to amyloid beta (Aβ) and Tau, is considered a core feature in the neuropathophysiology of Alzheimer's disease (AD) (Akiyama et al., 2000; Kinney et al., 2018; Krstic and Knuesel, 2013). The function of neuroinflammatory mechanisms in AD is important particularly during the early phases, when it affects neuropathological processes leading to subsequent clinical manifestations (Cuello, 2017). Numerous studies on the associations of peripheral and cerebrospinal fluid (CSF) immune markers of AD have been conducted to identify potential biomarkers and gain insights into the multifaceted neuroimmune mechanisms of early AD. In this respect, findings from

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a recent meta-analysis showed that the majority of studies on this subject found significant changes in levels of peripheral and CSF immune markers in patients with mild cognitive impairment (MCI) and AD dementia (Shen et al., 2019). Peripheral and CSF inflammatory makers that reflect the pathophysiological processes of AD pathology have the potential to improve early diagnosis and facilitate monitoring of treatment strategies (Molinuevo et al., 2018).

Neutrophil gelatinase-associated lipocalin (NGAL) has gained growing interest for its association with neurodegenerative diseases, including AD. NGAL is an acute phase protein and also referred to as Lipocalin 2, 24-kDa superinducible protein (SIP24), siderocalin, 24p3 and uterocalin. It is a 25 kDa member of the lipocalin protein family and plays a pivotal role in the host innate response against bacterial infections (Flo et al., 2004; Goetz et al., 2002). Investigations with human post mortem brain tissues showed that NGAL protein levels are significantly increased in the AD brain, with a regional distribution pattern of AD neuropathology, that is high levels particularly in the hippocampus (Dekens et al., 2017; Naudé et al., 2012). Interestingly, decreased CSF NGAL levels were found in MCI and AD dementia patients, compared to cognitively healthy controls (Dekens et al., 2017; Naudé et al., 2012). Its circulating levels, on the other hand, was found to be increased in patients with MCI as compared to cognitively healthy controls and AD dementia patients (Choi et al., 2011). This finding was supported in a recent study with cognitively healthy participants that were characterized as pre-clinical AD based on CSF amyloid beta 1-42 ($A\beta_{1\text{-}42}$) levels (Eruysal et al., 2019). Moreover, increased serum NGAL levels found in people with Down syndrome (Dogliotti et al., 2010; Naudé et al., 2015), whom are at high risk to develop dementia due to AD (Mann, 1988; Zigman and Lott, 2007). However, no significant differences in serum NGAL between MCI patients and cognitively healthy controls was found in another study (Naudé et al., 2012).

Existing studies collectively thus suggest that NGAL is associated with AD neuropathology and may function as a potential biomarker for the progression of MCI to AD dementia. However, investigations on the longitudinal associations of blood or CSF NGAL levels in the conversion from MCI to AD dementia are still lacking. The current study used a standardized longitudinal multicentre academic memory clinic population to evaluate the following study aims: (1) to compare baseline serum and CSF NGAL levels between baseline clinically diagnosed subjects with subjective cognitive decline (SCD), MCI and AD dementia, (2) to determine if baseline serum and CSF NGAL levels predict the conversion of MCI to AD dementia after a 2-year follow-up with CSF $A\beta_{1\text{-}42}$, total Tau (t-Tau), and Tau phosphorylated at threonine 181 (p-Tau) as reference markers, (3) to evaluate the associations of baseline serum and CSF NGAL levels with CSF neuropathological biomarkers of AD; $A\beta_{1\text{-}42}$, total t-Tau and p-Tau, and (4) to determine the associations of serum and CSF NGAL levels with measures of cognitive decline over time.

2. Materials and methods

2.1. . Study population

In this study, we used baseline serum and CSF samples, and follow-up data from study participants of the Dutch Parelsnoer Institute (PSI) Neurodegenerative Diseases Consortium. The PSI is a longitudinal multicentre study between the eight university medical centres in the Netherlands (https://www.health-ri.nl/initiatives/parelsnoer), in which data are prospectively and uniformly collected. Standardized operating procedures between the centres are used for the collection of clinical data, cognitive assessments, clinical diagnosis, magnetic resonance imaging (MRI) and procurement of blood, and CSF samples (Aalten et al., 2014). The objective of the PSI Neurodegenerative Diseases Consortium is to study biomarkers in the early stage, differential diagnosis, and prognosis of neurodegenerative diseases, in particular AD (Aalten et al., 2014). Eligible for inclusion were individuals referred to 1 of the 8 academic memory clinics for the evaluation of cognitive complaints, with a Clinical Dementia Rating scale (Morris, 1993) of 0, 0.5, or 1, and a Mini-Mental State Examination (MMSE) (Folstein et al., 1975) of 20 or higher. Syndrome diagnosis were made in multidisciplinary meetings and included; SCD, MCI, or AD dementia (Aalten et al., 2014). The diagnosis of dementia was based on the diagnostic and statistical manual of mental disorders 4th edition (DSM-IV) criteria (APA, 1994). Clinical diagnoses for AD were made according to National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria (APA, 1994; McKhann et al., 1984). MCI diagnosis was based on Petersen's criteria (Petersen et al., 1999). Additionally, all patients fulfilled the National Institute of Aging and Alzheimer's Association (NIA-AA) core clinical criteria for MCI or dementia due to AD (Albert et al., 2011). Patients were considered to have SCD when cognitive functioning was found to be normal from clinical evaluation and did not fulfil the diagnosis for MCI or dementia.

For the present study, we used PSI data from n = 268 study participants with SCD (n = 82), MCI (n = 98) and AD (n = 88) at baseline. Serum samples were missing from 10 participants, and CSF samples from 2 study participants. For this study, we used 2-year follow-up syndrome diagnosis and measures of the MMSE. The PSI Neurodegenerative Diseases Consortium was approved by the Medical Ethics Review Committee of the Amsterdam University Medical Centre. All local Medical Ethical Committees approved the local performance of the study. The research is performed according to the principles of the Declaration of Helsinki. All patients enrolled in the study gave their written informed consent.

2.2. Follow-up measures

Follow-up measures were obtained 2 years after baseline (mean = 26.0 months, SD = 3.0). Follow-up measures were available in n = 68 (69.4 %) of the 98 participants that were diagnosed with MCI at baseline. After this 2-year interval, n = 27 converted to dementia, of which n = 22 were clinically diagnosed as AD dementia and n = 5 were diagnosed with other forms of dementia (n = 1 vascular dementia, n = 1 frontotemporal dementia, n = 1 progressive aphasia and n = 2 other). These five participants were excluded from the analysis because NGAL levels may differ in other forms of dementia (Llorens et al., 2020) compared to AD dementia and the aim of this study was to evaluate NGAL as a predictor for the conversion to AD dementia.

MMSE scores at baseline were available in n = 261 (97.4 %) of the total study sample and 2-year follow-up measures were available in n = 149 (55.6 %) of the study participants.

2.3. Demographic variables

Educational level, lifestyle factors and medical history were obtained in an interview with the patient and his/her caregiver (Aalten et al., 2014). Educational level was classified as low (i.e., no education, primary education, or lower vocational education), intermediate (i.e., intermediate general secondary education, intermediate vocational education, or higher general secondary education), or high (i.e., higher vocational education or university). Current alcohol consumption was classified as none or any. Smoking behaviour was categorized into current smoker, former smoker, or never smoking. Cardiovascular disease was defined as (a history

of) hypertension, hyperlipidaemia, cardiac arrhythmias, angina pectoris, myocardial infarction, coronary artery disease, carotid artery stenosis, and/or peripheral artery disease. Cerebrovascular disease was defined as a history of transient ischemic attack, reversible ischemic neurologic deficit, and/or stroke. The presence of kidney disease and diabetes were determined by self-report during the interview.

2.4. Blood and CSF samples

A total of 3 mL CSF was collected by lumbar puncture at the L3/L4 or L4/L5 intervertebral space and stored at −80°C in 0.5 mL aliquots until analysis. Blood samples were collected via venepuncture into serum tubes. The tubes were kept at room temperature for 30 minutes to allow for clotting and were subsequently centrifuged at 2,000 × g, aliquoted into cryo vials and immediately stored at -80°C until analyses. Biological samples were collected at baseline of the study during the visit when demographic and clinical variables were collected. Blood and CSF collections were performed during visiting hours without prior fasting.

2.5. Laboratory analyses

NGAL in serum and CSF samples were quantified with sandwich ELISA (Cat# DY1757; R&D Systems) according to the manufacturer's protocol. Serum samples were diluted 1:150 for analyses and CSF samples were not diluted. The intra- and inter-assay coefficients of variation were ≤5% and ≤8%, respectively. A$\beta_{1\text{-}42}$, *t*-Tau, and *p*-Tau concentrations were measured using commercially available single-parameter ELISA methods (respectively Cat# 81583; Innotest beta-amyloid (1–42), Cat# 81572 Innotest hTAU-Ag and Cat# 81581; Innotest phospho-TAU (181P); Innogenetics, Ghent, Belgium) as previously described (Aalten et al., 2014). The analyses of all serum and CSF markers were performed blinded for clinical data.

DNA was isolated at each individual site. DNA isolation was performed robotically, based on a salting out method. After isolation and a quality control, the DNA was stored in four cups at −80°C. Isolated DNA was taken from the local biobanks and transported to the department of Clinical Genetics of the Maastricht University Medical Centre. Apolipoprotein E (APOE) genotype was determined on genomic DNA using the polymerase chain reaction (PCR) technique (Bekers et al., 2002). Genotyping was done blinded for all clinical data.

Measures for the biomarkers; A$\beta_{1\text{-}42}$ (n = 80), *t*-Tau (n = 81), *p*-Tau (n = 80) and APOE (n = 85) were missing in the whole study sample. Values for the study numbers that were used for the analyses in the whole study sample and separated study groups are provided in Tables 2–4.

2.6. Covariates

Age and sex were selected *a priori* as covariates based on their potential effects on syndrome diagnosis and, serum and CSF NGAL levels (Choi et al., 2011; Eruysal et al., 2019; Naudé et al., 2014). Even though increased levels of NGAL in renal disease is well studied (Buonafine et al., 2018), it was not included as a covariate in this study due to the low number of participants with reported kidney diseases (n = 7 in all of the participants and n = 3 in the MCI group).

2.7. Statistical analyses

All analyses were conducted using SPSS (version 26, IBM, USA). *p*-values were considered statistically significant at a value of less than 0.05. The raw values of *t*-Tau and *p*-Tau did not fulfil the normality assumption and all the biomarkers were natural logarithm (ln)-transformed. This resulted in acceptable skewness and kurtosis of the data, which were used for further statistical analyses. For the description statistics of study participant demographics, analysis of variance (ANOVA) was performed for continuous variables, and Pearson's chi squared tests for categorical variables between the syndromes. The homogeneity of variation was assessed with Levene's test. Levene's test was used to test the homoscedasticity of variances and Welch's ANOVA was used if this assumption was violated. ANOVA with Levene's homogeneity test, and Tukey post-hoc test for pair-wise comparisons (in case of an overall effect between the three groups), was performed to evaluate differences in serum and CSF NGAL levels between the diagnostic groups (i.e., SCD, MCI, and AD) at baseline. Analysis of covariance (ANCOVA) with a Bonferroni post-hoc adjustment test was subsequently performed to adjust for age and sex in the comparison of serum or CSF NGAL levels between the study groups (SCD, MCI and AD) at baseline. Univariate and multivariate logistic regression analyses were used to determine the odds ratios according to the likelihood ratio test for the conversion of MCI to AD dementia after 2-years follow-up. To enable direct comparable effect sizes of the associations across the biomarkers, the data was *Z*-score transformed data. Ln-transformed CSF NGAL and A$\beta_{1\text{-}42}$ values were inverted so that lower levels imply higher risk. Predictor variables included baseline ln-transformed (serum NGAL, CSF *t*-Tau, or *p*-Tau) and inverted ln-transformed (CSF NGAL and A$\beta_{1\text{-}42}$) biomarkers. The multivariate model adjusted for age and sex. Goodness of fit for the logistic regression models was evaluated with the Hosmer-Lemeshow test. Associations between baseline ln-transformed biomarkers and trajectories of MMSE scores over the 2-year follow-up were assessed with linear mixed models and adjusted for age and sex. Continues MMSE scores were used as the dependent variable. All models included a random intercept and slope to account for the within-patient correlation between MMSE scores and the variability of MMSE scores over time. The Akaike information criteria (AIC) value indicated first-order autoregressive covariance structure as the most appropriate fit and it was used to account for the follow-up relationship between MMSE scores with the independent variables.

3. Results

3.1. Study characteristics

Table 1 presents demographic and clinical characteristics of the total study sample, stratified according to syndrome diagnosis at baseline. The total study sample had a mean age of 66 years and 34.3% female participants. People with SCD were younger compared to MCI and AD ($F = 15.69$, df = 2, $p < 0.001$). People with SCD had a higher education compared to the MCI and AD dementia study groups ($X^2 = 6.07$, df = 2, $p < 0.05$). No significant differences for alcohol use and smoking was observed between the study groups ($X^2 = 4.35$, df = 2, $p = 0.11$). The MCI and AD dementia groups had higher proportions of people with diabetes, compared to the SCD group ($X^2 = 6.11$, df = 2, $p < 0.05$). Cerebrovascular disease, cardiovascular disease and kidney disease were equally distributed throughout the study groups. A total of 59 SCD participants had 2-year follow-up measures of which, 10 participants converted to MCI and 2 participants converted to AD. We explored the demographics of only the participants that had measures of CSF A$\beta_{1\text{-}42}$, total *t*-Tau and *p*-Tau, to determine potential selection bias (Supplementary Table 1). Differences in demographical and clinical characteristics between the study groups remained similar compared to that reported in Table 1, except for

Table 1
Study demographic and clinical characteristics

	Total (n = 268)	SCD (n = 82)	MCI (n = 98)	AD (n = 88)	Group differences (*p*-value)
Age years, mean (SD)	66.18 (9.56)	61.51 (9.14)	68.51 (8.84)	67.92 (9.27)	<0.001
Sex, female, N (%)	92 (34.3 %)	29 (35.4 %)	29 (29.6 %)	34 (38.6 %)	0.419
Education level, N (%)					0.048
- *Low*	66 (24.9 %)	12 (15.0 %)	29 (29.3 %)	25 (28.4 %)	
- *Middle*	93 (35.1 %)	31 (38.8 %)	30 (30.9 %)	32 (36.4 %)	
- *High*	106 (40.0 %)	37 (46.3 %)	38 (39.2 %)	31 (35.2 %)	
Smoking					0.113
- *Never*	108 (42.4 %)	27 (35.5 %)	38 (40.0 %)	43 (51.2 %)	
- *Former*	110 (43.1 %)	37 (48.7 %)	42 (44.2 %)	31 (36.9 %)	
- *Current*	37 (14.5 %)	12 (15.8 %)	15 (15.8 %)	10 (11.9 %)	
Alcohol consumption, N (%)	200 (75.5 %)	68 (84.0 %)	71 (73.2 %)	61 (70.1 %)	0.092
Cardiovascular disease, N (%)	89 (33.2 %)	29 (35.4 %)	34 (34.7 %)	26 (29.5 %)	0.432
Cerebrovascular disease, N (%)	35 (13.4 %)	14 (17.1 %)	12 (12.9 %)	9 (10.3 %)	0.238
Kidney disease, N (%)	7 (2.6 %)	0 (0.0 %)	3 (3.1 %)	4 (4.5 %)	0.203
Diabetes status, N (%)	29 (10.8 %)	4 (4.9%)	16 (16.3 %)	9 (10.2 %)	0.047
MMSE score (baseline), mean (SD)	26.27 (2.89)	28.23 (1.82)	26.67 (2.36)	23.45 (2.75)	<0.001
MMSE score (follow-up), mean (SD)	25.18 (4.83)	28.52 (1.66)	25.98 (3.38)	20.02 (5.21)	<0.001
APOE ε4 carrier, N (%)	103 (54.8 %)	25 (43.1 %)	43 (60.6 %)	35 (59.3 %)	0.098
CSF Amyloid-$\beta_{1\text{-}42}$ (ng/mL), median (IRQ)	604.00 (461.00-833.25)	801.00 (608.00-1014.00)	595.00 (463.00-869.00)	509.00 (419.50-604.00)	<0.001
CSF p-Tau (ng/mL), median (IRQ)	51.00 (35.00-71.00)	35.00 (22.00-52.25)	52.00 (38.50-71.00)	66.50 (46.50-87.75)	<0.001
CSF t-Tau (ng/mL), median (IRQ)	375.50 (223.50-594.50)	225.00 (158.00-357.00)	420.00 (242.00-599.00)	533.00 (356.00-839.25)	<0.001

Key: AD, Alzheimer's disease; APOE, apolipoprotein E; CSF, cerebrospinal fluid; IRQ, interquartile range; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; p-Tau, phosphorylated tau; SCD, subjective cognitive decline; SD, standard deviation; t-Tau, total tau.

Table 2
Logistic regression analysis of baseline serum and CSF NGAL levels with the conversion of MCI to AD after 2 years follow-up.

			Unadjusted		Adjusted	
	Total	Converters/non-converters	Odds ratio (95% CI)	*p*-value	Odds ratio (95% CI)	*p*-value
Serum NGAL	63	22/41	1.16 (0.63 – 2.12)	0.638	1.18 (0.62 – 2.24)	0.622
*CSF NGAL	61	20/41	2.01 (1.01 – 3.64)	**0.017**	2.27 (1.15 – 4.46)	**0.014**
*CSF A$\beta_{1\text{-}42}$	50	15/35	3.41 (1.41 – 8.25)	**0.007**	4.71 (1.55 – 14.34)	**0.006**
CSF t-Tau	50	15/35	2.78 (1.13 – 6.87)	**0.027**	3.00 (1.15 – 7.81)	**0.024**
CSF p-Tau	50	15/35	2.74 (1.13 – 6.60)	**0.025**	2.79 (1.13 – 6.86)	**0.026**

All values in bold indicate significant findings.
Odds ratios of Z scores are reported for comparison of effect sizes between biomarkers.
Key: A$\beta_{1\text{-}42}$, amyloid beta 1-42; CI, confidence interval; CSF, cerebrospinal fluid; NGAL, neutrophil gelatinase-associated lipocalin; p-Tau, Tau phosphorylated at threonine 181; t-Tau, total Tau.
* Values were inverted so that hazard ratios are comparable.

the non-significance of education level and marginal significance of diabetes status between the groups. Because the group differences in education were less pronounced in the sub-sample of participants with these available CSF markers, a possible selection bias is unlikely to affect the interpretation of the identified associations found in the different study groups.

3.2. Serum and CSF NGAL levels in baseline syndrome diagnosis

No significant differences in serum NGAL levels between the syndromes at baseline were found (ANOVA, F = 1.42, df = 2, $p = 0.24$) (Fig. 1 A). Significant differences for CSF NGAL were found between the baseline syndromes (ANOVA, F = 3.24, df = 2, $p = 0.04$) (Fig. 1B). Further p*ost-hoc* testing showed that CSF NGAL levels were decreased in people with MCI at baseline compared to people with SCD ($p = 0.03$). However, no significant differences were found between MCI and AD dementia ($p = 0.45$) or AD dementia compared to SCD ($p = 0.38$). CSF NGAL levels remained significantly decreased (ANCOVA, F = 4.57, df = 2, $p = 0.01$) in people with MCI compared to SCD ($p = 0.008$) after adjusting for age and sex as covariates with ANCOVA and *post-hoc* analyses. Due to age differences between the study groups, a sensitivity analysis was performed for CSF NGAL levels by only including participants age between 60 – 80 years, which provided non-significant age differences between the groups (Supplementary Table 2). In this sensitivity analysis we found that CSF NGAL levels remained significantly decreased (ANCOVA, F = 3.58, df = 2, $p = 0.03$) in people with MCI compared to SCD ($p = 0.048$). However, it should be noted that the effect size weakened compared to the analysis with all ages included.

3.3. Association of baseline serum and CSF NGAL levels with MCI converters at 2-year follow-up

The associations between the progression from MCI to AD dementia after 2 years follow-up and baseline ln-transformed biomarkers are presented in Table 2. After adjusting for age and sex in the multivariate logistic regression analysis the progression from MCI to AD dementia was associated with lower CSF NGAL (odds ratio (OR), 2.27; 95% CI; 1.15 – 4.46; $p = 0.01$) and lower CSF A$\beta_{1\text{-}42}$ levels (OR, 4.71; 95% CI; 1.55 – 14.34; $p = 0.006$). Higher levels of CSF *t*-Tau (OR, 3.00; 95% CI; 1.15 – 7.81; $p = 0.02$) and CSF *p*-Tau (OR, 2.79; 95% CI; 1.13 – 6.86; $p = 0.03$) levels were associated with the conversion to AD dementia over 2 years follow-up. The Hosmer-Lemeshow test proved adequate model performance (all $p > 0.05$) for univariate and multivariate logistic regression analyses. Demographics of MCI participants that completed follow-up measures versus participants without follow-up measures was

Table 3
Associations of baseline serum and CSF NGAL levels with CSF biomarkers for AD.

	Total				SCD				MCI				AD			
Serum NGAL	n	B (95 % CI)	β	p	n	B (95 % CI)	β	p	n	B (95 % CI)	β	p	n	B (95 % CI)	β	p
Unadjusted																
CSF Aβ_{1-42}	186	-0.12 (-0.23 – 0.01)	-0.16	**0.026**	58	-0.20 (-0.41 – 0.01)	-0.24	0.063	68	-0.02 (-0.20 – 0.16)	-0.03	0.820	59	-0.20 (-0.44 – 0.04)	-0.21	0.105
CSF t-Tau	185	0.00 (-0.07 – 0.07)	0.00	0.990	58	0.00 (-0.14 – 0.14)	0.00	0.984	68	-0.02 (-0.10 – 0.14)	0.04	0.726	59	-0.05 (-0.21 – 0.12)	-0.08	0.555
CSF *p*-Tau	184	-0.00 (-0.08 – 0.08)	-0.00	0.984	57	0.017 (-0.15 – 0.18)	0.03	0.835	68	0.01 (-0.13 – 0.15)	0.02	0.903	59	-0.06 (-0.25 – 0.13)	-0.08	0.546
Adjusted																
CSF Aβ_{1-42}	186	-0.09 (-0.19 – 0.02)	-0.11	0.126	58	-0.30 (-0.52 – -0.08)	-0.36	**0.009**	68	0.01 (-0.16 – 0.18)	0.01	0.904	59	-0.12 (-0.35 – 0.11)	-0.13	0.301
CSF t-Tau	185	-0.05 (-0.12 – 0.02)	-0.10	0.174	58	-0.02 (-0.18 – 0.14)	-0.03	0.841	68	-0.03 (-0.16 – 0.09)	-0.07	0.589	59	-0.07 (-0.22 – 0.08)	-0.11	0.344
CSF *p*-Tau	184	-0.05 (-0.13 – 0.04)	-0.08	0.290	57	0.01 (-0.17 – 0.18)	0.01	0.923	68	-0.03 (-0.17 – 0.11)	-0.05	0.677	59	-0.07 (-0.24 – 0.10)	-0.10	0.419
CSF NGAL	n	B (95 % CI)	β	p	n	B (95 % CI)	β	p	n	B (95 % CI)	β	p	n	B (95 % CI)	β	p
Unadjusted																
CSF Aβ_{1-42}	184	0.11 (-0.04 – 0.25)	0.11	0.149	57	-0.19 (-0.51 - 0.13)	-0.16	0.235	68	0.28 (0.05 – 0.51)	0.28	**0.020**	58	0.16 (-0.11 – 0.43)	0.25	0.245
CSF t-Tau	185	-0.10 (-0.19 – 0.01)	-0.16	**0.031**	57	-0.20 (-0.40 - -0.01)	-0.26	**0.047**	68	-0.08 (-0.24 – 0.08)	-0.125	0.30	58	-0.05 (-0.24 -0.13)	-0.08	0.555
CSF *p*-Tau	184	-0.12 (-0.23 - -0.01)	-0.16	**0.032**	57	-0.259 (-0.49 – 0.03)	-0.29	**0.030**	68	-0.15 (-0.34 –0.04)	-0.188	0.12	58	0.02 (-0.19 – 0.24)	0.03	0.819
Adjusted																
CSF Aβ_{1-42}	184	0.13 (-0.02 – 0.28)	0.13	0.098	57	-0.26 (-0.63 - 0.11)	-0.20	0.169	68	0.30 (0.08 – 0.53)	0.31	**0.010**	58	0.23 (-0.04 – 0.50)	0.22	0.089
CSF t-Tau	185	-0.14 (-0.23 – 0.04)	-0.21	**0.006**	57	-0.28 (-0.51 - -0.05)	-0.35	**0.018**	68	-0.12 (-0.29 – 0.05)	-0.18	0.175	58	-0.07 (-0.25 -0.11)	-0.10	0.441
CSF *p*-Tau	184	-0.15 (-0.26 - -0.03)	-0.19	**0.011**	57	-0.33 (-0.59 – 0.08)	-0.35	**0.012**	68	-0.17 (-0.36 – 0.03)	-0.21	0.094	58	0.02 (-0.19 – 0.22)	0.02	0.887

All values in bold indicate significant findings.
Analysis was performed with ln-transformed values.
Key: Aβ1-42, amyloid beta 1-42; AD, Alzheimer's disease; CI, confidence interval; CSF, cerebrospinal fluid; MCI, mild cognitive impairment; NGAL, neutrophil gelatinase-associated lipocalin; p-Tau, Tau phosphorylated at threonine 181; SCD, subjective cognitive impairment; t-Tau, total Tau.

Table 4
Associations of baseline ln-transformed biomarkers with the change in MMSE scores over 2 years adjusted for age and sex

	Total				SCD				MCI				AD			
	n	Coefficient	95 % CI	*p*	n	Coefficient	95 % CI	*p*	N	Coefficient	95 % CI	*p*	n	Coefficient	95 % CI	*p*
Unadjusted																
Serum NGAL	136	0.63	-0.50 – 1.75	0.276	40	0.52	-0.47 – 1.51	0.304	61	-1.30	-2.66 – 0.55	0.060	35	1.18	-1.08 – 3.43	0.303
CSF NGAL	141	1.06	0.25 – 1.87	**0.010**	40	-0.11	-0.79 – 0.58	0.758	64	1.14	0.00 – 2.27	**0.049**	37	0.82	-0.67 – 2.31	0.274
CSF A$\beta_{1\text{-}42}$	114	2.82	1.98 – 3.66	**<0.001**	36	0.85	-0.06 – 1.76	0.067	50	0.21	-1.04 – 1.46	0.738	28	2.11	0.31 – 3.90	**0.022**
CSF t-Tau	114	-2.14	-2.68 - -1.59	**<0.001**	36	-0.02	-0.58 – 0.54	0.950	50	-1.07	-1.99 - -0.16	**0.021**	28	-1.72	-2.99 - -0.44	**0.009**
CSF *p*-Tau	114	-2.44	-3.07 - -1.81	**<0.001**	36	-0.10	-0.75 – 0.55	0.768	50	-1.40	-2.44 - -0.35	**0.010**	28	-1.20	-2.76 – 0.35	0.128
Adjusted																
Serum NGAL	136	0.53	-0.57 – 1.62	0.344	40	0.38	-0.61 – 1.37	0.455	61	-1.05	-2.44 – 0.34	0.137	35	-0.16	-2.42 – 2.10	0.888
CSF NGAL	141	1.26	0.45 – 2.07	**0.002**	40	0.06	-0.63 – 0.75	0.868	64	1.41	0.26 – 2.55	**0.016**	37	1.02	-0.52 – 2.55	0.191
CSF A$\beta_{1\text{-}42}$	114	2.39	1.54 – 3.25	**<0.001**	36	0.88	-0.09 – 1.84	0.074	50	-0.15	-1.25 – 1.22	0.981	28	1.67	-0.15 – 3.49	0.072
CSF t-Tau	114	-1.90	-2.49 - -1.31	**<0.001**	36	0.35	-0.32 – 1.01	0.299	50	-0.75	-1.73 – 0.23	0.134	28	-1.48	-2.73 - -0.23	**0.021**
CSF *p*-Tau	114	-2.00	-2.67 – 1.32	**<0.001**	36	0.18	-0.54 – 0.89	0.621	50	-1.14	-2.21 - -0.07	**0.037**	28	-1.07	-2.56 – 0.43	0.159

All values in bold indicate significant findings.
Key: Aβ1-42, amyloid beta 1-42; AD, Alzheimer's disease; CSF, cerebrospinal fluid; CI, confidence interval; MCI, mild cognitive impairment; n, number; NGAL, neutrophil gelatinase-associated lipocalin; p-Tau, Tau phosphorylated at threonine 181; SCD, subjective cognitive decline; t-Tau, total Tau.

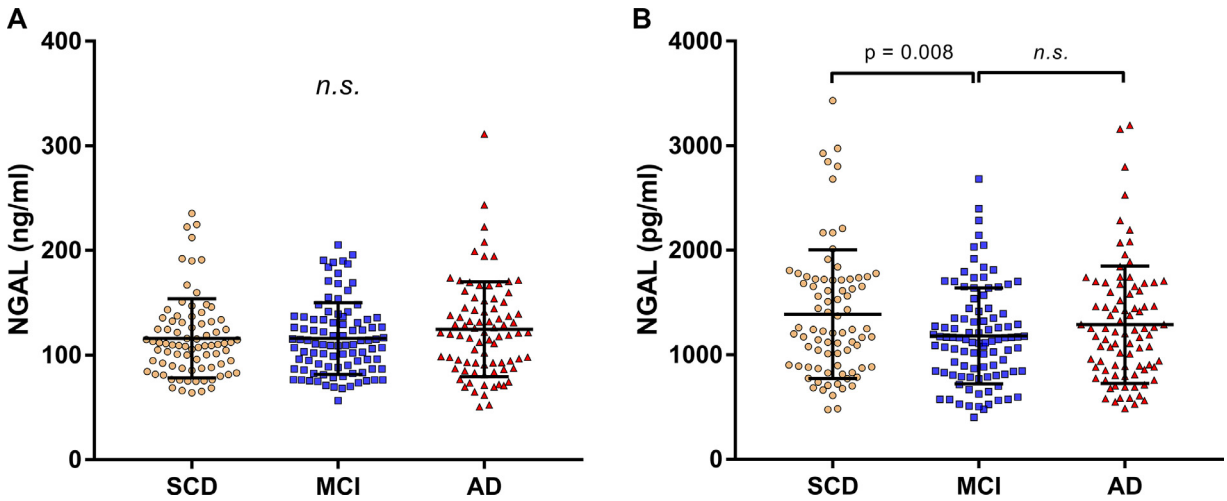

Fig. 1. Differences in (A) serum NGAL and (B) CSF NGAL between diagnostic groups at baseline, adjusted for age and sex. Bars indicate the median protein concentrations in the different study groups and are expressed as mean and standard deviation of the mean. Abbreviations: AD, Alzheimer's disease; CSF, cerebrospinal fluid; MCI, mild cognitive impairment; NGAL, neutrophil gelatinase-associated lipocalin; n.s., non-significant; SCD, subjective cognitive decline. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article

also explored for potential bias. Differences in the study demographics between MCI participants with follow-up versus without follow-up measures were non-significant, except that a higher proportion of the MCI participants with follow-up measures were APOE ε4 carriers (Supplementary Table 3).

3.4. Associations of baseline serum and CSF NGAL with biomarkers of AD

Higher serum NGAL levels were significantly associated with lower CSF A$\beta_{1\text{-}42}$ in the whole study group (B = -0.12, 95% CI = -0.23 – 0.01, Beta = -0.16, p = 0.03), however significance was lost after controlling for age and sex (B = -0.09, 95% CI = -0.19 – 0.02, Beta = -0.11, p = 0.13). Elevated serum NGAL was inversely correlated with CSF A$\beta_{1\text{-}42}$ in the SCD group after correcting for covariates (B = -0.03, 95% CI = -0.52 - -0.08, Beta = -0.36, p = 0.009) (Table 3). Lower CSF NGAL levels were associated with higher t-Tau (B = -0.14, 95% CI = -0.23 – 0.04, Beta = -0.21, p = 0.01) and p-Tau (B = -0.15, 95% CI = -0.26 – 0.03, Beta = -0.19, p = 0.01) in the whole study sample and with higher t-Tau (B = -0.28, 95% CI = -0.51 – -0.05, Beta = -0.35, p < 0.02) and p-Tau (B = -0.33, 95% CI = -0.59 – 0.08, Beta = -0.35, p = 0.01) in people with SCD after controlling for age and sex. Lower CSF NGAL levels were significantly associated with CSF A$\beta_{1\text{-}42}$ in participants with MCI (B = 0.30, 95% CI = 0.08 – 0.53, Beta = 0.31, p = 0.01), adjusted for age and sex.

The effect of APOE ε4 carriers on serum and CSF NGAL levels was also explored in the whole study sample and stratified according to baseline syndrome diagnosis (Supplementary Table 4). No significant differences were found for either serum or CSF NGAL levels with the presence of the APOE ε4 allele in the whole study sample and the different groups at baseline.

3.5. Serum and CSF NGAL levels with changes in MMSE scores between baseline and 2-year follow-up

Table 4 demonstrates the results of linear mixed models that were used to assess the associations between ln-transformed biomarkers (serum NGAL, CSF NGAL, A$\beta_{1\text{-}42}$, t-Tau and p-Tau) and the decline in MMSE scores, in unadjusted and adjusted models for age, sex and time. In the adjusted analyses, lower baseline CSF NGAL levels were associated with a decline in MMSE in the whole study sample (coefficient = 1.26, 95% CI = -0.63 – 0.75, p = 0.002) and in people with MCI (coefficient = 1.41, 95% CI = 0.26 – 2.55, p = 0.016). No significant associations between baseline serum NGAL levels and changes in MMSE scores over 2 years were found in the total study group or in the separate study groups (SCD, MCI and AD).

To compare our results with existing studies we have performed cross-sectional analyses for baseline ln-transformed biomarkers with MMSE scores at baseline (Supplementary Table 5). No significant associations for serum and CSF NGAL levels with MMSE scores at baseline were found in the whole study sample or in the separate study groups.

4. Discussion

Results from this longitudinal multicentre academic memory clinic population show that (1) CSF NGAL levels were decreased in people with MCI at baseline compared to people with SCD, (2) lower baseline CSF NGAL levels in MCI participants were associated with the progression to AD dementia after 2-years follow-up, (3) CSF NGAL levels were positively correlated with markers of Aβ neuropathology in study participants with MCI and (4) lower CSF NGAL levels in MCI study participants were associated with a decline in MMSE performance after 2-year follow-up.

To our best knowledge, this is the first follow-up study to evaluate NGAL levels as a predictor for the conversion of MCI to AD dementia. Our results show that lower baseline CSF NGAL levels significantly predicted MCI converters to clinically diagnosed AD dementia after 2-year follow-up. In relation to the reference CSF biomarkers for AD dementia, it was observed that lower CSF NGAL levels corresponded with lower A$\beta_{1\text{-}42}$ levels and an inverse relationship with t-Tau and p-Tau in the association for the conversion of MCI to AD dementia. NGAL's lower CSF levels in MCI is of interest since it contrasts with higher CSF levels found for the majority of other neuroinflammatory-associated markers, for example YKL-40 (also known as chitinase-3-like protein) and soluble triggering receptor expressed on myeloid cells2 (sTREM2) as recently reported in a meta analyses (Shen et al., 2019). Moreover, increased CSF NGAL levels was reported to discriminate vascular dementia from AD dementia with high accuracy (Llorens et al., 2020), suggesting that NGAL levels may reflect different underlying neuropathologies of neurodegenerative diseases. Given the po-

tential effects of various factors (e.g. depression, age, medication use and comorbid diseases) on NGAL production, it may be limited as a diagnostic biomarker as compared to the classically used CSF biomarkers of AD; A$\beta_{1\text{-}42}$, total *t*-Tau and *p*-Tau. Nevertheless, the results from this study indicate that CSF NGAL levels may function as a potential neuroinflammatory biomarker of neuropathological processes in prodromal AD.

The lower CSF NGAL levels found in the MCI study participants at baseline as compared to the SCD participants, support previous findings from an explorative study by our group (Naudé et al., 2012), in which CSF NGAL levels were significantly decreased in MCI participants compared to healthy cognitive controls. However, no significant differences in serum NGAL levels between baseline syndromes was found. Existing evidence on peripheral blood NGAL levels in prodromal stages of AD dementia remains inconclusive. A study by Choi et al. (2011) was the first to show that plasma NGAL levels were increased in MCI patients (n = 41) compared to AD dementia (n = 62) and healthy control participants (n = 38) (Choi et al., 2011). A more recent study concurrently showed that plasma NGAL levels were increased in healthy community-dwelling volunteers without cognitive impairment that were characterized as pre-clinical AD dementia (n = 38) based on CSF A$\beta_{1\text{-}42}$, *t*-Tau and *p*-Tau levels as compared to volunteers whom did not fulfil this biomarker criteria (n = 118) (Eruysal et al., 2019). However, no significant differences in serum NGAL levels between control (n = 26), MCI (n = 28) and AD dementia (n = 28) participants was found in an exploratory study by our group (Naudé et al., 2012). The discrepancies between these findings may be due to the relatively small numbers of participants in each of the studies with limited statistical power. Furthermore, differences in study designs may also be a limiting factor to directly compare the outcomes for peripheral circulating NGAL levels. For example, the studies by Eruysal et al., (Eruysal et al., 2019) and Naudé et al., (Naudé et al., 2012) included participants without comorbid diseases that may pose a potential risk for AD dementia. The study by Choi et al., (Choi et al., 2011) excluded participants with comorbidities that may produce dementia symptoms, but included a general score for comorbidity for other somatic diseases. In the present study, comorbid diseases were not controlled for in the statistical analyses due to the limited number of study participants. It should also be noted that the studies by Choi et al., and Eruysal et al., (Choi et al., 2011; Eruysal et al., 2019) measured NGAL in blood plasma, whereas serum samples were used in our previous work (Naudé et al., 2012) and in the present study. It was shown that NGAL concentrations varied between paired serum and plasma samples (Itenov et al., 2014), which may further contribute to the inconsistent outcomes reported between these studies. Future work is needed to determine whether serum or plasma should be used as the preferred method for NGAL measurements. Finally, the studies by Choi et al., and Eruysal et al., (Choi et al., 2011; Eruysal et al., 2019) measured NGAL in plasma samples from fasted participants, whereas non-fasted collections of blood was used in this study. NGAL expression and its circulating levels are influenced by metabolic status, insulin levels and food intake (Petropoulou et al., 2020; Tan et al., 2009; Zhang et al., 2014), which may have mitigated the group differences of serum NGAL levels in this study.

In accordance with the study of Eruysal et al., (Eruysal et al., 2019) that showed a positive association of increased plasma NGAL with CSF A$\beta_{1\text{-}42}$ levels in control and pre-clinical AD cognitively normal community dwelling participants, we found that increased serum NGAL levels were correlated with decreased CSF A$\beta_{1\text{-}42}$ levels in the SCD group. Moreover, the significant associations of lower CSF NGAL levels with lower CSF A$\beta_{1\text{-}42}$ levels in people with MCI indicate that lower CSF NGAL levels may reflect AD-associated neuropathological processes during the prodromal stages of AD dementia. In this respect, findings from preclinical research have shown that NGAL production and transport may be closely related to that of A$\beta_{1\text{-}42}$. Megalin (low density lipoprotein-related protein 2 (LRP2)), one of the known receptors of NGAL, functions as its transporter across cell membranes (Hvidberg et al., 2005) and is also a transporter for A$\beta_{1\text{-}42}$ (Hammad et al., 1997). Because megalin is decreased on the choroid plexus in the human brain with AD neuropathology (Pascale et al., 2011), it can contribute to the decreased transport of NGAL and A$\beta_{1\text{-}42}$ from the brain into the CSF and contribute to their decreased CSF levels. The lack of significance between CSF A$\beta_{1\text{-}42}$ and NGAL levels in the AD group, together with the marginally higher CSF NGAL levels in the AD group compared to the MCI group shown in this study may be attributed by an altered secretory activity of a damaged choroid plexus in the advanced stages of the disease (Balusu et al., 2016).

NGAL may play a contributing function in the pathophysiology of AD. Increased NGAL levels were found in the human AD brain with a regional distribution that reflects that of Aβ accumulation (Dekens et al., 2017; Naudé et al., 2012). In this regard, A$\beta_{1\text{-}42}$ may directly contribute to NGAL production in the brain. Studies with primary choroid plexus and astrocyte cell cultures from mice and rats showed that A$\beta_{1\text{-}42}$ stimulated the production of NGAL proteins (Dekens et al., 2020; Mesquita et al., 2014). Furthermore, in a study with mice it was shown that an intracerebroventricular injection of A$\beta_{1\text{-}42}$ led to increased expression of NGAL protein levels in the hippocampus and choroid plexus (Steeland et al., 2018). NGAL can also exacerbate A$\beta_{1\text{-}42}$ induced toxic effects in primary neuron cell cultures (Naudé et al., 2012) and astrocytes (Mesquita et al., 2014). However, a systemic absence of NGAL in knockout mice that were cross-bred with an Aβ mouse model for AD did not exhibit differences in memory functioning, amyloid beta plaque load or glia activation as compared to AD mice with NGAL (Dekens et al., 2018). Of note, extrapolation of results from mice to the humans should be interpreted with caution because mouse NGAL share 62% amino acid sequence homology with human NGAL (Kjeldsen et al., 2000), which may result in differences of their biological functions. The associations found between lower CSF NGAL with higher levels of *t*-Tau and *p*-Tau in the whole study sample and the in the SCD participants is intriguing since their biological interrelations is still largely unknown. A recent study with a murine model of non-alcoholic steatohepatitis showed that increased upregulation of NGAL protein levels significantly correlated with *p*-Tau (phosphorylated at serine 396) in the cerebral cortex (Mondal et al., 2020). The mechanistic functions of NGAL in the biological functions of Tau and *vice versa*, is an interesting yet unexplored pathway for further investigations.

Lower baseline CSF NGAL levels predicted a decline in MMSE scores over the 2-year follow-up in the whole study sample and in people with MCI. The cross-sectional analyses showed no significant associations between baseline serum and CSF NGAL levels with baseline MMSE scores. Similarly, a cross-sectional study found no significant associations of plasma NGAL with MMSE in pre-clinical stages of AD dementia (Eruysal et al., 2019), whereas another study found a positive correlation in MCI and AD dementia patients (Choi et al., 2011). Based on our results we speculate that earlier CSF NGAL levels may indicate the progress of impaired cognitive performance at a later stage, considering that CSF NGAL levels are associated with early-stage AD-associated pathophysiological processes.

NGAL has excellent storage stability (Han et al., 2009; Pedersen et al., 2010) and is resistant to proteolytic degradation (Kjeldsen et al., 1993). Moreover, the diurnal variations of NGAL levels in CSF (Naudé et al., 2017) and in the circulation (Eidson et al., 2017; Naudé et al., 2017) remain at stable concentrations during the day in older people. Thus, the nominal ef-

fects of the circadian rhythm on NGAL levels during the daytime and its stable biochemical properties makes it a useful marker for biomarker investigations.

Limitations of this study should be considered for the interpretation of the results. First, this study included study participants with subjective cognitive impairments as a control group. Because these participants are at risk for the development MCI and AD, it may have a diminishing effect on the cross-sectional comparisons of NGAL levels between the study groups at baseline. Second, missing data at 2-year follow-up may increase the risk of selection bias. Third, due to missing values for APOE ε4 carrier, CSF A$\beta_{1\text{-}42}$, *t*-Tau and *p*-Tau, we did not characterize pre-clinical dementia based on CSF A$\beta_{1\text{-}42}$, *t*-Tau and *p*-Tau values. Fourth, other unidentified co-existing neuropathologies cannot be ruled out, which may affect serum and CSF NGAL levels. Fifth, some of the participants in MCI non-converters group may have converted to AD after the 2-year follow-up, which possibly led to a reduced effect size in our outcomes. Sixth, the strength of the effect size for the comparison of CSF NGAL levels between the study groups may be overemphasized by the age differences between the study groups. Future studies should take age into consideration when investigating NGAL as a biomarker.

5. Conclusions

CSF NGAL levels may reflect early neuropathological processes during the stages of prodromal AD. Therefore, NGAL may be a prognostic neuroinflammatory biomarker candidate for studies aiming to investigate treatment outcomes of therapies that target Aβ and Tau for the prevention of AD. The associations of peripheral circulating NGAL with prodromal AD remain inconclusive. Further research with multi-cohort studies is required to validate the potential of CSF NGAL levels as biomarker for prodromal AD.

Disclosure statement

The authors have no actual or potential conflicts of interest.

Submission declaration and verification

This report has not been published or accepted for publication elsewhere, nor is it under editorial review by any other journal. The authors of this article are responsible for all contents of the article and had authority over manuscript preparation and are fully aware of this submission. All listed authors have approved of the submission of this manuscript to the Journal, Neurobiology of Aging. This manuscript does not have any relation to any other published, submitted or proposed papers reporting the same or overlapping data. All of the individuals listed have contributed sufficiently to the project to be included as authors, and all those who are qualified to be authors are listed in the author byline. The authors hereby further verify that if accepted, the content of this manuscript will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2021.07.001.

CRediT authorship contribution statement

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