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Serum NGAL is Associated with Distinct Plasma Amyloid-β Peptides According to the Clinical Diagnosis of Dementia in Down Syndrome

Petrus J.W. Naudé[a,b,*,1], Alain D. Dekker[a,c,1], Antonia M.W. Coppus[d,e,f], Yannick Vermeiren[c], Ulrich L.M. Eisel[b,g], Cornelia M. van Duijn[e], Debby Van Dam[c] and Peter P. De Deyn[a,c]
[a]*Department of Neurology and Alzheimer Research Center, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands*
[b]*Department of Molecular Neurobiology, University of Groningen, Groningen, The Netherlands*
[c]*Laboratory of Neurochemistry and Behaviour, Institute Born-Bunge, University of Antwerp, Wilrijk, Antwerp, Belgium*
[d]*Dichterbij, Center for the Intellectually Disabled, Gennep, The Netherlands*
[e]*Department of Epidemiology, Erasmus University Medical Center, Rotterdam, The Netherlands*
[f]*Department of Primary and Community Care (152 ELG), Radboud University Medical Center, Nijmegen, The Netherlands*
[g]*University Center of Psychiatry & Interdisciplinary Center of Psychopathology of Emotion regulation, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands*

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Abstract.
Background: The majority of people with Down syndrome (DS) develop dementia due to Alzheimer's disease (AD). Neuropathological features are characterized by an accumulation of amyloid-β (Aβ) deposits and the presence of an activated immune response. Neutrophil Gelatinase-Associated Lipocalin (NGAL) is a newly identified (neuro)inflammatory constituent in AD.
Objective: This study examines NGAL as an inflammatory marker in DS and its associations with plasma Aβ peptides according to the follow-up clinical diagnosis of dementia.
Methods: Baseline serum NGAL and plasma $A\beta_{40}$, $A\beta_{42}$, $A\beta_{n40}$, and $A\beta_{n42}$ were quantified in 204 people with DS. The diagnosis of dementia in DS was established by follow-up clinical assessments. The following study groups were characterized: DS with AD at baseline ($n=67$), DS without AD ($n=53$), and non-demented DS individuals that converted to AD ($n=84$). Serum NGAL was analyzed in 55 elderly non-DS, non-demented people.

[1] These authors contributed equally to this work.

*Correspondence to: Petrus J.W. Naudé, Department of Neurology and Alzheimer Research Center, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9700 RB Groningen, The Netherlands. Tel.: +31 50 363 2363; Fax: +31 50 363 2331; E-mail: p.j.w.naude@umcg.nl.

Results: Serum NGAL levels were significantly increased in DS subjects compared to non-DS people. Serum NGAL levels were not associated with clinical dementia symptoms in DS. However, NGAL was positively associated with $A\beta_{42}$ and $A\beta_{n42}$ in demented DS individuals and with $A\beta_{40}$ and $A\beta_{n40}$ in the non-demented DS group. NGAL was negatively associated with $A\beta_{42}/A\beta_{40}$ and $A\beta_{n42}/A\beta_{n40}$ ratios in converted DS subjects. These associations persisted for $A\beta_{n40}$, $A\beta_{42}/A\beta_{40}$, and $A\beta_{n42}/A\beta_{n40}$ after adjusting for demographics measures, apolipoprotein E $\varepsilon 4$ allele, platelets, and anti-inflammatory medication.
Conclusion: Serum NGAL levels are increased in DS and associated with distinct species of Aβ depending on the progression of dementia as diagnosed by baseline and follow-up clinical assessments.

Keywords: Alzheimer's disease, amyloid-β, apolipoprotein E, biomarker, down syndrome, inflammation, lipocalin 2, platelets

INTRODUCTION

The prevalence of Down syndrome (DS), or trisomy 21, is approximately 1 in 700–1200 live births [1, 2] and is the most common genetic incidence of intellectual disability in humans [3]. A vast majority of people with DS develop Alzheimer's disease (AD) pathology, which is mainly characterized by amyloid-β (Aβ) depositions in the brain [4]. A high prevalence of the clinical diagnosis of dementia (50–70%) in DS is respectively found in mid- to late life [5, 6]. This phenomenon is due to a triplication of the human chromosome 21 (HSA21) that harbors several genes, i.e., amyloid-β protein precursor (AβPP) and β-site AβPP cleaving enzyme 2, that are responsible for the increased production of Aβ [4]. In addition to increased brain Aβ levels, individuals with DS have increased plasma Aβ levels compared to people without DS [7–9].

Inflammatory-associated genes on HSA21 are likely overexpressed in DS and have been suggested to contribute to an aberrant immune regulation that is characterized by a pro-inflammatory environment [10, 11]. Increased pro-inflammatory cytokines have been identified in brain tissue of people with DS [12] as well as in their circulation [13, 14], which might even be present during their early adolescence [15]. Furthermore, increased neuroinflammatory processes have been suggested to play an important role in the pathophysiological processes of DS and AD [10, 11]. This study focuses on Neutrophil Gelatinase-Associated Lipocalin (NGAL), a newly introduced inflammatory constituent in the pathophysiology of AD [16]. NGAL is a 25 kDa acute phase protein that is also known as Lipocalin-2, Siderocalin, 24p3, or Uterocalin [17]. Human studies showed that increased blood NGAL levels are associated with risk factors for AD: mild cognitive impairment [18], late-life depression [19], and elderly depressed females with impaired recall memory [20]. Serum NGAL is also increased in adult and elderly DS people compared to adult people without DS [21]. Primary neuronal cell cultures studies showed that NGAL mRNA and protein production is increased by $A\beta_{42}$ [22] and $A\beta_{40}$ [23]. Furthermore, NGAL impairs neuroprotective mechanisms in neurons and exacerbates $A\beta_{42}$-mediated neuronal cell death [16, 22]. These studies in essence indicate that NGAL is an important inflammatory marker that is involved in the pathophysiology of AD.

The aims of this study were: 1) to validate if NGAL levels are elevated in DS individuals compared to non-DS controls; 2) to determine whether baseline serum NGAL levels are associated with the clinical diagnosis of dementia in DS, i.e., DS subjects with established AD at baseline (demented), without AD (non-demented), and non-demented DS people that converted to dementia over time; and 3) to associate serum NGAL with plasma $A\beta_{40}$, $A\beta_{42}$, $A\beta_{42}/A\beta_{40}$, $A\beta_{n40}$, $A\beta_{n42}$, or $A\beta_{n42}/A\beta_{n40}$ in these groups.

MATERIALS AND METHODS

Study population

In total, 204 people with DS were included in this study. All participants were enrolled between 1 December 1999 and 1 December 2003 at an age of 45 years or older and are part of the previously published Rotterdam DS cohort [24–27]. Fasting venous blood samples were obtained in the morning, once at baseline of the study. Blood was directly processed and plasma and serum were stored at -80°C and -20°C, respectively. Ethical approval for this study was granted by the ethical review board of Erasmus MC Rotterdam (METc protocol number: MEC 185.974/1999/202). Written informed consent to participate and to provide blood samples was obtained from legal representatives (relatives and/or caretakers), after written information was provided. Written consent was also obtained from persons with DS who had the mental capacity to consent. To determine whether NGAL levels are increased in DS compared to healthy

non-DS persons, serum samples from 55 healthy non-DS persons were obtained from the Antwerp Biobank of the Institute Born-Bunge. These volunteers did not have any illness, clinical variables nor did they use any medication which may have interfered with NGAL levels. Ethics approval for human sample collection of serum was granted by the Medical Ethical Committee of the Middelheim General Hospital (Antwerp, Belgium) (Approval numbers 2805 and 2806). The study was also conducted in compliance with the Helsinki Declaration.

Clinical AD assessment

As previously described [24, 28], AD was assessed at baseline using the International Classification of Diseases (ICD)-10 from the World Health Organization [29], according to the guidelines of the Special Interest Research Group on Aging of the International Association for the Scientific Study of Intellectual Disabilities (IASSID) to diagnose dementia in adults with intellectual disabilities [29–31]. These criteria emphasize on non-cognitive symptoms, which are often prominent signs of dementia in adults with intellectual disabilities. Importantly, ICD-10 criteria have been modified for use in adults with intellectual disabilities. It has been shown that the AD criteria of the ICD-10 and the Diagnostic and Statistical Manual of Mental Disorders (Fourth Edition) diagnosed dementia in the same adults with DS [32] and that these diagnostic criteria show 'substantial reliability and satisfactory validity' in other intellectual disabilities as well [33].

In our study, study participants were systematically screened for dementia and examined in person by a clinician. The demented individuals met the ICD-10 criteria at intake and had an insidious and progressive course of the disease. In addition, validated functional questionnaires such as the Dementia Questionnaire for persons with an intellectual disability (DMR) [34], Social Competence Rating Scale for persons with an intellectual disability (SRZ) [35], and, Vineland adaptive behavior scales [36] were prospectively completed by family or caretakers every twelve months (continues until present if the person is still alive). Three diagnostic groups were defined based on the AD assessment (ICD-10) and annual follow-up (DMR, SRZ, and Vineland): demented at baseline ($n = 67$), converted ($n = 84$), and non-demented ($n = 53$) DS subjects. DS people that converted to AD, was clinically established before January 2007, thus within 3 to 7 follow-up years after intake and blood sampling. All of the DS participants in this study were assessed annually from baseline until January 2013 and were therefore followed for 10–14 years since baseline of this study. Body mass index (BMI) at baseline was computed as weight in kilograms divided by height in square meters.

Analyses of blood samples

Blinded analysis of serum NGAL [16], plasma $A\beta_{40}$, $A\beta_{42}$, and truncated $A\beta_{n40}$, and $A\beta_{n42}$ [26] and apolipoprotein E (ApoE) genotype [25] was performed as previously described.

Blood (20 ml) obtained via the antecubital vein was collected in tubes containing K_2-EDTA and immediately processed for platelet preparation. Platelet-rich plasma and blood cell fractions were separated by centrifugation. Platelet-rich plasma was removed and centrifuged again to obtain platelet pellets. Platelets were suspended in sucrose containing 5% dimethylsulfoxide to maintain membrane integrity and stored at –80°C until use.

Covariates

Age, gender, and BMI were included as covariates based on previous findings [19]. The presence of the ApoE $\varepsilon 4$ allele was included as covariate as well since it can affect serum inflammatory markers [37] and possibly plasma Aβ levels [38]. Furthermore, blood platelets were included as final confounding factor, since previous studies described them as an importance source of plasma $A\beta_{40}$ and $A\beta_{42}$ [39, 40]. Recently, in a large cohort with elderly participants we showed that increased NGAL levels were associated with the use of anti-inflammatory medication [19]. Therefore, the use of non-steroidal anti-inflammatory drugs (NSAIDs) was included as final covariate. Only three DS people used corticosteroids and they were therefore not included as covariate.

Statistical analyses

In order to obtain a normal distribution of the serum NGAL levels, four identified outliers were trimmed to 304.19 ng/ml resulting in a skewness of 0.65 and kurtosis of −0.25. As some covariates had missing data, we imputed the mean value of the other subjects in case of continuous variables or the most frequent score in case of dichotomous or nominal data. Variables with missing values in the whole sample were: BMI ($n = 5$), ApoE ($n = 4$), platelets ($n = 9$), $A\beta_{40}$ ($n = 11$), $A\beta_{42}$ ($n = 10$), $A\beta_{42}/A\beta_{40}$ ($n = 11$), $A\beta_{n40}$ ($n = 11$),

$A\beta_{n42}$ ($n = 22$), and $A\beta_{n42}/A\beta_{n40}$ ($n = 22$). Missing Aβ variables were due to insufficient plasma volumes for the analyses of Aβ peptides. First, for the description statistics of study participant demographics, analysis of variance (ANOVA) was performed for continuous variables (with a Tukey *post-hoc* test for pair-wise comparisons in case of an overall effect between the three groups, i.e., age), and Pearson's chi squared tests for categorical variables. ANOVA with Tukey *post hoc* test for pair-wise comparisons was used to determine differences of NGAL protein levels between non-DS controls, demented, converted, and non-demented DS people. This was followed by analyses of covariance (ANCOVA) with Bonferroni *post hoc* test with serum NGAL levels as dependent variable to analyze NGAL levels between the studied groups, adjusted for age and gender as confounding factors. First, ANCOVA was performed to determine the interaction of $A\beta_{40}$, $A\beta_{42}$, $A\beta_{42}/A\beta_{40}$, $A\beta_{n40}$, $A\beta_{n42}$, or $A\beta_{n42}/A\beta_{n40}$ with diagnostic status of dementia (non-demented, converted, and demented at baseline) with NGAL as the dependent variable. A p-value of less than 0.1 was considered as statistically significant for interaction terms [41]. Since an interaction effect was found, linear regression analyses were conducted separately within each DS study group, with NGAL as the dependent variable, to examine its associations with serum $A\beta_{40}$, $A\beta_{42}$, $A\beta_{42}/A\beta_{40}$, $A\beta_{n40}$, $A\beta_{n42}$, or $A\beta_{n42}/A\beta_{n40}$. Subsequently, multiple regression models were performed separately for the three different DS groups, with NGAL as dependent variable, to examine the associations of plasma $A\beta_{40}$, $A\beta_{42}$, $A\beta_{42}/A\beta_{40}$, $A\beta_{n40}$, $A\beta_{n42}$, or $A\beta_{n42}/A\beta_{n40}$ with serum NGAL concentrations adjusted for confounding variables. *P*-values for were considered statistically significant at a value of less than 0.05. All analyses were conducted with SPSS version 22.0.

RESULTS

Population demographics

Demographics and clinical information of non-DS controls and DS persons are shown in Table 1. Non-DS controls were older than DS people and DS subjects with dementia at baseline and people whom converted to dementia during follow-up were older than the non-demented DS group. No significant differences were observed for gender, BMI, ApoE ε4 allele, or platelet numbers between groups. Significant differences in NGAL levels were found between the non-DS people and the DS groups. While the presence of ApoE ε4 allele have been associated with increased blood pro-inflammatory cytokines in humans [37, 42], results from this study show that NGAL levels were not significantly associated with the presence of the ApoE ε4 allele (unpaired t-test, $t(198) = 0.416$; $p = 0.321$).

Serum NGAL levels in healthy non-DS volunteers compared to DS individuals

Differences in NGAL levels between the studied groups was further explored, since significant differences in NGAL levels between non-DS controls, demented, converted and non-demented DS groups (ANOVA, $F = 10.12$, df = 3, $p < 0.001$) were found. NGAL levels were significantly lower in non-DS individuals 114.35 (37.5) ng/ml compared to demented 162.5 (61.9) ng/ml ($p < 0.001$), converted 155.2 (53.6) ng/ml ($p < 0.001$), and non-demented DS 163 (63.7) ng/ml ($p < 0.001$) subjects (Fig. 1). Moreover, analysis with ANCOVA ($F(3, 253) = 8.69$, $p < 0.001$) and Bonferroni *post hoc* tests showed that serum NGAL levels were increased in demented

Table 1
Demographics and clinical info of study participants

Characteristics	Non-DS controls ($n = 55$)	Demented DS ($n = 67$)	Converted DS ($n = 84$)	Non-demented DS ($n = 53$)	Statistics for DS participants
Gender, female *n* (%)	25 (46)	26 (39)	33 (39)	21 (40)	$\chi^2 = 0.71$, df = 3, $p = 0.87$
Age (y), mean (SD)	75.5 (9.4)[a]	54.5 (5.9)[b]	53.1 (5.3)[c]	49.7 (4.3)	$F(3, 255) = 190.38$, $p < 0.001$
BMI, mean (SD)	–	25 (4.8)	25.7 (3.9)	25.4 (3.8)	$F(2, 198) = 0.41$, $p = 0.67$
ApoE ε4 allele, *n* (%)	–	22 (33.8)	21 (25.6)	14 (26.4)	$\chi^2 = 1.36$, df = 2, $p = 0.51$
Platelets, mean (SD)	–	232.1 (78.4)	224.7 (90.9)	232.1 (73.6)	$F(2, 192) = 0.19$, $p = 0.83$
NSAID, *n* (%)	–	11 (19)	10 (12.8)	3 (6.3)	$\chi^2 = 4.40$, df = 2, $p = 0.36$
NGAL, mean (SD)	114.4 (52.2)	162.5 (37.5)	155.2 (53.6)	163.8 (63.7)	$F(3, 255) = 10.12$, $p < 0.001$

[a]Non-DS controls versus demented at baseline, converted and non-demented $p < 0.001$, [b]demented versus non-demented $p < 0.001$, [c]AD converted versus non-demented $p = 0.013$. *n*, number; y, years; SD, standard deviation; BMI, body mass index; ApoE, Apolipoprotein E; NSAID, non-steroidal anti-inflammatory drugs; NGAL, neutrophil gelatinase-associated lipocalin; AD, Alzheimer's disease; DS, Down syndrome.

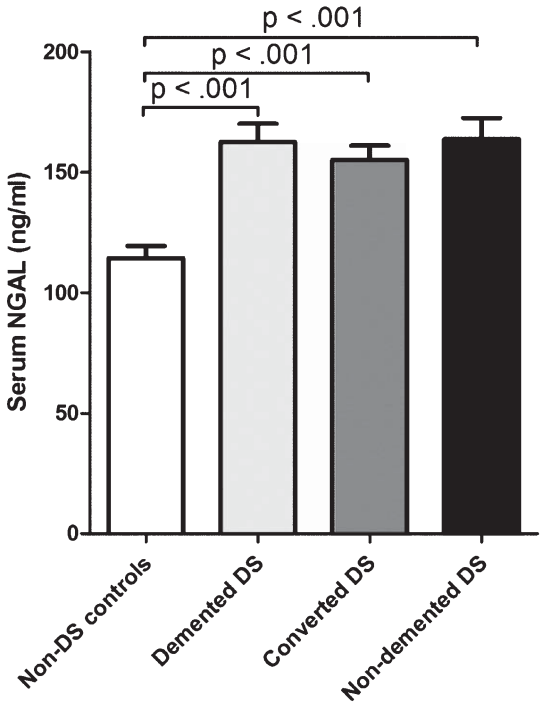

Fig. 1. Adjusted marginal mean values of serum NGAL levels in healthy non-DS controls versus demented DS at baseline, converted DS, and non-demented DS, including *p*-values of analysis of variance (ANOVA). Bars indicate the mean protein concentrations in the different study groups and are expressed ± standard error of the mean (s.e.m.).

converted and non-demented compared to non-DS controls ($p < 0.001$ in all groups) after including age and gender as covariates.

Association of NGAL with Aβ levels, characterized by diagnosis of dementia in DS

Firstly, ANCOVA analyses were performed to determine the interaction of $A\beta_{40}$, $A\beta_{42}$, $A\beta_{42}/A\beta_{40}$, $A\beta_{n40}$, $A\beta_{n42}$, or $A\beta_{n42}/A\beta_{n40}$ with the diagnostic status of dementia (demented, converted, and non-demented), with NGAL as dependent variable, to verify if associations of NGAL with Aβ should be performed separately in the DS groups based on dementia diagnosis. Outcomes from ANCOVA showed the following interactions; $A\beta_{40}$ ($p = 0.838$), $A\beta_{42}$ ($p = 0.050$), $A\beta_{42}/A\beta_{40}$ ($p = 0.435$), $A\beta_{n40}$ ($p = 0.053$), $A\beta_{n42}$ ($p = 0.007$), or $A\beta_{n42}/A\beta_{n40}$ ($p = 0.071$). Since the majority showed a significant interaction, as the *p*-value was considered significant less than 0.1, the presented results are stratified by dementia diagnosis. As shown in Table 2, higher serum NGAL levels were significantly associated with higher plasma $A\beta_{40}$ levels in the non-demented DS group, which remained significant after adjustments for confounding factors; age, gender, BMI, ApoE ε4, and platelets, but the significant association was lost after including NSAIDs as confounding factor. Linear regression analyses showed a significant association of higher NGAL levels with higher $A\beta_{42}$ levels. However, significance was lost after correcting for confounding factors. Higher NGAL levels were significantly associated with a lower $A\beta_{42}/A\beta_{40}$ ratio in converted DS people. This association lost significance after adjusting for age, gender, BMI, ApoE ε4, and platelets, however remained significant after including NSAIDs as covariant. Higher NGAL showed a strong association with higher $A\beta_{n40}$ levels in non-demented DS people independent of confounding factors. A significant association of higher NGAL levels with higher $A\beta_{n42}$ levels was found in the demented DS group, which remained significant after correcting for age, gender, BMI, ApoE ε4, and platelets. Inclusion of NSAIDs as covariant consequently resulted in a significant association of increased NGAL levels with increased $A\beta_{n42}$ levels in the demented and non-demented DS individuals and decreased $A\beta_{n42}$ levels in the converted DS people. Increased NGAL levels were significantly associated with a decreased $A\beta_{n42}/A\beta_{n40}$ ratio in converted DS subjects. This association remained marginally significant ($p = 0.055$) after correcting for age, gender, BMI, and ApoE ε4. Accordingly, the association remained significant after inclusion of platelet levels and NSAIDs.

DISCUSSION

The current study shows that serum NGAL levels were increased in elderly DS subjects compared to healthy, non-DS controls. Furthermore, serum NGAL levels were not associated with the clinical symptoms of dementia in DS. However, definite associations of NGAL levels with $A\beta_{40}$, $A\beta_{42}$, their truncated species, and their ratios depended on the follow-up clinical diagnosis of dementia. Therefore, these results support the notion that a pro-inflammatory environment is present in DS and that NGAL is an inflammatory marker that is significantly associated with distinct species of Aβ, moderated by the presence or absence of the clinically established dementia diagnosis over time.

Table 2
Association of serum NGAL levels with plasma amyloid-β species, including covariates, per diagnostic DS group

	$A\beta_{40}$			$A\beta_{42}$			$A\beta_{42}/A\beta_{40}$			$A\beta_{n40}$			$A\beta_{n42}$			$A\beta_{n42}/A\beta_{n40}$		
	B(SE)	β	p	B(SE)	β	p	B(SE)	β	p	B(SE)	β	p	B(SE)	β	p	B(SE)	β	p
Unadjusted																		
Demented	0.43 (0.23)	0.24	0.064	**3.25 (1.36)**	**0.30**	**0.021**	24.35 (135.82)	0.024	0.858	0.37 (0.21)	0.23	0.077	**4.66 (1.86)**	**0.32**	**0.015**	77.06 (146.64)	0.07	0.60
Converted	0.30 (0.16)	0.21	0.056	−1.09 (1.23)	−0.10	0.38	**−192.75 (96.68)**	**−0.22**	**0.05**	0.25 (0.18)	0.16	0.16	−2.33 (1.47)	−0.18	0.12	**−313.57 (135.90)**	**−0.26**	**0.024**
Non-demented	**0.44 (0.21)**	**0.28**	**0.042**	2.75 (1.66)	0.23	0.10	−103.80 (108.33)	−0.13	0.34	**1.04 (0.27)**	**0.48**	**<0.001**	4.47 (2.66)	0.24	0.099	−424.03 (249.41)	−0.25	0.096
Model 1																		
Demented	0.25 (0.25)	0.14	0.31	2.29 (1.36)	0.20	0.097	35.53 (139.20)	0.034	0.80	0.20 (0.22)	0.12	0.36	**4.82 (1.99)**	**0.29**	**0.019**	141.78 (150.29)	0.13	0.35
Converted	0.27 (0.16)	0.20	0.084	−1.04 (1.29)	−0.09	0.42	−190.90 (98.71)	−0.22	0.057	0.17 (0.18)	0.13	0.33	−2.35 (1.49)	−0.18	0.12	−273.24 (139.86)	−0.22	0.055
Non-demented	**0.44 (0.22)**	**0.28**	**0.049**	2.61 (1.75)	0.21	0.14	−107.33 (113.71)	−0.14	0.35	**1.03 (0.29)**	**0.48**	**0.001**	4.80 (2.76)	0.26	0.090	−441.50 (265.94)	−0.26	0.11
Model 2																		
Demented	0.23 (0.24)	0.13	0.96	2.13 (1.36)	0.19	0.12	34.97 (137.50)	0.033	0.80	0.21 (0.22)	0.13	0.32	**4.23 (2.01)**	**0.25**	**0.041**	119.60 (147.72)	0.11	0.42
Converted	0.21 (0.16)	0.16	0.19	−1.40 (1.29)	−0.13	0.28	−175.00 (99.20)	−0.21	0.082	0.15 (0.18)	0.10	0.42	−2.75 (1.49)	−0.22	0.069	**−300.19 (139.21)**	**−0.25**	**0.035**
Non-demented	**0.45 (0.22)**	**0.29**	**0.049**	2.64 (1.81)	0.21	0.15	−115.99 (116.02)	−0.15	0.32	**1.02 (0.29)**	**0.47**	**0.001**	4.51 (2.83)	0.24	0.12	−447.54 (268.93)	−0.26	0.11
Model 3																		
Demented	0.24 (0.25)	0.15	0.35	2.14 (1.37)	0.22	0.13	12.36 (143.62)	0.013	0.93	0.21 (0.21)	0.15	0.33	**4.30 (2.01)**	**0.28**	**0.039**	130.00 (147.75)	0.13	0.38
Converted	0.19 (0.17)	0.14	0.26	−2.08 (1.35)	−0.19	0.129	**−211.36 (101.89)**	**−0.25**	**0.042**	0.12 (0.18)	0.08	0.50	**−3.70 (1.28)**	**−0.29**	**0.005**	**−387.35 (118.74)**	**−0.33**	**0.002**
Non-demented	0.44 (0.23)	0.29	0.062	3.81 (1.92)	0.31	0.055	−105.16 (117.89)	−0.14	0.38	**1.02 (0.31)**	**0.48**	**0.002**	**6.84 (2.99)**	**0.36**	**0.029**	−299.11 (295.59)	−0.17	0.32

Model 1: Adjusted for age, gender, BMI, and ApoE ε4 allele. Model 2: Model 1, added with platelet levels. Model 3: Model 1 and 2, added with use of NSAID. NGAL, neutrophil gelatinase-associated lipocalin; Aβ, amyloid-β; BMI, body mass index; NSAID, non-steroidal anti-inflammatory drugs; DS, Down syndrome. The bold values indicate significant associations.

Serum NGAL levels in DS and healthy non-DS subjects: The role of Aβ

As mentioned above, our results show that serum NGAL levels in older DS people were significantly increased compared to healthy elderly non-DS people. This finding is in accordance with a study by Dogliotti and colleagues showing that serum NGAL levels are significantly increased in adults and elderly people with DS compared to adult non-DS healthy controls [21]. Because NGAL is encoded on human chromosome 9 [43], increased NGAL levels may not be directly attributed to the triplication of HSA21. Importantly, studies with neuronal cell cultures have shown that NGAL protein and mRNA production is stimulated by $A\beta_{42}$ [22] and $A\beta_{40}$ [23]. In this regard, a robust increase of NGAL protein levels is present in postmortem brain tissue of AD patients with a similar regional distribution pattern as the Aβ pathology [16]. These studies, therefore, indicate that increased NGAL production may be the result of Aβ accumulation that is characteristically present in DS brain, already at a young age. NGAL thus may be related to Aβ-related pathophysiological processes in the development of dementia in DS. Correspondingly, the association of serum NGAL with different plasma Aβ species was further investigated in this study population.

Associations between serum NGAL levels and different plasma Aβ species

Increased serum NGAL levels were: 1) positively associated with $A\beta_{42}$ and $A\beta_{n42}$ in the demented DS group; 2) positively associated with $A\beta_{40}$ and $A\beta_{n40}$ in non-demented DS subjects; and 3) negatively associated with $A\beta_{42}/A\beta_{40}$ and $A\beta_{n42}/A\beta_{n40}$ ratios in those non-demented DS individuals that converted to dementia over time. These findings are of interest considering the neuropathological regulation of Aβ accumulation in DS during lifetime. Neuropathological studies in DS demonstrated that sequential changes of Aβ plaque formation occur during the lifespan in people with DS, which can provide insights concerning the associations of NGAL with Aβ found in this study. Intraneural $A\beta_{42}$, but not $A\beta_{40}$, has been reported in very young DS people (3 years old) [44]. With increasing age, extracellular $A\beta_{42}$ plaques gradually accumulate and mature [44, 45]. Extracellular deposition of $A\beta_{42}$ in senile plaques precedes the presence of $A\beta_{40}$ by approximately a decade [45, 46]. During the later stages in life (around 50 years), $A\beta_{40}$ accumulation gradually increases in mature plaques and, moreover, it is the predominant Aβ species in cerebral amyloid angiopathy in DS [45, 47]. Although almost all individuals with DS have Aβ deposition resembling AD neuropathology [48, 49], there is a wide variation in the age at onset of dementia. This is due to complex mechanisms that are involved in Aβ regulation during the progression to dementia [50]. In this respect, alterations in the ratio between $A\beta_{42}$ and $A\beta_{40}$ may function as a significant predictor for the development of dementia due to AD [51, 52].

The positive association of increased NGAL with $A\beta_{40}$ in non-demented DS subjects may indicate that $A\beta_{40}$ has not yet accumulated into plaques in the brain resulting in a positive correlation with NGAL in the peripheral blood circulation. This association remained significant after adjustments for confounding factors were made. On the other hand, the association of increased NGAL with $A\beta_{42}$ in the demented DS group may be explained by microglial processes during later stages of Aβ pathology in DS. It was shown that activated microglia and astrocytes were present in diffuse and neuritic plaques [53] and microglia cells can clear $A\beta_{42}$ from the brain to compensate for Aβ pathology [54]. Alternatively, increased inflammatory processes associated with microglia activation may induce an increase in AβPP and consequently an increase in $A\beta_{42}$ production [10]. Both of these abovementioned processes can lead to increased levels of circulating $A\beta_{42}$ peptides. However, this significant association diminished after adjustments for age, gender, BMI, and ApoE ε4 allele. Interestingly, increased NGAL levels were negatively associated with the $A\beta_{42}/A\beta_{40}$ ratio in the converted DS group. This association remained marginally significant after the adjustments for age, gender, BMI, and ApoE ε4 were made. Considering changes of $A\beta_{40}$ and $A\beta_{42}$ in the brain described in the abovementioned neuropathological studies and the association of increased serum NGAL with plasma $A\beta_{40}$ in non-demented and $A\beta_{42}$ in demented DS subjects, it is reasonable to speculate that NGAL is associated with a shift in Aβ regulation present in people with DS whom are in process of converting to dementia. Moreover, it has been previously shown that truncated Aβ increases in parallel to their full length peptides in DS brain [55]. Similar associations of NGAL with full length Aβ and their truncated isoforms can therefore be expected. Indeed, our findings persisted for $A\beta_{n40}$ and $A\beta_{n42}$, similarly to their full-length isoforms. Generally, the association of NGAL levels was even stronger with truncated forms of Aβ than with full length Aβ.

The association of NGAL levels with $A\beta_{42}/A\beta_{40}$ and $A\beta_{n40}/A\beta_{n42}$ ratio strengthened after adjusting for NSAIDs as confounding factor. In addition, the associations of NGAL levels with $A\beta_{n42}$ levels became significant in all of the DS groups. In a previous cohort with a large population of elderly participants, we found that increased NGAL levels were associated with the use of anti-inflammatory medication, which may be explained by underlying somatic conditions [19]. Therefore, the increase in significance of associations after correcting for NSAIDs may be due to correcting for underlying physical ailments related to inflammatory conditions, explaining additional variance in NGAL levels unrelated to levels of Aβ peptides.

The relationship between NGAL, neurodegeneration, and DS

Fundamental research indicates that NGAL plays a role in several mechanisms involved in the pathophysiology of AD. Cell culture studies have shown that NGAL induces pro-apoptotic signaling cascades in neurons and exacerbates oligomeric $A\beta_{42}$-mediated neuronal cell death [16, 22]. In addition, NGAL can aggravate oxidative damage to neuronal cells [22, 56]. This is of importance since people with DS have an increased susceptibility for oxidative stress due to an extra copy of superoxide dismutase 1 [5]. Furthermore, NGAL exerts neuro-immunomodulatory effects. Increased NGAL induces astrocytes and microglia to a pro-inflammatory phenotype and silences their anti-inflammatory functioning [57, 58], whereas elimination of NGAL reduced neuroinflammation and neuronal damage after neuronal injury in mice [59, 60]. As basal NGAL levels increase with age in DS [21], it could increase the sensitivity toward toxic forms of Aβ and oxidative stress and, therefore, contribute to neurodegeneration and, consequently, the development of clinical symptoms of dementia that occur mid- to late life in DS.

Plasma Aβ as a potential biomarker for dementia conversion in DS

Blood-based biomarkers that can predict the conversion to dementia in DS are much desired because they would provide a valuable tool to enable and plan optimal adaptive caregiving. In addition, biomarkers can improve our knowledge of aberrant physiological processes involved during the disease progression. Several studies have investigated the association of plasma Aβ in DS and their potential as diagnostic markers for dementia with inconsistent results [61]. A possible explanation for these discrepancies is that changes of plasma Aβ concentrations in relation to the status of dementia might not be large enough for its use as a biomarker. In this respect, results from this study indicate that the association of NGAL with Aβ species may provide an indication of changes in Aβ accumulation during the progression to dementia in DS.

Strengths and limitations

This study has several strengths worth mentioning. This study consisted of a large DS population group. In addition to AD diagnosis at baseline using the ICD-10 criteria, follow-up clinical assessment in this DS population using validated questionnaires for dementia in DS enabled the identification of those DS individuals that remained non-demented or converted to dementia over time. Several important confounding factors were included that were shown to have potential associations with NGAL and Aβ. The role of circadian influences on blood markers was minimized by obtaining fasting morning blood samples. In addition, NGAL possesses great storage stability, i.e., NGAL can be subjected to several freeze-thaw cycles without affecting outcomes of its analyses which make it suitable for application as a biomarker [62].

In order to properly interpret the results presented in this study, study limitations ought to be acknowledged. ANCOVA analysis did not show a significant interaction of $A\beta_{40}$ and $A\beta_{42}/A\beta_{40}$ with the diagnosis of dementia, with NGAL as dependent variable and therefore, outcomes from these findings should be interpreted with caution. Increased significant associations of NGAL levels with $A\beta_{42}/A\beta_{40}$ and $A\beta_{n40}/A\beta_{n42}$ ratio and $A\beta_{n42}$ after correcting for NSAIDs may be due to underlying ailments that were not documented in this study. Results of this study are based on baseline blood sampling, but longitudinal studies with clinical assessments of dementia in DS accompanied with follow-up blood collection is warranted. Of particular interest would be to follow DS people from a younger age (<40 years) to accurately evaluate the association of NGAL with Aβ in the progression to dementia.

CONCLUSIONS

In conclusion, this study confirmed that serum NGAL levels are increased in elderly DS subjects compared to elderly non-DS controls and strengthens the notion that an increased pro-inflammatory condition is present in people with DS. Furthermore, NGAL

was not associated with either diagnosed dementia or progression to dementia in DS. However, serum NGAL levels were associated with different plasma Aβ species according to the clinical symptoms of dementia. Therefore, the association of serum NGAL with plasma Aβ may reflect the neuropathological regulation of Aβ accumulation and circulation in accordance to the clinical symptoms of dementia in DS. Finally, the measurement of circulating NGAL levels may improve the sensitivity of plasma Aβ as a biological marker for dementia in DS that merits further investigation.

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