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ABSTRACT
Numerous reports over the years have described neuroinflammatory events and vascular changes in neurodegenerative diseases such as Alzheimer´s disease (AD) and dementia with Lewy bodies (DLB). Interestingly, recent reports from other research areas suggest that inflammatory and vascular processes are influenced by gender. These findings are intriguing from the perspective that women show a higher incidence of AD and warrant investigations on how gender influences various processes in neurodegenerative dementia. In the current study we measured the cerebrospinal fluid (CSF) and plasma concentrations of hyaluroinic acid (HA), an adhesionmolecule known to regulate both vascular and inflammatory processes, in AD and DLB patients as well as in healthy elders. Our analysis showed that male AD and DLB patients had almost double the amount of HA compared to female patients whereas no gender differences were observed in the controls. Furthermore, we found that CSF levels of HA in foremost female AD patients correlated with various AD related biomarkers. Correlations between HA levels and markers of inflammation and vascular changes were only detected in female AD patients but in both male and female DLB patients. We conclude that HA may be linked to several pathological events present in AD, as reflected in CSF protein concentrations. The HA profile in CSF, but not in plasma, and associations to other markers appear to be gender-dependent which should be taken into account in clinical examinations and future biomarker studies.

Keywords: Alzheimer´s disease, dementia with Lewy bodies, gender, hyaluronic acid, inflammation, vascular changes

INTRODUCTION

Today it is well-known that Alzheimer's disease (AD) and dementia with Lewy bodies (DLB), two neurodegenerative dementia diseases that inevitably lead to severe impairment of cognitive functions and premature death, are characterized by specific neuropathological findings. AD patients display both senile plaques (accumulation and extracellular deposition of aggregated Aβ) and intraneuronal neurofibrillary tangles (NFTs) (hyperphosphorylation and aggregation of tau protein) whereas Lewy bodies and Lewy neurites (intraneuronal accumulations of mainly α-synuclein) are characteristic of DLB (1-3). A growing number of reports have also described neuroinflammatory events as hallmarks of these two diseases. In AD, cerebrospinal fluid (CSF) concentrations of various inflammation biomarkers are altered (including cytokines, chemokines, complement proteins, acute phase reactants and prostaglandin generating cyclooxygenases (4-7)) and inflammation-related events have been identified in vulnerable brain regions of both AD and DLB patients (5, 8). Moreover, several epidemiological studies have shown that the risk of AD is reduced in patients taking anti-inflammatory drugs (steroids and non-steroidal anti-inflammatory drugs (NSAIDs))(9). In recent years another cofounding factor has gained much attention as cerebrovascular changes have increasingly been recognized as a contributing factor to the generation of amyloid pathology, neurodegeneration and cognitive decline in AD. As many as 50% of AD cases show vascular co-pathology and conditions associated with vascular changes such as diabetes, atherosclerosis or high blood pressure increase the risk for late-onset AD (for review see (10, 11)).

Interestingly, recent studies suggest that gender may play a significant role in several processes related to AD. Clinical studies on inflammatory processes linked to acute conditions have demonstrated that males, compared to females, have higher incidence of septic complications and a higher proinflammatory cytokine profile after severe injury and surgery (12-15). In contrast, it seems like the frequency of complications related to chronic inflammatory conditions instead is greater in females (16, 17). These findings indicate that inflammatory responses in both acute and chronic complications, to some extent, differ between men and women. Gender seems to play a role also when it comes to vascular changes. Women generally have better cardiac function and have a lower risk of cardiovascular diseases such as atherosclerosis (18). The gender aspect is particular interesting considering that approximately 75% of the patients affected by AD are women. Whether the gender differences are due to the fact that women live longer than men, is still a subject of debate, however several meta-studies have shown that women indeed have an increased risk of developing AD (19-21). Moreover, there are two studies showing that men with AD respond better to cholinesterase inhibitors treatment than women (22, 23). Dementia with Lewy bodies, on the other hand, is a male-dominant disease (2), which further indicates that the mechanisms underlying these two neurodegenerative diseases differ. Studies on the relationship between gender, inflammation and neurodegenerative dementia are therefore of interest.

As described before, neuropathological studies have demonstrated the presence of several inflammation-related events in the brain of AD and DLB patients. One of these events is the upregulation of hyaluronic acid (HA), found in several brain areas of AD patients (24, 25). The HA molecule is a polysaccaride made of repeated non-sulphated glycoaminoglycans (GAGs) produced by various cell types in most organs

(26). The molecule is often regarded as an adhesionmolecule which major function is to take part in the orientation and organization of the extracellular matrix (ECM). It is highly expressed in skin and soft connective tissues and it is known to be the stabilizing backbone of the perineuronal network (PN) in the brain (27, 28). Its regulatory role in inflammatory processes has repeatedly been described before. In response to proinflammatory cytokines, such as tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1), endothelial cells up-regulate HA. Further, by binding to the cell-surface receptor CD44, HA recruits and activates leukocytes (29). Hyaluronic acid has also been described as a regulator of endothelial proliferation and blood vessel function (30) and it has become apparent that HA together with other adhesionmolecules, plays a key role in atherosclerosis (31). These finding together with the above mentioned links between inflammatory events, vascular changes and neurodegenerative diseases raise the question whether HA possibly could play a role in neurodegenerative dementia.
In the current study we therefore aimed to investigate if: i) HA levels in CSF and plasma differ between healthy elders and patients with AD, AD patients with documented vascular alterations (ADv) and patients with DLB ii) HA concentrations differ between male and female patients considering that gender seems to be a cofounding factor in processes involved in AD and DLB iii) HA can be linked to previously described disease biomarkers for AD and DLB as well as biomarkers indicative of vascular changes and inflammation.

Material and methods

Patients

The studied groups are randomized, selected samples of the earlier described Malmö Alzheimer Study (6, 7) and consisted of n=100 AD and n=37 DLB patients, and n=38 non-demented controls. Clinical diagnoses were made according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, by the American Psychiatric Association (DSM-IV, 1994) combined with NINCDS-ADRDA diagnostic criteria (32) for probable AD. Patients with AD were sub-grouped into 'pure AD' (n=64) and AD with a vascular component (ADv) (n=36). The category ADv was defined as fulfilment of the clinical criteria for AD and a history with at least one suspected cerebrovascular insult and/or minor ischemic insult on computerized tomography without any clear causative effect on the development of clinical dementia. None of the patients fulfilled the NINCDS-AIREN or DSM-IV criteria for vascular dementia. Diagnosis of probable dementia with Lewy bodies (DLB) was made according to the DLB consensus criteria (2, 33). Cognitive status of patients and controls was evaluated using the Mini Mental State Examination (34). The basic CSF AD-biomarker (Aβ1-42, T-tau, P-tau181) profile of the subjects included in the Malmö Alzheimer Study has been described before (6). The ethics committee of Lund University approved the study and the study procedures were in accordance with the Helsinki declaration of 1975 (revised in 2000). All individuals (or their nearest relatives) gave informed consent to participate in the study.

Analysis of plasma and CSF parameters

Matched blood and lumbar CSF samples were collected as described before (6). Cerebrospinal fluid from all individuals (n=175) was analysed, however due lack of matching plasma samples, the number of individuals included in the plasma analysis was reduced to n=149. The ratio of CSF/serum albumin was used as a measure of

blood brain barrier (BBB) function. Levels of albumin in serum and CSF were determined by nephelometry using the Behring Nephelometer Analyzer (Behringwerke AG, Marburg, Germany). The upper reference limit for the CSF/serum albumin ratio is 10.2 for individuals over 45 years of age (35). The plasma and CSF levels of soluble inter-cellular adhesionmolecule-1 (sICAM-1) and soluble vascular cell adhesionmolecule-1 (sVCAM-1) were determined using a commercially available quantitative enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions as described before (6). Plasma and CSF levels of α1-antichymotrypsin (ACT) and α1-antitrypsin (AAT) were determined using rocket immunoelectrophoresis as described by Laurell (1966) with in-house modifications (7). Complete results of the sICAM-1, sVCAM-1, ACT and AAT determinations of samples from patients included in the Malmö Alzheimer Study have previously been published (6, 7).

Quantification of Hyaluronic acid
The plasma and CSF levels of total HA were determined using commercially available quantitative enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Minneapolis, USA) according to the manufacturer's instructions. Assays were performed in duplicate and the optical density at 450 nm, with a background correction at 570 nm, was determined using a microplate reader (Labsystems iEMS Reader MF). The readings for each standard and sample were averaged, and the average of the zero standard was subtracted.

Statistical analysis
Statistical analysis was performed using the SPSS software (version 18.0 for Windows, SPSS Inc., Chicago, IL, USA). Normal distribution of the variables was tested using the Kolmogorov–Smirnov test and in case of non-normal distribution log transformation was used to yield normal distribution. The independent sample t-test was used for comparisons between two groups. For comparisons between more than two groups the one-way ANOVA or ANCOVA (controlling for age), followed by Bonferroni post-hoc test was used (comparisons, n=6). Correlations were investigated using the Pearson correlation test or partial correlation test (controlling for age). The two-sided χ^2 test was used to test frequency differences among the groups. Results are presented as medians or means ± standard error deviation or range. A $p < 0.05$ was considered significant. All presented p-values, in relation to multiple comparisons, are Bonferroni corrected.

Results
Characteristics of individuals included in the CSF analysis
Table 1 gives the demographic data, MMSE scores and APOE4 allele frequency of the investigated dementia patients and non-demented controls included in the CSF study (table 1). As expected, dementia patients had significantly lower MMSE scores ($p<0.001$), compared to controls, and both patient groups had a higher frequency of one or two APOE4 alleles ($p < 0.05$) (Table 1). No significant difference, between studied groups, was found with smoking and the occurrence of one or more of the chronic inflammatory diseases arteriosclerosis, chronic obstructive pulmonary disease (COPD), and rheumatoid disease was similar in the four investigated groups regardless of gender. Also the use of anti-hypertensive medication and NSAIDs were similar in all groups (data not shown). Patients in the ADv group were significantly older than control ($p=0.005$) and AD patients ($p=0.009$).

Table 1. Demographic data of individuals included in the CSF analysis

Diagnosis	Sex	*n*	Age at Investigation[a]	MMSE[a]	APOE4 carriers (%)
Controls	M	14	73 (60 - 84)	29 (27 - 30)	43%
	F	24	72 (60 - 87)	29 (26 - 30)	13%
	M+F	38	72 (60 - 87)	29 (26 - 30)	24 %
AD	M	32	71 (56 - 79)	***22 (9 - 29)	*70%
	F	32	75 (60 - 85)	***22 (6 - 29)	***78%
	M+F	64	73 (56 - 85)	***22 (6 - 29)	***74%
ADv	M	16	77 (71 - 86)	***22 (15 - 28)	*75%
	F	20	77 (66 - 85)	***23 (15 – 29	***70%
	M+F	36	77 (66 - 86)	***22 (15 – 29)	***72%
DLB	M	19	75 (62 - 84)	***22 (14 - 29)	37%
	F	18	75 (54 - 84)	***21 (10 - 28)	***67%
	M+F	37	75 (54 - 84)	***21 (10 - 29)	*51%

[a] Data are presented as medians and (range). *** Indicates a significant difference at the $p<0.001$ level compared to controls, * indicates a significant difference at the $p<0.05$ level compared to controls.

CSF levels of HA in AD and DLB patients differ between females and males

Due to the significant difference in age between patients with ADv, AD and controls, we evaluated whether HA CSF levels were associated with age in the group of non-demented elders by use of correlation analysis, prior to conducting group comparisons. We found a strong negative correlation between CSF HA levels and age in the male non-demented subjects ($r = -0.710$, $p=0.004$). A possible analysis bias due to age could thus not be ruled out. Therefore, age was added as a covariant factor in the statistical analyses of CSF HA levels in male controls and patients.

No significant difference (ANOVA $p=0.066$) in CSF HA levels was found between the controls and the three patient groups when all patients were included (males and females). Since earlier reports have suggested gender-specific differences in inflammatory responses we subdivided the controls and the patients groups into males and females. The subdivision of all groups based on gender revealed that males in both the AD and DLB groups had significantly higher CSF HA concentrations compared to female patients in the same groups as assessed by use of the t-test (108 % higher, $p<0.05$ and 77 %, higher $p<0.001$, respectively). No such differences were found within the controls and only a trend indicating gender-related differences in CSF HA levels was seen in the ADv group ($p=0.081$) (see figure 1). To investigate whether vascular changes exerted a significant effect on CSF HA levels between AD and ADv, female and male patients of both groups were compared using the t-test. Hyaluronic acid concentrations were significantly higher in females of the ADv patients compared to female AD patients ($p=0.013$) whereas no such difference was seen when comparing the male subjects across the two groups. Interestingly, no significant alteration of CSF HA was detected when females were compared across the investigated groups (controls, AD, ADv and DLB, ANOVA $p=0.095$). On the contrary, comparisons between the males of the different groups indicated that CSF HA levels varied between the groups (ANCOVA $p=0.030$). Although, both male DLB and ADv patients exhibited twice as high concentrations as the male controls, only CSF HA levels found in male ADv differed significantly from the HA levels found in the male controls ($p=0.044$) (see table 2).

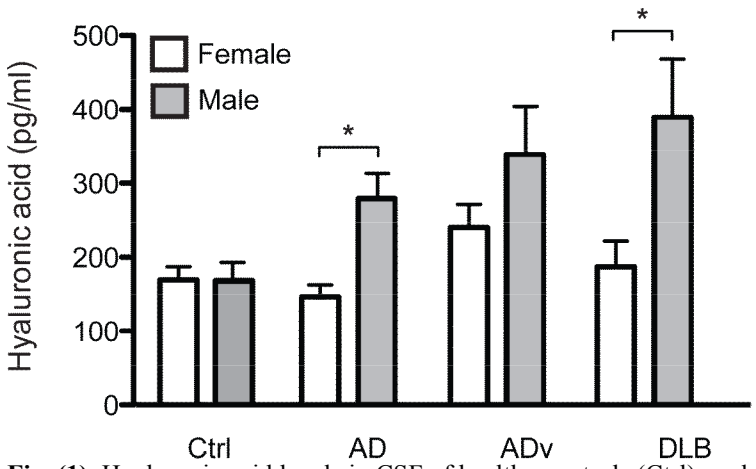

Fig. (1). Hyaluronic acid levels in CSF of healthy controls (Ctrl), and patients with Alzheimer´s disease (AD), Alzheimer´s disease with vascular changes (ADv) and dementia with Lewy bodies (DLB) divided based on gender. Each bar represents the mean ± SEM. Data was analysed with independent t-test. * indicates a significant difference at $p< 0.05$ level.

Table 2. CSF Hyaluronic acid levels (pg/ml) based on gender

Diagnosis	Male	increase (%)	Female	increase (%)
Controls	168.53 ± 24.78		171.33 ± 18.39	
AD	259.38 ± 27.57	53[a]	146.38 ± 16.38	-----
ADv	*346.64 ± 63.59	106[a]	227.97 ± 30.10	56[b]
DLB	389.46 ± 78.64	131[a]	187.23 ± 34.49	-----

Data are presented as means ± SEM. * indicates a significant difference at the corrected $p<0.05$ level compared to male controls. Data are analysed by ANCOVA controlled for age. a = indicates comparisons with male control subjects, b= indicates comparisons with female AD patients

Correlations between CSF HA, AD biomarkers and cognitive function

To investigate potential links between the quantified CSF HA levels, cognitive function (total MMSE scores) as well as various well-studied AD biomarkers (Aβ1-42, T-tau and P-tau) we used correlation analysis. Age was controlled for when HA levels in males were analysed. Interestingly, we found a positive correlation between HA CSF levels and the AD biomarker $A\beta_{1\text{-}42}$ in females with AD and ADv as well as in female controls. In addition, a negative correlation between the AD biomarker P-tau and HA was found in the female AD group, a relationship that was not detected in the other groups (see table 3). No correlations between the AD biomarkers and CSF HA levels were found in males of any group, even after adjustment for age (see table 3). No correlation with the AD biomarker T-tau or MMSE was detected in any of the investigated groups (data not shown).

Table 3. Variables linked to levels of HA in CSF

Variables	Ctrl	AD	ADv	DLB
	Female			
AAT mg/l	—	**0.697	*0.602	*0.642
ACT, mg/l	—	*0.425	—	*0.515
$A\beta_{1\text{-}42}$, ng/l	*0.538	*0.352	*0.491	—
P-tau, ng/l	—	*-0.371	—	—
sICAM1,µg/l	—	*0.382	*0.502	—
sVCAM1,µg/l	**0.577	—	—	—
Q alb	*0.502	**0.767	**0.671	*0.522

Variables	Ctrl	AD	ADv	DLB
	Male			
AAT mg/l	——	——	——	*0.495[a]
ACT, mg/l	**0.786[a]	——	——	**0.625 [a]
$A\beta_{1\text{-}42,}$ ng/l	——	——	——	——
P-tau, ng/l	——	——	——	——
sICAM1,μg/l	——	——	——	*0.508 [a]
sVCAM1,μg/l	——	——	——	*0.509 [a]
Q alb	*0.681[a]	*0.418[a]	——	***0.829 [a]

AAT=α1-antitrypsin; ACT=α1-antichymotrypsin; P-tau=tau phosphorylated at threonine 181; sICAM1= soluble intercellular adhesion molecule-1; sVCAM1=soluble vascular cell adhesion molecule; Q alb= CSF/serum albumin ratio. *** Indicates correlation is significant at 0.001, ** Indicates correlation is significant at 0.01. * Indicates correlation is significant at 0.05, ——Indicates no significant correlation. a indicates adjusted for age.

Correlations between CSF HA and markers of inflammation and vascular changes

Considering the proposed links between HA and inflammatory processes (28, 29), we investigated if CSF HA levels were associated with CSF levels of inflammatory markers linked to neurodegenerative dementia. The acute-phase reactants ACT and AAT have been shown to be associated with inflammatory processes and Aβ plaque formation (36, 37). The ACT and AAT concentrations, quantified in the CSF and plasma from the individuals included in the Malmö Alzheimer Study, has previously been reported (7). In the current study correlation analysis showed that AAT in all female patients strongly correlated with CSF HA and that ACT correlated with CSF HA in female AD and DLB patients. However, no links between CSF HA and the two acute-phase proteins were found in female controls. Partial correlation analysis of CSF HA levels in males revealed a relationship between CSF HA and ACT in male controls and male DLB patients (see table 3). No association between CSF concentrations of HA and C-reactive (CRP) levels was found in any of the investigated groups (data not shown).

Previous studies suggest that HA is involved in endothelial proliferation and vascular changes (31, 38). Therefore we investigated the potential relationship between the concentrations of HA in CSF and the CSF/serum albumin ratio (Q Alb), a crude indicator of BBB function. We found a strong correlation between the albumin ratio and CSF HA levels in all patient and controls with the exception for male ADv patients (see table 3). We also investigated the relationship between HA and the concentrations of the soluble versions of two molecules involved in endothelial cell function (39), sVCAM-1 and sICAM-1. Results from the CSF and plasma analyses for sVCAM-1 and sICAM-1 have previously been described for the Malmö Alzheimer Study (6). In the present study the CSF levels of sICAM-1 and HA correlated within the female ADv group and the sVCAM-1 levels correlated with HA levels in the female AD. Both VCAM-1 and ICAM-1 levels were, when adjusted for age, associated with HA levels in male DLB patients (see table 3).

Concentrations of HA in plasma

The strong association between CSF HA levels and the albumin ratio indicated that BBB function could affect CSF levels of HA. To be able to exclude the possibility that the CSF concentration of HA was influenced by blood-derived HA, we measured the plasma concentrations of HA in the majority of the patients included in the CSF analysis. Table 4 gives the demographic data of the included patients. The difference

in age between ADv and controls remained significant (p=0.025) also for the subset of patients included in the plasma analysis. Therefore we analysed the correlation between HA levels and age in the different groups, but found no correlation between HA plasma levels and age in the control group regardless of gender. Thus we did not consider age as a covariate in the statistical analysis of HA plasma levels. Importantly, plasma levels of HA did not correlate with HA levels in CSF in any of the investigated groups (data not shown). In contrast to the results obtained in the CSF investigation no gender-differences were found in plasma HA levels when comparing the genders in the various groups (data not shown). However, DLB patients demonstrated significantly lower HA plasma levels compared to controls (by 53 %, p=0.020) (see table 5) and compared to ADv (44 %, p=0.009) Neither the AD group nor the ADv group showed HA plasma levels differences compared to controls or each other.

Table 4. Demographic data of individuals included in the plasma analysis.

Diagnosis	Sex (F/M)	*n*	Age (yrs)[a]	MMSE[a]	APOE4 carriers (%)
Controls	24/14	38	72 (60-87)	29 (26-30)	23.7
AD	32/27	59	74 (56-85)	***22 (6-29)	***74.6
ADv	18/14	32	76 (66-85)	***22 (15-29)	***71.9
DLB	11/9	20	77 (71-84)	***21 (14-28)	**60.0

[a] Data are presented as medians and (range). *** Indicates a significant difference at the p=0.001 levels compared to controls. ** Indicates a significant difference at the p=0.01 levels compared to controls.

Associations between plasma HA, cognitive function, AD biomarkers and markers of inflammation and vascular changes

We found no links between plasma levels of HA, cognitive function (total MMSE score) and BBB integrity (albumin ratio). Furthermore, no associations were found between plasma HA and plasma levels of sVCAM-1 and ACT, and CSF concentrations of the AD markers P-tau, T-tau and $A\beta_{1-42}$ (data not shown). However, plasma HA and plasma sICAM-1 were significantly correlated in the AD patient group (r=0.257, p=0.011) and a negative correlation was found between plasma concentrations of HA and AAT in the control group (r = -0.326, p=0.043).

Table 5. HA levels in individuals included in the plasma analysis.

Diagnosis	HA (pg/ml)[a]	range	decrease (%)
Controls	16.93	3.80 – 74.13	-------
AD	14.23	2.75 – 177.82	-------
ADv	17.78	3.46 – 194.98	-------
DLB	*9.12	4.47 – 20.89	53[b]

[a] Data are presented as geometric means. * Indicates a significant difference at the p<0.05 levels compared to controls. b= indicates comparisons with control subjects. Data is analysed with one-way ANOVA and Bonferroni test (n=6).

Effect of the APOE4 allele, NSAID treatment and inflammatory diseases on CSF and plasma HA concentrations

Carriers of the APOE4 allele did not exhibit any difference in CSF HA or in plasma HA concentrations versus APOE4 non-carriers in any of the investigated groups. We

also found no difference in levels of HA in CSF or plasma when comparing subjects who had arterosclerosis, COPD or rheumatoid disease to subjects without these disorders. Levels of HA were not significantly changed in smokers or subjects treated with NSAID.

Discussion

The first aim of this study was to investigate whether HA could potentially function as an additional biomarker for AD and DLB. Although previous studies on brain tissue have shown an increase in HA expression in patients with AD, our result revealed no significant change in CSF levels of HA when comparing patients groups and non-demented controls. These findings suggest that CSF HA levels alone are not useful as a diagnostic tool to distinguish patients with AD and DLB from healthy individuals

Considering that gender might play an important role in processes also involving HA, such as inflammation and vascular changes, we investigated whether HA levels were influenced by gender. We found that male AD and DLB patients displayed significantly higher HA CSF levels than their female counterparts and a trend towards increased levels in all male patient groups compared to the male control group. Our result also revealed that HA CSF levels in female patient groups, as a contrast to male patients (particularly AD and DLB), remained unchanged compared to the female control group. The underlying mechanisms for this gender difference is yet to be determined. Previous studies have suggested that HA production increases with inflammation and thus it is tempting to speculate that the elevated levels of HA in male patients are a direct response to the inflammatory processes linked to the neurodegeneration. However, although previous studies have shown that males with inflammatory complications display a higher proinflammatory cytokine profile compared to females (12-15), this gender difference does not explain why we do not find elevated HA levels also in female patients. Therefore we suggest two different interpretations: Firstly, it is possible that the increased HA levels in male patients are not a consequence of AD and DLB related neurodegenerative changes but merely a result of other secondary inflammatory processes more pronounced in the male patients. Previous studies have shown that HA production is affected by several parameters such as inflammatory diseases (38). To exclude the possibility that such parameters affect our result, we compared HA levels in CSF from subjects with documented inflammatory disease like arteriosclerosis, COPD or rheumatoid disease to subjects without those conditions. We found no significant difference between subjects with and without inflammatory diseases in any of the investigated groups. Although, there may be other conditions not included in our study that could affect the HA, we suggest that the increased HA levels in males are due to either direct or indirect pathways linked to the neurodegenerative disorders. The second way to interpret our results is that unchanged HA levels in female patients signal a dysfunctional HA inflammatory response to neurodegeneration. Support for the latter theory is found in studies showing a regulatory role of estrogen on HA release and/or metabolism. Estrogen is a well-documented important modulator of the immune system (40). It is known to have protective properties during neuroinflammatory and neurodegenerative processes in the brain (41). The HA regulatory effect of estrogen has been described in studies showing altered levels of HA in serum from healthy women treated with estrogen (42) and preclinical studies have shown that estrogen stimulates the production of HA in mammary glands (43), dermis (44) and bladder/urethra (45) of ovariectomize rats. Although there are no (to our knowledge)

studies describing the influence of estrogen on HA production in the brain, we can not exclude the possibility that the hormone affects the presence of HA also in this part of the body. Thus it is tempting to conclude that the unchanged HA levels in female patients are caused by a dysfunctional inflammatory response due to the pronounced decline of estrogen in the menopausal women. However, it may well be that the HA levels in female patients reflect a normal response to the neuropathological changes seen in AD and DLB and that it is instead the high HA levels found in male patients that signal dysfunctional HA upregulation.
To further complicate the interpretation of our results we have to take into account the fact that HA can appear in different lengths and that size determines its regulatory properties. In normal steady-state conditions and in tissue healing HA appears as a high molecular weight molecule (HMW HA, size larger than 400 kDa) with anti-angiogenic and anti-inflammatory properties. In case of injury and inflammation, enzymes degrade HMW HA into low molecular weight products (LMW HA, smaller than 3 kDa). These small fragments have instead pro-angiogenic and proinflammatory properties (46). Immunoassays for quantification of HA, as the ELISA we used in the current study, measure the total amount of HA and thus do not distinguish between HMW and LMW. Given this limitation and since the two different HA forms plausibly vary in solubility, we can not differentiate whether the increase in HA levels in CSF of male patients is due to increased production or a result of an increased defragmentation of existing HA, or both. Further studies on the specific LMW and HMW profile in male and female patients are required in order to in-depth explain the gender-dependent variation in HA levels observed in the AD and DLB patients.

Our third aim was to investigate whether HA levels could be associated with the well-established AD biomarkers and thus be linked to the specific neurodegenerative processes assumed to be reflected by these AD markers. Interestingly, we found a positive correlation between HA levels and $A\beta_{1-42}$ CSF concentrations in the female control group as well as in the female AD and ADv patient group. In addition, we found a negative correlation between HA and P-tau in the female AD group. These results should be viewed from the perspective that clinical studies have repeatedly demonstrated lower levels of Aβ and elevated levels of P-tau in CSF of AD patients, suggested to be indicative of higher Aβ retention in the brain tissue i.e. higher plaque load, and degeneration of neurons (4). Thus, our biomarker results indicate that female AD patients with higher levels of HA have less plaque load and less neurodegeneration, which in turn could suggests a neuroprotective role for HA. Support for this idea is found in *in vitro* studies showing that neurons ensheated with PN, whereof HA is the major component, are protected against $A\beta_{1-42}$ induced neurotoxicity (47) and oxidative stress (48). Additionally, it has been shown that intrastriatal injections of Aβ induce a significant reduction of HA in the rat brain (49) and that there is a focal loss of HA in the core and the coronal zone of Aβ plaque in transgenic Tg2576 AD mice (50). Given these results, we speculate that increased plaque load in female AD patients reduces the protective HA content, which in turn leads to increased neurodegeneration. The positive correlation between HA and $A\beta_{1-42}$ found in non-demented female elders further suggests that HA plays a role in Aβ dynamics also under conditions of normal aging, which needs to be further investigated preferably using a neuropathological approach. The potential gender differences in HA regulation (such as the hormonal influence discussed above) could possibly be explained by the lack of correlations between HA and AD markers in male controls. Consequently, if this hypothesis holds true, it may well be that the

increased HA levels in male patients are induced by male dominant pathways secondary to the neurodegenerative processes and thus could potentially hide a relationship between AD makers and HA in male patients.

To elucidate whether HA could be linked to AD and DLB related inflammatory processes we aimed to study relationships between HA and other inflammatory biomarkers known to be linked to neurodegenerative dementia. Both ACT and AAT are acute-phase reactants found in amyloid plaques in the brain of AD patients (36, 37). Previously we have reported that AAT and ACT CSF levels were significantly higher in AD and DLB patients (all included in the Malmö Alzheimer Study) compared to healthy controls and that, interestingly, the AAT and ACT concentrations were significantly higher in men compared to women in all investigated groups (7). In our current study we found a correlation between the two acute-phase proteins and HA in almost all female patients groups but also in the male DLB patients group. The correlation between HA and ACT is especially interesting since previous studies have shown that activated astrocytes, the major source of ACT (51), upregulate the HA receptor CD44 (52) when they encircle early Aβ plaques.

Further, we aimed to investigate the relationship between HA CSF levels and vascular changes in individuals included in the study. Our result showing a significant increase in CSF HA levels in female ADv compared to female AD as well as a significant increase in male ADv compared to male controls indicates that HA expression is altered in response to vascular changes. Interestingly, research on ischemia and HA has led to similar results. Preclinical studies have shown that occlusion of the middle cerebral artery in rat, is followed by an increased HA production (53). Postmortem studies have shown that the expression of total HA as well as HA degrading enzymes are significantly increased in brain tissue of stroke patients (54) and finally, analysis of CSF from patients with cerebral infarctions show an increase in HA levels compared to healthy controls (55). These findings correspond well with ours, where the inclusion criteria for ADv was at least one suspected cerebrovascular insult and/or minor ischemic insult.
To further investigate the relationship between HA and vascular changes we correlated HA levels with levels of markers known to be linked to vascular changes in neurodegenerative disorders. ICAM-1 and VCAM-1 are adhesionmolecules upregulated in response to inflammation in the brain. When upregulated, the adhesion molecules interact with leukocytes and reduce vasodilation and thereby the permeability of the microvasculature in a similar fashion as HA (for review see (56)). We have previously shown that both plasma and CSF levels of sICAM-1 and sVCAM-1 are increased in AD and DLB patients (included in the Malmö Alzheimer Study) compared to healthy elders (6) and others have shown that ICAM-1 is both present in neuritic plaques and increased in cerebrovascular endothelium of AD patients (33). Furthermore, previous studies have shown that production of sICAM-1 and sVCAM-1 are influenced by HA (57, 58). When investigating the correlation between CSF HA levels and sICAM-1 and sVCAM-1 CSF levels we found that only females in the control and AD, ADv groups showed a correlation between HA and sICAM-1 and sVCAM-1, whereas only males in the DLB group showed correlations between HA and the mentioned adhesion molecules. Our analysis also suggested a strong relationship between the serum/CSF albumin ratio and CSF HA in almost all examined groups. These results of increased levels of HA in female patients with

ADv and correlations between HA and biomarkers for vascular changes suggest that HA levels could to some extent reflect vascular changes in elders.

Surprisingly, despite the positive association between higher serum/CSF albumin ratios and higher CSF HA we found no link between HA levels in plasma and CSF. Moreover, the HA profile differed between the two compartments. Plasma levels of HA found in DLB patients were significantly lower than those found in the controls and in the AD patients. The turnover of HA in blood is partly regulated by degradation in the liver. Therefore levels of circulating HA serve as a valuable tool for the estimation of liver function in patients with liver failure and patients under post-operational evaluation of their liver transplants (28). However, HA levels fluctuate diurnal and the highest levels are seen one hour after the patient has left the bed, probably due to HA accumulation in the tissues during night and release into the circulation upon muscle activation after bedrest (28). Although, the collection of plasma in our study was performed at approximately the same time during the day, we can not exclude that circumstances such as the fact that DLB patients commonly are affected by sleep disturbances (59) and therefore possible move less, may underlie the significant reduction of plasma HA levels seen in DLB patients. If this holds true is it important to consider the possibility that mobility could influence also the HA levels in brain. In the current study we however found no correlation between levels of HA in plasma and levels of HA in CSF, which leaves a potential influence of mobility on HA CSF levels as rather unlikely.

Conclusions

We conclude that HA levels in CSF do not differ between non-demented elders and patients with AD and DLB and thus CSF HA is unfit to serve as a specific disease biomarker for the investigated diagnostic groups. Further, females and males within the AD and DLB diagnostic groups differed in respect to CSF HA, but not plasma HA, supporting previous findings of gender-specific alterations in events associated with AD and DLB. In support, levels of several inflammatory markers were positively correlated with CSF HA levels in female patients but not in male patients. Last, the presence of vascular alterations in patients with AD appeared to abolish the difference in HA levels between genders, as found in the pure AD group. Therefore we propose that AD patients with vascular alterations might present a different biomarker profile compared to AD with less vascular co-pathology. Our results also emphasize the importance of investigating a potential gender-effect when analysing inflammation-related markers in CSF as gender divergence could not only influence diagnostic interpretations, but should also influence future gender-specific treatment strategies.

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References

1. Braak H, Braak E. Staging of Alzheimer's disease-related neurofibrillary changes. Neurobiol Aging. 1995 May-Jun;16(3):271-8; discussion 8-84.
2. McKeith IG, Dickson DW, Lowe J, Emre M, O'Brien JT, Feldman H, et al. Diagnosis and management of dementia with Lewy bodies: third report of the DLB Consortium. Neurology. 2005 Dec 27;65(12):1863-72.
3. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, et al. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology. 1991 Apr;41(4):479-86.
4. Blennow K, Hampel H, Weiner M, Zetterberg H. Cerebrospinal fluid and plasma biomarkers in Alzheimer disease. Nat Rev Neurol. Mar;6(3):131-44.
5. McGeer PL, McGeer EG. Inflammation, autotoxicity and Alzheimer disease. Neurobiol Aging. 2001 Nov-Dec;22(6):799-809.
6. Nielsen HM, Londos E, Minthon L, Janciauskiene SM. Soluble adhesion molecules and angiotensin-converting enzyme in dementia. Neurobiol Dis. 2007 Apr;26(1):27-35.
7. Nielsen HM, Minthon L, Londos E, Blennow K, Miranda E, Perez J, et al. Plasma and CSF serpins in Alzheimer disease and dementia with Lewy bodies. Neurology. 2007 Oct 16;69(16):1569-79.
8. Mackenzie IR. Activated microglia in dementia with Lewy bodies. Neurology. 2000 Jul 12;55(1):132-4.
9. McGeer PL, Schulzer M, McGeer EG. Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies. Neurology. 1996 Aug;47(2):425-32.
10. de la Torre JC. Impaired cerebromicrovascular perfusion. Summary of evidence in support of its causality in Alzheimer's disease. Annals of the New York Academy of Sciences. [Review]. 2000;924:136-52.
11. Breteler MM. Vascular risk factors for Alzheimer's disease: an epidemiologic perspective. Neurobiology of aging. [Review]. 2000 Mar-Apr;21(2):153-60.
12. Majetschak M, Flohe S, Obertacke U, Schroder J, Staubach K, Nast-Kolb D, et al. Relation of a TNF gene polymorphism to severe sepsis in trauma patients. Ann Surg. 1999 Aug;230(2):207-14.
13. Oberholzer A, Keel M, Zellweger R, Steckholzer U, Trentz O, Ertel W. Incidence of septic complications and multiple organ failure in severely injured patients is sex specific. J Trauma. 2000 May;48(5):932-7.
14. Schroder J, Kahlke V, Staubach KH, Zabel P, Stuber F. Gender differences in human sepsis. Arch Surg. 1998 Nov;133(11):1200-5.
15. Wichmann MW, Muller C, Meyer G, Adam M, Angele MK, Eisenmenger SJ, et al. Different immune responses to abdominal surgery in men and women. Langenbecks Arch Surg. 2003 Feb;387(11-12):397-401.
16. Lockshin MD. Sex differences in autoimmune disease. Lupus. 2006;15(11):753-6.
17. Whitacre CC. Sex differences in autoimmune disease. Nat Immunol. 2001 Sep;2(9):777-80.
18. Perez-Lopez FR, Larrad-Mur L, Kallen A, Chedraui P, Taylor HS. Gender differences in cardiovascular disease: hormonal and biochemical influences. Reprod Sci. [Research Support, Non-U.S. Gov't

Review]. 2010 Jun;17(6):511-31.
19. Andersen K, Launer LJ, Dewey ME, Letenneur L, Ott A, Copeland JR, et al. Gender differences in the incidence of AD and vascular dementia: The EURODEM Studies. EURODEM Incidence Research Group. Neurology. 1999 Dec 10;53(9):1992-7.
20. Azad NA, Al Bugami M, Loy-English I. Gender differences in dementia risk factors. Gend Med. 2007 Jun;4(2):120-9.
21. Gao S, Hendrie HC, Hall KS, Hui S. The relationships between age, sex, and the incidence of dementia and Alzheimer disease: a meta-analysis. Arch Gen Psychiatry. 1998 Sep;55(9):809-15.
22. Wattmo C, Wallin AK, Londos E, Minthon L. Predictors of long-term cognitive outcome in Alzheimer's disease. Alzheimer's research & therapy. 2011 Jul 20;3(4):23.
23. MacGowan SH, Wilcock GK, Scott M. Effect of gender and apolipoprotein E genotype on response to anticholinesterase therapy in Alzheimer's disease. International journal of geriatric psychiatry. [Clinical Trial Research Support, Non-U.S. Gov't]. 1998 Sep;13(9):625-30.
24. Jenkins HG, Bachelard HS. Glycosaminoglycans in cortical autopsy samples from Alzheimer brain. J Neurochem. 1988 Nov;51(5):1641-5.
25. Shimizu H, Sato S, Ohishi H, Mori O, Mori T, Ohami H. [Proteoglycans in senile dementia of Alzheimer's type]. Nippon Ronen Igakkai Zasshi. 1997 Jun;34(6):461-7.
26. Dityatev A, Fellin T. Extracellular matrix in plasticity and epileptogenesis. Neuron Glia Biol. 2008 Aug;4(3):235-47.
27. Bignami A, Hosley M, Dahl D. Hyaluronic acid and hyaluronic acid-binding proteins in brain extracellular matrix. Anat Embryol (Berl). 1993 Nov;188(5):419-33.
28. Laurent TC, Fraser JR. Hyaluronan. FASEB J. 1992 Apr;6(7):2397-404.
29. Siegelman MH, DeGrendele HC, Estess P. Activation and interaction of CD44 and hyaluronan in immunological systems. J Leukoc Biol. 1999 Aug;66(2):315-21.
30. Genasetti A, Vigetti D, Viola M, Karousou E, Moretto P, Rizzi M, et al. Hyaluronan and human endothelial cell behavior. Connect Tissue Res. 2008;49(3):120-3.
31. Karangelis DE, Kanakis I, Asimakopoulou AP, Karousou E, Passi A, Theocharis AD, et al. Glycosaminoglycans as key molecules in atherosclerosis: the role of versican and hyaluronan. Curr Med Chem. [Review]. 2010;17(33):4018-26.
32. McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Jr., Kawas CH, et al. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimer's & dementia : the journal of the Alzheimer's Association. [Research Support, Non-U.S. Gov't]. 2011 May;7(3):263-9.
33. McKeith IG, Galasko D, Kosaka K, Perry EK, Dickson DW, Hansen LA, et al. Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the consortium on DLB international workshop. Neurology. 1996 Nov;47(5):1113-24.

34. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res. 1975 Nov;12(3):189-98.
35. Blennow K, Fredman P, Wallin A, Gottfries CG, Karlsson I, Langstrom G, et al. Protein analysis in cerebrospinal fluid. II. Reference values derived from healthy individuals 18-88 years of age. Eur Neurol. 1993;33(2):129-33.
36. Gollin PA, Kalaria RN, Eikelenboom P, Rozemuller A, Perry G. Alpha 1-antitrypsin and alpha 1-antichymotrypsin are in the lesions of Alzheimer's disease. Neuroreport. 1992 Feb;3(2):201-3.
37. Gooptu B, Lomas DA. Conformational pathology of the serpins: themes, variations, and therapeutic strategies. Annu Rev Biochem. 2009;78:147-76.
38. Jiang D, Liang J, Noble PW. Hyaluronan as an immune regulator in human diseases. Physiological reviews. [Research Support, N.I.H., Extramural Review]. 2011 Jan;91(1):221-64.
39. Cotran RS, Mayadas-Norton T. Endothelial adhesion molecules in health and disease. Pathol Biol (Paris). 1998 Mar;46(3):164-70.
40. Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. Hum Reprod Update. 2005 Jul-Aug;11(4):411-23.
41. Vegeto E, Benedusi V, Maggi A. Estrogen anti-inflammatory activity in brain: a therapeutic opportunity for menopause and neurodegenerative diseases. Front Neuroendocrinol. 2008 Oct;29(4):507-19.
42. Tuomikoski P, Aittomaki K, Mikkola TS, Ropponen A, Ylikorkala O. Effect of oral and transdermal hormone therapy on hyaluronic acid in women with and without a history of intrahepatic cholestasis of pregnancy. Am J Obstet Gynecol. 2008 Apr;198(4):375 e1-5.
43. Sunil N, Srinivasan N, Aruldhas MM, Govindarajulu P. Impact of oestradiol and progesterone on the glycosaminoglycans and their depolymerizing enzymes of the rat mammary gland. Acta Physiol Scand. 2000 Mar;168(3):385-92.
44. Raine-Fenning NJ, Brincat MP, Muscat-Baron Y. Skin aging and menopause : implications for treatment. Am J Clin Dermatol. 2003;4(6):371-8.
45. de Deus JM, Girao MJ, Sartori MG, Baracat EC, Rodrigues de Lima G, Nader HB, et al. Glycosaminoglycan profile in bladder and urethra of castrated rats treated with estrogen, progestogen, and raloxifene. Am J Obstet Gynecol. 2003 Dec;189(6):1654-9.
46. Bollyky PL, Falk BA, Wu RP, Buckner JH, Wight TN, Nepom GT. Intact extracellular matrix and the maintenance of immune tolerance: high molecular weight hyaluronan promotes persistence of induced CD4+CD25+ regulatory T cells. J Leukoc Biol. 2009 Sep;86(3):567-72.
47. Miyata S, Nishimura Y, Nakashima T. Perineuronal nets protect against amyloid beta-protein neurotoxicity in cultured cortical neurons. Brain Res. 2007 May 30;1150:200-6.
48. Morawski M, Bruckner MK, Riederer P, Bruckner G, Arendt T. Perineuronal nets potentially protect against oxidative stress. Exp Neurol. 2004 Aug;188(2):309-15.
49. Genedani S, Agnati LF, Leo G, Buzzega D, Maccari F, Carone C, et al. beta-Amyloid fibrillation and/or hyperhomocysteinemia modify striatal patterns of hyaluronic acid and dermatan sulfate: Possible role in the pathogenesis of Alzheimer's disease. Curr Alzheimer Res. Mar;7(2):150-7.

50. Morawski M, Pavlica S, Seeger G, Grosche J, Kouznetsova E, Schliebs R, et al. Perineuronal nets are largely unaffected in Alzheimer model Tg2576 mice. Neurobiology of aging. [Comparative Study
Research Support, Non-U.S. Gov't]. 2010 Jul;31(7):1254-6.
51. Abraham CR. Reactive astrocytes and alpha1-antichymotrypsin in Alzheimer's disease. Neurobiology of aging. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.
Review]. 2001 Nov-Dec;22(6):931-6.
52. Akiyama H, Tooyama I, Kawamata T, Ikeda K, McGeer PL. Morphological diversities of CD44 positive astrocytes in the cerebral cortex of normal subjects and patients with Alzheimer's disease. Brain Res. 1993 Dec 31;632(1-2):249-59.
53. Al Qteishat A, Gaffney JJ, Krupinski J, Slevin M. Hyaluronan expression following middle cerebral artery occlusion in the rat. Neuroreport. 2006 Jul 31;17(11):1111-4.
54. Al'Qteishat A, Gaffney J, Krupinski J, Rubio F, West D, Kumar S, et al. Changes in hyaluronan production and metabolism following ischaemic stroke in man. Brain. 2006 Aug;129(Pt 8):2158-76.
55. Laurent UB, Laurent TC, Hellsing LK, Persson L, Hartman M, Lilja K. Hyaluronan in human cerebrospinal fluid. Acta Neurol Scand. 1996 Sep;94(3):194-206.
56. Ewers M, Mielke MM, Hampel H. Blood-based biomarkers of microvascular pathology in Alzheimer's disease. Experimental gerontology. [Research Support, N.I.H., Extramural
Research Support, Non-U.S. Gov't
Review]. 2010 Jan;45(1):75-9.
57. Jung EM, Kwon O, Kwon KS, Cho YS, Rhee SK, Min JK, et al. Evidences for correlation between the reduced VCAM-1 expression and hyaluronan synthesis during cellular senescence of human mesenchymal stem cells. Biochem Biophys Res Commun. Dec 7.
58. Karatay S, Kiziltunc A, Yildirim K, Karanfil RC, Senel K. Effects of different hyaluronic acid products on synovial fluid levels of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in knee osteoarthritis. Ann Clin Lab Sci. 2004 Summer;34(3):330-5.
59. Ferman TJ, Boeve BF. Dementia with Lewy bodies. Neurol Clin. 2007 Aug;25(3):741-60, vii.