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Levels of Retinal Amyloid-β Correlate with Levels of Retinal IAPP and Hippocampal Amyloid-β in Neuropathologically Evaluated Individuals

Nina Schultz[a,b], Elin Byman[a], the Netherlands Brain Bank[c] and Malin Wennström[a,*]
[a]*Department of Clinical Sciences Malmö, Clinical Memory Research Unit, Lund University, Malmö, Sweden*
[b]*Department of Neurology, University of California, San Francisco, CA, USA*
[c]*Netherlands Institute for Neuroscience, Amsterdam, the Netherlands*

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Abstract.
Background: Previous studies have used immunohistology to demonstrate Alzheimer's disease (AD) characteristic accumulation of amyloid-β (Aβ) in the retina of AD patients, a finding indicating retina examination as a potential diagnostic tool for AD pathology.
Objective: To further explore this idea by investigating whether levels of $A\beta_{42}$ and $A\beta_{40}$ in retina are associated with corresponding levels in hippocampus, neuropathological assessments, apolipoprotein E (*APOE*) genotype, and levels of islet amyloid polypeptide (IAPP).
Methods: Levels of high molecular weight (HMW) $A\beta_{42}$, $A\beta_{40}$, and IAPP in ultra-centrifuged homogenates of retina and hippocampus from patients with AD, multiple sclerosis, AD with Lewy bodies, and non-demented controls were analyzed using Mesoscale Discovery electrochemiluminescence technology employing immunoassay and enzyme-linked immunosorbent assay.
Results: Higher levels of retinal and hippocampal $A\beta_{42\text{-HMW}}$, $A\beta_{40\text{-HMW}}$, and $IAPP_{\text{-HMW}}$ were found in individuals with high neuropathological scores of Aβ plaques and in individuals carrying the *APOE* ε4 allele. The retinal levels of $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ correlated with corresponding levels in hippocampus as well as with neurofibrillary tangles (NFT) and Aβ scores. Retinal $IAPP_{\text{-HMW}}$ correlated with retinal levels of $A\beta_{42\text{-HMW}}$ and with NFT and Aβ scores.
Conclusion: These results show that different isoforms of Aβ can be detected in the human retina and moreover support the growing number of studies indicating that AD-related pathological changes occurring in the brain could be reflected in the retina.

Keywords: Alzheimer's disease, amyloid-β, hippocampus, IAPP, retina

INTRODUCTION

The highly vascularized light sensitive retina originates as an outgrowth of the developing brain and consists of several layers of neurons, which, similarly to brain neurons, are supported by glial cells [1]. This brain-eye interlink has led to the hypothesis that pathological changes occurring in the brain could be reflected in the retina. If this holds true, it opens up for new non-invasive methods to monitor and diagnose brain pathology. The research field of Alzheimer's disease (AD) has started to explore this possibility and a number of studies have shown an

*Correspondence to: Malin Wennström, Inga Marie Nilssons gata 53, 214 28 Malmö, Sweden. Tel.: +46 40335733; E-mail: malin.wennstrom@med.lu.se.

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association between AD pathology and pathological changes in the retina [2–6]. Most striking is the finding of amyloid-β (Aβ) depositions in retina of AD patients, as brain depositions of this peptide is widely known to be one of the hallmarks of AD. The presence of retinal Aβ depositions has foremost been revealed by immunohistological stainings using either antibodies directed against the $A\beta_{42}$ or the $A\beta_{40}$ peptides (the two Aβ variants foremost associated with AD) or curcumin [3, 4], a dye binding to amyloid peptides in general. The retinal Aβ is found primarily within the neuronal cell layer [4], both intracellularly [5] and extracellularly as well as outside vessels [7], but the peptide has also been found within retinal drusen [8]. However, even though previous studies have demonstrated increased levels of retinal $A\beta_{42}$ in AD patients and transgenic AD mice [9] as well as a correlation between retinal Aβ depositions and AD severity [4], there are no studies verifying whether levels of Aβ correlate between brain and retina in humans. Thus, in order to search for further support of the potential use for retina observation as a diagnostic tool, the current study aims to investigate whether levels of $A\beta_{42}$ and $A\beta_{40}$ in retina correlate with the corresponding levels in hippocampus of individuals with low and high Aβ scores [non-demented controls (NC)) and patients with either multiple sclerosis (MS), AD, or AD with Lewy bodies (AD+LB)]. Since, we found it particularly interesting to investigate whether there is support for an association between Aβ depositions in the two organs, we ultra-centrifuged homogenates of retinas and hippocampi in order to yield high molecular weight samples before analyzing levels of $A\beta_{42}$ and $A\beta_{40}$. Since recent studies have suggested that islet amyloid polypeptide (IAPP), a pancreatic derived hormone peptide can seed Aβ [10] and thereby contribute to the Aβ plaque formation in the brain [11], we further found it interesting to investigate the potential relationship between IAPP and Aβ peptides in the retina. Thus, we also measured levels of IAPP in the ultra-centrifuged samples and analyzed its association with Aβ levels, neuropathological assessments, and Apolipoprotein E (*APOE*) genotype.

MATERIAL AND METHODS

Individuals included in the study

Frozen samples of midlevel hippocampus and retina (right eyeball) from clinically and postmortem verified patients with AD ($n = 5$), MS ($n = 4$), AD+LB ($n = 3$), and NC ($n = 4$) from the Netherlands Brain Bank (NBB) were included in the study. All individuals were included in the retinal and hippocampal $A\beta_{40}$ and $A\beta_{42}$ analysis and the hippocampal IAPP analysis. Five of these individuals (AD $n = 1$, MS $n = 3$, and NC $n = 1$) were missing in the retinal IAPP analysis. Single nucleotide polymorphisms at position rs429358 and rs7412 of the *APOE* genotype were determined by polymerase chain reactions using allele-specific primers as previously described [12]. Clinical diagnosis, *APOE* genotype, demographic data, and neuropathological assessment (Braak stages for presence of neurofibrillary tangles (NFT) and ABC stages for presence of Aβ burden) are presented in Table 1. Written consent for the use of tissue and clinical data for research purposes was obtained from all patients or their next of kin in accordance with the International Declaration of Helsinki. The medical ethics review committee of VU medical center, Amsterdam, has approved the procedures of tissue collection and the regional ethical review board in Lund has approved the study. All human data was analyzed anonymously.

Tissue extraction

The right eyeball of the patients was enucleated and the anterior segment (including the lens and cornea) as well as the vitreous was removed. Thereafter the eyecups were filled with Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA), snap frozen with isopentane cooled with liquid nitrogen and kept in –80°C until used. Retina samples (cut in 1.0×0.5 cm pieces 0.5 cm from the optic nerve in the far peripheral superior part) and hippocampal samples (CA1 and molecular layer) (approximately 30 mg), were homogenized in cell-lysis buffer (Sigma Aldrich, St. Louis, MO) together with protease inhibitors at 4°C and ultra-centrifuged $350{,}000 \times g$ for 1 h 4°C. The pellet was re-homogenized in 100% formic acid (10 μl/mg tissue), incubated 1 h room temperature and then centrifuged at $13{,}000 \times g$ for 20 min 4°C. The supernatant was lyophilized and then re-dissolved in DMSO to generate the high-molecular weight fraction (HMW) (for $A\beta_{42}$ and $A\beta_{40}$ analysis). The HMW fraction was then further processed by dissolving the fraction in guanine hydrochloride (GuHCl) (1:1) to create the HMW-GuHCl fraction (for IAPP analysis).

Table 1
Demographic data, apolipoprotein E (APOE) genotype, neuropathological evaluation, and cause of death of the individuals included in the study

Clinical diagnosis	Gender	Age (y)	Neuropathol. evaluation (NFT/Aβ/LB)	*APOE* genotype	Cause of death	Postmortem delay
NC	M	70	1/O/3	3/2	Pneumonia, cardiogenic shock	6h 20min
NC	M	81	3/C/0	4/3	Pancreas carcinoma	4h 30min
NC	F	92	3/O/0	3/4	Heart failure	6h 35min
NC	F	60	0/O/0	3/2	Mammacarcinoma	8h 10min
AD	F	85	5/C/0	3/4	Pneumonia	8h 15min
AD	F	70	6/C/0	4/3	Atrioventricular block	6h 10min
AD	F	91	6/C/0	4/4	Cachexia, severe AD	4h 20min
AD	F	78	6/C/0	4/4	Cachexia, severe AD	4h 45min
AD	F	64	4/C/0	3/3	Cerebrovascular accident	6h 37min
MS	F	60	0/A/na	3/4	Multiple sclerosis	7h 25min
MS	M	78	2/O/na	3/3	Euthanasia, Multiple sclerosis	8h 45min
MS	F	87	2/A/na	3/3	Renal insufficiency	9h 30min
MS	F	81	4/A/na	3/3	Cardiac asthma	9h 35min
AD+LB	F	83	4/C/6	3/3	Gastro-enteritis, dementia	6h 15min
AD+LB	F	88	5/C/5	3/3	Cachexia, vascular dementia	5h 40min
AD+LB	M	63	4/C/6	4/4	Hepatic insufficiency, metastases	4h 55min

Individuals neuropathologically diagnosed as non-demented controls (NC) or patients with Alzheimer's disease (AD), multiple sclerosis (MS), and Alzheimer's disease with Lewy bodies (AD+LB) included in the study. Braak staging of neurofibrillary tangles (NFT) and Lewy bodies (LB) and ABC stages of amyloid (Aβ). F, female; M, male; na, not analyzed.

Analysis of $A\beta_{40\text{-}HMW}$ and $A\beta_{42\text{-}HMW}$ in retina and brain tissue

Levels of $A\beta_{38\text{-}HMW}$, $A\beta_{40\text{-}HMW}$, and $A\beta_{42\text{-}HMW}$ of retina and brain tissue were analyzed using MesoScale Discovery V-plex Aβ Peptide Panel 1 kit with electrochemiluminescence detection technology (MesoScale Discovery, Rockville, MD) according to manufacturers' protocol. The electrochemiluminescence signal was quantified using a MesoScale Discovery SECTOR Imager 6000. The majority of the $A\beta_{38}$ values was below detection and will therefore not be reported in this study. The dynamic range for $A\beta_{40}$ were 2.26-14900 pg/ml and for $A\beta_{42}$ 0.482-1380 pg/ml. Protein concentrations were assessed using BCA Protein Assay (Thermo Fischer, Waltham, MA) according to the manufactures' instructions, and used to normalize Aβ levels. Retinal $A\beta_{42}$ and $A\beta_{40}$ values from two individuals (NC $n=1$ and MS $n=1$) and hippocampal $A\beta_{42}$ values from one individual (MS $n=1$) were below detection and were set to the lowest detected normalized value divided by two.

Analysis of IAPP in retina and brain tissue

Levels of IAPP in HMW-GuHCl fraction of retina and brain tissue were analyzed using Amylin EIA Kit (Peninsula Laboratories, San Carlos, CA) according to manufacturers' instructions. The absorbance was quantified using a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT). Protein concentrations were assessed using BCA Protein Assay (ThermoFischer) according to the manufactures instructions and used to normalize IAPP.

Statistical analysis

Statistical analysis was performed using SPSS software 24.0 (SPSS Inc., Chicago, IL). The Wilk-Shapiro test was used to assess normal distribution. Levels of $A\beta_{40\text{-}HMW}$ and $A\beta_{42\text{-}HMW}$ were not normally distributed and logarithmic transformations were therefore used to correct for the skewed data distributions. Differences in levels of $A\beta_{40\text{-}HMW}$, $A\beta_{42\text{-}HMW}$, and $IAPP_{HMW}$ were analyzed by use of student *t*-test (when two groups were compared) and ANOVA followed by Tukey HSD test (when four groups were compared). Differences in levels of $A\beta_{40\text{-}HMW}$ and $A\beta_{42\text{-}HMW}$ were also analyzed using ANCOVA analysis with *APOE* as covariate. Correlations between the investigated variables were examined using two-tailed Pearson correlation test, except for in the correlation analyses involving NFT scores and Aβ scores, which were performed using Spearman correlation test. Results are presented as mean ± standard deviation. A $p<0.05$ was considered significant.

RESULTS

Individuals included in the study

The demographic data on individuals included in the study is showed in Table 1. No significant differences in age were found between the groups (NC: 76 ± 14, AD: 78 ± 11, MS: 77 ± 12, AD+LB: 78 ± 13 years). Both females and males were included in the NC, MS, and AD+LB groups, whereas the AD patient group contained only females. AD and AD+LB patients had disorder-characteristic high amyloid plaque burden (stage C) and high numbers of NFT (Braak stages 4 and above). The MS patients displayed low or none of the AD or AD+LB characteristic neuropathological alterations. The individuals included in the NC group showed presence of NFT (stage 3 and below) and two individuals also showed either high amyloid plaque load (stage C) or presence of Lewy bodies (stage 3). Finally, 4 out of 5 AD patients as well as 2 out of 4 NCs were *APOE* ε4 carriers, whereas only 1 out of 3 AD+LB and 1 out of 4 MS patients carried the *APOE* ε4 allele.

Levels of high molecular weight Aβ in hippocampus

Statistical analysis of hippocampal samples showed significantly higher levels of $A\beta_{42\text{-HMW}}$ in both AD patients and AD+LB patients compared to NC and patients with MS (Fig. 1A). One of the NC showed $A\beta_{42\text{-HMW}}$ levels in the same range as AD patients (Fig. 1A). This NC had also high Aβ scores, explaining its divergent $A\beta_{42\text{-HMW}}$ value as well as indicating a relationship between high Aβ scores and hippocampal $A\beta_{42\text{-HMW}}$ levels. To further investigate this relationship, we divided the individuals into groups based on Aβ scores. Comparison analysis of these groups showed significantly higher hippocampal $A\beta_{42\text{-HMW}}$ levels in individuals with high Aβ scores (stage C) compared to individuals with low Aβ scores (stage O or A) (Fig. 1B).

Hippocampal $A\beta_{40\text{-HMW}}$ levels were also significantly higher in AD patients compared to MS patients, whereas the higher levels in AD+LB patients compared to MS patients did not reach significance. The individual with high Aβ scores in the NC group also showed high $A\beta_{40\text{-HMW}}$ levels and thus there were no differences between NC and the other patient groups (Fig. 1C). Comparison analysis based on Aβ scores showed that individuals with high Aβ scores (C) had significantly higher hippocampal $A\beta_{40\text{-HMW}}$ levels compared to those with low Aβ scores (O or A) (Fig. 1D). Finally, analysis of $A\beta_{\text{-HMW}}$ levels in *APOE* ε4 carriers across the groups showed that individuals carrying the *APOE* ε4 allele displayed higher, but not significant, levels of both $A\beta_{42\text{-HMW}}$ (457.5 ± 459.6 versus 159.4 ± 211.5) and $A\beta_{40\text{-HMW}}$ (202.4 ± 411.4 versus 133.6 ± 137.6).

Levels of high molecular weight Aβ in retina

Levels of $A\beta_{42\text{-HMW}}$ in retina were, in similarity to the corresponding levels in hippocampus, sig-

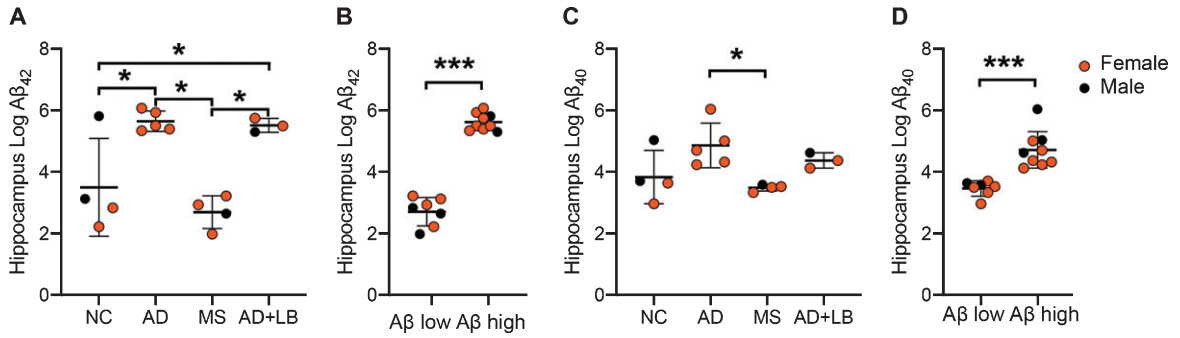

Fig. 1. Levels of $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ in hippocampus of individuals included in the study. Image in (A) shows a column scatter graph demonstrating higher log $A\beta_{42\text{-HMW}}$ in patients with Alzheimer's disease (AD) and Alzheimer's disease with Lewy bodies (AD+LB) compared to both non-demented controls (NC) and to patients with multiple sclerosis (MS). Image in (B) shows a column scatter graph demonstrating higher log $A\beta_{42\text{-HMW}}$ in individuals with high Aβ scores (C) compared to individuals with low Aβ scores (O or A). Image in (C) shows a column scatter graph demonstrating higher log $A\beta_{40\text{-HMW}}$ in AD patients compared to patients with MS. Image in (D) shows a column scatter graph demonstrating higher log $A\beta_{40\text{-HMW}}$ in individuals with high Aβ scores compared to individuals with low Aβ scores. Data is analyzed using ANOVA followed by Tukey HSD (A and C) and student *t*-test (B and D). Values are presented as mean value ± SD. Of note, $A\beta_{42}$ values from one MS patient were below the detection limit and set to the lowest detected normalized value divided by two before logistic transformation. *Significant correlation at $p < 0.05$ level. ***Significant correlation at $p < 0.001$ level.

nificantly higher in AD patients compared to MS (Fig. 2A). The $A\beta_{42\text{-HMW}}$ levels in AD+LB patients did not significantly differ compared to MS or NC and $A\beta_{42\text{-HMW}}$ levels in NC did not differ from levels in AD patients due to the one NC with high $A\beta_{42\text{-HMW}}$ levels and high Aβ scores (Fig. 2A). Comparison analysis based on Aβ scores showed significantly higher retinal $A\beta_{42\text{-HMW}}$ levels in individuals with high Aβ scores compared to individuals with low Aβ scores (Fig. 2B). Individuals carrying one or two *APOE4* ε4 alleles further displayed significantly higher retinal $A\beta_{42\text{-HMW}}$ levels compared to *APOE* ε4 non-carriers (Fig. 2C). Retinal levels of $A\beta_{40\text{-HMW}}$ showed a similar pattern as $A\beta_{42\text{-HMW}}$ levels, i.e., higher (although not significant) $A\beta_{40\text{-HMW}}$ levels in AD patients compared to MS but not to NC, due to high $A\beta_{40\text{-HMW}}$ levels in the individual with high Aβ scores (Fig. 2D). Levels of $A\beta_{40\text{-HMW}}$ were also significantly higher in individuals with high Aβ scores compared to individuals with low Aβ scores (Fig. 2E). Finally, individuals carrying one or two *APOE* ε4 alleles displayed significantly higher levels also of retinal $A\beta_{40\text{-HMW}}$ compared to *APOE* ε4 non-carriers (Fig. 2F). When the association to *APOE* was accounted for in an ANCOVA analysis (*APOE* as covariate) of $A\beta\text{-}_{HMW}$ levels in diagnostic groups, no significant differences between groups were found ($A\beta_{42\text{-HMW}}$ $p = 0.078$; $A\beta_{40\text{-HMW}}$ $p = 0.341$). The differences remained however significant when individuals with high versus low Aβ scores were compared ($A\beta_{42\text{-HMW}}$ $p = 0.006$; $A\beta_{40\text{-HMW}}$ $p = 0.011$).

Correlations between retinal and hippocampal levels of high molecular weight Aβ

Retinal levels of both $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ correlated with the corresponding levels in hippocampus (Fig. 3A, B). Moreover, NFT scores and Aβ scores correlated significantly with retinal

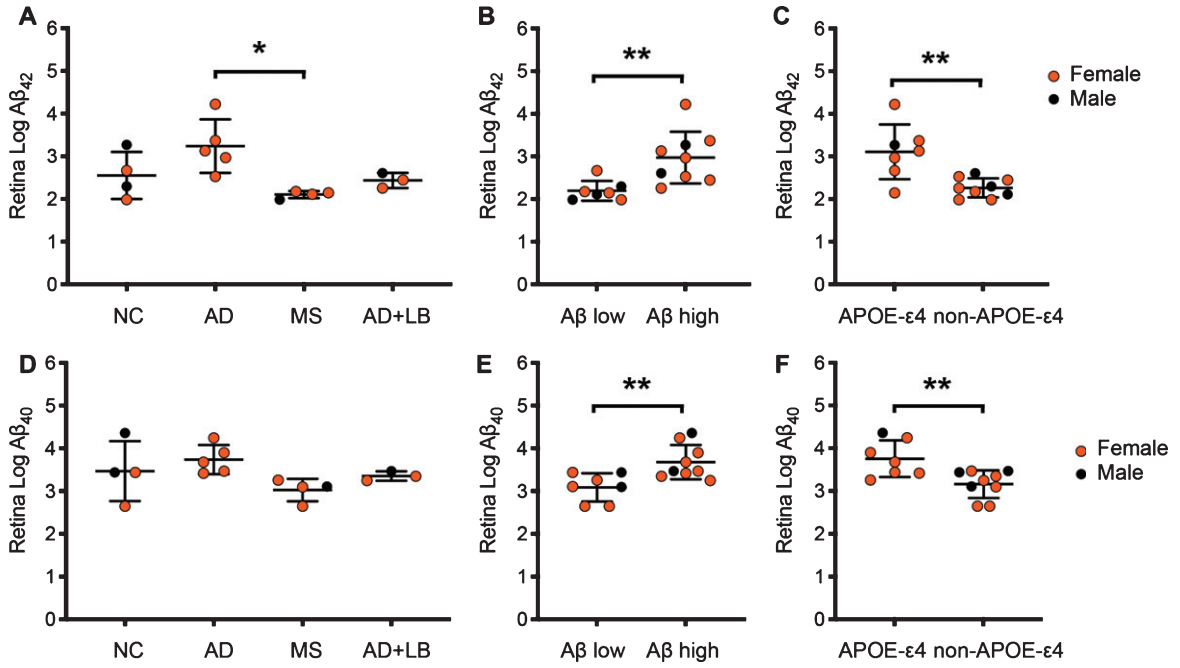

Fig. 2. Retinal levels of $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ of individuals included in the study. Image in (A) shows a column scatter graph demonstrating higher log $A\beta_{42\text{-HMW}}$ in patients with Alzheimer's disease (AD) compared to patients with multiple sclerosis (MS). The column scatter graph in (B) demonstrates the higher log $A\beta_{42\text{-HMW}}$ in individuals with high Aβ scores (C) compared to individuals with low Aβ scores (O or A). The column scatter graph in (C) shows higher log $A\beta_{42\text{-HMW}}$ in individuals carrying the *APOE* ε4 allele compared to *APOE* ε4 non-carriers. Image in (D) shows a column scatter graph demonstrating no significant differences in log $A\beta_{40\text{-HMW}}$ between diagnosis groups. The column scatter graph in (E) shows higher log $A\beta_{40\text{-HMW}}$ in individuals with high Aβ scores compared to individuals with low Aβ scores and the column scatter graph in (F) demonstrates higher log $A\beta_{40\text{-HMW}}$ in individuals carrying the *APOE* ε4 allele compared to *APOE* ε4 non-carriers. Data is analyzed using ANOVA followed by Tukey HSD (A and D) and student *t*-test (B, C, E, and F). Values are presented as mean value ± SD. Of note, $A\beta_{42}$ and $A\beta_{40}$ values from one NC and one MS patient were below the detection limit and set to the lowest detected normalized value divided by two before logistic transformation. *Significant correlation at $p < 0.05$ level. **Significant correlation at $p < 0.01$ level.

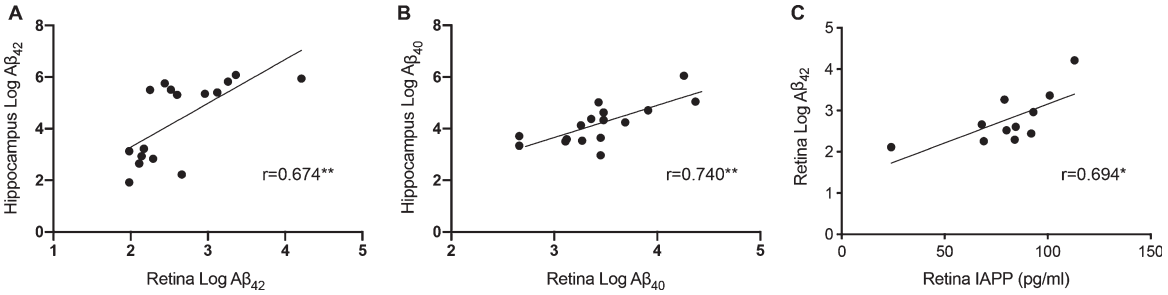

Fig. 3. Correlations between retinal and hippocampal levels of $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ and between retinal $A\beta_{42\text{-HMW}}$ and IAPP-$_{\text{HMW}}$. Scatter plot in (A) demonstrates correlations between retinal levels of $A\beta_{42\text{-HMW}}$ and hippocampal levels of $A\beta_{42\text{-HMW}}$. Scatter plot in (B) shows the relationship between retinal levels of $A\beta_{40\text{-HMW}}$ and hippocampal levels of $A\beta_{40\text{-HMW}}$. Scatter plot in (C) shows the association between retinal $A\beta_{42\text{-HMW}}$ and retinal IAPP-$_{\text{HMW}}$. Data was analyzed with Pearson correlation test. Of note, retinal $A\beta_{42}$ and $A\beta_{40}$ values from one NC and one MS patient as well as hippocampal $A\beta_{42}$ values from one MS patient were below the detection limit and set to the lowest detected normalized value divided by two before logistic transformation. *Significant correlation at $p<0.05$ level. **Significant correlation at $p<0.01$ level.

levels of $A\beta_{42\text{-HMW}}$ ($r=0.776$, $p=0.001$ and $r=0.693$, $p=0.004$, respectively) and $A\beta_{40\text{-HMW}}$ ($r=0.664$ $p=0.007$ and $r=0.630$, $p=0.012$, respectively). As expected, NFT scores and Aβ scores also correlated significantly with hippocampal levels of $A\beta_{42\text{-HMW}}$ ($r=0.682$, $p=0.005$ and $r=0.850$, $p=0.00006$, respectively) and $A\beta_{40\text{-HMW}}$ ($r=0.718$, $p=0.003$ and $r=0.850$, $p=0.00006$, respectively). The ratio between retinal and hippocampal $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ levels did not significantly differ although both the AD and AD+LB groups displayed lower retinal/hippocampus ratio of $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ compared to MS and NC ($A\beta_{42\text{-HMW}}$: 0.57 ± 0.09, 0.44 ± 0.04, 0.80 ± 0.16 and 0.79 ± 0.28, respectively; $A\beta_{40\text{-HMW}}$: 0.77 ± 0.08, 0.77 ± 0.02, 0.87 ± 0.06 and 0.92 ± 0.18, respectively). The $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ ratios were, however, significantly lower in individuals with high Aβ scores compared with low Aβ scores ($A\beta_{42\text{-HMW}}$: 0.52 ± 0.09 versus 0.84 ± 0.21, $p=0.001$, $A\beta_{40\text{-HMW}}$: 0.79 ± 0.66 versus 0.90 ± 0.14, $p=0.045$). No differences in either Aβ ratios were seen when comparing *APOE* ε4 carriers with *APOE* ε4 non-carriers ($A\beta_{42\text{-HMW}}$: 0.70 ± 0.23 versus 0.63 ± 0.21, $A\beta_{40\text{-HMW}}$: 0.86 ± 0.16 versus 0.81 ± 0.073). No associations between the different variables (retinal and hippocampal $A\beta_{42\text{-HMW}}$, $A\beta_{40\text{-HMW}}$, or Aβ ratios) and age or gender were found.

Levels of islet amyloid polypeptide in retina and hippocampus

No significant alterations in levels of retinal IAPP-$_{\text{HMW}}$ were found between any of the patient groups. However higher, but not significant, retinal IAPP-$_{\text{HMW}}$ levels were seen in individuals with high Aβ scores compared to those with low Aβ scores (69.1 ± 15.5 versus 44.9 ± 28.1). In the corresponding hippocampal fraction, significantly higher levels of IAPP-$_{\text{HMW}}$ in AD+LB patients compared to AD (106.0 ± 35.6 versus 54.8 ± 19.2, $p=0.040$) were found. No significant differences were seen between AD or MS patients compared to NCs (54.8 ± 19.2 versus 57.7 ± 7.0; 65.5 ± 7.7 versus 57.7 ± 7.0, respectively) and the hippocampal IAPP-$_{\text{HMW}}$ levels did not differ between individuals with low or high Aβ scores (72.1 ± 33.9 versus 62.6 ± 8.4). No significant changes in retinal IAPP-$_{\text{HMW}}$ levels or hippocampal IAPP-$_{\text{HMW}}$ levels were seen when *APOE* ε4 carriers were compared to *APOE* ε4 non-carriers (74.2 ± 16.0 versus 52.6 ± 21.2 and 55.8 ± 16.3 versus 81.6 ± 31.1, respectively).

Correlations between retinal levels of IAPP-$_{HMW}$ and $A\beta_{42\text{-HMW}}$

Finally, correlation analysis showed that retinal levels of $IAPP_{HMW}$ correlated with retinal levels of $A\beta_{42\text{-HMW}}$ (Fig. 3C), NFT ($r=0.822$, $p=0.002$) and a tendency with Aβ scores ($r=0.581$, $p=0.061$). No such correlations were found between retinal IAPP-$_{\text{HMW}}$ levels and retinal $A\beta_{40\text{-HMW}}$ levels or between hippocampal IAPP-$_{\text{HMW}}$ levels and hippocampal levels of $A\beta_{42\text{-HMW}}$ or $A\beta_{40\text{-HMW}}$.

DISCUSSION

In the current study, we demonstrate higher levels of $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ in both retina and

hippocampus from individuals with high Aβ scores and individuals carrying the *APOE* ε4 allele. The retinal levels of both amyloid peptides correlated with hippocampal levels of the same as well as with neuropathological evaluations of NFT and Aβ. We also demonstrate a correlation between levels of $A\beta_{42\text{-HMW}}$ and IAPP$_{\text{-HMW}}$ in the retina but not in the hippocampus. Retinal IAPP$_{\text{-HMW}}$ levels were higher in individuals with high Aβ scores and correlated positively with neuropathological assessments. No differences in hippocampal IAPP$_{\text{-HMW}}$ levels were seen and *APOE* ε4 carriers did not show altered retinal or hippocampal IAPP$_{\text{-HMW}}$ levels.

The number of analyzed individuals in each group was small with only 4 individuals in the NC group, 4 individuals in the MS group, 3 individuals in the AD+LB group, and 5 individuals in the AD group. Such a small sample size can undeniably lead to both under and over interpretations of the results and it is important to emphasize that the study needs to be repeated in a larger group of individuals. To increase the power and reduce potential misinterpretations, we have chosen to group the individuals based on their neuropathological staging for Aβ (high = stage C and low = stage O or A) instead of based on diagnosis and will in this discussion only reflect upon the results yielded after such grouping.

The analysis of hippocampal homogenates showed expected higher levels of $A\beta_{40\text{-HMW}}$ and $A\beta_{42\text{-HMW}}$ in individuals with high Aβ scores and individuals carrying the *APOE* ε4 allele, verifying that our tissue extraction and analysis is appropriate. Analysis of retinal samples further showed that individuals with high Aβ scores also had significantly higher retinal $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ levels, suggesting that individuals with high hippocampal Aβ burden also have high levels of Aβ present in the retina. This idea was confirmed by our correlation analysis demonstrating an association between retinal and hippocampal $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ levels. It is thus plausible that accumulation of Aβ in retina can reflect the corresponding accumulation in the brain, as suggested previously [4, 7]. In the current study we chose to compare Aβ levels in retina with the same levels in hippocampus. The rationale for choosing this brain region for the comparison analysis is the notion that hippocampus is affected by AD pathology [13], which eventually leads to the characteristic memory loss seen in AD patients. Inarguable, it would be of interest to also compare retinal Aβ levels with Aβ levels in other brain regions affected by AD pathology (for example, precuneus which is affected by AD at the earliest stages [14]) in order to further understand the temporal association between retina and brain accumulation of Aβ. Nevertheless, since the retinal $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ levels also correlated with NFT scores, it is tempting to speculate that retinal Aβ reflects general AD pathology. This idea is supported by several transgenic AD mice studies demonstrating a correlation between increased disease progression and retinal plaque load/$A\beta_{42}$ levels [4, 9, 15, 16]. A similar correlation between retinal Aβ plaque load and AD severity has also been found in a human postmortem study [3].

The significant lower ratio of retinal and hippocampal $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$ in individuals with high Aβ scores compared low Aβ scores suggests that Aβ in the retina does not form high molecular weight structures proportional to similar formation in the hippocampus. We thus draw the conclusion that Aβ accumulates and possibly aggregates at a higher degree in hippocampus compared to retina.

Our study further showed that individuals carrying one or two *APOE* ε4 alleles had significantly higher retinal levels of both $A\beta_{42\text{-HMW}}$ and $A\beta_{40\text{-HMW}}$, a finding not previously reported. Interestingly, *APOE* has been shown to be important for vascular development and previous *in vivo* studies have demonstrated vascular and synaptic impairments in the retina of *APOE* ε4 mice [17]. However, even though no histological differences in retina were seen between native *APOE* ε4 or *APOE* ε3 mice, the previous study showed that *APOE* ε4 mice have a stronger inflammatory response after laser driven injury compared to *APOE* ε3 mice [18]. Although we can only speculate upon the significance of *APOE* ε4 in retinal Aβ accumulation, it is most likely that retinal *APOE* ε4, just like brain *APOE* ε4, affects metabolism, aggregation and clearance of Aβ (for review, see [19]).

Previous experimental studies have shown that IAPP can seed Aβ and thus the peptide has been suggested to be involved in Aβ plaque formation in the brain of AD patients. We have previously shown increased levels of low molecular weight IAPP and decreased levels of high molecular weight IAPP in the retina of AD patients. The ELISA that we used in the former study detects only IAPP with an intact disulfide bond and a N-terminal amide group, i.e., the biologically active version of IAPP considered to be less prone to form toxic aggregates. In the current study, we instead used an EIA assay, where the IAPP in the samples competitively is bound to antibodies directed against both intact and modified versions of IAPP. Using this kit, we detected higher levels of

retinal $IAPP_{HMW}$ in individuals with high Aβ scores, and moreover a correlation between these levels and retinal levels of $Aβ_{42\text{-}HMW}$. Since the $Aβ_{\text{-}HMW}$ samples theoretically foremost contain aggregates as in plaques, we draw the conclusion that our results support the idea that IAPP is associated with Aβ plaque formation as previously suggested [20].

Although previous studies have demonstrated increased levels of IAPP in temporal cortex of AD patients [21, 22], we found no alterations in hippocampal $IAPP_{\text{-}HMW}$ levels in individuals with high Aβ scores and no correlations between levels of retinal $IAPP_{\text{-}HMW}$ and hippocampal $IAPP_{\text{-}HMW}$. We did however notice a positive correlation between levels of retinal $IAPP_{\text{-}HMW}$ and NFT as well as Aβ scores. These findings are in line with our previous study demonstrating unaltered hippocampal IAPP levels in AD patients, but positive correlations between retinal low molecular weight IAPP, NFT, and Aβ scores. The results are interesting as they could indicate that the presence of IAPP and its impact on Aβ plaque formation differ between brain regions and that IAPP only (if any) plays a minor seeding role in hippocampus. If this holds true, the association between retinal IAPP levels and neuropathological assessments could indicate that IAPP along with retinal IAPP is increased in other brain areas of AD patients. Further studies on homogenates from different brain regions are however required to verify this hypothesis. Another possibility is that IAPP accumulation in retina precedes the same in hippocampus, but this idea goes against the hypothesis that IAPP is involved in Aβ plaque formation.

Finally, our study analyses levels of $Aβ_{HMW}$ in homogenates of retina and hippocampus and does thus not provide information regarding the cellular localization of the analyzed peptide. However, when collecting the retina, we were careful to not include the retinal pigment epithelium cell layer in the samples. We can thus conclude that the $Aβ_{40}$ and $Aβ_{42}$ levels found in our samples most likely represent intracellular or extracellular Aβ in the retina and do not derive from amyloid depositions in drusen.

To conclude, our studies show that both $Aβ_{40}$ and $Aβ_{42}$ can be found in the human retina. They also indicate that elevated levels of high molecular weight $Aβ_{40}$ and $Aβ_{42}$ in hippocampus are associated with increased levels of the same in the retina, a finding supporting the idea that the retina can mirror senile plaque formation in the hippocampus. Moreover, since our studies also indicated that levels of $Aβ_{42\text{-}HMW}$ are associated with *APOE* ε4 and levels of $IAPP_{HMW}$, we conclude that these two factors might be implicated in the accumulation of Aβ in the retina of individuals with high Aβ scores.

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