#PYROGLUTAMATE AND ISOASPARTATE MODIFIED AMYLOID-BETA IN AGEING AND ALZHEIMER'S DISEASE#

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**Abstract:**
Alzheimer's disease (AD) is the most common cause of dementia among older adults. Accumulation of amyloid-β (Aβ) in the brain is considered central in AD pathogenesis and its understanding crucial for developing new diagnostic and therapeutic approaches. Recent literature suggests that ageing may induce post translational modifications in Aβ, in the form of spontaneous amino acid modifications, which enhance its pathogenic properties, contributing to its aggregation.

In this study, we have investigated whether the isoaspartate (IsoD-Aβ) and pyroglutamate (pE3-Aβ) modified forms of Aβ are significantly associated with AD pathology or represent markers of ageing. Cerebral neocortex of 27 AD cases, 32 old controls (OC) and 11 young controls (YC) was immunostained for pE3-Aβ and IsoD-Aβ, quantified as protein load and correlated with other Aβ forms and p-TAU. IsoD-Aβ and pE3-Aβ were detected at low levels in non-demented controls, and significantly increased in AD (p≤0.001), with a characteristic deposition of IsoD-Aβ in blood vessel walls and pE3-Aβ within neurons. Both AD and OC showed positive associations between IsoD-Aβ and Aβ (p=0.003 in AD and p=0.001 in OC) and between IsoD-Aβ and pE3-Aβ (p=0.001 in AD and OC). This last association was the only significant pE3-Aβ correlation identified in AD, whereas in the control cohorts pE3-Aβ also correlated with Aβ and AβPP (p=0.001 in OC and p =0.010 in YC).

Our analyses suggest that IsoD-Aβ accumulation starts with ageing; whereas pE3-Aβ deposition is more closely linked to AD. Our findings support the importance of age-related modifications of Aβ in AD pathogenesis.

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PYROGLUTAMATE AND ISOASPARTATE MODIFIED AMYLOID-BETA
IN AGEING AND ALZHEIMER’S DISEASE

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Abstract

Alzheimer's disease (AD) is the most common cause of dementia among older adults. Accumulation of amyloid-β (Aβ) in the brain is considered central in AD pathogenesis and its understanding crucial for developing new diagnostic and therapeutic approaches. Recent literature suggests that ageing may induce post translational modifications in Aβ, in the form of spontaneous amino acid modifications, which enhance its pathogenic properties, contributing to its aggregation.

In this study, we have investigated whether the isoaspartate (IsoD-Aβ) and pyroglutamate (pE3-Aβ) modified forms of Aβ are significantly associated with AD pathology or represent markers of ageing. Cerebral neocortex of 27 AD cases, 32 old controls (OC) and 11 young controls (YC) was immunostained for pE3-Aβ and IsoD-Aβ, quantified as protein load and correlated with other Aβ forms and p-TAU. IsoD-Aβ and pE3-Aβ were detected at low levels in non-demented controls, and significantly increased in AD (p≤0.001), with a characteristic deposition of IsoD-Aβ in blood vessel walls and pE3-Aβ within neurons. Both AD and OC showed positive associations between IsoD-Aβ and Aβ (p=0.003 in AD and p=0.001 in OC) and between IsoD-Aβ and pE3-Aβ (p=0.001 in AD and OC). This last association was the only significant pE3-Aβ correlation identified in AD, whereas in the control cohorts pE3-Aβ also correlated with Aβ and AβPP (p=0.001 in OC and p =0.010 in YC).

Our analyses suggest that IsoD-Aβ accumulation starts with ageing; whereas pE3-Aβ deposition is more closely linked to AD. Our findings support the importance of age-related modifications of Aβ in AD pathogenesis.
KEY WORDS: IsoAspartate, pyroglutamate, Alzheimer’s disease, amyloid-beta, ageing.
Introduction

Alzheimer’s disease (AD) is the most common cause of dementia in the elderly population. The molecular pathogenesis of this disease is only partially understood and the comprehension of new key aspects of its neurobiology is crucial for the future development of early diagnostic approaches and effective treatments [34]. AD is characterized by the intracellular and extracellular accumulation in the brain of aggregated amyloid-β (Aβ), and of hyperphosphorylated tau (p-TAU) within the neurons [32]. Aβ misfolding, resulting in its aggregation into oligomers, protofibrils and fibrils, is considered crucial in the development of AD [33] and synergistic with tau pathology [24]. Aβ is a family of peptides generated by β- and γ-secretase sequential cleavages of the amyloid precursor protein (APP) and currently regarded as one of the major therapeutic targets in AD [34].

Ageing is considered the main risk factor for AD, but the pathway through which ageing contributes to Aβ misfolding remains unclear. In particular, we still do not know whether ageing induces molecular changes in Aβ, driving its accumulation in the brain. Recent literature suggests that ageing may induce post-translational modifications, in particular spontaneous Aβ amino acid modifications, defined as age-related modifications, which enhance its pathogenic properties [9, 21, 23, 27, 44], leading to the “Protein ageing Hypothesis of AD” [23]. It is conceivable that accumulation of age-related amino acid modifications could start early in the ageing process [21]. Consequently, the understanding of the relationship between age-related-modifications of Aβ and AD pathology could potentially identify new diagnostic and therapeutic approaches in early AD.

Prominent examples of these age-modified forms of Aβ include isomerization (isoD-Aβ) and racemization of aspartate residues, and pyroglutamate formation at the N-
terminal of Aβ (pE-Aβ) [14]. IsoD-Aβ, similarly to the racemized form of aspartate, is the result of a chemically spontaneous and non-enzymatic reaction that introduces an additional methylene group in the peptide backbone of Aβ [37]. The formation of pE-Aβ is the consequence of a truncation at the level of a N-terminal glutamate, followed by the dehydration catalyzed by Glutaminyl Cyclase to form the cyclic pyroglutamate [11]. Evidence supports a direct role of these modifications in altering the intrinsic properties of Aβ, as to accelerate its deposition, or to impair its clearance and degradation [23, 37]. In vitro studies have shown that IsoD-Aβ was associated with accelerated Aβ aggregation and fibril formation [23, 36]; and known mutations, where aspartic acid of Aβ is replaced by asparagine and then modified into isoD, are associated with early-onset AD and high levels of Aβ deposition [3, 6, 42]. Similar observations were reported for pE-Aβ, in particular with the modification at the glutamate in position 3 of Aβ (pE3-Aβ). It is toxic in primary culture of neurons and astrocytes [28], and its expression in mouse and Drosophila brains acts as an important source of toxicity, displaying an accelerated aggregation, enhanced synaptic toxicity, high stability and resistance to degradation [19, 28, 31, 38]. Moreover, it was demonstrated that small amounts of pE-Aβ oligomers are sufficient to trigger the aggregation of unmodified Aβ1-42, leading to the formation of hypertoxic Aβ1-42 oligomers [22]. Of note, passive immunization with a pE-Aβ monoclonal antibody in APPswe/PS1ΔE9 AD mouse model, was able to lower Aβ plaque burden and prevent cognitive impairment [4]. Therefore, the formation of pE- and IsoD-Aβ may have a role in the pathological process of Aβ aggregation and accumulation.

In this study, we have addressed the question whether these Aβ modifications are significantly associated with AD pathology or if they represent physiological markers.
of ageing, using post-mortem brain tissue from AD cases compared to non-
neuropathological old and young controls.

Material and methods

Case selection

Seventy post-mortem cases were investigated divided among 3 cohorts as follows: 27
AD cases, 11 young controls defined as with no significant neuropathological
abnormality (YC, <63 years old) and 32 old control cases with no significant
neuropathological abnormality (OC, ≥ 63 years old), age-matched with the AD
cohort. A summary of the cohorts is presented in Table 1 with additional information
available in table S1 (Additional file 1). All AD cases had a clinical diagnosis of
probable Alzheimer’s disease according to NINCDS–ADRDA criteria and cases with
concomitant pathology were excluded. Diagnosis was made during life by an
experienced clinician and post-mortem neuropathological consensus criteria for AD
were satisfied, including Braak stage, by an experienced neuropathologist.

Immunohistochemistry

Four μm formalin-fixed paraffin-embedded sections from the inferior parietal lobule
(Brodmann area 40) were retrieved from the Brain banks for all cases. Protocols for
tissue fixation and processing were similar in both brain banks. In addition, the
staining was performed in batches with each batch including cases from all 3 cohorts
to ensure compatibility of the staining. The following primary mouse monoclonal
antibodies were used: 22C8 against 1-12 Aβ with 1,7 IsoAspartate modification
(IsoD-Aβ) provided by Elan Pharmaceuticals Inc., US [29, 30]; 337.48 specific to Aβ
with pyroglutamate at the third glutamate position (pE3-Aβ, BioLegend, US); 4G8,
specific for the amino acid residues 17-24 of Aβ, which reacts to the abnormally processed isoforms, as well as precursor forms (AβPP) [1, 2, 12](BioLegend, US); 82E1, an Aβ N-terminal specific antibody, which does not cross react with non-β secretase cleaved APP (IBL, Japan, [8]); AT8, directed against the human tau, phosphorylated at Ser202 and Thr205 (p-TAU) (ThermoFisher Scientific, US) (Figure 1 and Table 2).

Immunohistochemistry was performed using the appropriate antigen retrieval method (Table 2). Biotinylated secondary antibody (rabbit anti-mouse) was from Dako (Denmark), and normal serum and avidin–biotin complex from Vector Laboratories (UK). Bound antibody was visualized using the avidin–biotin–peroxidase complex method (Vectastain Elite ABC) with 3,3′-diaminobenzidine as chromogen and 0.05% hydrogen peroxide as substrate (both from Vector Laboratories, UK). All sections were dehydrated before mounting in DePeX (VWR International, UK). Sections incubated in the absence of the primary antibody were included as negative controls.

**IsoD-Aβ and pE3-Aβ quantification**

Quantification was performed blind to the experimental group and identity of the cases. For each antibody and case, 30 images of cortical grey matter were taken using a x20 objective lens, in a zigzag sequence in order to ensure that all cortical layers were represented in the quantification. The sampling pattern between all cases was consistent, starting at the depth of the sulcus and progressing up the sulcal wall to the gyral surface. The acquired images were analysed using ImageJ version 1.49 software (developed by Wayne Rasband NIH, US) with a threshold applied to the image to select and measure the total amount of specific immunostaining. The same threshold setting was maintained for all images of all cases stained for the same
antibody, and the area fraction of the measure function provided the proportion (%) of
the stained area related to the total area of the image (expressed as protein load).

**Semi Quantitative assessment**

Immunodetection for Iso-DAβ and pE3-Aβ was assessed as present or absent
according to the staining being defined as: intraneuronal deposits [5], dense-core
plaques, diffuse plaques and vessel wall deposits (i.e. cerebral amyloid angiopathy: CAA) [35]. The staining was independently reviewed by two operators.

**Statistical analysis**

To compare the protein load of the different Aβ forms and p-TAU between the
cohorts, the normality of each marker was assessed through examination of quantile-
quantile plots (not shown). As the data were non-parametric, the Kruskal-Wallis test
was performed for comparison among the three groups for each marker. To assess
whether the presence of pE3-Aβ and IsoD-Aβ immunostaining in intraneuronal
deposits, dense-core plaques, diffuse plaques and CAA differed significantly between
AD and OC and OC and YC, the Fisher's Exact Test was used.

To investigate the relationship of isoD-Aβ and pE3-Aβ with the other Aβ forms and
p-TAU, correlations were performed using the Pearson or Spearman correlation
coefficients, as determined by the normality of the markers. The threshold for
statistical significance was set at 5% for intergroup comparisons and Fisher’s test and
1% for correlations, as determined by the use of SPSS 21.0 (IBM, US).
Results

Quantification of IsoD and pE3-Aβ

The quantification of Iso-D-Aβ, independently of the location of the staining, revealed that Iso-D-Aβ load was significantly higher in AD compared to OC (p<0.001) and AD vs YC (p=0.001) cohorts. No significant difference was identified between OC and YC groups (Figure 2a).

Similarly, the pE3-Aβ load was significantly higher in AD vs OC (p=0.001) and AD vs YC (p<0.001) cases; while no significant difference was found between OC and YC groups (Figure 2e).

IsoD-Aβ and pE3-Aβ immunodetection and locations

IsoD-Aβ and pE3-Aβ immunostaining was observed in the commonly identified Aβ locations in the brain: intraneuronal deposits, diffuse deposits, dense-core plaques, and CAA (Figure 3a-d and f-i). However significant differences were observed between OC and AD regarding the presence of some of these neuropathological features (Figure 3e and 3j).

Overall, the comparison between AD and OC cases revealed that IsoD-Aβ immunostaining in core plaques and CAA was significantly more frequent in the AD vs OC cases (core plaques: AD=96% vs OC=44%, p<0.0001; CAA: AD=63% vs OC=28%, p=0.009) (Figure 3e). IsoD-Aβ intraneuronal deposits were also significantly more present in AD than OC; however this feature was limited to only a minority of AD cases (AD=18%; OC=0%, p=0.039). No significant difference was observed instead in relation to IsoD-Aβ stained diffuse deposits (p= 0.118) (Figure 3e).
No IsoD-Aβ staining was identified in YC (Figure 5j), with the exception of 2 cases among the oldest, who were 59 and 62 years old and had some diffuse and core plaques (Figure 5l).

pE3-Aβ immunostaining was significantly more present in AD vs OC cases in intraneuronal deposits (AD=52% vs OC=9%, p<0.001); in diffuse plaques (AD=89% vs OC= 44 %, p<0.001); and core plaques (AD=100% vs OC=63%, p<0.001) (Figure 3j). No significant difference was observed instead in relation to pE3-Aβ CAA (p = 0.065).

pE3-Aβ was negative in the majority of YC (Figure 5k). Only two YC cases, the same cases which have sparse isoD-Aβ deposits, also had sparse pE3-Aβ-positive immunostaining mainly as diffuse plaques, with the case aged 62 also having few core plaques (Figure 5m).

**Relationship between IsoD, pE3-Aβ and hallmarks of AD**

Correlations were performed in the 3 cohorts between IsoD and pE3-Aβ loads each other, and respectively, with AβPP (clone 4G8), Aβ (clone 82E1), and p-TAU (AT8 antibody). All analyses are presented in Table 3 and figure 4 and the representative pictures in figure 5. In the AD group, two significant positive correlations were found for IsoD-Aβ with pE3-Aβ (ρ= 0.582, p=0.001) and Aβ (ρ= 0.545, p=0.003). In the OC cohort, significant positive associations were identified for IsoD-Aβ with pE3-Aβ (ρ= 0.557, p=0.001), Aβ (ρ= 0.578, p=0.001) and AβPP (ρ= 0.591, p<0.001).

For pE3-Aβ in OC cohort, in addition to the correlation identified with IsoD-Aβ, there were positive significant associations with Aβ (ρ= 0.609, p<0.001) and AβPP (ρ=0.570, p=0.001). Within the YC group, a positive correlation was observed between AβPP and pE3-Aβ load (ρ= 0.736, p=0.010).
Discussion

In this study, we have investigated two post-translational modifications of Aβ, IsoD-Aβ and pE3-Aβ, normally defined as age-related modifications [11, 21, 25], and explore whether their accumulation is significantly increased in AD patients compared with age-matched controls and younger controls. Our findings show that both IsoD-Aβ and pE3-Aβ can be detected at a low level in non-demented age-matched controls but both are at significantly higher levels in AD.

It is possible that these modified forms of Aβ start to form and aggregate years before other molecular lesions and symptoms of disease clearly appear. Indeed pE3-Aβ has been detected in the past not only in plaques but also in dispersible and soluble Aβ aggregates outside the plaques and described in pathologically preclinical AD cases [26]. Consequently in our study, some of non-demented age-matched controls might have actually died after IsoD-Aβ and pE3-Aβ accumulation in the brain had started, but before the formation of a significant Aβ burden.

The higher level of IsoD-Aβ in AD may be explained by the properties of the amino acid of Aβ. There is evidence that IsoD forms most easily at sequences in which the side chain of the C-flanking amino acid is relatively small and hydrophilic [36]. The most favourable C-flanking amino acids are Glycine (G), Serine (S), and Histidine (H). The Aβ sequence has S and G residues at the C-term of aspartate 7 (D7), rendering this residue most likely to be spontaneously transformed to IsoD with ageing. The other aspartate residue analysed, D1, is the first amino acid of the Aβ sequence, which is also the β-secretase site of APP. D1 does not have C-term residues favourable to spontaneous isomerization. However, its isomerization in AD can be driven by other chemical conditions, e.g. oxidative stress and production of
radicals [18] as demonstrated for D-racemization [13, 40], occurring through the same chemical intermediate of D-isomerization [21]. Therefore, it is reasonable to hypothesize that in AD, IsoD-Aβ accumulates at a significantly higher level than during ageing due to faster isomerization of D7 or to a possible additional modification of D1; processes that are likely not to be mutually exclusive.

The increased pE3-Aβ in AD may be explained by increased expression of Glutaminyl cyclase (QC), the enzyme that catalyses the conversion of glutamate into pyroglutamate. QC was found in the cortex of AD patients [20] and its levels correlated with insoluble pE3-Aβ aggregates, and not with unmodified Aβ peptides. Noteworthy, in the same patients, the insoluble pE3-Aβ correlated with the cognitive decline better than elevated level of unmodified Aβ [20].

As expected, in our study, the majority of YC cases did not have IsoD- or pE3-Aβ forms. Of note, the only cases that had some immunostaining were among the oldest, aged 59 and 62 years, and thus close to the old control group.

Similar observations were reported in a study focused on brain tissues of cases with Down Syndrome (DS). Trisomy 21 is associated with the progressive development of AD neuropathology as observed in DS people in their forties. A study reported that deposits containing pE3-Aβ were not detectable in DS cases under 27 years old, while they were present in older cases [16].

Interestingly, our assessment of different features of Aβ deposits shows that the differences between AD and controls are not only quantitative, but also related to the specific compartments of brain parenchyma and vasculature where IsoD-Aβ and pE3-Aβ accumulate. IsoD-Aβ was largely extracellular, in core plaques as well as in blood vessel walls, in accordance with previous published data [14, 36]. While the majority of AD cases had both these features, they were less prevalent in the OC
group. In AD, IsoD-Aβ in core plaques and CAA was more prevalent than IsoD-Aβ within neurons. This is in agreement with the results of a previous study focused on DS and small group of AD and OC with IsoD-Aβ detected in amyloid cores and vascular amyloid [10]. The limited intraneuronal detection of IsoD-Aβ is consistent with the location in the cytoplasm and endoplasmatic reticulum of L-isoaspartyl methyltransferase, the enzyme able to repair IsoD to L-aspartyl residue. This explains that possibilities to repair extracellular IsoD are very limited [36], implying that once extracellular IsoD-Aβ deposits are formed, differently from intraneuronal deposits, they might likely remain unrepaired.

We detected pE3-Aβ in the vasculature, as previously reported [17, 41], but in our larger cohorts the prevalence of this feature was not significantly greater in AD than controls. However, pE3-Aβ in AD was found in diffuse deposits, core plaques and intraneuronal deposits, in agreement with previous studies [17, 45] and in these specific locations, pE3-Aβ was significantly more frequent in AD than OC. Of note, pE3-Aβ in core plaques was the only feature observed in the majority of both AD and OC. A larger difference in the comparison of pE3-Aβ neuropathological features between OC and AD was related to intraneuronal pE3-Aβ, which was limited to few OC cases while present in 52% of AD. This suggests that intraneuronal accumulation of pE3-Aβ might be characteristic of AD, compatible with an increased somatic activity of QC enzyme. The presence of pE3-Aβ in plaques, might result instead from the possibility of secretion of this enzyme into the extracellular space, as observed in some experimental studies [7].

Our correlation analyses showed a number of relationships between IsoD and pE3-Aβ and hallmarks of AD. We observed positive associations of IsoD-Aβ with pE3-Aβ and Aβ (peptide with no N-terminal modifications) in AD. Of note, the same
relationships were found in OC, although they had less IsoD-Aβ. This suggests that
the presence of IsoD is associated with a process of accumulation of Aβ forms,
already in progress with ageing. This is indeed in agreement with studies showing
that isoaspartate post-translational modifications affect the fibrillization and toxicity
properties of Aβ [3], possibly with a role in seeding the accumulation of Aβ species
[43]. Interestingly in OC, both IsoD- and pE3-Aβ showed associations with AβPP
and Aβ loads. In AD, despite the increased accumulation of IsoD-Aβ and pE3-Aβ,
there was no association of the two modified forms with AβPP and only IsoD-Aβ
correlated with Aβ load, while pE3-Aβ was independent from it. This may imply that
different mechanisms control the accumulation of these Aβ modifications in ageing
and in AD, with the existence of a direct link between IsoD- / pE3-Aβ accumulation
and AβPP only during ageing.

p-TAU was associated with pE3-Aβ in both AD and OC cohorts, but not with the
same strength as the other described associations. This relationship was already
described in a study performed in 18 AD and 23 age-matched controls [17], where the
data of both cohorts were combined. Accordingly, when we pooled AD and OC data,
our association became stronger ($\rho=0.6704$, $p<0.0001$) and similar to the previous
published study, as shown in Figure S1 (Additional file 1). Therefore, it is reasonable
to hypothesize that the presence of pE3-Aβ in the brain leads to further
neuropathological lesions, like the hyperphosphorylation of tau.

In summary, we have observed that high levels of IsoD- and pE3-Aβ are present in
AD brain. IsoD-Aβ accumulation appears to be mainly related to ageing and, both in
old and AD brains, this modification is associated with the build-up of unmodified Aβ
as well as pE3-Aβ. In AD, IsoD-Aβ load is significantly higher than in OC and also
accumulates significantly more in blood vessel walls. In contrast, pE3-Aβ appears to
be more specifically linked to the disease process. In AD brain, pE3-Aβ deposition is significantly higher than in controls with a characteristic location in neurons.

However, both post-translational modifications cannot be related at the moment to any temporal sequences of events.

**Conclusions**

These findings underline the importance of age-related post-translational modifications of Aβ in relation to AD. They complement the studies that have revealed the complexity of Aβ chemistry and activity [3, 15]. In addition, our observations support the relevance of investigating IsoD-Aβ and pE3-Aβ levels as potential biomarkers for AD and the impact of the different Aβ-targeted therapies on these modified forms of Aβ. This research could benefit in the future from the development and optimization of new analytical methods [46], able to separate with high resolution the different forms of Aβ peptides in biological fluids.

**List of abbreviations**


**Declarations**
Ethics approval and consent to participate

The study was covered by the ethical approval from the Brain banks that provided the tissue. The YC cohort was sourced from BRAIN UK (NRES Committee South Central Hampshire B, REC reference: 14/SC/0098), OC and AD cases from the South West Dementia Brain bank (NRES Committee South West Central Bristol, REC reference: 08/H0106/28+5). All donors have given informed consent for autopsy and use of their brain tissue for research purposes.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions
MLM, AM and DB designed the study. MLM, ASP, KG, CP performed all experiments. JARN provided assistance with the neuropathological assessments; MLM and DB analysed and interpreted the data. MLM wrote the manuscript and DB, JARN and AM reviewed the manuscript. All authors read and approved the final manuscript.

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**Additional files**

Additional file 1 contains all supplemental information mentioned in this manuscript: table S1 and its description and figure S1 and its description.
References


Legends

**Fig. 1 Regions of APP recognized by the antibodies used in this study.**

Top of the figure: Illustration of APP. Aβ region is labelled in yellow and β and γ-secretase cleavage sites are indicated in red.

Bottom of the figure: Illustration of the Aβ and APP regions recognized by 22C8, 337.48, 82E1, 4G8 clones. The positions of IsoD- and pE3-Aβ modifications recognized, respectively by clones 22C8 and 337.48, are indicated with triangles.

**Fig. 2 Quantification of IsoD-Aβ and pE3-Aβ loads**

Both IsoD-Aβ (A) and pE3-Aβ (E) loads are significantly higher in the AD cases than in the OC and YC groups. Illustrations of immunostaining for IsoD-Aβ (b-d) and pE3-Aβ (f-h) in the 3 cohorts. Scale bar = 50 μm

**Fig. 3 Location of IsoD-Aβ and pE3-Aβ immunostaining**

Illustrations of the different neuropathological features detected by immunostaining for IsoD-Aβ (upper panel) and pE3-Aβ (lower panel): intraneuronal (a, f*), diffuse deposits (b, g), core plaques (c, h), and cerebral amyloid angiopathy (CAA) (d, i). The assessment of the different neuropathological features shows that: (e) IsoD-Aβ in CAA, core plaques and neurons was significantly more frequent in AD vs OC; however intraneuronal IsoD-Aβ was limited to only 18% of AD cases; (j) pE3-Aβ was significantly more frequent in neurons, diffuse deposits and core plaques in AD compared to OC cases. Scale bar (a) = 50 μm; (b-i)= 25 μm

**Fig. 4 Correlation analysis**
Representation of the most significant correlations (p< 0.01) observed in the AD, OC and YC cohorts

Fig. 5 Pictures of IsoD-Aβ, pE-Aβ, Aβ, AβPP and p-TAU in AD, OC, and YC

Representative pictures of IsoD-Aβ (a), pE3-Aβ (b), Aβ (c), p-TAU (d) staining of AD cases, the significant correlations of which are described in table 4.

Representative pictures of IsoD-Aβ (e), pE3-Aβ (f), Aβ (g), AβPP (h) and p-TAU (i) staining of OC cases, the significant correlations of which are described in table 4.

IsoD-Aβ (j) and pE3-Aβ (k), as well as Aβ, AβPP and p-TAU staining were negative in the majority of YC.

Only in two YC cases, IsoD-Aβ (l), pE3-Aβ (m), Aβ (n), AβPP (o) deposits were detectable. Scale bar = 50 μm
Table 1: Summary of the AD, old and young cohorts.

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<td>26-62</td>
<td>n/d</td>
<td>n/d</td>
<td>n/a</td>
</tr>
</tbody>
</table>

n/a : non-applicable
n/d: non-determined
### Table 2: Antibodies and immunohistochemistry conditions

<table>
<thead>
<tr>
<th>Clone</th>
<th>Specificity</th>
<th>Company</th>
<th>Modified amino acid</th>
<th>Antigen Abbreviation</th>
<th>Dilution</th>
<th>Antigen retrieval method</th>
</tr>
</thead>
<tbody>
<tr>
<td>22C8</td>
<td>IsoAspartate Aβ</td>
<td>Elan Pharmaceuticals</td>
<td>Aspartate in position 1,7</td>
<td>IsoD-Aβ</td>
<td>1:10,000</td>
<td>Formic acid 80% for 30 minutes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Formic acid 100% for 3 min +EDTA buffer and microwave</td>
</tr>
<tr>
<td>337.48</td>
<td>pyroglutamate Aβ</td>
<td>BioLegend</td>
<td>Glutamate in position 3</td>
<td>pE3-Aβ</td>
<td>1:100</td>
<td>Formic acid 80% for 30 minutes</td>
</tr>
<tr>
<td>4G8</td>
<td>Aβ and APP</td>
<td>BioLegend</td>
<td>n/a</td>
<td>AβPP</td>
<td>1:2,000</td>
<td>Formic acid 80% for 30 minutes</td>
</tr>
<tr>
<td>82E1</td>
<td>N-terminus end specific</td>
<td>IBL</td>
<td>n/a</td>
<td>Aβ</td>
<td>1:100</td>
<td>Formic acid 80% for 30 minutes</td>
</tr>
<tr>
<td>AT8</td>
<td>Phosphorylated tau</td>
<td>ThermoFisher Scientific</td>
<td>Ser202 and Thr205</td>
<td>p-TAU</td>
<td>1:500</td>
<td>Sodium citrate buffer and pressure cooker</td>
</tr>
</tbody>
</table>
Table 3: Results of correlation analyses within the 3 cohorts

<table>
<thead>
<tr>
<th>AD cases</th>
<th>pE3-αβ</th>
<th>AβPP</th>
<th>Aβ</th>
<th>p-TAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>IsoD-αβ</td>
<td>ρ=0.582 p=0.001</td>
<td>p=-0.026 p=0.897</td>
<td>p=0.545 p=0.003</td>
<td>p=0.336 p=0.086</td>
</tr>
<tr>
<td>pE3-αβ</td>
<td>ρ=0.119 p=0.554</td>
<td>p=0.098 p=0.628</td>
<td>p=0.389 p=0.045</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OC cases</th>
<th>pE3-αβ</th>
<th>AβPP</th>
<th>Aβ</th>
<th>p-TAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>IsoD-αβ</td>
<td>ρ=0.557 p&lt;0.001</td>
<td>p=0.591 p&lt;0.001</td>
<td>p=0.578 p=0.001</td>
<td>p=0.289 p=0.115</td>
</tr>
<tr>
<td>pE3-αβ</td>
<td>ρ=0.570 p=0.001</td>
<td>p=0.609 p&lt;0.001</td>
<td>p=0.380 p=0.038</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>YC cases</th>
<th>pE3-αβ</th>
<th>AβPP</th>
<th>Aβ</th>
<th>p-TAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>IsoD-αβ</td>
<td>ρ=0.097 p=0.778</td>
<td>p=0.145 p=0.670</td>
<td>p=0.247 p=0.465</td>
<td>p=0.382 p=0.246</td>
</tr>
<tr>
<td>pE3-αβ</td>
<td>ρ=0.736 p=0.010</td>
<td>p=0.663 p=0.026</td>
<td>p=0.107 p=0.755</td>
<td></td>
</tr>
</tbody>
</table>

bold ** correlation significant at the 0.01 level (2-tailed).
italic: Pearson’s ρ and p-value
non-italic: Spearman’s ρ and p-value
REGIONS of APP RECOGNIZED BY THE ANTIBODIES USED IN THIS STUDY

- β
- Aβ
- Aβ
- Aβ

Antibodies:
- 22C8
- 337.48
- 82E1
- 4G8

Figure 1
Click here to download Figure Moro et al 2017 fig 1.tif
Click here to access/download

**Supplementary Material**

Moro et al ADDITIONAL FILE  1412.doc
14 December 2017

Dear Professor Attems,

Revision: Pyroglutamate and isoaspartate modified amyloid-beta in ageing and Alzheimer’s disease – ANEC-D-17-00149

We thank you and the reviewers for your comments, and the recognition of the importance of our work. We are grateful for the opportunity to respond to them. We have addressed the reviewers’ comments, and have made changes to the manuscript in MS Word, which are discussed below and highlighted in blue text in the manuscript.

Reviewer 1
Moro et al. report the presence of IsoD-Abeta in the brains of AD and non-demented elderly cases. IsoD-AspAbeta was found to occur earlier than pyroglutamate modified pE3-Abeta in non-demented brains. The authors used an antibody produced by Elan pharmaceuticals. No reference was given in "Material and Methods" for the antibody characterization. Blots were not shown to characterize the antibody. The authors carried out only immunohistochemistry and quantified their results. The detection of IsoD-Abeta as the first form of Abeta being deposited is a very important and interesting finding. However, based on the data provided the report requires some more data to become convincing to me.

Specific point 1

*The antibody against IsoD-Abeta must be characterized by western and dot blot analysis to demonstrate specificity. It must be tested whether there is cross reactivity with other forms of D- or L-Asp-Abeta (IsoLAsp, DAsp, LAsp) and truncated forms of Abeta. Western blots from brain homogenates have to be carried out to demonstrate that the antibody detects a 4kDa protein and whether there is substantial cross reactivity in the brain. If the characterization of the antibody is already published elsewhere an appropriate citation in the "Material and Methods" section is sufficient."

Response

The characterisation of the IsoAspartate antibody 22C8 was performed by Elan Pharmaceuticals and published in 2004 and 2011 in their United States patent applications for, respectively, “Humanized and chimeric N-terminal amyloid beta-antibodies” and “prevention and treatment of amyloidogenic diseases”. The references for these citations have been added to the Material and Methods, in the section “Immunohistochemistry” on page 6.

Specific point 2

*Double label immunohistochemistry to demonstrate the relationship between IsoD-Abeta and 4G8, 82E1 and pE3-stained Abeta in cases with early Abeta deposition should be shown to document that indeed IsoD-Abeta is the first deposited form of Abeta."

Response

We agree with the reviewer that IsoAsp-Aβ detection as the first form of Aβ being deposited could be a very important and interesting finding. However, this is not what we claim to have shown in this current study. This is exactly what we aim to study in the future, extending our investigation on post-translational modifications of Aβ to the analysis of biological fluids, at
different stages of disease progression, as highlighted in the conclusion: “This research could benefit in the future from the development and optimization of new analytical methods, able to separate with high resolution the different forms of Aβ peptides in biological fluids”.

Our data and the current literature support the hypothesis of a possible role for IsoAsp-Aβ accumulation with ageing (and thus potentially seeding the accumulation of Aβ species); whereas pE3-Aβ appears to be more specifically linked to the disease process. However both peptides cannot be related at the moment to any temporal sequence of events, and this point has been clarified on page 15: “In summary, we have observed that high levels of IsoD- and pE3-Aβ are present in AD brain. IsoD-Aβ accumulation appears to be mainly related to ageing and, both in old and AD brains, this modification is associated with the build-up of unmodified Aβ as well as pE3-Aβ. In AD, IsoD-Aβ load is significantly higher than in OC and also accumulates significantly more in blood vessel walls. In contrast, pE3-Aβ appears to be more specifically linked to the disease process. In AD brain, pE3-Aβ deposition is significantly higher than in controls with a characteristic location in neurons. However, both post-translational modifications cannot be related at the moment to any temporal sequences of events.”

We have also been very cautious throughout the text to mention that our findings, based on the correlations, “suggest” and “support” the current hypothesis but do not demonstrate it. Indeed, post-mortem human brain tissue in the current context is not appropriate for experiments on Aβ seeding, looking at one specific time point only per case. We have reviewed the text accordingly.

**Specific point 3**

*Some more relevant reports about pE3-Abeta and DAsp-Abeta in non-demented cases should be discussed: Iwatsubo 1996 (Am J Pathol), Lemere 1995 (Neurobiol Disease), Upadhaya (Brain 2014).*

**Response**

We thank the reviewer for suggesting these three reports. We have added them to our discussion on pages 11, 12 and 13 of the manuscript.

- Page 11: “It is possible that these modified forms of Aβ start to form and aggregate years before other molecular lesions and symptoms of disease clearly appear. Indeed pE3-Aβ has been detected in the past not only in plaques but also in dispersible and soluble Aβ aggregates outside the plaques and described in pathologically preclinical AD cases [Upadahaja Brain 2014]. Consequently in our study, some of non-demented age-matched controls might have actually died after IsoD-Aβ and pE3-Aβ accumulation in the brain had started, but before the formation of a significant Aβ burden”.

- Page 12: “Similar observations were reported in a study focused on brain tissues of cases with Down Syndrome (DS). Trisomy 21 is associated with the progressive development of AD neuropathology as observed in DS people in their forties. A study reported that deposits containing pE3-Aβ were not detectable in DS cases under 27 years old, while they were present in older cases [Lemere Neurobiol Disease 1996]”.

- Page 13: “This is in agreement with the results of a previous study focused on DS and small group of AD and OC with IsoD-Aβ detected in amyloid cores and vascular amyloid [Iwatsubo Am J Pathol 1996]”.

**Reviewer #2**
In this study Moro and colleagues investigated post-translational modifications (PTMs) of β amyloid (namely isoaspartate and pyroglutamate modified β amyloid) in human post-mortem tissue in patients with Alzheimer's disease (AD), aged matched controls, and compared findings to young controls (<63 years). It is an interesting study, which highlights the importance of PTMs of β amyloid in ageing and Alzheimer's disease. The rationale is sound; the conclusions are representative of the results and are clear and concise and in line with current literature. However, I do have some points for consideration:

**Specific point 1**

Post-mortem tissue for this study was obtained from two different brain banks, therefore can the authors confirm that all tissue included in the study was sampled from the same anatomical location within the parietal lobe (inclusion of specific Brodmann areas would be helpful). A comment should be included on any differences in fixation length/procedures which may have an influence on the effectiveness of the antibody staining and affect subsequent results.

**Response**

The same anatomical area within the parietal lobe was retrieved from both Brain banks for all cases. The area investigated was the inferior parietal lobule (Brodmann area 40). This point has now been clarified in the Material and Methods, page 6 as follows: “Four µm formalin-fixed paraffin-embedded sections from the inferior parietal lobule (Brodmann area 40) were retrieved from the Brain banks for all cases.”

In relation to tissue fixation and processing this point has been clarified in the Material and Methods, page 6: “Protocols for tissue fixation and processing were similar in both brain banks. In addition, the staining was performed in batches with each batch including cases from all 3 cohorts to ensure compatibility of the staining”.

**Specific point 2**

In the case selection the authors state neuropathological consensus criteria for AD were satisfied. Braak neurofibrillary tangle (NFT) stages are provided, but as 3 of the AD cases were classified as Braak stage IV, the demographics table would benefit (if available) from the inclusion of CERAD scores, Thal phases (especially as this manuscript is focused on β amyloid) and the degree of neuropathologic change as determined by the NIA-AA criteria. Braak NFT stages have not been provided for the young controls, however as tau pathology can be observed in up to 90% of individuals under the age of 30, this information should be included. Were there any specific selection criteria applied (i.e did any cases have concomitant Lewy body or cerebrovascular pathology?).

**Response**

We have modified the table 1 (summary of the cases) and prepared a new table (Table S1 in supplementary file 1) with additional information for the Alzheimer’s cohort and the old control group provided by the brain banks. The oldest controls (including the young control cases) and AD cases did not have the requested information available.

Cases were selected based on the post-mortem report and any cases with concomitant pathology were excluded from our selection. The text has been amended on page 6 under case selection: “A summary of the cohorts is presented in Table 1 with additional available information in Table S1 (Additional file 1). All AD cases had a clinical diagnosis of probable Alzheimer’s
disease according to NINCDS–ADRDA criteria and cases with concomitant pathology were excluded.”

**Specific point 3**

*When quantifying protein deposits, the authors account for sampling bias by utilizing a zigzag sequence to ensure all cortical layers are represented in the data collection. However, there have been differences observed in the density of pathological lesions between gyri and sulci. I recommend a sentence is added to the description accounting for this.*

**Response**

The following sentence has been added on page 7: “The sampling pattern between all cases was consistent, starting at the depth of the sulcus and progressing up the sulcal wall to the gyral surface.”

**Specific point 4**

*It is stated that images were taken at x20 magnification, this is not particularly accurate for quantitative analysis, did the authors use a x20 objective lens with a x10 eyepiece lens, which seems more likely?*

**Response**

This has been corrected accordingly on page 7: “using a x20 objective lens”

**Specific point 5**

*In the 2 graphs in figure 3 (e and j) the order of the deposition subtype (e.g CAA, core plaques) differs between graphs, it would make the graphs easier to look at if they were in the same order in each of the graphs.*

**Response**

The order of the deposition subtypes in the graphs has been corrected as suggested in figure 3.

**Specific point 6**

*In the text referring to figure 5 on page 10 of the results section (lines 5-10) the authors state that only 2 of the young controls have pyroglutamylated β amyloid deposits, and refer to figure 5k and 5m, however as far as I can see there are no pathological depositions in figure 5k. This differs from the figure legend where it states that pyroglutamylated β amyloid staining is negative in figure 5k. Please can the authors ensure the text in the manuscript is concordant with the figure and figure legend.*

**Response**

The text on page 10 of the manuscript has been modified as follows: “pE3-Aβ was negative in the majority of YC (figure 5k). Only two YC cases, the same cases which have sparse isoD-Aβ deposits, also had sparse pE3-Aβ-positive immunostaining mainly as diffuse plaques, with the case aged 62 also having few core plaques (Figure 5m).”

**Specific point 7**
Are there any clinical data available for these cases, as it would be interesting to see if pathological burden of each of the PMTs is associated with clinical parameters such as MMSE rather than dichotomizing cases as AD or controls.

Response

We agree that this is an interesting point, but unfortunately clinical data are not available for the cases of this study.

Once again, we thank you for your comments, which have clarified our findings and improved the manuscript. We believe it will be of value to your readership and a welcome addition to the literature on ageing and Alzheimer’s disease.

Yours sincerely

Maria Luisa Moro and Delphine Boche