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Late age increase in soluble amyloid-beta levels in the APP23 mouse model despite steady-state levels of amyloid-beta-producing proteins

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Abstract

Age is considered the most important risk factor for Alzheimer's disease. Soluble amyloid-beta (Aβ) has been implicated as the primary neurotoxic agent in Alzheimer's disease pathology. The link between ageing and Aβ, however, remains unclear. In this study, we aimed to investigate the evolution of soluble Aβ over various age groups in the APP23 amyloidosis mouse model and correlate these changes to alterations in the levels of proteins involved in Aβ production. We found a distinct pattern with an initial buildup of Aβ which could be linked to an increase in amyloid precursor protein (APP). Following this increase, Aβ concentrations remained stable until a surge in $Aβ_{1-42}$ at 18 months. This rise was followed by an increase in $Aβ_{1-40}$ and overall Aβ levels. The rise in Aβ at later age did not correlate to changes in the levels of APP, presenilin, and β-secretase and is suggested to result from a decrease in clearance. The APP23 model could provide an interesting tool for future research regarding ageing and Aβ clearance.

Keywords: Alzheimer's disease; Amyloid-beta; aAgeing; bBeta-secretase; gGamma-secretase; eClearance

Abbreviations: Aβ, amyloid-beta; AD, Alzheimer's disease; EOAD, early-onset Alzheimer's disease; LOAD, late-onset Alzheimer's disease; APP, amyloid precursor protein; PS, presenilin; BACE1, beta-site APP-cleaving enzyme 1; CTFβ, C-terminal fragment beta; HET, heterozygous; WT, wild type; BCA, bicinchoninic acid

1.1 Introduction

Dementia is one of the leading causes for dependence and disability in later life. While 2–10% of all dementia cases develop before the age of 65, the majority commences after 65. From the age of 65, the prevalence actually doubles with every 5 year increment in age, increasing exponentially to 40% or more in people aged 85 years and older, making age the most important risk factor for dementia [1]. Alzheimer's disease (AD) is the most common cause of dementia, responsible for 50–75% of all dementias [1]. This progressive, neurodegenerative disorder is neuropathologically characterized by neuronal loss, gliosis, dystrophic neurites, amyloid plaques, and neurofibrillary tangles of hyperphosphorylated tau [2].

Based on the age of onset, AD can be subdivided into two subtypes: early-onset AD (EOAD) and late-onset AD (LOAD). LOAD is the most frequently occurring type of AD and has an age of onset of 65 years or older. Although several genetic risk factors for LOAD have been identified in genome-wide association studies, no clear cause for the disease has been found. Consequently, LOAD is assumed to be a polygenic/multifactorial disorder and is also termed sporadic AD [3]. EOAD, on the other hand, accounts for about 5–10% of all AD cases and is generally caused by mutations in one of the following three genes: amyloid precursor protein (*APP*) [4–6], presenilin 1 [7], and presenilin 2 [8,9]. Mutations in these genes tend to lead to a characteristic younger age of onset, usually between 35 and 65 years of age [10]. The three genes are connected by one common feature, namely, they are all involved in the processing of APP and the resulting

production of the amyloid-beta (Aβ) peptide.

The Aβ peptide is produced by the sequential cleavage of APP, a type I transmembrane protein, by β- and γ-secretase. Initially, APP is cleaved by β-secretase at the N-terminal end of the Aβ peptide, releasing an extracellular fragment, soluble APPβ [11]. Next, the remaining membrane-bound fragment, C-terminal fragment β (CTFβ), is cut into an intracellular fragment (amyloid intracellular domain) and an extracellular fragment (Aβ) by the γ-secretase complex [12]. The resulting Aβ peptide has been found to vary in length between 37 and 43 amino acids. The most commonly produced Aβ species is $A\beta_{40}$, but $A\beta_{42}$ is more prone to aggregation and considered to be the most pathogenic [13–16]. Presenilins (PS) have been indicated as the catalytic site of the γ-secretase complex [17–21] and AD-related mutations in their genes tend to cause a shift in Aβ production from $A\beta_{1-40}$ to $A\beta_{1-42}$ [22–25]. Whereas, mutations in the *APP* gene can affect Aβ production by either shifting the Aβ production from $A\beta_{1-40}$ to $A\beta_{1-42}$ [26] or by increasing the total amount of Aβ [27,28]. In addition to this genetic evidence linking Aβ to AD, research has demonstrated the neurotoxic effects of Aβ both in vitro and in vivo (As reviewed in [29]). The Aβ peptide is also the main component of amyloid plaques, one of the key features of AD pathology. Together, these findings have led to the development of the amyloid cascade theory, which states that Aβ plays a central role in AD pathology [30].

Initially, scientists believed the neuronal damage observed in AD was caused by the deposition of amyloid into plaques in the brain, but the plaque load correlates poorly with disease severity [31,32]. The brain levels of soluble Aβ, on the other hand, do correlate well with the amount of synaptic loss and cognitive deficits [33–35]. Therefore, the current predominant theory states that soluble aggregates of Aβ are the primary neurotoxic agents in AD. In this study, we aimed to further explore the relationship between the Aβ pathology and ageing [36]. To this end, we examined the evolution in the soluble Aβ levels in the APP23 mouse model for AD over a wide range of ages. The APP23 mouse model shows a 7-fold overexpression of human APP_{751} with the K670M/N671L Swedish double mutation compared to the levels of endogenous murine APP [37]. This Swedish mutation renders APP more accessible to β-secretase and gives rise to an overall increase in Aβ production [28]. The first scarce amyloid plaques in this model appear at the age of 6 months, and the first cognitive deficits have been observed as early as 3 months of age [37,38]. We investigated whether the changes in soluble Aβ levels also correlated with these previously observed changes in pathology and symptomatology. Finally, we also explored if alterations in soluble Aβ levels could be correlated to changes in the levels of proteins involved in Aβ production, i.e. APP, β- and γ-secretase.

~~2.~~2 Materials and methods

~~2.1.~~2.1 Animals

Male heterozygous (HET) APP23 mice, overexpressing human APP_{751} carrying the Swedish double mutation (K670M/N671L) [37], were used for all experiments. Their male wild-type (WT) littermates were included as a control group. Genotypes were determined through PCR. All mice were bred within our facilities on a C57Bl/6J background and group-housed in standard mouse cages under conventional laboratory conditions with a 12:12 h light–dark cycle (light on at 8:00 AM, light off at 8:00 PM), constant room temperature (22 ± 2 °C), humidity level (55 ± 5%), and food and water available ad libitum. Based on established milestones in the progression of AD pathology within the model (e.g. first appearance of plaques, cognitive deficits), six age groups were selected for the study: 1.5, 3, 6, 12, 18, and 24 months [39]. Experiments were conducted in accordance with the European Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes, and the Animal Ethics Committee of the University of Antwerp approved all procedures.

~~2.2.~~2.2 Tissue collection

Animals were euthanized at the desired age through cervical dislocation. The brain was harvested and dissected on ice into three parts: two hemi-forebrains and the cerebellum (the olfactory bulbs were discarded). After dissection, the brains were immediately stored at − 80 °C until use.

~~2.3.~~2.3 Protein extraction

Hemi-forebrains were thawed on ice and weighed to determine the wet weight prior to extraction. Next, 900 μl of ice-cold TNT-buffer [50 mM Tris-base (Thermo Fisher Scientific), 150 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM 1,10-Phenanthroline Monohydrate (Merck Millipore), 1X protease inhibitor cocktail (Sigma–Aldrich), pH 7.40] was added. The tissue was carefully mechanically dissociated using a 1-ml syringe, followed by further finer dissociation with a 1-ml syringe and 20 gauge needle and with a 1-ml pipet. Another 900 μl of ice-cold TNT-buffer was added and the sample was centrifuged for 30 min at 25,000 RCF to pellet cell debris, cellular membranes, and organelles. The supernatant was collected and centrifuged a second time for 30 min at 25,000 RCF. The resulting supernatant contained the protein fraction from the soluble subcellular compartment, still soluble after 25,000 RCF centrifugation, and will hereafter be referred to as the soluble fraction. The supernatant was collected, briefly sonicated, and stored at − 20 °C. The pellet from the first centrifugation step was resuspended in 900 μl cold RIPA-buffer [50 mM Tris-base (Thermo Fisher Scientific), 150 mM NaCl, 0.5% Triton X-100, 1 mM Ethylenediaminetetraacetic acid, 3% SDS, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 2 mM 1,10-Phenanthroline Monohydrate (Merck Millipore), 1X protease inhibitor cocktail (Sigma–Aldrich), pH 7.40]. The resuspended sample was first dissociated vigorously with a 1-ml pipet. Then, another 900 μl of RIPA-buffer was added and the sample was vortexed for 20 s. The sample was placed on a rotating platform for 15 min at 4 °C and centrifuged for 30 min at 25,000 RCF. The resulting pellet contains the insoluble fraction and was stored at − 20 °C. The supernatant was centrifuged a second time for 30 min at 25,000 RCF. The final supernatant contained the membrane-bound protein fraction and was again briefly sonicated and stored at − 20 °C. Information on the evaluation of the separation

in the various subcellular fractions can be found in the supplementary data.

~~2.4.~~2.4 Bicinchoninic acid protein assay

The overall protein content in the protein extracts was determined with the ~~B~~bicinchoninic acid (BCA) protein assay. The standard microplate procedure was used according to the manufacturer's instructions (Thermo Scientific Pierce). All samples were diluted to within the detection limits of the test, and 25 µl of the diluted samples was pipetted in duplicate into the microplate. Next, 200 µl of working reagent was added to each well and the plate was placed on a plate shaker for 30 s. After incubation for 30 min at 37 °C, the plate was cooled and the absorbance was measured on a plate reader at 550 nm. This test was performed as a quality control for the reproducible and efficient execution of the extraction protocol and to ensure equal amounts of total protein were loaded on the gel for SDS-PAGE.

~~2.5.~~2.5 ELISA

The concentration of $A\beta_{1-x}$, $A\beta_{1-40}$, and $A\beta_{1-42}$ in the soluble protein fraction was determined by ELISA measurements using the human amyloid β (1–x) ~~A~~assay kit, the human amyloid β (1–40) ~~A~~assay kit, and the human amyloid β (1–42) ~~A~~assay kit (IBL International). All samples were diluted to within the detection limits of the test and analy~~s~~zed in duplicate according to the manufacturer's instructions. The amyloid β (1–x) assay detects all Aβ variants with an intact N-terminus and a length of more than 16 amino acids. The amyloid β (1–40) assay shows ≤ 0.1% cross-reactivity with other human Aβ species, but does show 16.3% cross-reactivity with endogenous $A\beta_{1-40}$. The amyloid β (1–42) assay shows ≤ 0.1% cross-reactivity with other human Aβ species and endogenous Aβ. The following sample sizes were used for the various age groups: 1.5 months (n = 9), 3 months (n = 10), 6 months (n = 10), 12 months (n = 11), 18 months (n = 10), 24 months (n = 8 for Aβ1–x measurements and n = 9 for other ELISA measurements).

~~2.6.~~2.6 SDS-PAGE and ~~western blotting~~Western blotting

The membrane-bound protein fraction was thawed and all samples were diluted to the same total protein concentration based on the BCA assay results. NuPAGE® LDS Sample Buffer and reducing agent were added to the samples which were subsequently heated to 95 °C for 10 min for denaturing, reducing SDS-PAGE. A constant amount of total protein was loaded on NuPage® 4–12% Bis-Tris gels (Life Technologies). Each gel contained at least one sample of each age group to allow for relative quantification and to minimize inter-test variation. The gels were run in the Xcell SureLock Mini-Cell system (Life Technologies) for 45 min at 200 V. All gels were run in duplicate. After electrophoresis, the proteins were blotted for 1 h to Immobilon®-PSQ membrane (Millipore) in the XCell II™ Blot Module (Life Technologies) using the standard manufacturer's protocol. After blotting, the membranes were blocked for 2 h in ~~t~~Tris-buffered saline with 0.1% tween-20 and 5% milk. Next, the membranes were incubated overnight at 4 °C in the same block buffer with primary antibody. Three primary antibodies were used: mouse monoclonal anti-$A\beta_{1-16}$ (diluted 1/2000, 6E10, Covance), rabbit monoclonal anti-PS1 (diluted 1/5000, EP2000Y, Novus biologicals), and rabbit monoclonal anti-beta-site APP-cleaving enzyme 1 (BACE1) (diluted 1/5000, EPR3956, Novus biologicals). Following overnight incubation, the membranes were washed with ~~t~~Tris-buffered saline with 0.1% tween-20 and incubated for 1 h at room temperature in block buffer with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Dako). After a final wash step, the membrane was treated with the SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Fisher Scientific) to visualize the protein bands. Blots were imaged with the G:Box imager equipped with Genesnap software (Syngene) and analy~~s~~zed with the Image Studio Lite v4.0 software (Li-Cor). PS1 and BACE1 levels were determined for 7 mice per genotype in each age group. For APP and CTF-β, the sample size was 8 mice per group, with the exception of the 12-month group which contained 9 mice.

~~2.7.~~2.7 Statistical analysis

Due to the relatively small size of the test groups, non-parametrical statistical analyses with exact significance values were used for all group comparisons. Comparisons between the genotype groups were performed using the independent-samples Mann–Whitney U test. Differences between the various age groups within the HET group were evaluated with the independent-samples Kruskal–Wallis test. Post-hoc analysis between specific age groups was performed using the independent-samples Mann–Whitney U test with a Bonferroni correction for multiple comparisons (significance level $p < 0.0033$). All p-values compared to this Bonferroni-corrected significance level will be marked throughout the text as p_{bon}. Outliers with a high coefficient of variation (≥ 20%) between duplicate measurements were excluded from statistical analysis. This resulted in the exclusion of one WT and one HET from the 6-month group for the $A\beta_{1-42}$ analysis and the $A\beta_{1-42}/A\beta_{1-40}$ ratio analysis. All statistical tests were performed using SPSS statistics software v22.0 (IBM). All graphs were created using Graphpad Prism v5.03 (Graphpad). As ELISA measurements of WT mice usually resulted in values below the lower detection limit, the WT groups were omitted from all graphs displaying ELISA results.

~~3.~~3 Results

~~3.1.~~3.1 ~~T~~The evolution in soluble $A\beta_{1-x}$ content

Initially, the overall Aβ levels in the soluble protein fraction were determined for the various age and genotype groups using the human amyloid β (1–x) ELISA. Given the fact that the WT mice do not contain the human APP construct, the $A\beta_{1-x}$ measurements resulted in background levels below or near the test's lower detection limit. Consequently, a clear difference could be observed between the WT and HET mice in all age groups ($p < 0.001$). The Aβ levels of the HET animals also displayed a

distinct evolution over the various age groups (Fig. 1A, $p < 0.001$). The youngest age group, 1.5 months old, showed no significant difference with the 3-month-old group ($p = 0.008$), but did show significantly lower levels than all other age groups ($p_{bon} < 0.001$). The Aβ levels of the 3 months group displayed a borderline significant difference with the 6 months ($p_{bon} = 0.003$) and 12 months ($p_{bon} = 0.002$) age group and a distinct difference with the 18 months and 24 months group ($p_{bon} < 0.001$). No significant differences could be found between ages of 6 months, 12 months, and 18 months ($p_{bon} > 0.05$), whereas the 24 months age group showed levels that were significantly higher than all other age groups ($p_{bon} < 0.001$).

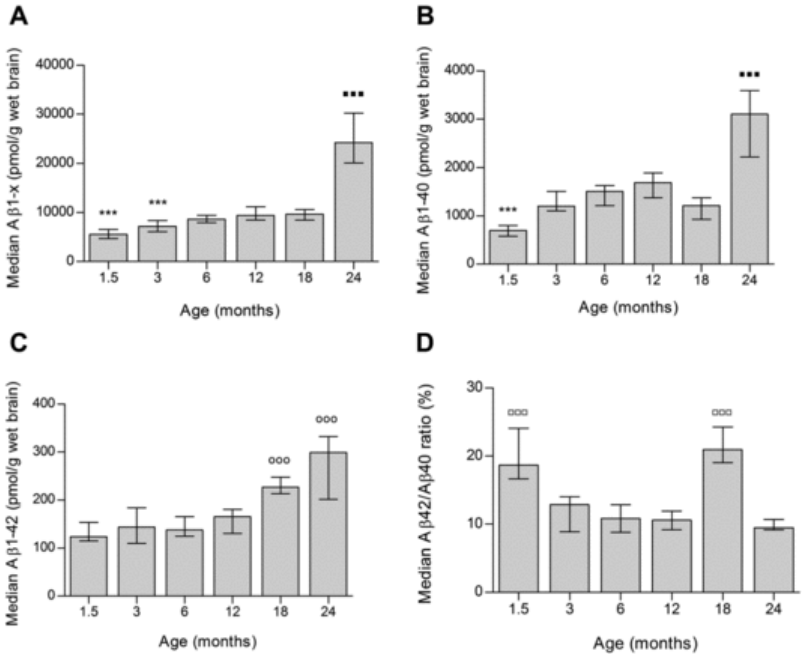

Figure 1: Fig. 1 Results of the ELISA measurements of various Aβ species in heterozygous APP23 mice ranging from 1.5 months to 24 months in age. Group medians are displayed in the graphs (error bars: interquartile range). (A) Comparison of $Aβ_{1-x}$ concentrations between the various age groups: 1.5 months ($n = 9$), 3 months ($n = 10$), 6 months ($n = 10$), 12 months ($n = 11$), 18 months ($n = 10$), and 24 months ($n = 8$). (B) Comparison of $Aβ_{1-40}$ concentrations between the various age groups: 1.5 months ($n = 9$), 3 months ($n = 10$), 6 months ($n = 10$), 12 months ($n = 11$), 18 months ($n = 10$), and 24 months ($n = 9$). (C) Comparison of $Aβ_{1-42}$ concentrations between the various age groups: 1.5 months ($n = 9$), 3 months ($n = 10$), 6 months ($n = 9$), 12 months ($n = 11$), 18 months ($n = 10$), and 24 months ($n = 9$). (D) Comparison of $Aβ_{1-42}/Aβ1_{-40}$ ratio between the various age groups: 1.5 months ($n = 9$), 3 months ($n = 10$), 6 months ($n = 9$), 12 months ($n = 11$), 18 months ($n = 10$), and 24 months ($n = 9$). Symbols indicate a significant difference compared to specific age groups (post hoc Mann–Whitney with Bonferroni correction for multiple comparisons; significance level $p < 0.0033$). Solid squares = compared to all younger age groups, asterisks = compared to 6,12,18, and 24 months, open circles = compared to 1.5, 3, 6, and 12 months, open squares = compared to 3, 6, 12, and 24 months. Abbreviations: Aβ, amyloid-beta.

3.2.3.2 Changes in the soluble $Aβ_{1-40}$ and $Aβ_{1-42}$ content and the $Aβ_{1-42}/Aβ_{1-40}$ ratio

In addition to the global $Aβ_{1-x}$ analysis, we also specifically investigated the levels of soluble $Aβ_{1-40}$ and $Aβ_{1-42}$. As with the $Aβ_{1-x}$ analysis, the values of the WT group were mostly situated below the lower detection limit and a clear difference could be observed between the WT and HET in all age groups for both $Aβ_{1-40}$ and $Aβ_{1-42}$ ($p < 0.001$). When comparing the $Aβ_{1-40}$ levels for HET between the various age groups (Fig. 1B), the youngest age group displayed significantly lower levels than all other ages ($p_{bon} < 0.001$). The levels of the 24 months group, on the other hand, were significantly elevated compared to all other ages ($p_{bon} < 0.001$). No statistically significant differences could be found between the $Aβ_{1-40}$ levels of the 3, 6, 12, and 18-month-old group. The 3-month-old group did show slightly lower levels than the 12-month-old group ($p_{bon} = 0.020$) and the 18-month-old values were also found to be lower than the levels at 6 months ($p_{bon} = 0.023$) and 12 months ($p_{bon} = 0.013$), but these differences did not reach the Bonferroni-corrected post hoc significance level of 0.0033. The $Aβ_{1-42}$ levels in the APP23 model seemed to remain constant from 1.5 months to 12 months on (Fig. 1C, $p_{bon} > 0.050$). At 18 months and 24 months, the levels appeared to be significantly elevated compared to the younger ages ($p_{bon} < 0.001$), but no difference was found between the two oldest age groups ($p_{bon} = 0.278$). Finally, we also analysed how the changes in the individual levels of $Aβ_{1-40}$ and $Aβ_{1-42}$ affect the ratio between the two Aβ variants (Fig. 1D). In most age groups, the amount of $Aβ_{1-42}$ was about 10% of the amount of $Aβ_{1-40}$. However, at the age of 1.5 and 18 months, a significant spike in the $Aβ_{1-42}/Aβ_{1-40}$ ratio could be observed ($p_{bon} < 0.001$) and the $Aβ_{1-42}$ levels reached around 20% of the $Aβ_{1-40}$ levels.

3.3.3.3 Quantitative comparison of APP, CTFβ, and Aβ levels between age groups

In order to further investigate the underlying causes for the changes in Aβ levels, we explored whether these changes were also reflected in the levels of the precursor protein for Aβ and in the levels of CTFβ, an intermediary product in the production of Aβ. The two characteristic bands of immature and mature (or post-translationally modified) APP were analyszed together to obtain the total level of full-length APP. Control WT samples were run on each blot, but as they showed no bands for human APP or CTFβ, they were not included in the statistical analysis. All protein levels were determined as the relative percentage compared to the protein level at 1.5 months. Overall, a significant difference could be found for the relative APP levels between the various age groups (Fig. 2A and 2B, $p_{bon} = 0.002$). Between the age of 6 months and 24 months, however, the levels remained constant ($p_{bon} > 0.05$). At 1.5 months, significantly lower APP levels were observed compared to the levels at 18 and 24 months of age ($p_{bon} < 0.001$). The levels at 1.5 months were also slightly lower than at 6 ($p_{bon} = 0.006$) and 12 months ($p_{bon} = 0.042$), but these differences did not reach the 0.0033 Bonferroni-corrected significance level. Similar lower levels could be observed between the 3-month-old group and 6 months ($p_{bon} = 0.007$), 18 months ($p_{bon} = 0.007$), and 24 months groups ($p_{bon} = 0.007$). No difference was found between the 1.5 months and the 3 months group ($p_{bon} = 0.404$). The CTFβ levels displayed a clear evolution in time (Fig. 3A and 3B, p, p < 0.001). CTFβ content was found to be significantly lower in the youngest age group compared to all other ages ($p_{bon} < 0.001$), except for 3 months ($p_{bon} = 0.083$). Between 3 and 12 months, no changes in CTFβ levels could be observed ($p_{bon} > 0.05$). At 18 months, a significant rise in CTFβ could be observed compared to all younger ages ($p_{bon} < 0.0033$). This rise in CTFβ continues, resulting in even higher levels at 24 months ($p_{bon} < 0.001$). Finally, we also observed that the rise in CTFβ levels at 18 and 24 months coincides with appearance of Aβ monomer and low n-oligomer bands on the western blotWestern blot (Fig. 3A). As these bands are not present at younger ages, no relative quantification was performed.

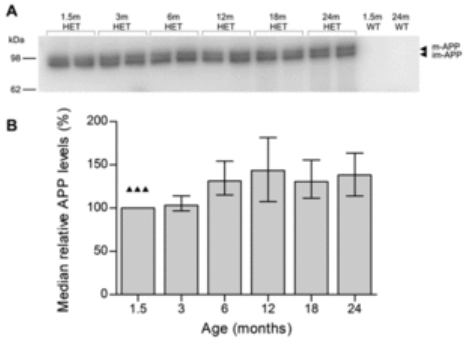

Figure 2:Fig. 2 Results from western blotWestern blot analysis of full-length APP levels in heterozygous APP23 mice ranging from 1.5 months to 24 months in age. (A) Image displaying western blotWestern blot results from heterozygous mice of all age groups. Two wild-type samples were run as negative controls. (B) Graph displaying relative quantification of APP levels compared to the APP level of 1.5-month-old APP23 mice (group medians with interquartile range error bars, n = 9 for the 12 months group and n = 8 for all other age groups). Solid triangles indicate significantly lower APP levels compared to 18 and 24 months (post hoc Mann–Whitney with Bonferroni correction for multiple comparisons; significance level p < 0.0033). Abbreviations: m-APP, mature amyloid precursor protein; im-APP, immature amyloid precursor protein; HET, heterozygous; WT, wild type.

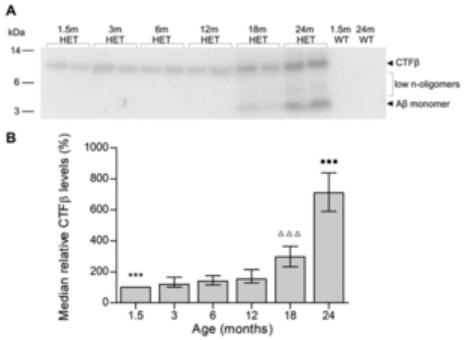

Figure 3:Fig. 3 Results from western blotWestern blot analysis of CTFβ, Aβ monomer, and low n-oligomer levels in heterozygous APP23 mice ranging from 1.5 months to 24 months in age. (A) Image displaying western blotWestern blot results from heterozygous mice of all age groups. Two wild-type samples were run as negative controls. (B) Graph displaying relative quantification of CTFβ levels compared to the CTFβ level of 1.5-month-old APP23 mice (group medians with interquartile range error bars n = 9 for the 12 months group and n = 8 for all other age groups). Symbols indicate a significant difference compared to specific age groups (post hoc Mann–Whitney with Bonferroni correction for multiple comparisons; significance level p < 0.0033). Solid squares = compared to all younger age groups, asterisks = compared to 6,12,18, and 24 months, open triangles = compared to 1.5, 3, 6, 12, and 24 months. Abbreviations: Aβ, amyloid-beta; CTFβ, C-terminal fragment beta; HET, heterozygous; WT, wild type.

3.4.3.4 Evaluating PS1 and BACE1 levels between different age groups

In the end, we investigated whether the observed changes in Aβ levels correlated to changes in the levels of proteins playing a key role in the production of Aβ, PS1, and BACE1. We determined PS1 (Fig. 4) and BACE1 (Fig. 5) levels in both WT and

HET animals of each age group and expressed them as relative percentages compared to the protein level of WT mice at the age of 1.5 months. Overall, none of the various age groups displayed differences between the HET and WT for both PS1 ($p > 0.05$) and BACE1 ($p > 0.05$). No evolution over time could be found in the levels of PS1 and BACE1 for both WT ($p_{bon} > 0.05$) and HET ($p_{bon} > 0.05$) animals.

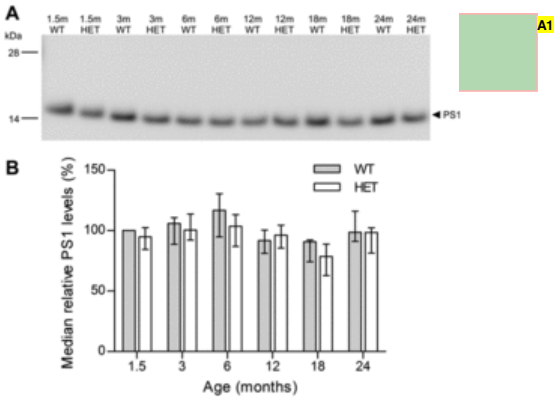

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Figure 4:Fig. 4 Results from ~~western blot~~Western blot analysis of PS1 levels in heterozygous APP23 mice and wild-type littermates ranging from 1.5 ~~months~~ to 24 months in age. (A) Image displaying ~~western blot~~Western blot results from HET and WT mice of all age groups. (B) Graph displaying relative quantification of PS1 levels compared to the PS1 level of 1.5-month-old WT mice (group medians with interquartile range error bars, n = 7 for all test groups). Abbreviations: PS1, presenilin 1; HET, heterozygous; WT, wild type.

Annotations:

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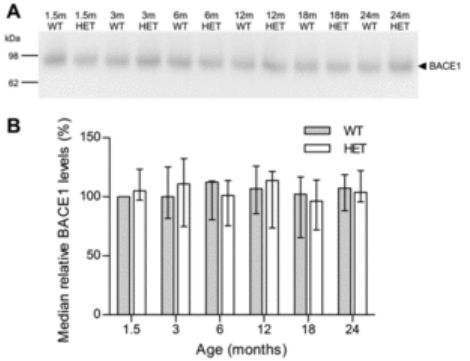

Figure 5:Fig. 5 Results from ~~western blot~~Western blot analysis of BACE1 levels in heterozygous APP23 mice and wild-type littermates ranging from 1.5 ~~months~~ to 24 months in age. (A) Image displaying ~~western blot~~Western blot results from HET and WT mice of all age groups. (B) Graph displaying relative quantification of BACE1 levels compared to the BACE1 level of 1.5-month-old WT mice (group medians with interquartile range error bars, n = 7 for all test groups). Abbreviations: BACE1, beta-site APP-cleaving enzyme 1; HET, heterozygous; WT, wild type.

~~4~~ 4 Discussion

The aim of this study was to investigate the evolution of soluble Aβ levels with increasing age in the APP23 mouse model. The overall Aβ levels displayed a specific pattern with an initial buildup in the soluble Aβ levels, followed by more or less constant levels up to 18 months and ending in a substantial increase at an older age. In general, the evolution in $A\beta_{1-40}$ levels appeared to follow a pattern similar to the $A\beta_{1-x}$ pattern. The $A\beta_{1-42}$ levels deviated from the global pattern as they did not show the initial increase in the younger age groups and a significant increase in $A\beta_{1-42}$ can already be observed at 18 months. These findings are in line with the results found in several other studies regarding the Aβ pathology in APP23 and other mouse models [38,40–42]. While the pattern differs significantly from the pattern that has been observed in the APPPS1 double mouse model [40], it shows a lot of similarities with previous findings in APP23 and other APP-mutation models. The APPPS1 model, which contains a mutation in the APP gene, as well as in the PS1 gene, displays a continuous increase in Aβ levels [40]. APP23, APP24, and APP51 mice, on the other hand, all appear to share the pronounced jump in Aβ levels at an older age [40,41]. The steady state in soluble Aβ levels, however, could not be confirmed by these studies. One of the studies already showed modest increase starting at the age of 8 months [41], but this study did not focus solely on soluble Aβ. The researchers used the formic acid soluble fraction, which also contains the Aβ present in brain plaques. As the APP23 model starts to develop plaques from the age of 6 months onwards

[37,38,41], the observed rise in Aβ could be due to solubilized Aβ from plaques. Another study did focus on soluble Aβ, but did not include ages between 6 and 16 months. As such, the steady state around 12 months could not be verified [40]. As the other studies also did not include the 1.5-month-age group, no comparison could be made for the initial rise in overall Aβ and $Aβ_{1-40}$. The absence of this increase in the $Aβ_{1-42}$ levels has been previously observed [38]. With regard to the increase in soluble $Aβ_{1-42}$ at 18 months, one research group found a similar increase at 16 months, but this group also found a significant increase in $Aβ_{1-40}$ at this age. Despite the difference in the absolute measurement of $Aβ_{1-40}$ levels, they did find a similar increase in the $Aβ_{1-42}$/$Aβ_{1-40}$ ratio at 16 months. The difference in the $Aβ_{1-40}$ measurement could be due to differences in the protein extraction method, the breeding colony, or the selected subpopulation of mice. Additionally, since the $Aβ_{1-42}$/$Aβ_{1-40}$ ratio has been shown to influence the Aβ aggregation kinetics and an increased ratio has been linked to more severe AD pathology [43–46], this could actually prove to be the more pathologically significant parameter. In general, our results seem to suggest that $Aβ_{1-42}$ levels appear to rise faster than $Aβ_{1-40}$ leading to an increase in the $Aβ_{1-42}$/$Aβ_{1-40}$ ratio at 1.5 and 18 months. The fact that $Aβ_{1-42}$ and $Aβ_{1-40}$ levels can change independently from each other is a curious and relatively unexpected finding. Unlike other mutations, the mutation in the APP23 model does not cause γ-secretase to preferentially cleave at the $Aβ_{1-42}$ site. The change in ratio may, therefore, not be due to differences in the production of the Aβ species, but rather a consequence of variability in aggregation, degradation, and/or removal. Interestingly, the increased ratios precede distinct cognitive deterioration in the APP23 model. After the first spike at 1.5 months, cognitive deterioration occurs, and at the next time point (3 months), cognitive deficits can be observed in complex cognitive tasks, like the Morris water maze [38,47]. Applying simpler tasks, like small-sized Morris water mazes or nonspatial passive avoidance learning, deficits in lower order cognitive functions can be detected at the age of 25 months [47], the first time point after the second increase in the $Aβ_{1-42}$/$Aβ_{1-40}$ ratio at 18 months. During the steady-state period between the two observed increases in the ratio, no changes in cognitive performance have been observed, but the period does coincide with the appearance of plaques and an increase in plaque load and insoluble Aβ [37,38,48]. This is in agreement with the theory that soluble Aβ is responsible for the cognitive deficits and not the Aβ plaques [29,34,49]. In fact, these findings appear to support the hypothesis that the formation of plaques could have a protective effect by sequestering free, soluble Aβ [50]. It is probable that, while Aβ is continuously produced in excess, the steady-state in the pool of soluble Aβ between 3 and 12 months is initially maintained by diverting Aβ towards the insoluble pool, resulting in the observed increase in plaque load from 6 months on. With the second increase in the $Aβ_{1-42}$/$Aβ_{1-40}$ ratio, a significant increase in plaques has also been reported [41,42]. We opted to use only males in this study, as we wanted to compare our results with previous behavioral and cognitive experiments where exclusively males were used to avoid influences of fluctuating hormonal levels. In general, conflicting findings have been published regarding gender differences in AD pathology. Where a gender effect has been reported for certain aspects of AD pathology (e.g. cognitive performance, atrophy) [reviewed in 51], no such effects have been found for others. For instance, a recent multicenter study showed no gender differences in CSF biomarker levels, including $Aβ_{1-42}$ [52]. Perhaps more importantly, a study in the 5xFAD mouse model showed no effect of gender on APP, BACE, and Aβ levels [53]. Together, these findings might suggest that the Aβ-producing pathway is not affected by gender and that our results could possibly be extrapolated to female APP23 mice as well.

In this study, we also wanted to explore the possible causes for the observed changes in Aβ levels. In general, the increase in the amount of soluble of Aβ in the brain can be caused by an increase in production, a decrease in clearance, or a combination of both. Here we investigated the concentration of four key proteins from the Aβ production pathway: APP, CTFβ, PS1, and BACE. APP showed slightly lower levels at a younger age, but the levels remained relatively stable in mature mice. The initial evolution could of course simply be part of the brain maturation process [54]. Alterations due to brain development could also contribute to the changes in Aβ levels at younger ages. However, these developmental changes do not provide an explanation for the second increase in Aβ levels at a later age. CTFβ, an intermediary product in the production of Aβ, does display the secondary increase at old age, as well as the initial buildup. No significant alterations could be found in the PS1 and BACE1 levels. In the past, studies have found that BACE activity can be increased with age and that this increase in activity is not always reflected by an elevated protein level [55,56]. However, so far no data exists about the evolution in BACE activity in APP23 model. While we cannot exclude the possibility that the activity of β- and γ-secretase is altered by post-translational modifications or other mechanisms, our study shows no clear correlation between the PS1 and BACE1 levels and the changes in Aβ concentrations. The APP levels, on the other hand, may provide some insight into the mechanism behind the initial increase in Aβ and CTFβ, but fail to explain the changes in Aβ and CTFβ levels at 18 months and older. The rise in concentration of these proteins and the appearance of Aβ monomers and low n-oligomers in the membrane-bound fraction is, therefore, possibly due to decreased clearance. This decrease in Aβ clearance has actually already been reported in several studies with AD patients [57–60]. In the APP23 mouse model a recent, in vivo PET imaging study also showed increased tracer retention at advanced ages [61]. In fact, while a general slower protein turnover has been reported with increasing age (~ 30–40% slower), it was recently established that the Aβ turnover actually slows by 60% [57]. Several of the mechanisms involved in Aβ clearance, like the glymphatic system, proteolytic degradation, and autophagy, have also been found to show age-dependent alterations [62–64]. Together, these findings suggest that alterations in the clearance of Aβ could play an important part in AD pathology, especially in sporadic cases of AD where no clear genetic evidence for changes in Aβ production is present. Until now, research has put a lot of emphasis on determining protein levels. These levels, however, are the result of a complex interaction between the processes responsible for production and removal. Therefore, future research should maybe focus more on the age-related changes in Aβ turnover and kinetics, exploring production and removal simultaneously where possible.

5.5 Conclusion

In this study, we found a distinct age-related pattern in the evolution of soluble Aβ levels in the APP23 mouse model. While this model already displays cognitive deficits and plaque pathology at a relatively early age due to the human APP construct and the initial buildup of human Aβ, we discovered a surge in Aβ levels at a later age which shows interesting similarities to alterations in Aβ levels and Aβ clearance observed in human patients. Owing to practical considerations and the increased demand in time and resources these older age groups are often underrepresented in experiments. However, our research shows that these age groups could provide valuable insights into AD pathology and its connection to ageing, for EOAD as well as sporadic AD.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.10.027.

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Transparency document

Multimedia Component 1

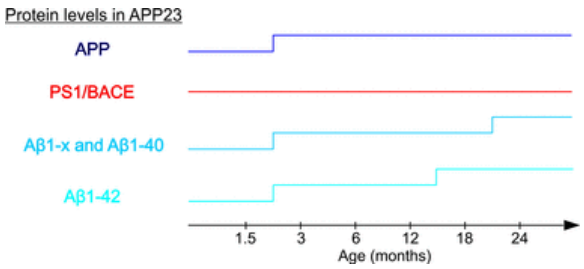
Transparency document

~~Appendix A.~~Appendix A. Supplementary data

Multimedia Component 2

Supplementary material

Graphical abstract



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Highlights

- APP23 mice display a distinct age-related evolution in Amyloid-β levels.
- The $A\beta_{1-42}/A\beta_{1-40}$ ratio spikes at 2 distinct ages: 1.5 months and 18 months.
- An initial build up in APP, CTFβ and Aβ is observed at a young age.
- An increase in $A\beta_{1-42}$ at 18 months is followed by an increase in $A\beta_{1-40}$.
- The late age increase is most likely due to a decrease in clearance.

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