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Citation for published version:
Rudinskiy, N, Fuerer, C, Demurtas, D, Zamorano, S, De Piano, C, Herrmann, AG, Spires-jones, TL, Oeckl, P, Otto, M, Frosch, MP, Moniatte, M, Hyman, BT & Schmid, AW 2016, 'Amyloid-beta oligomerization is associated with the generation of a typical peptide fragment fingerprint', *Alzheimer's & Dementia: The Journal of the Alzheimer's Association*. https://doi.org/10.1016/j.jalz.2016.03.011

Digital Object Identifier (DOI):
10.1016/j.jalz.2016.03.011

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Alzheimer's & Dementia: The Journal of the Alzheimer's Association

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Authors final peer reviewed manuscript as accepted for publication

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Download date: 12. Oct. 2024

Aβ oligomerization is associated with the generation of a typical peptide fragment fingerprint.

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Keywords: Alzheimer's disease; aggregation; autocleavage, oligomers, C-terminal amidation, mass spectrometry, selected reaction monitoring.

Author contributions: AWS conceived the project, designed experiments and directed the study. AWS, CF, CD and SZ performed mass spectrometry analysis. DD performed electron Microscopy imaging. AWS, CF and CD performed biochemical studies. PO and OM performed CSF analysis. NR performed immunohistochemistry. AH and TLS performed array tomography. MPF performed histological examination of brain tissue. BTH, MPF, NR, PO and OM provided biological samples. AWS, CF, NR, SZ, MM and BTH wrote the paper. All authors discussed the results and commented on the paper.

Conflict of interest: none

ABSTRACT

Amyloid-beta (Aβ) peptide oligomerization plays a central role in the pathogenesis of Alzheimer's disease (AD) and Aβ oligomers are collectively considered an appealing therapeutic target for the treatment of AD. However, the molecular mechanisms leading to the pathological accumulation of oligomers are unclear and the exact structural composition of oligomers is being debated. Using targeted and quantitative mass spectrometry, we reveal site-specific Aβ autocleavage during the early phase of aggregation, producing a typical Aβ fragment signature and that truncated Aβ peptides can form stable oligomeric complexes with full-length Aβ peptide. We show that the use of novel anti-Aβ antibodies raised against these truncated Aβ isoforms allows for monitoring and targeting the accumulation of truncated Aβ fragments. Antibody-enabled screening of transgenic models of AD, as well as human post-mortem brain tissue and cerebrospinal fluid revealed that, aggregation-associated Aβ cleavage is a highly relevant clinical feature of AD.

61 **1. INTRODUCTION**

62

63 Alzheimer disease (AD) is a progressive neurodegenerative disorder that is manifested as
64 a gradual decline in memory and cognitive function. A number of studies indicate that
65 soluble oligomers might account for the AD-associated decline in synaptic plasticity [1, 2]
66 and that inhibition of natural Aβ oligomerization rescues deficits in long-term potentiation
67 (LTP) [3]. Several types of Aβ assemblies of dimeric and trimeric [4] [1] [2] or dodecameric
68 (Aβ* 56) [5] nature have been observed *in vitro* and *in vivo* in transgenic mouse models,
69 human cerebrospinal fluid (CSF) [6], and post-mortem AD brain extracts [7] [8], with the
70 higher molecular weight species being considered the main neurotoxic culprit associated with
71 cognitive dysfunction. Collectively Aβ oligomers can be considered as an appealing
72 diagnostic and therapeutic target. However, the general morphological heterogeneity and, to
73 some extent, metastable structure renders an antibody based targeting and detection of
74 oligomers difficult. Therefore, the development of specific anti-oligomeric based therapeutics
75 remains challenging.

76 Cerebrospinal fluid (CSF) analyses from AD patients indicate that the presence of Aβ
77 oligomers correlates with a concomitant decrease in Aβ42 levels. CSF levels of total and
78 phosphorylated Tau protein [9], tissue transglutaminase (tTGase) [10], ubiquitin [11], Aβ
79 oligomers [12] as well as changes in Aβ1-42 concentration, together with the presence of
80 particular Aβ truncations [13] have been collectively suggested as useful biomarkers in AD.

81 Previously, mass spectrometry (MS) based analysis of CSF revealed a specific Aβ peptide
82 fragment signature in sporadic AD patients [14-16] and it has been reported that truncated Aβ
83 is known to represent more than 60% of all Aβ species found in non-demented as well as in
84 AD individuals [17]. These findings may suggest that Aβ oligomers could consist of a
85 heterogeneous morphological entity of full-length Aβ40 and Aβ42 as well as truncated Aβ

86 isoforms, of which the latter may serve as an important molecular seed during peptide
87 aggregation [18]. Similarly, a recent report showed that the aqueous phase of human AD
88 brain extracts contained SDS-stable Aβ species of a molecular weight range of 6-7kDa and
89 that these Aβ species may form part of larger Aβ aggregates [19].

90 In this work we sought to identify a "molecular crosstalk" during the lag phase of Aβ
91 peptide aggregation that typically precedes the pathological accumulation of neurotoxic
92 oligomers. Here, we have identified site specific autocleavage of Aβ peptide and report a
93 typical peptide fragment fingerprint, which may be associated with the early nucleation
94 process of Aβ aggregation. Using targeted and quantitative MS, we reveal a highly
95 reproducible Aβ fragment signature with a significant abundance of C-terminal peptide
96 amidation. Moreover, we show that these truncated Aβ peptides have a particularly high
97 propensity in forming SDS-stable low molecular weight oligomers of dimeric and trimeric
98 nature. These findings have enabled us to develop novel neo-epitope antibodies that
99 selectively bind to the gradual accumulation of truncated Aβ isoforms during the early phase
100 of peptide aggregation. Our targeted analysis of human brain tissue extracts and CSF
101 revealed that Aβ cleavage within the peptide's β-turn region is a highly relevant feature
102 observed in AD.

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111 **2. METHODS**

112 **2.1 Aβ peptide preparation.**

113 Full-length wild type (wt) Aβ peptides Aβ1-40, arctic mutant Aβ1-40 (Arc) and Aβ1-42
114 Aβ (Dr. James I. Elliott, Yale University, USA) were dissolved in 1,1,1,3,3,3-Hexafluoro-2-
115 propanol (HFIP) at a concentration of 1mg/ml, followed by a 10-min sonication to break any
116 preformed aggregates. HFIP solution was evaporated under a ventilated fume hood by
117 applying a light stream of N_2 gas. The HFIP film containing the Aβ peptide was either
118 directly re-suspended in 100% DMSO and further diluted to 1% DMSO in a new buffer or
119 stored dry at −20 °C until use. Aβ peptide fragments comprising of residues: 1-15, 1-22, 1-23,
120 1-24-NH_2, 1-25, 1-25-NH_2, 26-40, 24-40, (purity of ≥ 97%) were purchased from GenicBio
121 Ltd. (Shanghai, China). Aβ peptide concentrations were determined by UV absorbance using
122 the peptide's molar extinction coefficient at 280nm.

123 **2.2 Size Exclusion Chromatography of Aβ42 ADDLs and transgenic mouse brain tissue**
124 **extracts.**

125 Size exclusion chromatography (SEC) fractionation was carried out using an ÄKTA
126 Explorer FPLC (GE Healthcare) placed inside a cold (4 °C) chamber. A Superdex 200
127 10/300 GL column (GE Healthcare) was used and samples were eluted with either 25 mM
128 ammonium acetate (pH 8.5) or a Superdex 75 10/300GL with 20mM Tris 20mM NaCl
129 (pH7,5) (for aggregated Aβ1-25), at a flow rate of 0.5 ml/min. Prior to injection, samples
130 were centrifuged at 4°C 16,000 × g for 20 min and 0.5ml of sample supernatant was injected
131 onto the column. Aggregated Aβ1-25 peptide was filtered using 0.22µm filter devices prior to
132 injection to prevent from injecting any large, fibrillary aggregates. Peptide elution was
133 detected by absorbance at 280 nm, 275nm and 215nm and 0.5 ml fraction volumes were

134 collected. Eluted fractions were either used immediately or aliquoted (50ul) and stored at -
135 80°C. Where indicated, samples volumes were concentrated approximately 10x in a speed
136 vacuum.

137 **2.3 Matrix assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry**
138 **(MALDI TOF/TOF).**

139 Aliquots (2 µl) of samples were used for MALDI-TOF/TOF MS (ABI 4800 model,
140 Applied Biosystems) measurements. Matrix solution of α-cyano-4-hydroxycinnamic acid (7
141 mg/ml in ACN/0.1% TFA (1:1, v/v)) was used for sample deposition. The sample (1 µl) was
142 mixed with 1 µl of matrix solution and then 1 µl of this mixture was deposited in duplicates
143 on the target plate and allowed to air dry. Samples were analyzed in reflectron positive mode.

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145 **2.4 Digestion of Aβ peptides.**

146 Proteolytic digestion using LysN (2ng/ul) was performed overnight at 37 °C in 50 mM
147 ammonium bicarbonate, pH 10 (LysN buffer). For in-gel digestions, coomassie stained gel
148 bands were cut at the migration level of LMW Aβ oligomers (range: 6kDa - 14kDa) as
149 revealed by their immunoreactive bands in WB. Gel bands were destained and dried in a
150 speed vacuum prior to resuspension in Lys-N buffer containing 2ng/ul Lys-N protease
151 followed by overnight digestion at 37°C. Following digestion, the solution was recovered and
152 pooled with the peptides extracted from gels and concentrated by speed vacuum prior to LC-
153 MS measurements. Immunoprecipitated samples (IP), were reduced and alkylated followed
154 by in-solution digestion at 37°C using standard LysN buffer (50ul) and approximately 70ng
155 of LysN. Dried samples were resuspended in 10% DMSO and 5% FA as described below,
156 followed by LC-MS/MS or LC-SRM analysis.

157 **2.5 Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).**

158 For high resolution LC-MS/MS analysis, peptides were resuspended in 2% ACN, 0.1%
159 FA and separated by reversed-phase chromatography on a Dionex Ultimate 3000 RSLC
160 nanoUPLC system connected in-line with an Orbitrap Elite (Thermo Fischer Scientific,
161 Waltham, MA, USA). The instrument was operated in an information-dependent mode where
162 peptide masses for light and heavy lysine (K) labelled fragments Aβ16-23, Aβ16-24-NH_2,
163 Aβ16-25 and Aβ16-27 (purchased from Sigma Aldrich, Germany) were selected for
164 collision-induced dissociation (CID) to generate tandem mass spectra using a normalized
165 collision energy (CE) of 35. Samples were first captured on a homemade capillary pre-
166 column (Magic C18; 3 µm-200Å; 2 cm × 100 µm) prior to analytical separation. A 80-min
167 biphasic gradient was run starting from 100% A solvent (2% acetonitrile, 0.1% formic acid)
168 to 90% B solvent (100% acetonitrile, 0.1% formic acid) on capillary column (Nikkyo C18; 3
169 µm-100 Å; 15 cm × 75µm inner diameter at 250 nl/min).

170 **2.6 Quantitation of Aβ peptide fragments using Selected Reaction Monitoring (SRM)**
171 **mass spectrometry.**

172 All samples were analysed on a TSQ-Vantage triple quadrupole mass spectrometer
173 (Thermo Fisher Scientific). A 0.7-FWHM-resolution window for both Q1 and Q3 was set for
174 parent- and product-ion isolation. Fragmentation of parent ions was performed in Q2 at 1.5
175 mTorr, using collision energies calculated with the Pinpoint software (v1.1). Cycle times of
176 0.5s-1s were used for SRM runs with a minimum dwell time of 20ms.

177 Parent-ion selection was set for Lys-N digested peptides on the positively-charged parent
178 ions. CID fragmentation energies and the best transition selection were tested manually by
179 infusion on the TSQ using the synthetic peptide standards listed below. Mouse or human

brain and CSF samples were extracted and prepared for digestion as outlined below. Following overnight digestion at 37°C, samples were dried using a speed vacuum and stored at −20°C until analyses were performed. For Aβ peptide quantitation studies, a mixture of accurately quantified (by amino acid analysis) heavy isotope (lysine, K) labelled peptide standards (Sigma Aldrich, Germany) comprising of residues: Aβ16-23, Aβ16-25, Aβ16-27, Aβ28-38, Aβ28-40, Aβ28-42 and Aβ28-43, were spiked into each tube after resuspension of samples in the Lys-N digestion buffer. Aβ peptide fragments were initially resuspended in a solution containing 20% DMSO & 10% formic acid (FA) and further diluted to 5% DMSO & 2.5% FA prior to injection and analysis by LC-SRM. This solution provided maximum long-term stability of all peptide standards. Nano-LC-SRM parameters: Dried peptide aliquots were resuspended in 20µl DMSO (10%) with 5% FA. This preparation provided peptide solubility over two weeks without any significant changes in overall peptide recovery. Following resuspension, samples were briefly sonicated (3min) and allowed to settle for 1h to increase overall peptide solubility before analysis. Typically, 5µl of sample was loaded and captured on a homemade capillary precolumn (C18; 3 µm, 200 Å; 2 cm × 250 µm) before analytical LC separation (ACQUITY UPLC, Waters). Samples were separated using a 60min biphasic gradient starting from 100% solvent A (100% acetonitrile, 0.1% formic acid) to 90% solvent B (100% acetonitrile, 0.1% formic acid) on a Nikkyo (Nikkyo Technology) nano-column (C18; 3 µm, 100 Å; 150mm length and 100µm inner diameter; flow of 0.5µl/min). The gradient was followed by a wash for 8 min at 90% solvent B and column re-equilibration for 15 min at 100%.

204 **2.7 SDS-PAGE and Immunoblotting.**

205 Dried amples were mixed using standard SDS Lämmli sample buffer, Novex SDS sample
206 buffer and heated at 80°C for 5 min prior to loading onto gels. Three different commercially
207 available gels were used in order to compare the migration behaviour of Aβ peptide
208 fragments: Novex 16% Tris-Tricine gels, 1mm (Invitrogen), Biorad 10-20% Tricine gels
209 (Biorad, Switzerland) and Novex Nupage 4-12% Bis-Tris gels, 1mm (Invitrogen), of which
210 the latter type gels were used throughout the study. The following commercially available
211 running buffers were used: Novex Nupage Mes-SDS buffer (Invitrogen), Novex Tricine-SDS
212 buffer (Invitrogen) and Biorad Tricine buffer (Biorad, Switzerland). PAGE separated samples
213 were electroblotted onto nitrocellulose (0.22µm) membranes using standard protocols as
214 provided by the manufactures. Membranes were blocked for 1h at room temperature under
215 constant rocking using Odyssey blocking buffer (Li-COR Biosciences, Bad Homburg,
216 Germany) diluted 1:1 in PBS. Following blocking, membranes were incubated at 4 °C with
217 constant rocking overnight using the primary rabbit polyconal neo-epitope antibodies N-5ns,
218 N-5s, N-4, N-3s, D-4s, or D-6ns (0.28-0.5µg /ml), or the commercially available mouse
219 monoclonal antibodies 6E10 and 4G8 (0.5µg /ml) (Enzo, Life Sciences, Switzerland).
220 Membranes were washed four times with PBS-Tween (PBS containing 0.01% Tween 20),
221 followed by incubation with a goat anti-rabbit or anti-mouse secondary IgG antibody (highly
222 cross-adsorbed) (dilution, 1:5000) conjugated to Alexa Fluor 680 or 800 and scanned in a LI-
223 COR scanner at a wavelength of 700 nm and 800 nm respectively.

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227 **2.8 Dotblotting**.

228 Typically, 1ul samples were spotted onto a nitrocellulose membrane, which corresponded
229 to a total peptide load of 100ng (Aβ1-40) and 50ng (Aβ1-42) unless otherwise stated in the
230 figures. Samples were left to dry for 15min followed by blocking of the membrane (30min)
231 with LICOR buffer. Membrane strips were incubated with primary antibodies either for 2hrs
232 at room temperature or overnight at 4°C on a shaker. Identical solutions, antibody
233 concentrations and revelation procedures with secondary antibodies were used as described
234 for immunoblotting above.

235

236 **2.9 Generation of polyclonal antibodies.**

237 Briefly, a hepta to deca peptide sequence corresponding to the target neo-epitope
238 sequence of human Aβ peptide was conjugated to a KLH-linker and used for immunization
239 of rabbits (e.g. Aβ1-25: C+GG-VFFAEDVG-COOH). Antibodies (Table 1) were raised in
240 rabbits against a peptide identical to C-terminal residues Gly25 (N-5ns & N-5s), Asp23 (N-
241 3s), Val24-NH_2 (N-4), or N-terminal residues Val24 (D-4) and Ser26 (D-6) of human Aβ
242 peptide. All polyclonal antibodies were affinity-purified against the target Aβ neo-epitope
243 sequence using the carboxy- and amidated C-terminal form of the peptide sequence.
244 Antibody specificity and affinity was validated using direct ELISA with surface immobilized
245 (cross-inked to BSA) Aβ peptide sequences of normal and amidated C-termimus. All neo-
246 epitope antibodies were prepared by Eurogentec SA, (Liege, Belgium).

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251 **2.10 In vitro Aβ peptide aggregation studies.**

252 HFIP (1,1,1,3,3,3-Hexafluoro-2-propanol) (Sigma-Aldrich, Switzerland) dried Aβ peptide
253 films were solubilized in DMSO and further diluted with PBS (or 30mM Tris, 150mM NaCl)
254 to a final concentration of 0.1mg/ml for Aβ1-40 and 0.05mg/ml for Aβ1-42. Samples were
255 incubated at 37°C and left for spontaneous aggregation during either 0-20 hrs (short-term), 1-
256 5 days (intermediate) or 1-10 wk (long-term). Aggregated sample aliquots were drawn at
257 different time points and either analysed immediately using MS and/or DB or snap frozen in
258 liquid nitrogen and stored at -80°C. Typically, one microliter was spotted onto a
259 nitrocellulose membrane for dotblotting (50ng to100ng / spot) or mixed with alpha-cyano
260 matrix for MALDI-TOF/TOF analysis. For heavy water ($H_2{}^{18}O$, 97.0%, Cambridge Isotope
261 laboratories Inc., MA, USA) peptide aggregation studies, HFIP dried Aβ peptide films were
262 resuspended in anhydrous DMSO and diluted with $H_2{}^{18}O$ (containing Tris-NaCl
263 10mM/150mM) to a final DMSO concentration of ≤1%. The heavy $H_2{}^{18}O$ part of the final
264 reaction solution was estimated at approximately ≥95%.

265

266 **2.11 Immunoprecipitation of mouse and human brain tissue.**

267 Mouse and human brain tissue samples were serially extracted using TBS, 2% SDS or
268 formic acid (70-90%FA) as stated in the text. Briefly, tissue samples were homogenized (20
269 strokes on ice) in TBS and 5mM EDTA with protease inhibitor complex (Roche,
270 Switzerland) using a Teflon homogenizer. Samples were then subjected to centrifugation
271 (150,000g) during 45min and the supernatant was recovered as the TBS soluble fraction.
272 Protein pellets were subjected to an additional extraction using either SDS (2%) or FA (70%)
273 followed by centrifugation. Typically, pellets were extracted with FA overnight at 4°C (to
274 minimize formylation adducts) followed by centrifugation. SDS fractions were diluted to

275 ≤0.1% SDS final concentration and FA fractions were neutralized to pH 7.5 with 5M sodium
276 hydroxide (NaOH) solution prior to IP. All samples were initially depleted of endogenous
277 IgG's using a mixture of protein A&G agarose beads (Roche AG, Switzerland). Typically, 2-
278 4ug/ml rabbit polyclonal antibody (N-3s, N-4 or N-5ns) or 3-5ug/ml of 6E10 or 4G8 mouse
279 monoclonal was used for overnight IP under continues rotation (4rpm/min) at 4°C. Samples
280 were eluted with 40% ACN /H_2O & 0.1% TFA and dried in a speed vacuum. IP'ed samples
281 were either directly analysed by WB or MALDI-TOF/TOF or split and further digested
282 overnight using LysN proteolysis for LC-MS/MS or SRM analysis as outlined above. Human
283 CSF samples (500ul) were IP'ed with either N-5ns, or a mixture of the two commercial
284 antibodies 6E10 and 4G8.

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299 **3. RESULTS**

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301 **3.1 In vitro Aβ peptide aggregation is associated with autocleavage within the peptide's**
302 **β-turn vicinity resulting in the generation of a typical peptide fragment signature.**

303 We examined the aggregation behavior of synthetic Aβ42 peptide using MS and observed
304 that peptide aggregation is associated with a time dependent appearance of a typical peptide
305 fragment signature *in vitro*. To rule out the possibility of artefactual peptide hydrolysis during
306 sample preparation or MS analysis, we carried out aggregation studies using normal and
307 heavy oxygen labelled water ($H_2{}^{18}O$) to determine whether peptide cleavage is the result of
308 aggregation-induced peptide hydrolysis.

309 MS analysis of Aβ42 aggregation in both, normal and heavy (^{18}O) labeled water produced
310 identical MS spectra (Fig. 1A), revealing a highly reproducible Aβ peptide fragment
311 signature following short term (t=12h) aggregation. Under heavy water labelled conditions,
312 the majority of N-terminal fragment masses were shifted by 2 mass units as a result of a
313 hydrolysis induced ^{18}O atom incorporation at the newly formed C-terminus, as shown with
314 one of the most abundant N-terminal fragment of residues Aβ1-25 (Fig. 1A-G). Aβ1-25
315 represents one typical truncated Aβ isoform from a selection of several large N-terminal
316 fragments (Fig. 1B), where the monoisotopic peak was found to be shifted by 2.0012Da
317 (0.5003Da for m/z = 734.3513 $[M+4H]^{4+}$) as compared to the monoisotopic peak of normal
318 (m/z= 733.8463 $[M+4H]^{4+}$) (Fig. 1C, left) experimental conditions. No difference in mass
319 shift was observed for the complementary C-terminal fragment of residues 26-42 indicating
320 that hydrolysis-induced peptide cleavage resulted in stable ^{18}O incorporation only at the neo-
321 C-terminal residue Gly_{25} (Fig. 1C, right).

322 We also observed substantial C-terminal amidation ($CONH_2$) within the same isotopic
323 cluster of Aβ1-25, where the mass shift between the unmodified monoisotopic peak (m/z=

324 733.8509 $[M+4H]^{4+}$) and the amidated monoisotopic peak (m/z= 733.6037 $[M+4H]^{4+}$)
325 accounted for 0.988 Da (theoretical Δ mass = 0.984 Da) (Fig. 1C, right). The estimated
326 relative abundance of C-terminal amidation of fragment Aβ1-25-NH_2 has been estimated to
327 account of approximately 20% of the normal, carboxy C-terminal population (data not
328 shown).

329 Interestingly, Aβ1-24 was found to be predominantly amidated (Aβ1-24-NH_2) and only
330 minor levels of the normal carboxy C-terminus were detected (data not shown). Overall, we
331 observed that the gradual accumulation of the Aβ fragments of residues Aβ1-23, Aβ1-24-
332 NH_2, Aβ1-25 or Aβ1-25-NH_2 reflect a typical fragment signature during the early phase of
333 peptide aggregation because these large N-terminal fragments could be readily detected
334 following short term aggregation (≤1h) and typically preceded the accumulation of earlier
335 reported Aβ isoforms such as Aβ1-15 (Supplemental Fig.1). We also observed that some N-
336 terminal fragments have increased aggregation propensities, as seen by the rapid formation of
337 MS stable entities of dimeric and trimeric nature (Supplemental Fig.1 A&B) and that this in
338 turn may affect LC-MS analysis. Therefore, to monitor the generation of these Aβ isoforms
339 in a more reproducible manner, we processed samples using Lys-N proteolysis, which
340 generates the proteolytic cleavage product of residues Aβ16-25[20]. Lys-N digestion of Aβ
341 peptides resulted in highly increased solution stabilities and MS detection (100 fold) of the
342 proteolytic cleavage products as compared to non-digested Aβ1-25 (Supplemental Fig. 1D).
343 The change in overall charge distribution from multiply charged ions ($[M+2H]^{2+}$ to
344 $[M+5H]^{5+}$) for Aβ1-25 to a predominantly double charged ion ($[M+2H]^{2+}$) for the Lys-N
345 fragment Aβ16-25 would generally account for this significantly improved detection.

346 Quantitative MS analysis, using a spiked-in heavy lysine (K16) labelled surrogate peptide
347 indicated, that the relative abundance of fragment Aβ1-25 accounts for approximately 5% of
348 full-length Aβ42 (Fig. 1D). However, the absolute abundance of the here identified truncated

349 Aβ isoforms of Aβ1-23, Aβ1-24-NH_2 and Aβ1-25 as well as the C-terminal amidated form
350 would clearly exceed this level.

351 To further confirm a hydrolysis-induced peptide cleavage during *in vitro* aggregation, we
352 applied MS/MS using collision-induced dissociation (CID) to identify the site of stable ^{18}O
353 incorporation. MS/MS analysis of the Lys-N digested Aβ1-25 (16-25) fragment allowed for
354 unambiguous identification of the ^{18}O atom incorporation at Gly25 (Fig. 1E & F).

355

356

357 **3.2 N-terminal Aβ peptide fragments are highly prone to oligomerization.**

358 To determine the biophysical properties of truncated Aβ peptides, we investigated the
359 aggregation properties of four large N-terminal fragments: Aβ1-23, Aβ1-24-NH_2, Aβ1-25
360 and Aβ1-25-NH_2. Following *in vitro* aggregation of the synthetic Aβ fragments (t=1wk) we
361 observed significant changes in sodium dodecylsulfate polyacrylamide gel electrophoresis
362 (SDS-PAGE) migration behavior as seen by the appearance of several low molecular weight
363 (LMW) species in the range of 6kDa to 14kDa (Fig. 2A). High molecular weight (HMW)
364 oligomers were observed in the 49kDa to 62kDa range and this oligomeric entity was found
365 to be particularly characteristic for the amidated fragment Aβ1-25-NH_2. Immunoblot analysis
366 of some Aβ isoforms remains challenging using the conventional antibody 6E10, as seen for
367 Aβ1-23, which may result from conformation associated epitope masking [21], and therefore
368 a decreased sensitivity for 6E10 when compared with 4G8.

369 MS detection of LMW oligomers was mainly achieved at the dimer and trimer level, such
370 as shown with Aβ1-25, which may highlight the metastable structure found with soluble
371 oligomers during LC-MS analysis (Supplementary Fig. 1A & B). Moreover, prolonged aging
372 of the truncated Aβ isoforms Aβ1-25 or Aβ1-25-NH_2 resulted in the generation of shorter N-
373 terminal fragments such as the earlier reported fragments of residues Aβ1-14 and Aβ1-15

374 (Supplementary Fig. 1C & E). This observation was in agreement with prolonged Aβ42
375 aggregation experiments (t=7d), showing a time dependent decrease in MS detection of Aβ1-
376 25 together with the gradual appearance of shorter fragments, such as Aβ1-15
377 (Supplementary Fig. 2A).

378 Transmission electron microscopy (TEM) imaging indicated that N-terminal fragments
379 have a high propensity to form soluble, oligomeric aggregates following long-term
380 incubation. Aβ1-25 preferentially formed homogenous spherical aggregates, which was less
381 evident with the shorter N-terminal fragments of residues Aβ1-23 and Aβ1-24-NH_2 and only
382 very few clusters of fibrils were observed with the amidated form Aβ1-25-NH_2 (Fig. 2A and
383 Supplemental Fig. 2F). We further employed SEC (in Tris-NaCl) of aggregated Aβ1-25 and
384 show that the LMW structures typically observed with these truncated Aβ isoforms are true
385 observations and can therefore exclude a SDS-PAGE induced migration artefact (Fig. 2B).
386 SEC fractionation resulted in a clear separation of two Aβ structures centered at the migration
387 level of ≤ 6kDa (fraction: 12ml) and ≤ 3kDa (fraction: 15ml) and MS analysis of fraction
388 volume 12ml revealed the presence of stable Aβ1-25 dimers and trimers, whereas monomers
389 were mainly detected in fraction volume 15ml (data not shown). Because Aβ1-25 dimers
390 (5.8kDa) and trimers (8.7kDa) were highly enriched in SEC fractions corresponding to a MW
391 standard of ≤14kDa, we speculated that LMW Aβ1-25 structures of may form part of larger
392 Aβ entities, which dissociate during SDS-PAGE analysis.
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398 **3.3 Monitoring Aβ peptide aggregation using neo-epitope antibodies which specifically**
399 **target truncated Aβ isoforms.**

400 To create a simple analytical tool for the detection of site specific autocleavage during
401 aggregation we set out to develop an antibody enabled proof-of-principle tool for monitoring
402 Aβ cleavage. We therefore developed a panel of rabbit polyclonal antibodies (Table 1) with
403 high binding specificity for N-terminal fragments of residues Aβ1-23 (N-3s), Aβ1-24-NH_2
404 (N-4) and Aβ1-25 (N-5s & N-5ns) (Fig. 3 & Supplemental Fig 3A), as well as for two
405 complementary C-terminal fragments: Aβ24-42 (D-4s) and Aβ26-42 (D-6ns) (Supplementary
406 Fig. 3B).

407 Epitope binding was tested by dotblot (DB) using a repertoire of synthetic Aβ peptide
408 standards (1ug/spot) of different sequences or C-terminal endings and binding specificity was
409 compared with the conventional antibodies 6E10 and 4G8. None of the neo-epitope
410 antibodies detected full-length Aβ40 peptide, which highlights their unique binding
411 specificity for cleaved Aβ isoforms. Overall, 4G8 (17-24) showed higher specificity with
412 respect to its binding epitope as compared to 6E10 (1-16) because detection of fragment Aβ1-
413 15 was significantly reduced with 6E10, which is an observation reported before [22].

414 N-5s showed high specificity for the C-terminal Gly25 residue and N-3s selectively detected
415 fragments ending with C-terminal residue Asp23. The N-5ns binding epitope was found to
416 include residues spanning the neo-C-terminal region of residues 23-25, with preferential
417 binding properties for C-terminal Gly25 (Supplemental Fig. 3A) and binding was
418 significantly decreased or absent with the shorter fragments of Aβ1-22 and Aβ1-15
419 respectively. Similarly, antibody N-4 revealed high binding specificity for the amidated C-
420 teminal form of Val24 (Aβ1-24-NH_2) (Fig. 3B). Because of the analytical limitations
421 observed with DB (native conditions) we further compared the neo-epitope antibody
422 selectivity to 6E10 using immunoprecipitation (IP) of an Aβ fragment mixture along with

423 full-length Aβ40 (Fig. 3C) or in presence of a complex human proteome matrix (brain tissue
424 extract) (Supplementary Fig. 4C & D). All neo-epitope antibodies showed high selectivity for
425 their target fragments, whereas IP with 6E10 resulted in a pull-down of all Aβ fragments
426 together with full-length Aβ40.
427 The application of neo-epitope antibody N-5ns was further tested using ELISA and WB. At
428 working concentrations of ≤ 0.3ug/ml, N-5ns showed significantly lower affinity for the C-
429 terminal amidated form Aβ1-25-NH_2 (Fig. 3D) and WB revealed high selectivity for the
430 target fragments (Fig. 3E&F). In summary we can conclude that, by using a combination of
431 different conventional techniques, we were able to show that the above mentioned neo-
432 epitope antibodies have unique binding properties for truncated Aβ isoforms and may serve as
433 complementary tools for the analysis of biological samples.
434
435
436 **3.4 Aβ peptide cleavage is highly associated with changes in peptide secondary**
437 **structure.**
438 In order to understand the mechanisms associated with Aβ peptide cleavage during
439 aggregation we investigated peptide cleavage by stabilizing Aβ secondary structure in
440 different solutions. In PBS solution (or 40mM Tris: data not shown), Aβ40 showed a
441 predominantly unordered structure with considerable decrease in signal amplitude over the
442 time course of incubation, whereas incubation in 20% TFE solution induced a stable α-helical
443 structure during five days of incubation (Fig. 4A). Significant Aβ cleavage was observed in
444 PBS or Tris solutions, whereas cleavage was strongly attenuated in TFE (Fig. 4 B & C),
445 indicating that Aβ cleavage is highly associated with changes of the peptide's secondary
446 structure as a result of peptide aggregation. This observation was also true for Aβ42 (data not
447 shown).

448 Having established neo-epitope antibody specificity, we then selected and employed N-
449 5ns to monitor *in vitro* Aβ42 aggregation using DB and MS. We observed a robust increase
450 in N-5ns DB signal shortly after peptide incubation indicating a nearly instantaneous
451 accumulation of diagnostic Aβ fragments and TEM imaging of the peptide morphology
452 indicated that there was a correlation between the N-5ns signal saturation by DB and the
453 presence of typical oligomeric and protofibrilar aggregates (Fig. 4D).

454 We further studied the effects of different experimental conditions in Aβ aggregation.
455 Earlier findings provided strong evidence that the protein cross-linking activity of tissue
456 transglutaminase (tTGase) plays an important role in AD pathogenesis [23] [24]. We
457 therefore sought to investigate if tTGase induced Aβ aggregation mirrors the accumulation of
458 Aβ fragment fingerprints. Aβ40 aggregation increased significantly in the presence of tTGase
459 and was accompanied by the formation of low amounts of intra- and intermolecular cross-
460 links (data not shown) as reported previously [25] [18]. This change in aggregation behavior
461 could be detected by DB (Fig. 4E) and IP-MS analysis confirmed the presence of the N-5ns
462 target fragments Aβ1-23 and Aβ1-25 in samples subjected to different aggregation conditions
463 (Fig. 4F).

464 Since we were able to show that several peptide cleavage sites represent a typical hallmark
465 of early aggregation, we argued that the sum of several cleavage products would increase
466 overall DB screening sensitivity and showed, that the combination of several neo-epitope
467 antibodies indeed increased detection sensitivity during *in vitro* peptide aggregation
468 (Supplementary Fig. 4A). We further reasoned that neo-epitope antibody-based monitoring of
469 Aβ cleavage, prior to the accumulation of β-sheet enriched fibrils, would provide valuable
470 information for future screening natural inhibitory compounds (Supplementary Fig. 4B) [26]
471 [27] [28] [29]. This novel screening concept would be complementary to the typically
472 employed Thioflavin-T fluorescence measurements, since N-5ns would allow identification

473 of aggregation inhibitory compounds targeting an oligomerization fate [21] [30] rather than
474 advanced fibrillation.
475
476
477 **3.5 Aβ fragments form stable complexes with soluble oligomers.**
478 To better understand the biophysical properties of truncated Aβ in peptide
479 oligomerization, we investigated the aggregation kinetics of amyloid-derived diffusible
480 ligands (ADDL's) using a combination of SEC, DB, WB and MS. Following 20hrs of Aβ42
481 oligomerization, we observed a substantial increase in N-5ns signal using DB (Fig. 5A) and
482 this positive signal was associated with the presence of a typical oligomeric morphology
483 (data not shown). Following *in vitro* aggregation, the ADDL preparation was centrifuged in
484 order to precipitate large insoluble aggregates (pellet) and the supernatant containing soluble
485 Aβ species was subjected to SEC fractionation. To identify SEC fractions containing
486 truncated Aβ isoforms, 50ul fractions were dried by speed vacuum and re-suspended in 5ul of
487 PBS of which 1ul was spotted in duplicates onto a nitrocellulose membrane and probed by
488 DB using N-5ns and 6E10 respectively. SEC fraction probing with N-5ns allowed
489 identification of a substantial amount of truncated Aβ within the oligomeric fractions (8ml -
490 12ml) (Fig.5A). DB probing with 6E10 and N-5ns of the monomeric fraction (18ml)
491 indicated that this fraction contained a mixture of both, Aβ42 monomers as well as truncated
492 Aβ isoforms and WB analysis of these SEC fractions was in line with the findings from DB
493 (Fig. 5B). Moreover, WB also revealed the presence of HMW oligomers centered at the
494 49kDa to 62kDa range. The presence of low amounts of this HMW entity observed in the
495 monomeric fraction may indicate that truncated Aβ fragments may favor the formation of
496 these HMW structures as a result of sample concentration by speed-vacuum.

497 To further validate the N-5ns positive signals found by DB and WB, we analyzed
498 oligomeric fractions using IP-MS. MS analysis of the "crude" fraction volume 8ml revealed
499 that this fraction contained fragments Aβ1-23, Aβ1-24-NH_2, Aβ1-25 and Aβ1-25-NH_2 along
500 with some shorter fragments of Aβ1-17 to Aβ1-22 as well as full-length Aβ42 (Fig. 5C; top).
501 Similarly, MS analysis of LMW Aβ species extracted from SDS-PAGE gel bands (≤14kDa)
502 confirmed the presence of different truncated Aβ isoforms as well as their complementary C-
503 terminal fragments (Supplementary Fig.5).

504 IP with N-4 resulted in a specific pull-down of fragment Aβ1-24-NH_2 (Fig. 5C; center)
505 along with Aβ42 and N-5ns allowed a specific recovery of fragments Aβ1-25 but not Aβ1-
506 25-NH_2, as well as trace amounts of Aβ1-23 together with Aβ42 (Fig. 5C; bottom).

507 To further elucidate the importance of Aβ1-25 in oligomers, we analyzed two oligomeric
508 (11.5ml and 14ml) and a monomeric fraction (18ml) using IP-MS (N-5ns) (Fig. 5D) and
509 could identify Aβ1-25 in both, oligomeric and monomeric fractions. The relative abundance
510 of this particular fragment varied considerably, which was in line with our DB and WB
511 analysis. IP with N-5ns also resulted in a pull-down of Aβ42 indicating that oligomeric, as
512 well as monomeric fractions contained metastable complexes of fragment Aβ1-25 and Aβ42,
513 which may dissociate to a generally monomeric level during SDS-PAGE analysis. This data
514 collectively confirms the above reported observation that truncated Aβ can form stable
515 entities with soluble oligomers.

516 To provide quantitative values for Aβ1-25 and Aβ42 we used IP combined with LC-SRM
517 analysis. SEC fractions were split and one part was denatured with 70% FA over-night at 4°C
518 to allow gradual dissociation of large oligomeric species. We reasoned that this approach
519 would reduce the overall recovery (pull-down) of full-length Aβ42 stably bound to the
520 surface of large oligomers and therefore allow more accurate quantitation of the Aβ1-25
521 target fragment *per se*. SRM quantitation confirmed the significantly lower levels of Aβ1-25

522 (2-3pg/ul) (Fig. 5E) found in fraction volume 11.5ml as compared to the monomeric (18ml)
523 fraction (21-23pg/ul), which is also in line with our WB analysis. Moreover, we found that
524 FA dissociation of oligomers significantly reduced (40x) the amount of full-length Aβ42
525 stably bound to fragment Aβ1-25. IP-MS of the monomeric fraction, using a combination of
526 three different neo-epitope antibodies, revealed the presence of three truncated isoforms:
527 Aβ1-23, Aβ1-24-NH_2 and Aβ1-25 together with Aβ42 (Fig. 5F).

528 Because our initial mock IP's of freshly prepared Aβ fragment mixtures did not pull-down
529 full-length Aβ40 or Aβ42, we sought to provide direct evidence that detection of Aβ42 within
530 the monomeric fraction is the result of a collective pull-down due to stable interaction of
531 truncated Aβ and Aβ42. For this purpose, we denatured the first IP sample over-night using
532 90% FA, followed by a second IP using the same antibody cocktail. MS analysis of the
533 sequential IP (2nd) confirmed our assumption, because Aβ42 was no longer detected
534 following FA treatment (Fig. 5G).

535 MS analysis of the insoluble, pellet fraction (from ADDL prep.) indicated that large
536 insoluble aggregates also consist of heterogenic entities rich in N-terminal as well as C-
537 terminal truncated Aβ isoforms together with full-length Aβ42 (data not shown). This
538 observation was further corroborated by the finding that IP with antibody N-5s or N-5ns of
539 7M guanidine hydrochloride (GHCl) denatured Aβ42 fibrils, resulted in a specific recovery
540 of Aβ1-25 together with trace amounts of Aβ42 (Supplementary Fig. 6A & B).

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546 **3.6 N-terminal Aβ peptide fragments are present in the amyloid deposits in the human**
547 **AD brain.**

548 Immunohistochemical staining (both chromogenic and fluorescent) of the frontal cortical
549 tissue of human sporadic AD subjects (N = 6 AD cases and 3 age-matched non-demented
550 controls) using N-5ns resulted in robust labeling of thioflavin-S-positive amyloid plaques and
551 cerebral amyloid angiopathy (CAA) (Fig. 6A & B). Thioflavin-S-positive intraneuronal tau
552 tangles were not labelled with N-5ns (Fig. 6B) and no non-specific labeling was detected in
553 the control non-AD brains (Fig. 6C). Because one of the early and important effects of
554 oligomeric Aβ in AD is the binding of Aβ to synapses and the resultant synaptic dysfunction
555 and loss [31], we examined the presence of Aβ neo-epitopes in synapses. Tissue from human
556 subjects was prepared for high-resolution array tomography [32], allowing accurate detection
557 of individual synapses. As seen with the immunostaining of paraffin sections, N-5ns labeled
558 amyloid deposits that were positive for thioflavin-S and 6E10 (Fig. 6D). Both dense-core and
559 diffuse plaques were immuno-positive for N-5ns. We also observed staining of N-5ns at
560 individual pre- and postsynaptic puncta in the region of plaques, indicating that this Aβ
561 fragment may be important in synapse degeneration (Fig. 6E).

562

563

564 **3.7 Analysis of human AD brain and transgenic mice brain tissue highlights the**
565 **significant abundance of Aβ cleavage in AD.**

566 In order to further corroborate our *in vitro* observation on Aβ cleavage, we analyzed
567 human post-mortem brain tissue samples from human controls and AD subjects.
568 We employed IP-MS and WB analysis of TBS and FA tissue extractions to investigate
569 presence of Aβ truncation using the two conventional antibodies 6E10 and 4G8 and compared
570 Aβ peptide recovery with neo-epitope antibodies. IP with 6E10&4G8 resulted in the detection

571 of a band centered at the typical migration level of Aβ monomers (>3kDa) (Fig. 7A, top blot:
572 AD#1 lane: 1, AD#2 lane: 4), whereas IP with neo-epitope antibodies revealed a band
573 centered at the migration level of ≤6kDa for one AD subject (Fig. 7A top blot, lane: 5).
574 Interestingly, re-probing the membrane with a neo-epitope antibody cocktail revealed a band
575 centered at the migration level of ≥3kDa (Fig. 7A bottom blot: AD#1 lane: 2, AD#2 lane: 5),
576 indicating that both AD samples contained truncated Aβ isoforms, whereas 4G8 failed to
577 show similar specificity at this migration level, which is an observation also made with the
578 synthetic Aβ fragments (Fig. 3).
579 We further analyzed the same brain samples by MALDI-TOF/TOF MS. IP-MS (6E10&4G8)
580 analysis of the AD#2 brain tissue extract (FA) resulted in a pull-down of large Aβ fragments
581 Aβ36, Aβ37, Aβ38 and Aβ40 together with several N-terminal fragments Aβ1-13-NH_2, Aβ1-
582 13, Aβ1-20, Aβ1-22, Aβ1-23, Aβ2-24-NH_2 Aβ1-24-NH_2 and Aβ1-25 (Fig. 7B). IP-MS
583 analysis of the same AD brain, using a combination of three neo-epitope antibodies resulted
584 in a specific pull-down of the target fragments (Fig. 7C). The same samples were also
585 subjected to LC-MS/MS (Orbitrap) analysis for high resolution peptide mass confirmation
586 (Fig. 7D & E).
587 Moreover show that, IP with N-5ns enables a specific recovery of fragment Aβ1-25 from
588 Tg2576, APP/PS1 (Supplementary Fig. 6C & D) and 5xFAD (data not shown) transgenic
589 mice brains. The use of SDS (Supplemental Fig. 7), TBS or FA (Supplementary Fig. 8A & B)
590 extraction protocols all resulted in similar truncated Aβ recovery, however, the levels of Aβ1-
591 25 were found to be significantly increased in FA extracts as compared to TBS soluble
592 fractions (Supplementary Fig. 8B). LC-SRM analysis of TBS and FA brain extracts from
593 human controls indicated that the levels of cleaved Aβ isoforms are significantly reduced or
594 below the limit of detection (data not shown).
595

596 **3.8 Human CSF analysis reflects the accumulation of N-terminal fragments in AD**
597 **brain.**

598 We first measured levels of Aβ42 and total Tau (T-Tau) in AD patients and non-demented
599 (ND) controls using ELISA and observed significantly ($p<0.001$) decreased levels of Aβ42 as
600 well as increased T-Tau levels ($p<0.001$) (Supplementary Fig. 8C & D) in AD patients as
601 compared to ND controls, which is in line with earlier reported measurements of human CSF
602 samples [11, 33]. We were further interested in identifying Aβ cleave by applying
603 quantitative IP-SRM (N-5ns) to measure Aβ1-25 levels in CSF and could clearly confirm the
604 presence of Aβ1-25 in both; AD patients and age matched controls subjects (Supplementary
605 Fig. 8E). A large inter-subject variability of Aβ1-25 levels was generally observed in AD
606 patients (n=16) but the measured levels failed to show a statistical significant difference
607 ($p>0.05$) when compared to control subjects (n=14) (Supplementary Table II).

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611 **4. DISCUSSION**

612 The presence of particular Aβ peptide fragments *in vitro* [34, 35] and *in vivo* [36, 37] has
613 been reported before and the accumulation of some Aβ isoforms is thought to be associated
614 with a putative enzymatic activity [38-40]. Generally, it seems unlikely that either proteinases
615 or exopeptidases are responsible for the generation of truncated Aβ isoforms because *in vitro*
616 peptide cleavage still occurs in the presence of a metalloprotease inhibitor [35] or
617 bacteriostatic agents (data not shown). Overall, the exact mechanism associated with the
618 putative concerted enzymatic cleavage of Aβ still remains unclear. Regardless of proteinase
619 activity, earlier reports show that *in vitro* Aβ40 aggregation resulted in peptide cleavage at
620 residue Asp23 [41] and that the increased aggregation observed for Aβ40 in the presence of

621 tTGase was accompanied with significant cleavage at residues Glu22 and Gly25 respectively
622 [18]. Moreover, it has been suggested that Aβ25-35 as well as full-length Aβ40 mediated
623 toxicity may result from a peptide cleavage induced radicalisation of cell membranes, and
624 that prolonged incubation resulted in significant Aβ cleavage at the Gly25-Ser26 bond [42].
625 The here reported occurrence of cleavage at residue Gly25 is of particular interest, because
626 Aβ1-25 was earlier identified as a sphingolipid binding domain motif, which can be rapidly
627 internalized by neuronal cells [43].

628 We report here new, additional Aβ fragments and were able to demonstrate that the
629 gradual appearance of fragments Aβ1-23, Aβ1-24-NH_2 Aβ1-25 and its amidated form Aβ1-
630 25NH_2 can be associated with the early events of Aβ aggregation, because MS detection of
631 the aforementioned N-terminal fragments precedes the accumulation of shorter Aβ isoforms
632 (i.e Aβ1-15).

633 To our knowledge, the presence of substantial C-terminal amidation *in vitro* and *in vivo*
634 has not been reported before, suggesting that Aβ cleavage is the result of at least two distinct
635 molecular mechanisms. More importantly, we show that Aβ cleavage is strongly attenuated
636 when stabilizing the peptide in an α-helical structure. This suggests that the transition from an
637 unstructured, random-coil conformation to a β-sheet ordered structure triggers the cleavage
638 cascade typically observed during peptide aggregation. Moreover, the time-dependent
639 increase in Aβ1-15 abundance may indicate that shorter N-terminal fragments represent
640 cleavage products associated with a more advanced phase of aggregation, which is
641 corroborated by the observation that long-term incubation of the here described fragment of
642 Aβ1-25, or its more amyloidogenic form Aβ1-25-NH_2, give rise to shorter Aβ isoforms.
643 Generally, the detection of a particular Aβ fragment signature in different AD transgenic
644 models as well as human AD brains; collectively highlight the need for further understanding

645 the presence of Aβ fragment signatures in AD. Therefore, the exact molecular mechanism
646 leading to site specific hydrolysis of Aβ remains to be elucidated in future studies.

647 Soluble Aβ oligomers play a central role in AD pathogenesis, with dimers [44] and
648 dodecamers (Aβ* 56) having attracted most of the scientific attention in the past. We report
649 here that IP of soluble Aβ40 or Aβ42 oligomers resulted in a specific recovery of Aβ
650 assemblies with a gel migration range of ≥6kDa, and that similar Aβ assemblies could be
651 detected in TBS and FA lysates from human AD brains. Given by our findings, it is
652 conceivable that Aβ assemblies of putative dimeric or trimeric nature consist of a mixture of
653 truncated and full-length Aβ, which may form metastable complexes with HMW structures,
654 which is partly in agreement with earlier reports [19].

655 The detection of Aβ1-25 in CSF samples from human controls may suggest a significant
656 abundance of Aβ oligomers present in control subjects, which is in line with earlier reports
657 [45]. Interestingly, Holtta et al. (2013) showed that CSF oligomers were significantly
658 increased in patients with mild and moderate dementia when compared to controls, whereas
659 no significant difference was found in patients with severe dementia [12]. The here observed
660 lack of statistical significance in Aβ1-25 levels in AD patients may result from the relatively
661 small sample size and hence statistical power. It is also conceivable that CSF sample freeze-
662 thaw cycles together with other, earlier reported cofounding factors[46, 47] may have
663 collectively contributed to a rapid *ex vivo* Aβ aggregation in these samples.

664 In conclusion, we argue that neo-epitope antibodies would serve as appealing capture
665 antibodies for future ELISA developments because we were able to show that the here
666 described Aβ fragments can self-propagate to dimers and trimers or form stable entities with
667 large oligomers. However, measuring changes in levels of truncated Aβ isoforms merits
668 additional, future analytical improvements. We believe that monitoring pathological changes
669 in Aβ levels in human CSF [39] or plasma [48] requires the use of multiplexed approaches,

670 where truncated Aβ isoforms together with several earlier reported Aβ fragments [49] as well
671 as pathologically relevant post-translational modifications, such as pyroglutamate modified
672 Aβ [16], should be monitored simultaneously and longitudinally in human biofluids.
673
674
675 **RESEARCH IN CONTEXT**
676 ***Systematic Review***: Aβ dimers, trimers and dodecamers have received the most scientific
677 attention in the past, because these entities have been suggested to form the building blocks of
678 larger neurotoxic assemblies. However, the key molecular triggers associated with early Aβ
679 oligomerization are poorly understood and to date, the exact molecular structure of LMW
680 oligomers still remains a conundrum.
681 ***Interpretation***: We have identified here new truncated Aβ isoforms with high aggregation
682 propensities, which may serve as seeding units during early peptide aggregation. We provide
683 analytical evidence that these truncated Aβ isoforms are highly abundant in Aβ oligomers.
684 ***Future directions****:* We plan to further study the generation of truncated Aβ isoforms as
685 well as their significance to the pre-symptomatic accumulation of neurotoxic oligomers. The
686 use typical Aβ peptide fragment fingerprints for a pre-symptomatic diagnosis of subjects
687 suffering from MCI or other forms of dementia will be of particular interest. Furthermore, we
688 are interested in studying the structural properties and aggregation fate of the here described
689 truncated Aβ isoforms. This will help to identify and understand the structure homology
690 found in Aβ oligomers which in turn may improve the future development of oligomer
691 specific antibodies.
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ACKNOWLEDGMENTS

This work was partially funded by the "Enable Grant" kindly awarded by the Technology Transfer Office (TTO) (Dr. André Catana) of the Ecole Polytechnique Fédérale de Lausanne (EPFL), the Biomedizin-Naturwissenschaft-Forschung (BNF) program from the University of Bern, Switzerland, and internal funding of the EPFL. We would like to thank Mrs. Maria Anna Lauricella (Eurogentec SA, Liege, Belgium) for the technical expertise and support in antibody development, Prof. Hilal Lashuel, Laboratory of Molecular Neurobiology and Neuroproteomics (LMNN, EPFL), for kindly proving us with Aβ40/42 peptides. We thank Dr. Adam Swetloff (TTO, EPFL) for constructive discussions on the project. We are grateful to Dr. Bernard Schneider, Neurodegenerative Disease Laboratory (LEN, EPFL), for providing and helping us with APP-Swe/Arc mutant cell cultures and 5xFAD transgenic mouse tissue as well as Mr. Philippe Colin (LEN, EPFL) for his technical support in mouse tissue preparations. Immunohistochemistry and human brain sample preparation was supported by the Massachusetts Alzheimer Disease Research Center (NIHP50 AG005134).

SUPPLEMENTARY DATA

Supplementary figures 1-8 and tables I & II.

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