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### Amyloid-beta oligomerization is associated with the generation of a typical peptide fragment fingerprint

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1	$A\beta$ oligomerization is associated with the generation of a typical peptide fragment
2	fingerprint.
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4	Nikita Rudinskiy <sup>1+</sup> , Christophe Fuerer <sup>2+</sup> , Davide Demurtas <sup>3</sup> , Sebastian Zamorano <sup>2</sup> , Cyntia De Piano <sup>2</sup> ,
5	Ami Herrmann <sup>4</sup> , Tara. L. Spires-Jones <sup>4</sup> , Patrick Oeckl <sup>5</sup> , Markus Otto <sup>5</sup> , Matthew P. Frosch <sup>6</sup> ,
6	Marc Moniatte <sup>2</sup> , Bradley T. Hyman <sup>1</sup> , Adrien W. Schmid <sup>2*</sup> .
7	
8	<sup>1</sup> Harvard Medical School, Mass General Hospital, Massachusetts Boston, USA.
9	<sup>2</sup> Proteomics Core Facility, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland.
10	<sup>3</sup> Interdisciplinary Centre for Electron Microscopy (CIME), EPFL, Switzerland.
11	<sup>4</sup> The University of Edinburgh, Center for Cognitive and Neural Systems & Euan MacDonald Centre for
12	Motorneurone Disease, Edinburgh, Scotland.
13	<sup>5</sup> Department of Neurology, Ulm University Hospital, Ulm, Germany.
14	<sup>6</sup> Massachusetts General Hospital and Harvard Medical School, Massachusetts General Institute for
15	Neurodegenerative Disease, Charlestown, MA, USA.
16	<sup>+</sup> These authors contributed equally to this work.
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21	
22	*To whom correspondence should be addressed:
23	Adrien W. Schmid, Ph.D.
24	Proteomics Core Facility, Life Sciences (SV)
25	PCF-PTP, Station 15, EPFL
20 27	Tel: $+41(0)216930651$
28	Fax: $+41$ (0)21 693 18 88
29	E-mail: <u>adrien.schmid@epfl.ch</u>
30 31	
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33	amidation, mass spectrometry, selected reaction monitoring.
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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.

- 43
- 44 **Conflict of interest:** none
- 45

#### 46 ABSTRACT

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48 Amyloid-beta (AB) peptide oligomerization plays a central role in the pathogenesis of 49 Alzheimer's disease (AD) and AB oligomers are collectively considered an appealing 50 therapeutic target for the treatment of AD. However, the molecular mechanisms leading to 51 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 52 53 site-specific A $\beta$  autocleavage during the early phase of aggregation, producing a typical A $\beta$ fragment signature and that truncated Aß peptides can form stable oligomeric complexes with 54 55 full-length A $\beta$  peptide. We show that the use of novel anti-A $\beta$  antibodies raised against these 56 truncated AB isoforms allows for monitoring and targeting the accumulation of truncated AB fragments. Antibody-enabled screening of transgenic models of AD, as well as human post-57 58 mortem brain tissue and cerebrospinal fluid revealed that, aggregation-associated AB 59 cleavage is a highly relevant clinical feature of AD.

#### 61 1. INTRODUCTION

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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 soluble oligomers might account for the AD-associated decline in synaptic plasticity [1, 2] 65 66 and that inhibition of natural AB oligomerization rescues deficits in long-term potentiation (LTP) [3]. Several types of Aβ assemblies of dimeric and trimeric [4] [1] [2] or dodecameric 67  $(A\beta^* 56)$  [5] nature have been observed *in vitro* and *in vivo* in transgenic mouse models. 68 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 AB 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 oligomers difficult. Therefore, the development of specific anti-oligomeric based therapeutics 74 75 remains challenging.

Cerebrospinal fluid (CSF) analyses from AD patients indicate that the presence of  $A\beta$ oligomers correlates with a concomitant decrease in A $\beta$ 42 levels. CSF levels of total and phosphorylated Tau protein [9], tissue transglutaminase (tTGase) [10], ubiquitin [11], A $\beta$ oligomers [12] as well as changes in A $\beta$ 1-42 concentration, together with the presence of particular A $\beta$  truncations [13] have been collectively suggested as useful biomarkers in AD.

Previously, mass spectrometry (MS) based analysis of CSF revealed a specific  $A\beta$  peptide fragment signature in sporadic AD patients [14-16] and it has been reported that truncated  $A\beta$ is known to represent more than 60% of all  $A\beta$  species found in non-demented as well as in AD individuals [17]. These findings may suggest that  $A\beta$  oligomers could consist of a heterogeneous morphological entity of full-length  $A\beta40$  and  $A\beta42$  as well as truncated  $A\beta$ 

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

90 In this work we sought to identify a "molecular crosstalk" during the lag phase of  $A\beta$ 91 peptide aggregation that typically precedes the pathological accumulation of neurotoxic oligomers. Here, we have identified site specific autocleavage of AB peptide and report a 92 93 typical peptide fragment fingerprint, which may be associated with the early nucleation 94 process of A $\beta$  aggregation. Using targeted and quantitative MS, we reveal a highly 95 reproducible AB fragment signature with a significant abundance of C-terminal peptide 96 amidation. Moreover, we show that these truncated  $A\beta$  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 A<sub>β</sub> isoforms during the early phase 100 of peptide aggregation. Our targeted analysis of human brain tissue extracts and CSF 101 revealed that A $\beta$  cleavage within the peptide's  $\beta$ -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) A $\beta$  peptides A $\beta$ 1-40, arctic mutant A $\beta$ 1-40 (Arc) and A $\beta$ 1-42 114 Aß (Dr. James I. Elliott, Yale University, USA) were dissolved in 1,1,1,3,3,3-Hexafluoro-2propanol (HFIP) at a concentration of 1mg/ml, followed by a 10-min sonication to break any 115 116 preformed aggregates. HFIP solution was evaporated under a ventilated fume hood by 117 applying a light stream of N<sub>2</sub> 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. Aß peptide fragments comprising of residues: 1-15, 1-22, 1-23, 120 1-24-NH<sub>2</sub>, 1-25, 1-25-NH<sub>2</sub>, 26-40, 24-40, (purity of  $\ge$  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 AB1-25), 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 AB1-25 peptide was filtered using 0.22µm filter devices prior to injection to prevent from injecting any large, fibrillary aggregates. Peptide elution was 132 133 detected by absorbance at 280 nm, 275nm and 215nm and 0.5 ml fraction volumes were collected. Eluted fractions were either used immediately or aliquoted (50ul) and stored at 80°C. Where indicated, samples volumes were concentrated approximately 10x in a speed
vacuum.

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

Aliquots (2  $\mu$ l) of samples were used for MALDI-TOF/TOF MS (ABI 4800 model, Applied Biosystems) measurements. Matrix solution of  $\alpha$ -cyano-4-hydroxycinnamic acid (7 mg/ml in ACN/0.1% TFA (1:1, v/v)) was used for sample deposition. The sample (1  $\mu$ l) was mixed with 1  $\mu$ l of matrix solution and then 1  $\mu$ l of this mixture was deposited in duplicates 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.**

Proteolytic digestion using LysN (2ng/ul) was performed overnight at 37 °C in 50 mM 146 147 ammonium bicarbonate, pH 10 (LysN buffer). For in-gel digestions, coomassie stained gel 148 bands were cut at the migration level of LMW AB 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 AB16-23, AB16-24-NH<sub>2</sub>, 163 AB16-25 and AB16-27 (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  $\mu$ m-200Å; 2 cm × 100  $\mu$ m) 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  $\mu$ m-100 Å; 15 cm × 75 $\mu$ m inner diameter at 250 nl/min).

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

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

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

180 brain and CSF samples were extracted and prepared for digestion as outlined below. 181 Following overnight digestion at 37°C, samples were dried using a speed vacuum and stored 182 at  $-20^{\circ}$ C until analyses were performed. For A $\beta$  peptide quantitation studies, a mixture of 183 accurately quantified (by amino acid analysis) heavy isotope (lysine, K) labelled peptide 184 standards (Sigma Aldrich, Germany) comprising of residues: A\beta16-23, A\beta16-25, A\beta16-27, 185 AB28-38, AB28-40, AB28-42 and AB28-43, were spiked into each tube after resuspension of 186 samples in the Lys-N digestion buffer. Aß peptide fragments were initially resuspended in a 187 solution containing 20% DMSO & 10% formic acid (FA) and further diluted to 5% DMSO & 188 2.5% FA prior to injection and analysis by LC-SRM. This solution provided maximum long-189 term stability of all peptide standards. Nano-LC-SRM parameters: Dried peptide aliquots 190 were resuspended in 20µl DMSO (10%) with 5% FA. This preparation provided peptide 191 solubility over two weeks without any significant changes in overall peptide recovery. 192 Following resuspension, samples were briefly sonicated (3min) and allowed to settle for 1h to 193 increase overall peptide solubility before analysis. Typically, 5µl of sample was loaded and 194 captured on a homemade capillary precolumn (C18; 3  $\mu$ m, 200 Å; 2 cm  $\times$  250  $\mu$ m) before 195 analytical LC separation (ACQUITY UPLC, Waters). Samples were separated using a 60min 196 biphasic gradient starting from 100% solvent A (100% acetonitrile, 0.1% formic acid) to 90% 197 solvent B (100% acetonitrile, 0.1% formic acid) on a Nikkyo (Nikkyo Technology) nano-198 column (C18; 3 µm, 100 Å; 150mm length and 100µm inner diameter; flow of 0.5µl/min). 199 The gradient was followed by a wash for 8 min at 90% solvent B and column re-equilibration 200 for 15 min at 100%.

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#### 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 AB 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**.

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

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#### 236 **2.9 Generation of polyclonal antibodies.**

Briefly, a hepta to deca peptide sequence corresponding to the target neo-epitope 237 238 sequence of human AB peptide was conjugated to a KLH-linker and used for immunization of rabbits (e.g. A\beta1-25: C+GG-VFFAEDVG-COOH). Antibodies (Table 1) were raised in 239 240 rabbits against a peptide identical to C-terminal residues Glv25 (N-5ns & N-5s). Asp23 (N-3s), Val24-NH<sub>2</sub> (N-4), or N-terminal residues Val24 (D-4) and Ser26 (D-6) of human Aβ 241 242 peptide. All polyclonal antibodies were affinity-purified against the target Aß 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 (cross-inked to BSA) AB peptide sequences of normal and amidated C-termimus. All neo-245 246 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 A\beta1-40 and 0.05mg/ml for A\beta1-42. 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 matrix for MALDI-TOF/TOF analysis. For heavy water (H2<sup>18</sup>O, 97.0%, Cambridge Isotope 260 261 laboratories Inc., MA, USA) peptide aggregation studies, HFIP dried Aß peptide films were resuspended in anhydrous DMSO and diluted with H<sub>2</sub><sup>18</sup>O (containing Tris-NaCl 262 10mM/150mM) to a final DMSO concentration of  $\leq$ 1%. The heavy H<sub>2</sub><sup>18</sup>O part of the final 263 264 reaction solution was estimated at approximately  $\geq 95\%$ .

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

Mouse and human brain tissue samples were serially extracted using TBS, 2% SDS or 267 268 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%) followed by centrifugation. Typically, pellets were extracted with FA overnight at 4°C (to 273 274 minimize formylation adducts) followed by centrifugation. SDS fractions were diluted to

275	$\leq 0.1\%$ 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 <sub>2</sub> O & 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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#### **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.

We examined the aggregation behavior of synthetic A $\beta$ 42 peptide using MS and observed that peptide aggregation is associated with a time dependent appearance of a typical peptide fragment signature *in vitro*. To rule out the possibility of artefactual peptide hydrolysis during sample preparation or MS analysis, we carried out aggregation studies using normal and heavy oxygen labelled water (H<sub>2</sub><sup>18</sup>O) to determine whether peptide cleavage is the result of aggregation-induced peptide hydrolysis.

309 MS analysis of A $\beta$ 42 aggregation in both, normal and heavy (<sup>18</sup>O) labeled water produced 310 identical MS spectra (Fig. 1A), revealing a highly reproducible AB peptide fragment signature following short term (t=12h) aggregation. Under heavy water labelled conditions, 311 312 the majority of N-terminal fragment masses were shifted by 2 mass units as a result of a hydrolysis induced <sup>18</sup>O atom incorporation at the newly formed C-terminus, as shown with 313 314 one of the most abundant N-terminal fragment of residues AB1-25 (Fig. 1A-G). AB1-25 315 represents one typical truncated AB 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  $(0.5003\text{Da for m/z} = 734.3513 \text{ [M+4H]}^{4+})$  as compared to the monoisotopic peak of normal 317  $(m/z=733.8463 [M+4H]^{4+})$  (Fig. 1C, left) experimental conditions. No difference in mass 318 319 shift was observed for the complementary C-terminal fragment of residues 26-42 indicating that hydrolysis-induced peptide cleavage resulted in stable <sup>18</sup>O incorporation only at the neo-320 321 C-terminal residue Gly<sub>25</sub> (Fig. 1C, right).

322 We also observed substantial C-terminal amidation (CONH<sub>2</sub>) within the same isotopic 323 cluster of A $\beta$ 1-25, 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  $\Delta$  mass = 0.984 Da) (Fig. 1C, right). The estimated 326 relative abundance of C-terminal amidation of fragment A $\beta$ 1-25-NH<sub>2</sub> has been estimated to 327 account of approximately 20% of the normal, carboxy C-terminal population (data not 328 shown).

329 Interestingly,  $A\beta 1-24$  was found to be predominantly amidated ( $A\beta 1-24-NH_2$ ) 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 AB fragments of residues AB1-23, AB1-24-332 NH<sub>2</sub>, A $\beta$ 1-25 or A $\beta$ 1-25-NH<sub>2</sub> 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 ( $\leq$ 1h) and typically preceded the accumulation of earlier 335 reported AB isoforms such as AB1-15 (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 turn may affect LC-MS analysis. Therefore, to monitor the generation of these Aβ isoforms 338 339 in a more reproducible manner, we processed samples using Lys-N proteolysis, which generates the proteolytic cleavage product of residues A\beta16-25[20]. Lys-N digestion of A\beta 340 341 peptides resulted in highly increased solution stabilities and MS detection (100 fold) of the 342 proteolytic cleavage products as compared to non-digested A\beta1-25 (Supplemental Fig. 1D). The change in overall charge distribution from multiply charged ions  $([M+2H]^{2+})$  to 343  $[M+5H]^{5+}$ ) for A $\beta$ 1-25 to a predominantly double charged ion ( $[M+2H]^{2+}$ ) for the Lys-N 344 345 fragment A\beta16-25 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 A $\beta$ 1-25 accounts for approximately 5% of 348 full-length A $\beta$ 42 (Fig. 1D). However, the absolute abundance of the here identified truncated 349 A $\beta$  isoforms of A $\beta$ 1-23, A $\beta$ 1-24-NH<sub>2</sub> and A $\beta$ 1-25 as well as the C-terminal amidated form 350 would clearly exceed this level.

To further confirm a hydrolysis-induced peptide cleavage during *in vitro* aggregation, we applied MS/MS using collision-induced dissociation (CID) to identify the site of stable <sup>18</sup>O incorporation. MS/MS analysis of the Lys-N digested A $\beta$ 1-25 (16-25) fragment allowed for unambiguous identification of the <sup>18</sup>O atom incorporation at Gly25 (Fig. 1E & F).

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

358 To determine the biophysical properties of truncated AB peptides, we investigated the 359 aggregation properties of four large N-terminal fragments: AB1-23, AB1-24-NH<sub>2</sub>, AB1-25 360 and A\beta1-25-NH2. Following *in vitro* aggregation of the synthetic A\beta 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<sub>2</sub>. Immunoblot analysis 366 of some A $\beta$  isoforms remains challenging using the conventional antibody 6E10, as seen for 367 AB1-23, 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 A $\beta$ 1-25, 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 A $\beta$  isoforms A $\beta$ 1-25 or A $\beta$ 1-25-NH<sub>2</sub> resulted in the generation of shorter N-373 terminal fragments such as the earlier reported fragments of residues A $\beta$ 1-14 and A $\beta$ 1-15 (Supplementary Fig. 1C & E). This observation was in agreement with prolonged AB42

aggregation experiments (t=7d), showing a time dependent decrease in MS detection of A<sup>β</sup>1-

25 together with the gradual appearance of shorter fragments, such as A $\beta$ 1-15

Transmission electron microscopy (TEM) imaging indicated that N-terminal fragments

have a high propensity to form soluble, oligomeric aggregates following long-term

incubation. A\beta1-25 preferentially formed homogenous spherical aggregates, which was less

evident with the shorter N-terminal fragments of residues  $A\beta$ 1-23 and  $A\beta$ 1-24-NH<sub>2</sub> and only

very few clusters of fibrils were observed with the amidated form Aβ1-25-NH<sub>2</sub> (Fig. 2A and

Supplemental Fig. 2F). We further employed SEC (in Tris-NaCl) of aggregated A\beta1-25 and

show that the LMW structures typically observed with these truncated  $A\beta$  isoforms are true

observations and can therefore exclude a SDS-PAGE induced migration artefact (Fig. 2B).

SEC fractionation resulted in a clear separation of two A $\beta$  structures centered at the migration level of  $\leq 6$ kDa (fraction: 12ml) and  $\leq 3$ kDa (fraction: 15ml) and MS analysis of fraction volume 12ml revealed the presence of stable A $\beta$ 1-25 dimers and trimers, whereas monomers

were mainly detected in fraction volume 15ml (data not shown). Because A $\beta$ 1-25 dimers (5.8kDa) and trimers (8.7kDa) were highly enriched in SEC fractions corresponding to a MW standard of  $\leq$ 14kDa, we speculated that LMW A $\beta$ 1-25 structures of may form part of larger

392 A $\beta$  entities, which dissociate during SDS-PAGE analysis.

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(Supplementary Fig. 2A).

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#### 398 3.3 Monitoring AB peptide aggregation using neo-epitope antibodies which specifically 399 target truncated AB 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 A $\beta$  cleavage. We therefore developed a panel of rabbit polyclonal antibodies (Table 1) with 403 high binding specificity for N-terminal fragments of residues AB1-23 (N-3s), AB1-24-NH2 (N-4) and AB1-25 (N-5s & N-5ns) (Fig. 3 & Supplemental Fig 3A), as well as for two 404 405 complementary C-terminal fragments: AB24-42 (D-4s) and AB26-42 (D-6ns) (Supplementary 406 Fig. 3B).

407 Epitope binding was tested by dotblot (DB) using a repertoire of synthetic A<sup>β</sup> peptide 408 standards (lug/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 AB40 peptide, which highlights their unique binding 411 specificity for cleaved A<sub>β</sub> 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 A<sup>β1-</sup>

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 AB1-22 and AB1-15 419 respectively. Similarly, antibody N-4 revealed high binding specificity for the amidated C-420 teminal form of Val24 (AB1-24-NH<sub>2</sub>) (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 AB 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.

The application of neo-epitope antibody N-5ns was further tested using ELISA and WB. At working concentrations of  $\leq 0.3$ ug/ml, N-5ns showed significantly lower affinity for the Cterminal amidated form A $\beta$ 1-25-NH<sub>2</sub> (Fig. 3D) and WB revealed high selectivity for the target fragments (Fig. 3E&F). In summary we can conclude that, by using a combination of different conventional techniques, we were able to show that the above mentioned neoepitope antibodies have unique binding properties for truncated A $\beta$  isoforms and may serve as complementary tools for the analysis of biological samples.

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# 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  $A\beta$  peptide cleavage during 439 aggregation we investigated peptide cleavage by stabilizing AB secondary structure in 440 different solutions. In PBS solution (or 40mM Tris: data not shown), AB40 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  $\alpha$ -helical 443 structure during five days of incubation (Fig. 4A). Significant A<sup>β</sup> cleavage was observed in PBS or Tris solutions, whereas cleavage was strongly attenuated in TFE (Fig. 4 B & C), 444 445 indicating that AB 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 AB42 (data not 447 shown).

Having established neo-epitope antibody specificity, we then selected and employed N-5ns to monitor *in vitro* A $\beta$ 42 aggregation using DB and MS. We observed a robust increase in N-5ns DB signal shortly after peptide incubation indicating a nearly instantaneous accumulation of diagnostic A $\beta$  fragments and TEM imaging of the peptide morphology indicated that there was a correlation between the N-5ns signal saturation by DB and the presence of typical oligomeric and protofibrilar aggregates (Fig. 4D).

454 We further studied the effects of different experimental conditions in AB 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 AB aggregation mirrors the accumulation of 458 A $\beta$  fragment fingerprints. A $\beta$ 40 aggregation increased significantly in the presence of tTGase 459 and was accompanied by the formation of low amounts of intra- and intermolecular crosslinks (data not shown) as reported previously [25] [18]. This change in aggregation behavior 460 461 could be detected by DB (Fig. 4E) and IP-MS analysis confirmed the presence of the N-5ns 462 target fragments A $\beta$ 1-23 and A $\beta$ 1-25 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 A $\beta$  cleavage, prior to the accumulation of  $\beta$ -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 than474 advanced fibrillation.

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#### 477 **3.5 Aβ fragments form stable complexes with soluble oligomers.**

478 To better understand the biophysical properties of truncated  $A\beta$  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 Aß species was subjected to SEC fractionation. To identify SEC fractions containing 486 truncated A<sub>β</sub> 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, AB42 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 AB fragments may favor the formation of 496 these HMW structures as a result of sample concentration by speed-vacuum.

surface of large oligomers and therefore allow more accurate quantitation of the A $\beta$ 1-25

521 target fragment *per se*. SRM quantitation confirmed the significantly lower levels of Aβ1-25

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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<sub>2</sub>, Aβ1-25 and Aβ1-25-NH<sub>2</sub> 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 ( $\leq$ 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 A $\beta$ 1-24-NH<sub>2</sub> (Fig. 5C; center) 505 along with A $\beta$ 42 and N-5ns allowed a specific recovery of fragments A $\beta$ 1-25 but not A $\beta$ 1-506 25-NH<sub>2</sub>, as well as trace amounts of A $\beta$ 1-23 together with A $\beta$ 42 (Fig. 5C; bottom).

507 To further elucidate the importance of A $\beta$ 1-25 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 AB1-25 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 AB42 indicating that oligomeric, as 512 well as monomeric fractions contained metastable complexes of fragment A $\beta$ 1-25 and A $\beta$ 42, 513 which may dissociate to a generally monomeric level during SDS-PAGE analysis. This data 514 collectively confirms the above reported observation that truncated AB can form stable 515 entities with soluble oligomers.

To provide quantitative values for A\beta1-25 and A\beta42 we used IP combined with LC-SRM

analysis. SEC fractions were split and one part was denatured with 70% FA over-night at 4°C

to allow gradual dissociation of large oligomeric species. We reasoned that this approach

would reduce the overall recovery (pull-down) of full-length AB42 stably bound to the

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 A $\beta$ 42 525 stably bound to fragment A $\beta$ 1-25. IP-MS of the monomeric fraction, using a combination of 526 three different neo-epitope antibodies, revealed the presence of three truncated isoforms: 527 A $\beta$ 1-23, A $\beta$ 1-24-NH<sub>2</sub> and A $\beta$ 1-25 together with A $\beta$ 42 (Fig. 5F).

Because our initial mock IP's of freshly prepared A $\beta$  fragment mixtures did not pull-down full-length A $\beta$ 40 or A $\beta$ 42, we sought to provide direct evidence that detection of A $\beta$ 42 within the monomeric fraction is the result of a collective pull-down due to stable interaction of truncated A $\beta$  and A $\beta$ 42. For this purpose, we denatured the first IP sample over-night using 90% FA, followed by a second IP using the same antibody cocktail. MS analysis of the sequential IP (2<sup>nd</sup>) confirmed our assumption, because A $\beta$ 42 was no longer detected 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 A $\beta$  isoforms together with full-length A $\beta$ 42 (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 A $\beta$ 42 fibrils, resulted in a specific recovery 540 of A $\beta$ 1-25 together with trace amounts of A $\beta$ 42 (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 oligometric  $A\beta$  in AD is the binding of  $A\beta$  to synapses and the resultant synaptic dysfunction 555 and loss [31], we examined the presence of A $\beta$  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  $A\beta$ 561 fragment may be important in synapse degeneration (Fig. 6E).

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

In order to further corroborate our *in vitro* observation on Aβ cleavage, we analyzed
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 A $\beta$  truncation using the two conventional antibodies 6E10 and 4G8 and compared 570 A $\beta$  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 A $\beta$  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  $\leq 6kDa$  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  $\geq$ 3kDa (Fig. 7A bottom blot: AD#1 lane: 2, AD#2 lane: 5), 576 indicating that both AD samples contained truncated AB isoforms, whereas 4G8 failed to 577 show similar specificity at this migration level, which is an observation also made with the 578 synthetic A $\beta$  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 AB fragments 581 Aβ36, Aβ37, Aβ38 and Aβ40 together with several N-terminal fragments Aβ1-13-NH<sub>2</sub>, Aβ1-13, AB1-20, AB1-22, AB1-23, AB2-24-NH2 AB1-24-NH2 and AB1-25 (Fig. 7B). IP-MS 582 583 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 AB1-25 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 AB recovery, however, the levels of AB1-591 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 AB isoforms are significantly reduced or 594 below the limit of detection (data not shown).

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

598 We first measured levels of AB42 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 AB42 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 AB cleave by applying 603 guantitative IP-SRM (N-5ns) to measure AB1-25 levels in CSF and could clearly confirm the 604 presence of A $\beta$ 1-25 in both; AD patients and age matched controls subjects (Supplementary 605 Fig. 8E). A large inter-subject variability of A\beta1-25 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 AB peptide fragments in vitro [34, 35] and in vivo [36, 37] has 613 been reported before and the accumulation of some AB 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 A<sub>β</sub> 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 AB 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 AB40 in the presence of

tTGase was accompanied with significant cleavage at residues Glu22 and Gly25 respectively [18]. Moreover, it has been suggested that A $\beta$ 25-35 as well as full-length A $\beta$ 40 mediated toxicity may result from a peptide cleavage induced radicalisation of cell membranes, and that prolonged incubation resulted in significant A $\beta$  cleavage at the Gly25-Ser26 bond [42]. The here reported occurrence of cleavage at residue Gly25 is of particular interest, because A $\beta$ 1-25 was earlier identified as a sphingolipid binding domain motif, which can be rapidly internalized by neuronal cells [43].

We report here new, additional A $\beta$  fragments and were able to demonstrate that the gradual appearance of fragments A $\beta$ 1-23, A $\beta$ 1-24-NH<sub>2</sub> A $\beta$ 1-25 and its amidated form A $\beta$ 1-25NH<sub>2</sub> can be associated with the early events of A $\beta$  aggregation, because MS detection of the aforementioned N-terminal fragments precedes the accumulation of shorter A $\beta$  isoforms (i.e A $\beta$ 1-15).

633 To our knowledge, the presence of substantial C-terminal amidation in vitro and in vivo 634 has not been reported before, suggesting that AB cleavage is the result of at least two distinct 635 molecular mechanisms. More importantly, we show that A $\beta$  cleavage is strongly attenuated 636 when stabilizing the peptide in an  $\alpha$ -helical structure. This suggests that the transition from an 637 unstructured, random-coil conformation to a  $\beta$ -sheet ordered structure triggers the cleavage 638 cascade typically observed during peptide aggregation. Moreover, the time-dependent 639 increase in AB1-15 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 Aβ1-25, or its more amyloidogenic form Aβ1-25-NH<sub>2</sub>, give rise to shorter Aβ isoforms. 642 643 Generally, the detection of a particular AB 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 A $\beta$  fragment signatures in AD. Therefore, the exact molecular mechanism 646 leading to site specific hydrolysis of A $\beta$  remains to be elucidated in future studies.

647 Soluble A $\beta$  oligomers play a central role in AD pathogenesis, with dimers [44] and 648 dodecamers (A $\beta$ \* 56) having attracted most of the scientific attention in the past. We report 649 here that IP of soluble AB40 or AB42 oligomers resulted in a specific recovery of AB 650 assemblies with a gel migration range of  $\geq 6kDa$ , and that similar A $\beta$  assemblies could be 651 detected in TBS and FA lysates from human AD brains. Given by our findings, it is 652 conceivable that AB assemblies of putative dimeric or trimeric nature consist of a mixture of 653 truncated and full-length A $\beta$ , which may form metastable complexes with HMW structures, 654 which is partly in agreement with earlier reports [19].

655 The detection of A $\beta$ 1-25 in CSF samples from human controls may suggest a significant 656 abundance of AB 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 A $\beta$ 1-25 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* AB aggregation in these samples.

In conclusion, we argue that neo-epitope antibodies would serve as appealing capture antibodies for future ELISA developments because we were able to show that the here described A $\beta$  fragments can self-propagate to dimers and trimers or form stable entities with large oligomers. However, measuring changes in levels of truncated A $\beta$  isoforms merits additional, future analytical improvements. We believe that monitoring pathological changes in A $\beta$  levels in human CSF [39] or plasma [48] requires the use of multiplexed approaches, 670 where truncated A $\beta$  isoforms together with several earlier reported A $\beta$  fragments [49] as well

as pathologically relevant post-translational modifications, such as pyroglutamate modified

 $A\beta$  [16], should be monitored simultaneously and longitudinally in human biofluids.

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#### 675 **RESEARCH IN CONTEXT**

676 *Systematic Review*:  $A\beta$  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  $A\beta$ 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  $A\beta$  isoforms with high aggregation 682 propensities, which may serve as seeding units during early peptide aggregation. We provide 683 analytical evidence that these truncated  $A\beta$  isoforms are highly abundant in  $A\beta$  oligomers.

684 *Future directions*: We plan to further study the generation of truncated  $A\beta$  isoforms as 685 well as their significance to the pre-symptomatic accumulation of neurotoxic oligomers. The 686 use typical A<sub>β</sub> 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 are interested in studying the structural properties and aggregation fate of the here described 688 689 truncated A $\beta$  isoforms. This will help to identify and understand the structure homology 690 found in AB oligomers which in turn may improve the future development of oligomer 691 specific antibodies.

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