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

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Nikita Rudinskiy[1+], Christophe Fuerer[2+], Davide Demurtas[3], Sebastian Zamorano[2], Cyntia De Piano[2], Ami Herrmann[4], Tara. L. Spires-Jones[4], Patrick Oeckl[5], Markus Otto[5], Matthew P. Frosch[6], Marc Moniatte[2], Bradley T. Hyman[1], Adrien W. Schmid [2*].

[1]Harvard Medical School, Mass General Hospital, Massachusetts Boston, USA.
[2]Proteomics Core Facility, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland.
[3]Interdisciplinary Centre for Electron Microscopy (CIME), EPFL, Switzerland.
[4]The University of Edinburgh, Center for Cognitive and Neural Systems & Euan MacDonald Centre for Motorneurone Disease, Edinburgh, Scotland.
[5]Department of Neurology, Ulm University Hospital, Ulm, Germany.
[6]Massachusetts General Hospital and Harvard Medical School, Massachusetts General Institute for Neurodegenerative Disease, Charlestown, MA, USA.
[+]These authors contributed equally to this work.

*To whom correspondence should be addressed:

Adrien W. Schmid, Ph.D.
Proteomics Core Facility, Life Sciences (SV)
PCF-PTP, Station 15, EPFL
1015 Lausanne, Switzerland
Tel: +41 (0)21 693 06 51
Fax: +41 (0)21 693 18 88
E-mail: adrien.schmid@epfl.ch

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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.

1. INTRODUCTION

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

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

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

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 Aβ species of a molecular weight range of 6-7kDa and that these Aβ species may form part of larger Aβ aggregates [19].

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

2. METHODS

2.1 Aβ peptide preparation.

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

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

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

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

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

2.4 Digestion of Aβ peptides.

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

224

225

226

2.8 Dotblotting.

Typically, 1ul samples were spotted onto a nitrocellulose membrane, which corresponded to a total peptide load of 100ng (Aβ1-40) and 50ng (Aβ1-42) 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.

2.9 Generation of polyclonal antibodies.

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

2.10 In vitro Aβ peptide aggregation studies.

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

2.11 Immunoprecipitation of mouse and human brain tissue.

Mouse and human brain tissue samples were serially extracted using TBS, 2% SDS or formic acid (70-90%FA) as stated in the text. Briefly, tissue samples were homogenized (20 strokes on ice) in TBS and 5mM EDTA with protease inhibitor complex (Roche, Switzerland) using a Teflon homogenizer. Samples were then subjected to centrifugation (150,000g) during 45min and the supernatant was recovered as the TBS soluble fraction. 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 minimize formylation adducts) followed by centrifugation. SDS fractions were diluted to

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

3. RESULTS

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

We examined the aggregation behavior of synthetic Aβ42 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 ($H_2{}^{18}O$) to determine whether peptide cleavage is the result of aggregation-induced peptide hydrolysis.

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

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

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

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

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

Aβ isoforms of Aβ1-23, Aβ1-24-NH_2 and Aβ1-25 as well as the C-terminal amidated form 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 ^{18}O incorporation. MS/MS analysis of the Lys-N digested Aβ1-25 (16-25) fragment allowed for unambiguous identification of the ^{18}O atom incorporation at Gly25 (Fig. 1E & F).

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

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

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

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

Transmission electron microscopy (TEM) imaging indicated that N-terminal fragments have a high propensity to form soluble, oligomeric aggregates following long-term incubation. Aβ1-25 preferentially formed homogenous spherical aggregates, which was less evident with the shorter N-terminal fragments of residues Aβ1-23 and Aβ1-24-NH_2 and only very few clusters of fibrils were observed with the amidated form Aβ1-25-NH_2 (Fig. 2A and Supplemental Fig. 2F). We further employed SEC (in Tris-NaCl) of aggregated Aβ1-25 and show that the LMW structures typically observed with these truncated Aβ 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 Aβ structures centered at the migration level of ≤ 6kDa (fraction: 12ml) and ≤ 3kDa (fraction: 15ml) and MS analysis of fraction volume 12ml revealed the presence of stable Aβ1-25 dimers and trimers, whereas monomers were mainly detected in fraction volume 15ml (data not shown). Because Aβ1-25 dimers (5.8kDa) and trimers (8.7kDa) were highly enriched in SEC fractions corresponding to a MW standard of ≤14kDa, we speculated that LMW Aβ1-25 structures of may form part of larger Aβ entities, which dissociate during SDS-PAGE analysis.

3.3 Monitoring Aβ peptide aggregation using neo-epitope antibodies which specifically target truncated Aβ isoforms.

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

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

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

full-length Aβ40 (Fig. 3C) or in presence of a complex human proteome matrix (brain tissue extract) (Supplementary Fig. 4C & D). All neo-epitope antibodies showed high selectivity for their target fragments, whereas IP with 6E10 resulted in a pull-down of all Aβ fragments together with full-length Aβ40.

The application of neo-epitope antibody N-5ns was further tested using ELISA and WB. At working concentrations of ≤ 0.3ug/ml, N-5ns showed significantly lower affinity for the C-terminal amidated form Aβ1-25-NH_2 (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 neo-epitope antibodies have unique binding properties for truncated Aβ isoforms and may serve as complementary tools for the analysis of biological samples.

3.4 Aβ peptide cleavage is highly associated with changes in peptide secondary structure.

In order to understand the mechanisms associated with Aβ peptide cleavage during aggregation we investigated peptide cleavage by stabilizing Aβ secondary structure in different solutions. In PBS solution (or 40mM Tris: data not shown), Aβ40 showed a predominantly unordered structure with considerable decrease in signal amplitude over the time course of incubation, whereas incubation in 20% TFE solution induced a stable α-helical structure during five days of incubation (Fig. 4A). Significant Aβ cleavage was observed in PBS or Tris solutions, whereas cleavage was strongly attenuated in TFE (Fig. 4 B & C), indicating that Aβ cleavage is highly associated with changes of the peptide's secondary structure as a result of peptide aggregation. This observation was also true for Aβ42 (data not shown).

Having established neo-epitope antibody specificity, we then selected and employed N-5ns to monitor *in vitro* Aβ42 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 Aβ 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).

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

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

of aggregation inhibitory compounds targeting an oligomerization fate [21] [30] rather than advanced fibrillation.

3.5 Aβ fragments form stable complexes with soluble oligomers.

To better understand the biophysical properties of truncated Aβ in peptide oligomerization, we investigated the aggregation kinetics of amyloid-derived diffusible ligands (ADDL's) using a combination of SEC, DB, WB and MS. Following 20hrs of Aβ42 oligomerization, we observed a substantial increase in N-5ns signal using DB (Fig. 5A) and this positive signal was associated with the presence of a typical oligomeric morphology (data not shown). Following *in vitro* aggregation, the ADDL preparation was centrifuged in order to precipitate large insoluble aggregates (pellet) and the supernatant containing soluble Aβ species was subjected to SEC fractionation. To identify SEC fractions containing truncated Aβ isoforms, 50ul fractions were dried by speed vacuum and re-suspended in 5ul of PBS of which 1ul was spotted in duplicates onto a nitrocellulose membrane and probed by DB using N-5ns and 6E10 respectively. SEC fraction probing with N-5ns allowed identification of a substantial amount of truncated Aβ within the oligomeric fractions (8ml - 12ml) (Fig.5A). DB probing with 6E10 and N-5ns of the monomeric fraction (18ml) indicated that this fraction contained a mixture of both, Aβ42 monomers as well as truncated Aβ isoforms and WB analysis of these SEC fractions was in line with the findings from DB (Fig. 5B). Moreover, WB also revealed the presence of HMW oligomers centered at the 49kDa to 62kDa range. The presence of low amounts of this HMW entity observed in the monomeric fraction may indicate that truncated Aβ fragments may favor the formation of these HMW structures as a result of sample concentration by speed-vacuum.

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

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

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

To provide quantitative values for Aβ1-25 and Aβ42 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 Aβ42 stably bound to the surface of large oligomers and therefore allow more accurate quantitation of the Aβ1-25 target fragment *per se*. SRM quantitation confirmed the significantly lower levels of Aβ1-25

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

Because our initial mock IP's of freshly prepared Aβ fragment mixtures did not pull-down full-length Aβ40 or Aβ42, we sought to provide direct evidence that detection of Aβ42 within the monomeric fraction is the result of a collective pull-down due to stable interaction of truncated Aβ and Aβ42. 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^{nd}) confirmed our assumption, because Aβ42 was no longer detected following FA treatment (Fig. 5G).

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

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

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

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.

We employed IP-MS and WB analysis of TBS and FA tissue extractions to investigate presence of Aβ truncation using the two conventional antibodies 6E10 and 4G8 and compared Aβ peptide recovery with neo-epitope antibodies. IP with 6E10&4G8 resulted in the detection

of a band centered at the typical migration level of Aβ monomers (>3kDa) (Fig. 7A, top blot: AD#1 lane: 1, AD#2 lane: 4), whereas IP with neo-epitope antibodies revealed a band centered at the migration level of ≤6kDa for one AD subject (Fig. 7A top blot, lane: 5). Interestingly, re-probing the membrane with a neo-epitope antibody cocktail revealed a band centered at the migration level of ≥3kDa (Fig. 7A bottom blot: AD#1 lane: 2, AD#2 lane: 5), indicating that both AD samples contained truncated Aβ isoforms, whereas 4G8 failed to show similar specificity at this migration level, which is an observation also made with the synthetic Aβ fragments (Fig. 3).

We further analyzed the same brain samples by MALDI-TOF/TOF MS. IP-MS (6E10&4G8) analysis of the AD#2 brain tissue extract (FA) resulted in a pull-down of large Aβ fragments Aβ36, Aβ37, Aβ38 and Aβ40 together with several N-terminal fragments Aβ1-13-NH_2, Aβ1-13, Aβ1-20, Aβ1-22, Aβ1-23, Aβ2-24-NH_2 Aβ1-24-NH_2 and Aβ1-25 (Fig. 7B). IP-MS analysis of the same AD brain, using a combination of three neo-epitope antibodies resulted in a specific pull-down of the target fragments (Fig. 7C). The same samples were also subjected to LC-MS/MS (Orbitrap) analysis for high resolution peptide mass confirmation (Fig. 7D & E).

Moreover show that, IP with N-5ns enables a specific recovery of fragment Aβ1-25 from Tg2576, APP/PS1 (Supplementary Fig. 6C & D) and 5xFAD (data not shown) transgenic mice brains. The use of SDS (Supplemental Fig. 7), TBS or FA (Supplementary Fig. 8A & B) extraction protocols all resulted in similar truncated Aβ recovery, however, the levels of Aβ1-25 were found to be significantly increased in FA extracts as compared to TBS soluble fractions (Supplementary Fig. 8B). LC-SRM analysis of TBS and FA brain extracts from human controls indicated that the levels of cleaved Aβ isoforms are significantly reduced or below the limit of detection (data not shown).

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

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

4. DISCUSSION

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

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

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

The detection of Aβ1-25 in CSF samples from human controls may suggest a significant abundance of Aβ oligomers present in control subjects, which is in line with earlier reports [45]. Interestingly, Holtta et al. (2013) showed that CSF oligomers were significantly increased in patients with mild and moderate dementia when compared to controls, whereas no significant difference was found in patients with severe dementia [12]. The here observed lack of statistical significance in Aβ1-25 levels in AD patients may result from the relatively small sample size and hence statistical power. It is also conceivable that CSF sample freeze-thaw cycles together with other, earlier reported cofounding factors[46, 47] may have collectively contributed to a rapid *ex vivo* Aβ 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 Aβ fragments can self-propagate to dimers and trimers or form stable entities with large oligomers. However, measuring changes in levels of truncated Aβ isoforms merits additional, future analytical improvements. We believe that monitoring pathological changes in Aβ levels in human CSF [39] or plasma [48] requires the use of multiplexed approaches,

where truncated Aβ isoforms together with several earlier reported Aβ fragments [49] as well as pathologically relevant post-translational modifications, such as pyroglutamate modified Aβ [16], should be monitored simultaneously and longitudinally in human biofluids.

RESEARCH IN CONTEXT

Systematic Review: Aβ dimers, trimers and dodecamers have received the most scientific attention in the past, because these entities have been suggested to form the building blocks of larger neurotoxic assemblies. However, the key molecular triggers associated with early Aβ oligomerization are poorly understood and to date, the exact molecular structure of LMW oligomers still remains a conundrum.

Interpretation: We have identified here new truncated Aβ isoforms with high aggregation propensities, which may serve as seeding units during early peptide aggregation. We provide analytical evidence that these truncated Aβ isoforms are highly abundant in Aβ oligomers.

Future directions*:* We plan to further study the generation of truncated Aβ isoforms as well as their significance to the pre-symptomatic accumulation of neurotoxic oligomers. The use typical Aβ peptide fragment fingerprints for a pre-symptomatic diagnosis of subjects 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 truncated Aβ isoforms. This will help to identify and understand the structure homology found in Aβ oligomers which in turn may improve the future development of oligomer specific antibodies.

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SUPPLEMENTARY DATA

Supplementary figures 1-8 and tables I & II.

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