Clinical ApoA-IV amyloid is associated with fibrillogenic signal sequence.


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ABSTRACT

Apolipoprotein A-IV amyloidosis is an uncommon form of the disease normally resulting in renal and cardiac dysfunction. ApoA-IV amyloidosis was identified in sixteen patients attending the National Amyloidosis Centre and in eight clinical samples received for histology review. Unexpectedly, proteomics identified the presence of ApoA-IV signal sequence residues (p.18-43 to p.20-43) in 16/24 trypsin-digested amyloid deposits, but in only 1/266 non-ApoA-IV amyloid samples examined. These additional signal residues were also detected in the cardiac sample from the Swedish patient in which ApoA-IV amyloid was first described, and in plasma from a single cardiac ApoA-IV amyloidosis patient. The most common signal-containing peptide observed in ApoA-IV amyloid, p.20-43 and to far lesser extent the N-terminal peptide, p.21-43 were fibrillogenic in vitro at physiological pH generating Congo red positive fibrils. The addition of a single signal-derived alanine residue to the N-terminus has resulted in markedly increased fibrillogenesis. If this effect translates to the mature circulating protein in vivo, then the presence of signal may result in preferential deposition as amyloid, perhaps acting as seed for the main circulating native form of the protein; it may also influence other ApoA-IV associated pathologies.

Key words: ApoA-IV amyloidosis; ApoA-IV sequence coverage; ApoA-IV signal-containing peptide; amyloid proteomics; targeted mass spectrometry in serum; fibrillogenic ApoA-IV signal-containing peptide.
INTRODUCTION
Amyloidosis occurs when a diverse range of normal circulating proteins misfold and subsequently deposit as insoluble fibrillary masses in a range of tissues resulting in organ dysfunction and clinical disease [1]. There are over thirty known amyloidogenic proteins associated with human disease [2]. Amyloid deposits can be restricted to specific organs, for example fibrinogen Aα amyloid is invariably found in the kidney [3]; with other amyloidogenic proteins, such as transthyretin (TTR) or immunoglobulin light chains, amyloid can be detected in multiple organs [4–7]. In some cases, the development of the disease is associated with increased circulating concentrations of the precursor proteins: common examples being the kappa and lambda light chains arising from a monoclonal gammopathy [8] and serum amyloid A which is associated with a raised inflammatory response [9]. With other proteins, including lysozyme, transthyretin (TTR) and fibrinogen Aα, the presence of a variant can result in protein destabilisation and the subsequent formation of amyloid [10–12]. Amyloid deposits can also arise from normal circulating levels of wild type proteins. This is notable in the case of TTR, where amyloid deposits of wild type protein are commonly found in the hearts of the elderly [13,14], possibly as a result of age-related oxidative changes [15]. Apolipoprotein A-IV (ApoA-IV) was originally identified as a new amyloid fibril protein in fibrils extracted from cardiac tissue of a patient with senile systemic amyloidosis (now termed ATTR wildtype (ATTRwt) amyloidosis) [16]. Additional biochemical studies showed that the amyloidogenicity of ApoA-IV resided in the N-terminus. It was later shown by the same group that ApoA-IV and TTR were not co-deposited but occupied anatomically distinct regions, demonstrating that two types of amyloid can be present, and separately deposited, in the same tissue [17]. There were few further reports of ApoA-IV amyloid (AApoA-IV) since typing relied mainly on immunological techniques, and few pathology laboratories had access to the original high quality antibody for typing.

The observation that a mass spectrometric-based proteomics approach could be applied to identify proteins in formalin-fixed paraffin-embedded (FFPE) material [18–21] led to the development of proteomics as a clinical service for typing amyloid by Ahmet Dogan and colleagues at the Mayo Clinic [22–25]. Proteomics makes it relatively easy to identify (and potentially quantify) any of the currently known amyloidogenic proteins in clinical samples. It was then determined by the Mayo team that ApoA-IV was a common constituent of amyloid
deposits, and could be used as a signature protein, together with serum amyloid P component and apolipoprotein E, to identify the presence of amyloid [24]. Whilst this was beneficial in typing amyloid by proteomics, it caused some difficulty in the identification of ApoA-IV as an amyloid protein rather than an amyloid-associated signature protein. Proteomics was used successfully at the Mayo Clinic to identify cases of renal and cardiac ApoA-IV amyloidosis [26–29]. This was based primarily on an unusual clinical presentation coupled with a high number of ApoA-IV peptides identified by proteomics (spectral count) and the absence of other obvious amyloidogenic proteins. This became their new criteria for identifying ApoA-IV amyloid. In our centre we have developed further criteria for the identification of ApoA-IV amyloid based on clinical presentation together with morphological, immunohistochemical and proteomics behaviour. We have also identified peptides containing signal sequence (p.18-43, p.19-43 and p.20-43) in a number of clinical ApoA-IV amyloid samples, suggesting that this may influence amyloid deposition and also be of diagnostic value in identifying this rare form of amyloidosis.

Materials and methods

Ethical approval
Samples were collected and analysed as part of routine clinical examination. Informed consent was obtained from all patients and clinical care was in accordance with the Declaration of Helsinki. The material collection and use for the original Swedish sample (#25) was performed under a valid permit by the Ethical Committee at Uppsala University Hospital (Ups 01-083).

Patients and Samples
Fifteen patients with renal or cardiac dysfunction attending the UK NHS National Amyloidosis Centre (NAC) clinics between March 2016 and October 2020 were diagnosed with ApoA-IV amyloidosis (Table 1). Duodenal ApoA-IV amyloidosis was also diagnosed in a single patient. Samples of FFPE tissue or fat aspirates were obtained as part of routine diagnostic procedures and were evaluated as previously reported using Congo red staining, immunohistochemistry (IHC) with a panel of antibodies and proteomics analysis [30–32]. Serum was prepared from blood taken from two healthy volunteers and from three patients given an AApoA-IV diagnosis.
Eight tissue samples sent for histology review were also diagnosed with ApoA-IV amyloid based on immunohistochemistry (IHC) and the proteomics criteria. The FFPE block from the original patient diagnosed with ApoA-IV amyloidosis [16] was obtained from the Department of Immunology, Genetics and Pathology, Uppsala, Sweden and was re-cut to obtain 6 µm sections which were stained with Congo red, followed by IHC using an anti-TTR antibody [33] and an anti-ApoA-IV antipeptide antibody [17]. Two separate areas of TTR positive and ApoA-IV positive amyloid were micro-dissected and captured.

**Proteomics analysis**

Full experimental details of our proteomics approach, based on that of the Mayo clinic, have been reported previously [34]. Samples were originally analysed on a Thermo Velos Orbitrap mass spectrometer coupled to a Waters nanoACQUITY UPLC system, and more recently, on a Thermo Q-Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to a Dionex Ultimate 3000 UPLC system (Thermo Scientific, Bremen, Germany). Data were analysed by Mascot software (Matrix Science, London, UK) using the Swiss-Prot human database. Search parameters were charge states +2, +3 and +4, precursor mass tolerance of 10 ppm and 0.6 Da for HCD fragments; the more intense 13C ion was normally selected for dissociation. Trypsin was used as the *in silico* proteolytic enzyme, with semi-trypsin included to identify additional non-tryptic signal sequences. The significance level was set at p<0.05. Methionine oxidation was included as variable modification. Separate MS analyses were performed on the Q-Exactive Plus instrument using Parallel Reaction Monitoring (PRM) selecting for specific 12C ions of ApoA-IV peptides, p.18-43, p.19-43 and p.20-43 (MH3+ m/z 944.121, 892.087 and 868.408 respectively). Data are recorded in a separate proteomics database; in January 2021 this database contained ~2100 clinical entries excluding controls, external research samples and fats following decellularization treatment [35].

**ApoA-IV immunoprecipitation**

ApoA-IV protein was immunoprecipitated from serum collected from three ApoA-IV patient (#1, #3 and #11) and from two healthy controls. A mouse monoclonal antihuman ApoA-IV antibody (Santa Cruz Biotechnology, Texas, USA) was buffer-exchanged into PBS using BioSpin columns (Bio-Rad Laboratories, Hertfordshire, UK) and incubated overnight with rotation at 4 °C with
CNBR-activated Sepharose 4B beads (Sigma-Aldrich, St. Louis, MO). The antibody beads were used to immunoprecipitate ApoA-IV using the procedure described previously for TTR [34].

**Fibrillogenesis of ApoA-IV peptides p.18-43, 19-43, 20-43 and p.21-43**

Synthetic ApoA-IV peptides p.18-43, p.19-43, p.20-43 and the N terminal peptide, p.21-43 (peptide purity >95%) were purchased from Life Technologies Limited, Paisley, UK. They were dissolved at 200 µg/ml in phosphate buffered saline (pH 7.4) containing 10 µM thioflavin T (ThT) and placed in Costar 96-well plates (Fisher Scientific, Leicestershire, UK) in a BMG LABTECH, FLUOstar Omega double-orbital shaker (BMG LABTECH, Ortenberg, Germany). Three replicates for each peptide were incubated at 37 °C with shaking at 900 rpm, and ThT fluorescence (Ex440nm, Em480nm) was recorded for 24 h at 500 s intervals.

**Transmission Electron Microscopy (TEM)**

A drop of a suspension of fibrils, collected following 24h incubation, was loaded onto a formvar/carbon coated 300 mesh copper grid and excess solvent was allowed to dry at room temperature for 3 min before blotting with filter paper. After washing with water for 10 s, the dry sample was stained with 2% uranyl acetate solution for 1 min and examined in a JEOL JEM-1400 Flash transmission electron microscope (JEOL, Tokyo, Japan) operating at 120 kV.

**RESULTS**

Cardiac ApoA-IV amyloidosis was initially diagnosed in nine patients (all male, median age 77), renal amyloidosis in six patients (5 male and female, median age 68) and duodenal ApoA-IV amyloidosis in a single patient, all attending clinics at the National Amyloidosis Centre (Table 1). All renal patients had previous chronic kidney disease, with amyloid deposition but with no plasma cell dyscrasia or clone noted. On ¹²³I SAP scintigraphy no visceral amyloid deposits were detected. All patients went on to develop cardiac amyloid, from between 6 months and 7 years post renal amyloid diagnosis. Cardiac amyloid deposition was assessed using Tc-DPD scan on all patients, all resulting in Perugini grade 0. A clinical summary is shown in supplementary material, Table S1. The morphology in the renal and cardiac cases was different but characteristic in that amyloid presents as swathes of medullary sheets in renal biopsies, and can be minimal in the
cardiac tissue with scanty deposits surrounding myocytes or in vessels. IHC indicated the presence of ApoA-IV in 4 cases, and also in a separate, non-cardiac biopsy from patient #8. Other samples were not tested with the ApoA-IV antibody. There was no evidence for ApoA-IV immunostaining in any non-ApoA-IV amyloid despite high ApoA-IV Mascot scores. In each case, ApoA-IV was identified by proteomics as the top scoring protein together with a low probability for the presence of other candidate amyloid proteins. The Mascot scores, which use probability-based algorithm, were high: in only one sample the score was <800, which is 10 times the minimum score of 80 used in our algorithm to confidently identify proteins. This is in agreement with the criteria originally reported by Dasari and colleagues [26]. We re-examined the data in our clinical proteomics database for the presence of ApoA-IV amyloid using these criteria. ApoA-IV amyloid was identified in eight histology review samples from cardiac (4), renal (3) and oral mucosal (1) biopsies (#17–24). Distinct areas of TTR and ApoA-IV were observed by both IHC and proteomics in #20. In each of these additional cases ApoA-IV top scoring (highest probability) amyloid protein, with 7/8 scoring greater than 2400, which is 30-fold higher than the minimum acceptable score. With the exception of fibrinogen Aα and ApoA-I, which can be excluded by other criteria [32], there were no other amyloid candidates present. Clinical and morphological data, where available, were consistent with the diagnosis. These data are summarised in Table 1 and Figure 1 and also expressed as a Scaffold output (Proteome Software, Inc, Portland, Oregon, USA) in supplementary material, Figure S1. ApoA-IV was present with minimum Mascot score of 80 in 55% (1142/2075) of the samples in our database where ApoA-IV was not identified as the amyloid type (supplementary material, Table S2). This is not unexpected since it is one of the three currently acknowledged amyloid signature proteins. It was the top scoring amyloid protein in only 311 samples, with high scores (>2400) only observed in 8 (1.8%) of samples (supplementary material, Table S2, and Figure 1). Of the uncertain diagnoses with a top ApoA-IV score only 16 showed no evidence for other amyloid proteins; the ApoA-IV scores were relatively low (82–383) and there was no other reason to consider ApoA-IV as the amyloid protein.

During the analyses of the clinical and histology review ApoA-IV cases we observed that the tryptic peptide, p.20-43 (AEVSADQVATVMWDYFSQLSNNAK) was detected in 17/24 cases. This was unexpected since it corresponds to a tryptic cleavage after p.20R in the signal region. The raw data were reanalysed by Mascot using semi-trypsin as the in silico protease: additional
signal-containing peptides were observed corresponding p.18-43 (ARAEVSA..., n= 6) and p.19-43 (RAEVSA... n=12) together with single examples of p.17-43 (GARAEVSA...) and p.15-43 (VAGARAEVSA...). Signal-containing peptides were present in a post-mortem sample from a presumed cardiac TTR patient (#20) where distinct areas of both ApoA-IV and TTR immunostaining were observed (Table 1). The N-terminal peptide, p.21-43 (EVSA...) was present in all samples. There were also some examples of truncated N-terminal peptides, p.22-43 (VSA...), p.23-43 (SA...), but none without the expected p.43 lysine C-terminus which is generated by trypsin in the sample workup. This indicated that ApoA-IV amyloid was associated with the presence of signal sequence. A single signal peptide, p.19-43 was also identified in a fat sample from patient #3, which had been treated by the decellularisation method designed to remove non amyloid deposits and enhance proteomic identification [35]. As controls, all other non-ApoA-IV samples with an ApoA-IV score of >800 were examined (n=266); the N terminal peptide (p.21-43) was present in 27% of samples, however only a single sample of fat showed evidence of p.18-43, and this was identified from a single MSMS spectrum. The protein coverage of ApoA-IV for the ApoA-IV patients and controls with high ApoA-IV signature scores is shown in supplementary material, Figure S2. This indicates that ApoA-IV amyloid is also associated with a reduced C-terminal load.

The FFPE block of cardiac tissue from the original Swedish patient (#25) diagnosed with ApoA-IV amyloidosis was obtained, and sections recut and analysed by histochemistry: distinct areas were observed exhibiting ApoA-IV and TTR immunoreactivity respectively in agreement with the original data. Two areas of each immuno-positive region were laser dissected and prepared for proteomics using our standard procedure. In the ApoA-IV positive section proteomics identified ApoA-IV as the top scoring protein (Mascot scores 4245 and 2547), together with the signature proteins, SAP and ApoE. Other lower scoring (score <750) proteins were also observed including TTR, ApoA-I and kappa light chain. Three signal-containing ApoA-IV peptides, p.18-43, p.19-43 and p.20-43 were present together with the N-terminal peptide, p.21-43. In the TTR immuno-positive area, TTR was the top scoring protein (2753 and 2000); ApoA-IV was present with lower scores (<600) and there was no evidence for signal peptides, which is consistent with ApoA-IV in this region being a co-deposited signature protein.
To confirm the identity of these Mascot-identified signal-containing peptides, four authentic peptide samples (p.18-, p.19-, p.20- and p.21-43) were analysed under our standard LCMS conditions. Peptides p.15-43 and p.17-43 were not compared against standards and their identity is based on Mascot search analysis only. Peptides p.18-43 and p.19-43 essentially coeluted at 26.8 minutes, with p.20-43 and the N-terminal peptide, p.21-43 eluting later at 28.8 and 29.1 minutes respectively. Each peptide generated a similar set of y ions (y3 – y13) together with characteristic b ions. The clinical ApoA-IV samples and the Swedish cardiac sample showed the same relative retention times and elution pattern as the standards (Figure 2), and generated similar MSMS spectra (supplementary material, Figure S3). These data confirm that there is signal-containing protein in ApoA-IV amyloid.

Since any ApoA-IV deposited in amyloid must have come from the circulation, serum from patients #1,3 and 11 whose samples showed the high ApoA-IV scores, was immunoprecipitated with anti-ApoA-IV antiserum and the sample subjected to PRM mass spectrometric analysis targeting the signal peptides, p.18-43, p.19-43 and p.20-43. All three peptides were present only in the immunoprecipitate from patient #3 (supplementary material, Figure S4), although none were identified in the remaining two ApoA-IV patients, nor in serum from two healthy controls. These data are consistent with very low normal circulating concentration of signal-containing proteins.

To determine whether these additional signal residues could influence the deposition of amyloid, the authentic peptides were incubated in PBS buffer at 37 °C with gentle shaking, and fibril formation was measured by ThT fluorescence emission as previously described [36]. Although neither arginine-containing signal peptides (p.18-43 and p.19-43) showed any fibril formation, the most common signal–containing peptide, p.20-43, and to a much lesser extent, the N-terminal peptide p.21-43, were fibrillogenic under these mild conditions (Figure 3). The fibrils from the p.20-43 peptide exhibited the classic amyloid green birefringence following Congo red staining, and the electron micrographs were consistent with amyloid fibrils. Small specks of positive material could be also observed for the p.21-43 (N-terminal peptide) preparation, however there was no evidence for amyloid fibrils in the incubates of p.18-43 and p.19-43. The addition of a single alanine to the normal N-terminal peptide, p.21-43, has
markedly increased its fibrillogenicity; this effect is lost by further extension with arginine (p.18-43 and p.19-43).

Discussion

Mature ApoA-IV is a 376 residue (p.21-396) circulating protein which is involved in a number of physiological processes. ApoA-IV amyloid deposits were first recognised in a Swedish cardiac patient through traditional protein biochemistry analyses and the development of specific and complementary immunohistochemical methods [16]. The advent of diagnostic amyloid proteomics pioneered at the Mayo Clinic revolutionised the field and made it possible to rapidly type amyloid [37, 38]. ApoA-IV is a common constituent of amyloid tissue, therefore its identification alone is insufficient to identify the amyloid protein: here the proteomics criteria identified by the Mayo Clinic, using an abnormally high number of ApoA-IV spectra coupled with the absence of other amyloid proteins, are central to confirming ApoA-IV amyloid. In our centre we use the Mascot protein database search engine which generates a probability-based score dependent on the quality and number of individual mass spectra to identify proteins (http://matrixscience.com). Although it is not a quantitative measure, the score is a surrogate for protein amount and is helpful in distinguishing the amyloid protein from contaminants. Our algorithm identifies ApoA-IV where it is the top scoring protein, normally with scores in excess of 1000, in the absence of other candidate amyloid proteins. Whilst our data is in general agreement with that of the Mayo Clinic, we note that a low score (or low total spectral count) does not preclude the presence of ApoA-IV amyloid. Equally, the absence of other amyloid proteins may be insufficient to distinguish ApoA-IV amyloid from the amyloid signature. Here, additional clinical, morphological/IHC and proteomics features will increase confidence in the amyloid typing, and these were applied to identify 24 examples of ApoA-IV amyloid amongst over 2000 clinical samples in our current proteomics database. The presence of distinct areas of ApoA-IV and TTR amyloid in patient #20 are consistent with a diagnosis of two unrelated forms of amyloidosis: ApoA-IV together with age-related wild type cardiac ATTR amyloidosis; this was also observed in the original Swedish sample [17].

The most unexpected finding of our study was the identification of signal sequence containing peptides p.15-43, p.17-43, p.18-43, p.19-43 and, most commonly, p.20-43 in ApoA-IV amyloid
deposits. Some of these peptides have previously been identified in a number of top down proteomics analyses, for example in CSF [39] and breast cancer tissue [40], although no biological role was ascribed. There was also evidence for p.20-43 in the first proteomics identification of renal ApoA-IV report from the Mayo Clinic, where residue p.20A was highlighted as an identified tryptic peptide although its appearance did not receive any comment [26]. At least one signal peptide was present in 17/24 of the ApoA-IV amyloid cases, as well as in the original Swedish cardiac sample. The identity of the three main signal peptides, p.18-43, p.19-43 and p.20-43 in patient-derived samples was confirmed by chromatographic and mass spectrometric comparison with authentic peptides and does not rely simply upon a Mascot-generated identification. ApoA-IV is a circulating protein, synthesised in the intestine therefore it, and the associated signal-containing proteins present in amyloid should arise from the blood. Using targeted PRM analysis we could identify all three signal peptides present in blood of patient #3, although none were detected in two other patients and in two healthy controls. This would indicate that signal-containing proteins are not a major component of circulating ApoA-IV. The signal does not appear to be associated with signature ApoA-IV since only a single poor-quality signal peptide was found in only one of the 266 non-ApoA-IV cases with high scores (>800) examined. It was not possible in the current study to determine whether the signal-containing peptides identified in amyloid and in blood are part of the full protein or a smaller truncated protein, however the limited coverage data would suggest that the amyloidogenic ApoA-IV contains a lower proportion of C-terminal protein than signature ApoA-IV.

The major circulating form of ApoA-IV (p.147N) is inherently amyloidogenic [16], and so the presence of signal residues in ApoA-IV amyloid could simply be due to an increased circulating concentration. This could be caused by faulty processing, or secondary effects of the additional signal sequence whereby altered lipid binding could raise the free protein plasma concentration. Our in vitro fibrillogenesis data with peptides p.18-43 to p.21-43, showing that the addition of a single alanine residue to the normal N-terminus causes increased fibrillogenicity, raises the possibility that the presence of signal residues could exert a direct and deleterious effect on the circulating protein. This could be propagated through a number of mechanisms including direct destabilisation of the protein, altered binding to lipids and other proteins such as clusterin, or increased sensitivity to endogenous protease activity or mechanical forces [36,41,42].
Although there is a large gap between the behaviour of a small peptide in vitro and the stability of a protein in the complex milieu of blood, our data are suggestive of a potential causative relationship between the presence of signal sequence and enhanced ApoA-IV amyloidogenicity. It may be that the presence of these signal residues is no more than an additional marker that can be applied to increase confidence in ApoA-IV amyloid diagnosis, however, since ApoA-IV is involved in other human diseases [43], the presence of signal sequence in the circulating protein could have other deleterious pathophysiological effects.

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Author contributions statement

GWT, DC, VB, and JDG designed the study. GWT and DC analysed the data and wrote the paper. NBR, JAG and NB variously carried out the staining procedures, extraction (LCD), digestion and mass spectrometric analyses of the samples. DC and PN carried out the immunoprecipitation and PRM analysis, and with GV and AM, undertook the fibrillogenesis studies. PN and PPM carried out electron microscopy; AC gave insight into structural analysis; SG, LM contributed to discussion and preliminary data PW provided the original Swedish sample and advised on IHC analysis and ApoA-IV amyloidosis. JDG and PNH provided clinical interpretation, and DR advised on genetics. The clinical database algorithm was implemented and developed by AB. All authors provided interpretative input and reviewed the manuscript.

Data availability statement

Anonymised raw mass spectra data, Mascot mgf files and raw data of fibrillogenesis experiments are available on request from the corresponding author.
References


Table 1. Identification of the signal-containing peptides in FFPE clinical tissues.

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<td>ApoA-IV + TTR present</td>
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Mascot score, significant mass spectra (spectral matches/unique significant peptides, USP). Further clinical details are shown in supplementary material, Table S1. Patients 1–15 attended the NAC clinic and were diagnosed with cardiac or renal ApoA-IV amyloidosis. Patient 16 attended clinic with localised amyloid in the gut. FFPE blocks from patients 17–24 were received from other centres for histology review. Signal peptides were identified in 17/24 patient samples, with p.20-43 being the most common. Peptides p.15-43 and p.17-43 were not confirmed by coelution with standards. The block from the original ApoA-IV patient (#25) from Sweden was retrieved and re-analysed; ApoA-IV signal peptides were present. Both TTR and ApoA-IV were identified in separate regions of the tissue from patients #20 and #25. ApoA-IV signal sequence was also present in a fat biopsy and a blood sample collected from patient #3.
Figure legends

**Figure 1.** The ApoA-IV Mascot score histogram for all samples (light blue bars); the histogram for ApoA-IV clinical amyloid samples is shown as dark blue bars. The scores of the 24 clinical ApoA-IV patients are also shown as open blue circles together with that of the original Swedish sample (open diamond); in each of these cases, ApoA-IV was the top scoring amyloid protein. Signal sequence was detected in 17/24 samples (closed blue circles) and also in the Swedish sample (closed red circle diamond).

**Figure 2.** Normalised ion chromatograms (MH$_3^{3+}$) for the signal-containing peptides p.18-43, p.19-43 and p.20-43, together with the N terminal peptide, p.21-43 for (A) authentic standards and (B) patient #11. (C and D) show the MSMS spectra (MH$_3^{3+}$) for the p.18-43 standard and that from patient #11. The common y ion series (y3-y15) is highlighted.

**Figure 3.** (A) Fibrillogenesis at pH 7 and 37 °C of the signal-containing peptides, p.18-43, p.19-43 and p.20-43 and the N terminal peptide p.21-43 analysed at the same time. (B) Three separate experiments were performed for each peptide, and the mean (SD) of the change in ThT fluorescence emission between 0.25 h and 18 h are shown. Fluorescence intensity is shown in arbitrary units (a.u.). Panels (C1-4) show the bright field Congo red staining for the p.20-43 fibrils together with the fluorescence with and without the polarising filter, and the electron micrograph of the p.20-43 fibrils (after 24 h of incubation).