We report here a unique amyloidoma of the radial nerve which could not be subtyped by available techniques, including immunohistochemistry and standard clinical and laboratory evaluation. In order to identify the amyloid monomer, we developed a novel preparative procedure designed to optimize conditions for liquid chromatography tandem mass spectrometry analysis of formalin-fixed/paraffin-embedded (FFPE) tissue. Subsequent mass spectrometric analysis clearly identified kappa light chain as the monomer, with no evidence of lambda light chain. Manual interpretation of the matched spectra revealed no evidence of polyclonality. This study also enabled detailed characterisation of twelve likely amyloid matrix components. Finally, our analysis revealed extensive hydroxylation of collagen type I but, unexpectedly, an almost complete lack of hydroxylated residues in the normally heavily-hydroxylated collagen type VI chains, pointing to structural/functional alterations of collagen VI in this matrix that could have contributed to the pathogenesis of this very unusual tumour. Given the high quality of the data here acquired using a standard quadrupole-time of flight tandem mass spectrometer of modest performance, the robust and straightforward preparative method described constitutes a competitive alternative to more involved approaches using state-of-the-art equipment.

**Keywords:** Amyloidosis, protein extraction, AL, kappa light chain, collagen hydroxylation

**Abbreviations:** AA, amyloid A; AL, light chain amyloidosis; FFPE, formalin-fixed/paraffin-embedded; LCM, Laser Capture Microdissection; LC-MS/MS, Liquid Chromatography tandem Mass Spectrometry; MS, mass spectrometry;

**Introduction**

Amyloidosis is a disease process that encompasses a number of related conditions characterized by tissue deposition of specific amyloidogenic proteins, or ‘monomers’. The monomers form insoluble amyloid deposits comprising aggregates of amyloid fibrils embedded in a matrix containing additional proteins. Amyloid matrix proteins such as amyloid P protein, apolipoproteins and proteoglycans may contribute to amyloid deposition and stabilize already-formed amyloid deposits[1]. The most frequently-encountered types of amyloidosis are systemic and include those formed by monomers derived from immunoglobulin light chains (AL[2]), amyloid A (AA[3]), and transthyretin[4]. These systemic amyloidoses are serious diseases which generally carry poor prognosis as they are associated with the deposition of insoluble amyloid proteins in the extracellular matrix of various organs, including the heart and kidneys. The term ‘primary amyloidoma’ is applied to rare, tumour-like amyloid masses which, on occasion, can reach considerable size[5]. By contrast with systemic amyloidoses, patients with localized primary amyloidomas usually have an excellent prognosis if the tumours are removed before irreversible tissue damage has occurred.

Amyloidomas involving the peripheral nervous system (PNS) are extremely rare, with only a handful of case reports available. For unknown reasons, amyloidomas of the PNS are often located in the Gasserian ganglion of the trigeminal nerve[6–14]. PNS amyloidomas outside the Gasserian ganglion have, to our knowledge, been reported in...
the literature only four times previously[15–18]. Notably, the case presented here is the first report of an amyloidoma surrounding the radial nerve.

Amyloidomas are usually diagnosed on the basis of histo-

logical amyloid stains to confirm amyloid deposition, in com-
bination with immunohistochemical staining to determine the main type of amyloid protein involved. Molecular typing of the ‘monomer’ is an essential clinical step in the diagnostic work-up of all amyloidoses, given the prognostic implications and differing therapeutic options available for distinct molecular variants. Primary amyloidomas are commonly found to be of ALkappa or ALlambda type, but several cases of amy-

loidomas consisting mainly of deposited β2-microglobulin or AA have also been reported[19–22].

While Congo red staining is a reliable tool for screening of tissues for presence of amyloid, subsequent typing of the amyloid by immunohistochemistry can be notoriously unreliable[23]. In cases when immunohistochemical analysis is inconclusive, amyloid typing may be attempted by mass spectrometric (MS) analysis. A major hurdle here is that any excised tissue is usually formalin-fixed and embedded in paraffin (FFPE). This is necessary to enable histology and immunohistochemistry, but greatly complicates subsequent analysis by MS[24]. Several novel preparatory techniques have been reported to improve subsequent MS analysis of FFPE tissues[24–29]. In particular, the method described in[26] reportedly produces a comparable number of identified proteins from FFPE and frozen tissue. Another recently introduced approach to MS-assisted amyloid typing is the use of Laser Capture Microdissection (LCM[18,30]) of amyloid rich regions of tissue biopsies prior to protein extraction and MS analysis. Whilst clearly useful, this approach relies on specialized equipment and is also limited in terms of the amount of material that can be analysed. This may render LCM unsuitable for certain specimens, especially for investigations extending beyond typing of the main amyloidogenic protein.

The underlying cause for the development of amyloidomas is unclear. Therefore, increased knowledge of the identities and roles of both the specific monomers and amyloid stabilizing matrix proteins is likely to prove crucial in order to better understand their pathogenesis. Problematically, characterization of the protein constituents of amyloidomas rarely transcends immunohistochemical typing of the main amyloid, and detailed information of the protein composition of amyloidomas is typically not obtained. The recent advances in proteomic techniques have however begun to open up the possibility of more reliable amyloid typing, as well as characterization of the amyloid matrix proteins, through MS. In a recent LCM-assisted MS study of five NS amyloidomas, Rodriguez et al. reported all to contain lambda (but not kappa) light chain, in addition to a number of proteins likely constituting the amyloid matrix[18].

Here we describe a new method that we have developed and applied to the characterization of an unusual amyloidoma. This method may have application in the elucidation of other cases of difficult-to-diagnose amyloidoses.

**Case presentation**

A previously well 53-year-old Caucasian female presented with a 10-month history of a slowly increasing soft tissue mass on the radial aspect of her left forearm, and the remainder of her clinical examination was unremarkable. There was no family history of note. Ultrasound and MR imaging confirmed the presence of an 8.5 × 2.6 cm fusiform mass that was continuous with the radial nerve sheath. She subsequently proceeded to excision of the localized mass that proved to be an amyloidoma arising from the superficial branch of the left radial nerve (Figure 1). A postoperative MRI scan at 12 months showed no evidence of localized recurrence.

Relevant laboratory investigations including full blood count and erythrocyte sedimentation rate, serum protein electrophoresis and immunofixation, urine examination for immunoglobulin light chains, serum free light chain analysis, serum creatinine and calcium levels, and bone marrow aspirate and trephine were all normal (data not shown). CT imaging of the abdomen and pelvis revealed normal appearance of the liver, spleen and kidneys, and an echocardiogram showed normal left ventricular size and function, with normal serum BNP level (data not shown). Transthyretin gene analysis showed wild-type sequence only (not shown).

The amyloidoma was immediately preserved by formalin fixation and embedded in paraffin. Tissue sections from the excised mass showed peripheral nerve, with gross accumulation of amyloid in the perineurium which was Congo-red positive and which showed green fluorescence under polarized light (Figure 2). There were moderate numbers of lymphocytes and both kappa and lambda light chain-positive plasma cells. Immunohistochemical staining of the amyloid was negative for serum amyloid A, apolipoprotein A1, and kappa and lambda immunoglobulin light chains. Since the amyloidoma proved refractory to typing using standard clinical and laboratory approaches, the FFPE specimen was subjected to further analysis by MS, described in detail in the Methods section.

![Figure 1. Amyloidoma of the radial nerve visualized in situ during its surgical removal.](image-url)
Methods

Sample preparation and LC-MS/MS
Two cubes of tissue (each ~2 mm³) were excised from the central region of the FFPE biopsy specimen using a clean scalpel blade and placed separately in low-protein binding tubes (Axogen, Union City, CA). The tissue pieces were deparaffinised by immersing them in 0.5 mL of n-hexane followed by vortex-mixing for 10 s. The samples were then incubated for 1 h at room temperature (RT, ≈23°C), after which 25 μL of methanol was added and the samples were again mixed briefly and centrifuged at 16,000g for 2 min. After removal of the liquid layer, the resulting pellets were left to air dry at RT. Following the method outlined in[26], we then added 200 μL of sodium dodecylsulphate (SDS) reducing buffer (2% SDS, 20 mM dithiothreitol, 50 mM ammonium bicarbonate, pH 8) to one of the tubes. This sample was then sonicated on ice for 3 × 30 s using a Soniprep 150 operated at 10–15 microns and subsequently incubated for 1 h at 70°C with moderate shaking. The second pellet was extracted with 200 μL of a urea/thiourea-containing buffer (7 M urea, 2 M thiourea, 0.1% Surfact-Amps® X-100 (Pierce), 20 mM dithiothreitol, 50 mM ammonium bicarbonate, pH 8), sonicated as above and incubated for 1 h at 40°C with shaking. Both extracted pellets were then alkylated by addition of iodoacetamide to 50 mM final concentration and incubated for 1 h in the dark. Excess iodoacetamide was then quenched by the addition of equimolar dithiothreitol. Reduced and alkylated samples were diluted 10-fold (urea/thiourea extracted pellets) or 20-fold (SDS extracted pellets) in 50 mM ammonium bicarbonate (pH 8) and digested with 2 μg trypsin (Sequencing grade, Promega) at 37°C overnight. Prior to LC-MS/MS analysis, trypic digests were acidified and desalted on 10 mg Oasis HLB SPE cartridges and eluted with 60% acetonitrile. A portion of the eluted material was diluted 5-fold with 0.1% (v/v) formic acid and 10 μL of this was used for LC-MS/MS analysis. The HPLC system consisted of a 0.3 × 5 mm C18 Pepmap trap column (LC Packings) and a 0.3 × 100 mm Zorbax 300SB C18 column (Agilent, Palo Alto, CA). Peptides were separated using a gradient of 0.1% formic acid in 18 MΩ water (A) and 0.1% formic acid in acetonitrile (B) as follows: 0–3 min 10% B, 75 min 35% B, 77 min 95% B, 82 min 95% B, 83 min 10% B, 90 min 10% B. The column eluate was directed into the ion source of a QSTAR-XL hybrid quadrupole-time of flight tandem mass spectrometer (Applied Biosystems, Foster City, CA) whereupon a TOF-MS scan was performed from 300 to 1600 m/z, followed by the fragmentation of the three most abundant multiply-charged species.

Database searches
A protein sequence database was prepared by extracting all of the human sequences from NCBI as of 28 Oct 2008 (199,127 entries) using the FASTA Database Utilities within Bioworks Browser Rev 3.3 (Thermo Electron Corp., San Jose, CA). The generated data were then searched against this database using both the Mascot v2.0.05 search engine and the ProteinPilot 2.0.1 (Applied Biosystems) application. Search parameters for Mascot were as follows: Enzyme, semi-trypsin; Maximum missed cleavages, 1; Fixed modifications, carbamidomethyl (Cys); Variable modifications, deamidation (NQ), N-acetylation (Protein), pyro-glu (NH₂-terminal E/Q), hydroxylation (K/P); Peptide mass tolerance, 0.15 Da; Fragment mass tolerance, 0.15Da. Search parameters for ProteinPilot were as follows: Search effort, thorough; Cys alkylation, iodoacetamide; Digestion, trypsin; Special factors, urea denaturation; ID focus, amino acid substitutions. Those features in the base peak chromatogram representing multiply-charged species that were either not matched by the database searches, or were matched to kappa light-chain variants, were selected for manual interpretation of their fragment-ion spectra. In all cases where manual interpretation suggested a different sequence to that matched by ProteinPilot, manually obtained sequences were subjected to a protein database search using the online search tool BLAST freely available at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Protein hits scoring >56 in the forward search were considered significantly matched by Mascot, while for ProteinPilot we applied a cut-off at a total score ≥2. Some proteins, in particular immunoglobulin molecules, were matched by both Mascot and ProteinPilot with up to 14 entries with similar protein names and/or sequences. Alignment of these sequences using the web-based Kalign tool (http://www.ebi.ac.uk/Tools/
Results

LC-MS/MS following SDS-based extraction and the novel urea-thiourea based extraction protocol

Our first aim was to find a method that worked well for a homogenous sample of FFPE amyloid, which we anticipated to be extremely difficult to dissolve. To this end, we compared two approaches towards amyloid sample preparation in the stage prior to LC-MS/MS analysis: the SDS-based extraction described in[26], which has been reported to work very well for MS analysis of FFPE tissues, and a novel extraction buffer that we designed and which contained, amongst other things, a combination of urea and thiourea. We included these denaturants because thiourea is known to increase the solubility of hydrophobic proteins prior to two-dimensional electrophoresis[31] and may thus prove useful for solubilisation of amyloid.

In our hands, the SDS-based extraction resulted in few detectable peptide peaks and by using it we were unable to identify any of the proteins in the tissue samples under analysis here. By contrast, the novel urea/thiourea-based approach generated a wealth of peptide peaks that were useable for identification of multiple proteins in the suspected amyloidoma.

Database search of LC-MS/MS data

A Mascot search of the generated peptide peaks yielded 41 protein identifications with significant scores (>56). This initial list was condensed into 21 truly unique proteins after combining entries with very similar sequences (Table 1). For example, kappa light chain immunoglobulin was identified as 14 separate NCBI entries, all with highly similar sequences but with slight variations, particularly in the variable region, as would be expected for an immunoglobulin. The list of identified proteins from the ProteinPilot search with Total Scores ≥2.0 was similar, but not identical to that from the Mascot search (Table 1). Multiple hits referring to essentially the same protein were condensed as described for the Mascot search above, resulting in a total of 17 unique proteins that were matched with significant identification scores by ProteinPilot. Twelve proteins were identified by either Mascot or ProteinPilot but not both (Table 1). These significant but low-scoring hits were discounted on the basis of only being identified using one of the two search algorithms. The 13 proteins identified with significant scores by both Mascot and ProteinPilot were considered to be true constituents of the sample (in bold, Table 1). Data summaries containing detailed information on all matched spectra are available for both searches in Supplemental Table 1.

<table>
<thead>
<tr>
<th>Protein Name (Gene Name)</th>
<th>Representative NCBI entry</th>
<th>ProteinPilot (significant &gt;2)</th>
<th>Mascot (significant &gt; 56)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Immunoglobulin proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunoglobulin kappa light chain VIj region</td>
<td>gi</td>
<td>21669339</td>
<td>18.8</td>
</tr>
<tr>
<td>Unnamed protein product (Immunoglobulin heavy constant alpha 1)</td>
<td>gi</td>
<td>14042015 (GeneID: 3493)</td>
<td>12.9</td>
</tr>
<tr>
<td>Immunoglobulin J chain</td>
<td>gi</td>
<td>21489959</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Collagen proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prepro-alpha(I) collagen</td>
<td>gi</td>
<td>1418928</td>
<td>20.1</td>
</tr>
<tr>
<td>Alpha 2 type I collagen</td>
<td>gi</td>
<td>48762934</td>
<td>13.6</td>
</tr>
<tr>
<td>Alpha-1 (III) collagen</td>
<td>gi</td>
<td>930045</td>
<td>-</td>
</tr>
<tr>
<td>Collagen, type VI, alpha 1</td>
<td>gi</td>
<td>87196339</td>
<td>10.3</td>
</tr>
<tr>
<td>Type VI collagen alpha 2</td>
<td>gi</td>
<td>13603394</td>
<td>6.6</td>
</tr>
<tr>
<td>Alpha 3 type VI collagen</td>
<td>gi</td>
<td>55743106</td>
<td>36.6</td>
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<tr>
<td>Collagen, type XI, alpha 1</td>
<td>gi</td>
<td>119593317</td>
<td>-</td>
</tr>
<tr>
<td>Collagen, type XIV, alpha 1</td>
<td>gi</td>
<td>55743096</td>
<td>1.6</td>
</tr>
<tr>
<td><strong>Amyloid core protein</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum amyloid P component</td>
<td>gi</td>
<td>4502133</td>
<td>9.1</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum albumin</td>
<td>gi</td>
<td>6013427</td>
<td>21.2</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>gi</td>
<td>88853069</td>
<td>12.7</td>
</tr>
<tr>
<td>Unnamed protein product (Actin, gamma 1)</td>
<td>gi</td>
<td>140438064 (GeneID: 71)</td>
<td>3.7</td>
</tr>
<tr>
<td>Vimentin variant</td>
<td>gi</td>
<td>62896523</td>
<td>2.9</td>
</tr>
<tr>
<td>Alpha-2 antiplasmin</td>
<td>gi</td>
<td>39725934</td>
<td>2.0</td>
</tr>
<tr>
<td>Growth arrest-specific 2 like 2</td>
<td>gi</td>
<td>21281675</td>
<td>-</td>
</tr>
<tr>
<td>Laminin alpha 2 subunit</td>
<td>gi</td>
<td>62087424</td>
<td>-</td>
</tr>
<tr>
<td>ATPase type 13A5</td>
<td>gi</td>
<td>119598483</td>
<td>-</td>
</tr>
<tr>
<td>Diffuse panbronchiolitis critical region 1</td>
<td>gi</td>
<td>119623756</td>
<td>-</td>
</tr>
<tr>
<td>Titin isoform N2-A</td>
<td>gi</td>
<td>110349719</td>
<td>-</td>
</tr>
</tbody>
</table>

We did not apply a criterion of a minimum number of unique peptides contributing to protein identification; however all 13 proteins identified with a significant identification score by both search methods were identified by at least four unique, non-redundant peptides.

Identity of the amyloid monomer

Although the Mascot and ProteinPilot searches of the data successfully identified 13 proteins in the tissue sample, this approach did not provide information regarding the relative abundances of the identified proteins. Abundance information could however be derived from the base peak chromatogram, in which only the most intense of the >1000 matched and used spectra were discernable as separate peaks. We therefore sought to identify the proteins comprising the multiply-charged peaks that dominated this chromatogram.
(Figure 3, peaks A-L). The smallest of these, peak A, was mapped by ProteinPilot to collagen alpha-1(I) (Table 2), suggesting that this protein was a dominant component of the non-kappa light chain constituents of the amyloidoma. The nine most intense multiply-charged peaks in the chromatogram (Figure 3, peaks B-I, L) were however all matched by ProteinPilot to variations of kappa light chain immunoglobulin (Table 2). Manual interpretation of all major peaks matched by ProteinPilot to kappa light chain immunoglobulin revealed minor sequence variations from the best match.

Table 2. Peptides corresponding to the most intense peaks in the base peak chromatogram (Figure 3), as matched by ProteinPilot. For manually inspected sequences containing a leucine (L) or isoleucine (I), which are indistinguishable by the method used here, the sequence was denoted as ‘identical to ProteinPilot match’ if the sequence matched by ProteinPilot contained either I or L at the corresponding position and the manually inspected sequence was otherwise identical.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Protein name</th>
<th>NCBI entry</th>
<th>Score</th>
<th>Database peptide sequence</th>
<th>Modification suggested by ProteinPilot</th>
<th>Sequence as per manual sequencing/inspection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>prepro-alpha1(I) collagen</td>
<td>gi</td>
<td>1418928</td>
<td>99</td>
<td>GVGQPGGPGPR</td>
<td>Oxidation(P)@8</td>
</tr>
<tr>
<td>B</td>
<td>anti-tetanus toxoid</td>
<td>gi</td>
<td>58223306</td>
<td>99</td>
<td>SNLAWQHKPGQAPR</td>
<td>SN(I/L)AWQHKPGQAPR</td>
</tr>
<tr>
<td>C</td>
<td>immunoglobulin kappa light</td>
<td>gi</td>
<td>21669339</td>
<td>99</td>
<td>LLIYDASTR</td>
<td>(I/L)(I/L)YDASTR</td>
</tr>
<tr>
<td>D</td>
<td>chain VLJ region</td>
<td>gi</td>
<td>75707477</td>
<td>85</td>
<td>EIVMTQSPATLSLSPGER</td>
<td>Glu-&gt;pyro-Glu@N-term; Val-&gt;Glu@3</td>
</tr>
<tr>
<td>E</td>
<td>IGK@ protein</td>
<td>gi</td>
<td>16741061</td>
<td>99</td>
<td>FSGSGSTDFTLTISR</td>
<td>Asp-&gt;Gln@9</td>
</tr>
<tr>
<td>F</td>
<td>chain variable region¹</td>
<td>gi</td>
<td>75707477</td>
<td>90</td>
<td>EIVMTQSPATLSLSPGER</td>
<td>Protein Terminal Carbamyl@N-term; Dethiomethyl(M)@4; Deamidated(Q)@6</td>
</tr>
<tr>
<td>G</td>
<td>immunoglobulin kappa light</td>
<td>gi</td>
<td>21669339</td>
<td>90</td>
<td>LLIYDASTR</td>
<td>Carbamyl@N-term</td>
</tr>
<tr>
<td>H</td>
<td>chain VLJ region</td>
<td>gi</td>
<td>21669339</td>
<td>99</td>
<td>TVAAPSVFIFPPSDEQ</td>
<td>TVAAPSVF(I/L)FFPSDEQ</td>
</tr>
<tr>
<td>I</td>
<td>immunoglobulin kappa light</td>
<td>gi</td>
<td>21669339</td>
<td>99</td>
<td>TVAAPSVFIFPPSDEQLK</td>
<td>TVAAPSVF(I/L)FFPSDEQ</td>
</tr>
<tr>
<td>J</td>
<td>chain VLJ region</td>
<td>gi</td>
<td>21669339</td>
<td>85</td>
<td>TVAAPSVFIFPPSDEQL</td>
<td>TVAAPSVF(I/L)FFPSDEQ</td>
</tr>
<tr>
<td>K</td>
<td>not matched by ProteinPilot²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(I/L)QSEDVAAY²</td>
</tr>
<tr>
<td>L</td>
<td>immunoglobulin kappa light</td>
<td>gi</td>
<td>21669339</td>
<td>99</td>
<td>TVAAPSVFIFPPSDEQL</td>
<td>TVAAPSVF(I/L)FFPSDEQ</td>
</tr>
</tbody>
</table>

¹Identical or highly homologous to immunoglobulin kappa light chain VLJ region (gi|21669339).
²Best match by BLASTP of manually sequenced peptide: EIELTQSPATLSLSPGER from human immunoglobulin kappa light chain variable region L2 (gi|5731243).
³Manually sequenced, found to correspond to a porcine trypsin fragment.
⁴Best match by BLASTP of manually sequenced peptide: LQAEDVAAY from human immunoglobulin kappa light chain variable region (gi|98956210).

Figure 3. Base peak chromatogram showing the main peaks generated from the amyloid sample as detected by LC-MS/MS. The identities of the twelve most intense multiply charged peaks (A-L) were determined in order to establish the main protein constituent of the sample. (1+) denotes singly charged peaks that were therefore unlikely to be tryptic peptides, so were not automatically selected for MS/MS during data acquisition.

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by ProteinPilot for three of the kappa light chain main peaks (Table 2, Figure 4). BLAST searches of these manually-obtained sequences did however confirm kappa light chain variants to be the best match in each case. The two major peaks (J and K) not matched by ProteinPilot were also manually interpreted. Peak J was found by BLAST search to correspond to a region of porcine trypsin (introduced during tryptic digestion) and K was consistent with an additional region of kappa light chain immunoglobulin (Table 2, Figure 4). Hence, ten of the twelve major peaks were matched by ProteinPilot and/or BLAST to human kappa light chain immunoglobulin. As illustrated in Figure 4, the identified peptides were mainly from the variable region of kappa light chain immunoglobulin but sequences from the constant region were also represented. Furthermore, two sets of peaks corresponded to the same region but carried different elution properties due to their oxidation states (peaks D and F) or carbamylation status (peaks C and G). A further three peaks (H, L and I) were matched to the same region but differed in terms of number of C-terminal residues. All overlapping manually obtained sequences were found to be identical in terms of amino acid residues, hence no evidence of polyclonality was found. Comparison of the specific sequences obtained by manual interpretation (as displayed in Figure 4) to critical regions of the four subgroups of kappa light chain, in combination with BLAST searches, identified the kappa light chain as being of type III. In summary, these data show that kappa light-chain-derived peptides clearly comprised the main protein components of the tissue sample. The monoclonality of this amyloidoma hence comprised kappa light-chain-derived protein, and the diagnosis was ALkappa.

### Hydroxylation status of matrix-component collagen proteins

Database searches using both Mascot and ProteinPilot indicated that the two type I collagen molecules identified here were the main proteins containing hydroxylated peptides extracted/identified from the amyloidoma matrix. Of the two algorithms, ProteinPilot generated the more detailed output and therefore this dataset was selected for systematic analysis of the hydroxylation status of all five collagen molecules identified. Type I collagen molecules were heavily hydroxylated, with 14 of 16 and nine of ten unique peptides matched to types I alpha 1 and I alpha 2, respectively, containing one or more hydroxylated residue(s). In contrast, none of the six unique peptides matched to collagen VI alpha 1 or the five unique peptides matched to collagen VI alpha 2 contained any hydroxylated residues and only one of the 31 matched unique peptides from collagen VI alpha 3 was hydroxylated (Table 3).

### Discussion

This patient was diagnosed with a localized perineural AL (kappa type) amyloidoma of the radial nerve. This case is unique as it is the first report of an amyloidoma at this locus. The case has broader implications, however, since it is in several ways representative of the many suspected amyloidomas that are difficult to type using currently available procedures.

In order to identify the protein constituents in this deposit, we initially followed a recently published SDS-based method for protein extraction from FFPE tissue[26]; however, this approach did not yield interpretable data in our hands. We therefore developed and tested a new urea/thiourea-based protocol designed to optimize protein extraction and conditions prior to MS analysis. In marked contrast to the former approach, LC-MS/MS of the urea/thiourea-extracted sample generated an abundance of interpretable data, which enabled us to identify 17 (ProteinPilot) and 21 (Mascot) unique proteins in the sample with significant identification scores. Of these, 13 were identified by both search methods and therefore deemed to have been identified with a high probability, and were therefore carried forward for further analysis.

The main protein, as judged by mapping of the twelve most intense multiply-charged peaks in the base peak chromatogram, corresponded to immunoglobulin kappa light chain, type III. Searches of the NCBI database using both ProteinPilot and Mascot identified multiple variants of kappa light chain rather than one single database entry. This was expected, since immunoglobulin light chains exhibit a high degree of sequence variability between individuals as well as clones, and it is unsurprising that the database contained no exact match to some of the sequences that were obtained here. Careful examination of manually-obtained overlapping sequences revealed no variations within the major peaks, suggesting that the amyloidoma was likely of monoclonal origin. The identification of peptides from both the variable and constant regions is consistent with the recently-addressed role of the constant domain in AL amyloidosis[32].

The present case is consistent with the notion that localized amyloidomas are usually of the light chain type. However, most PNS amyloidomas reported to date have been of the lambda rather than kappa light chain type[8,16,18,33] and we cannot find in the available literature a previous record of a PNS amyloidoma solely of the kappa light chain type. The present case thus appears to be unique both in terms of locus and the particular amyloid monomer.

In addition to kappa light chain, we identified twelve probable components of the amyloidoma matrix. The designation of all these non-kappa light chain proteins as amyloid matrix components was made probable by their isolation from well within the substance of the well-defined...
Figure 4. Kalign (2.0) alignment in ClustalW format of the six different NCBI entries determined by ProteinPilot and/or BLASTp to best fit the ten major peaks matched to kappa light chain in the base peak chromatogram (Figure 3). Numbers represent NCBI gi accession numbers, with the uppermost sequence being the top scoring kappa light chain obtained by both ProteinPilot and Mascot. The sequences obtained by manual sequencing or inspection are shown beneath each matched region. While it was not possible to distinguish between I and L residues by the methods used here, we refrained from using the customary (I/L) designation in this figure in the interest of maintaining the alignment. Instead, either I or L was chosen for the manual sequence, based on whether I or L was present in the corresponding position in the best match for that specific peptide. Residues differing between the manually obtained sequence and the best match (both in yellow) are highlighted using red font. Letters inscribed in boxes refer to the major peaks in Figure 3; arrows indicate start and finish of the variable and constant regions of the top scoring kappa light chain entry, as per the NCBI protein database.
and membrane-delimited amyloidoma. Most of the identified matrix components have previously been recorded in amyloidomas[1,18,34–37], including the most abundant protein in the human body, type I collagen[18]. The less common type VI collagen has not, to the best of our knowledge, previously been reported in any amyloidoma but was here identified with the highest protein identification score by both search methods. Collagen VI (a heterotrimer of collagen chains VI alpha-1, -2, and -3) is thought to link to type I collagens through decorin, a small proteoglycan[38]. Post-translational hydroxylation of collagen chains is thought to be required for their correct oligomerization as well as tissue distribution, and types I and VI collagens are normally both heavily hydroxylated[39]. Indeed, we found most peptides matched to the type II collagen chains to be hydroxylated, showing that our method could identify peptides containing hydroxylated residues. By contrast, only one hydroxylated peptide was found amongst 42 unique peptides matched to the type VI collagen chains. This indicates that collagen VI was present in a mainly non-hydroxylated form in this amyloidoma matrix. It is clear that hydroxylation is critical for collagen folding[40,41]. Defective hydroxylation can reportedly lead to intracellular buildup of incorrectly folded collagen[42] and is also related to pathogenesis in severe connective tissue disorders[43]. Defective collagen has also been proposed to be a direct cause of primary cutaneous amyloid[44]. This suggests the possibility that locally-defective collagen hydroxylation could have contributed to the onset of the specific amyloid deposition in this case, thereby contributing to formation of this amyloidoma. Further studies are needed to test this hypothesis.

Conclusions

We here describe for the first time a new preparative procedure, which in combination with LC-MS/MS enabled high-resolution insight into the protein composition of a unique case of amyloidoma, and reliable typing of the monomer. While our approach was clearly superior to at least one previously published technique purported to handle FFPE tissues well[26], we have not yet conducted an exhaustive comparison of all available techniques for protein extraction. Thus it cannot be excluded that similarly useful data could be obtained following alternative protein extraction methods e.g. utilizing 6M guanidine-HCl[28]. The finding of non-hydroxylated collagen VI in the sample may point towards a possible contributory cause for the amyloid deposition in this case, and also illustrates the level of detail that can be obtained using our novel sample preparation protocol. Notably, we acquired these high quality data from FFPE tissue using a standard quadrupole-time of flight tandem mass spectrometer of modest performance and without the need for LCM. Our method thus provides a competitive alternative to more involved approaches using state-of-the-art equipment. We expect that this straightforward method will prove useful both as a diagnostic method and for future mechanistic investigation of amyloidoses, as well as other types of formalin-fixed tissues.

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