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Mass spectrometry-based proteomics in clinical practice amyloid typing: state-of-the-art from a French nationwide cohort

Amyloidosis refers to a large spectrum of diseases, all characterized by the deposition of extracellular misfolded proteins in the form of insoluble highly ordered amyloid fibrils in one (localized amyloidosis) or multiple tissues (systemic amyloidosis).[1-3] Amyloid deposits progressively disrupt tissue structure and exert a toxic effect on the adjacent cells that ultimately result in organ dysfunction. To date, 36 different proteins are known to form amyloid fibrils in humans.[1] The identification of the causal protein is of paramount importance for patient management because effective treatments are now available, especially for the main amyloid types derived from immunoglobulin light chain (AL) and transthyretin (ATTR).[2]

Although there is a predilection for particular organs depending on the amyloid type, clinical manifestations are heterogeneous and may overlap between the different types.[2,3] Therefore, determination of the amyloid type cannot rely on the sole clinical findings. Traditionally, amyloid typing is performed using immunofluorescence (IF) on frozen sections and immunohistochemistry (IHC) on paraffin sections. Interpretation of antibody labeling with modified proteins is often challenging despite experience.[3-5] Indeed, commercial antibodies are not optimized for recognizing mutant and truncated amyloid proteins and the β-pleated conformation may elicit non-specific positivity. Therefore, laser microdissection from formalin-fixed paraffin-embedded (FFPE) tissue combined with tandem mass spectrometry (LMD-MS/MS) has been developed and progressively implemented in routine practice.[6-13] The rationale for the application of this method is based on the relative abundance of amyloid protein that usually corresponds to one of the dominant proteins within the studied biopsy sample.[6-13] In recent years, MS-based proteomics has become the reference method because of its excellent identification capacity.[6-13] However, its performance on various tissues/organs remains still limited to a few expert centers worldwide.[6-13] In the present study, we document our experience of amyloid typing by LMD-MS/MS, over a 10-year period.

We conducted a retrospective study including 833 amyloidosis specimens retrieved from our collection from January 2010 to Sept 2021. The diagnosis of amyloidosis was established on biopsy specimens using Congo red (CR) staining. As Vrana *et al*. in their pioneering report in 2009, we studied two independent sets of amyloidosis specimens.[6] The first set was a training set that consisted of 92 tissue specimens (90 patients, 67.6±14.0 years, 29 female[F]/61 male[M]). Each case of this set was well classified using the IF/IHC method and the identified amyloid type was in keeping with the extensive clinical, biological, genetic and imaging workup. The training set included 42 ATTR, 38 AL (35 λ, 3 κ) and 12 AA amyloidosis. The second set was a validation set that consisted of 741 tissue specimens (686 patients, 68.6±13.3 years, 249 F/437 M). For each case, LMD-MS/MS was indicated because of inadequate or absence of frozen sample available for IF, negative IF/IHC, equivocal IF/IHC, and IF/IHC inconsistent with clinical, biological, genetic and imaging investigations. Patient consent was obtained according to the Institutional Review board of CHU de Toulouse.

We used a previously established proteomics method.[13,14] For each sample, a 10 µm-thick section of FFPE tissue was mounted on slides (Expression Pathology, USA) and stained with CR (Merck, Germany). One hundred thousand μm^2 of deposits were selected by laser microdissection under fluorescent light (Leica 6500, Germany). Proteins were extracted from the collected material in ammonium bicarbonate buffer, reduced with dithiothreitol, and alkylated with iodoacetamide. Then, proteins were digested into peptides with trypsin (SIGMA, France) and analyzed by nano-liquid chromatography (nanoLC) coupled to tandem MS (LMD-MS/MS) using an Ultimate 3000 RSLCnano system (Dionex, Netherlands) coupled to an LTQ-Orbitrap Velos or to a Q-Exactive Plus mass spectrometer (Thermo Fischer Scientific, Germany). Data were processed with Mascot against human entries of the SwissProt protein database. Validation of results was performed through a false discovery rate set to 1% at protein and peptide sequence match levels determined by target decoy search using the in-house-developed Proline software (http://proline.profiproteomics.fr/). The spectral count metrics (number of MS/MS spectra) was used to rank the proteins according to their relative abundance in the sample. The most abundant protein identified was considered to be the causative protein. This allowed us to determine the amyloid subtype and the presence/absence of four proteins usually associated with amyloid deposits: serum amyloid-P component (SAP), apolipoprotein E (ApoE), apolipoprotein A4 (ApoA4), and apolipoprotein A1 (ApoA1).[6,15] A minimum number of four MS/MS spectra per protein was considered clinically valid.

Univariate testing was performed using Fisher exact test,

Table 1. Amyloid types identified by mass spectrometry and their frequency (N=705).

Type	Precursor protein	N	%	Age in years	Sex, F/M
AL*	Immunoglobulin light chain	407#	57.7	67.3±11.9	171/236
ATTR	Transthyretin	182	25.8	76.6±10.9	36/146
AA	Serum amyloid A	43	6.1	63.0±15.7	17/26
AApoAI	Apolipoprotein A I	16	2.3	53.3±10.3	9/7
ASem1	Semenogelin 1	12	1.7	67.1±5.0	0/12
AApoAIV	Apolipoprotein A IV	8	1.1	67.1±11.5	2/6
AFib	Fibrinogen α	6	0.9	61.0±12.4	2/4
AKRT5-14	Keratin 5 and keratin 14	4	0.6	60.3±12.1	3/1
ALac	Lactoferrin	4	0.6	41.5±27.6	4/0
BGH3	Transforming growth factor-β-induced protein ig-h3	4	0.6	64.5±9.7	2/2
AIns	Insulin	4	0.6	37.5±13.4	2/2
ACal	Calcitonin	4	0.6	47.5±6.4	2/2
AApoCII	Apolipoprotein C II	3	0.4	74.7±6.1	1/2
Aβ2M	β2-microglobulin	2	0.3	53±5.7	1/1
APTH	Parathyroid hormone	2	0.3	52.5±6.4	1/1
AApoAII	Apolipoprotein A II	1	0.1	52	0/1
ALECT2	Leukocyte chemotactic factor-2	1	0.1	67	0/1
AANF	Atrial natriuretic factor	1	0.1	74	1/0
AH	Immunoglobulin heavy chain	1	0.1	70	1/0

*Co-deposition of a heavy chain (IGG, IGA, IGM or IGD) was found in 20% of cases; #194 κ and 213 λ. F: female; M, male.

with Benjamini-Hochberg adjustment for multiple comparisons. Multivariate adjustment was done using multivariate logistic regression with age, sex and tissue origin explicative covariables.

In the training set, we found that LMD-MS/MS successfully identified the amyloid type in all cases and the concordance rate between LMD-MS/MS and IF/IHC was of 100%. In the validation set, the indications of proteomic analysis were in order of frequency: absence of frozen sample available for IF (70.5%), equivocal IF/IHC (15.7%), negative IF/IHC (9.9%), and inconsistent result (3.9%). LMD-MS/MS successfully identified the amyloid protein in 95.0% with 19 different amyloid types. The main amyloid types were AL (n=407), ATTR (n=182) and AA (n=43) accounting for 89.6% of our cohort. The patient demographics and the frequency of the 19 amyloid types are reported in Table 1. The tissue/organ tropism and the tissue/organ amyloid protein identification rate are detailed in Figure 1. Specific analysis of the AL (n=101) and ATTR (n=58) subgroups with equivocal or negative IHC/IF revealed false-negative and false-positive staining in 48.5% and 41.5% for immunoglobulin light chain antibodies and, in 12.0% and 56.8% for TTR antibody, respectively. The universal amyloid signature SAP/ApoE/ApoA4 was present in 81.6% of cases with an overrepresentation of ApoA1 in AL amyloidosis compared to ATTR and AA amyloidosis (64.3% *vs.* 27.0% and 29.3% respectively, $P<0.001$), and an underrepresentation of ApoA4 in AA amyloidosis compared to AL and ATTR amyloidosis (56.1% *vs.* 91.6% and 90.4% respectively, $P<0.001$), that persisted after adjustment for age, sex and organ/tissue ($P<0.001$).

Overall, our study, based on one of the largest cohort ever reported,[6-13] confirms that MS-based proteomics after laser microdissection is the new gold standard for typing amyloidosis. In the literature, the identification rate of the amyloid protein ranged from 85% to 100%. In the largest series by the Mayo Clinic, Rochester, USA, 21 amyloid types were detected, the frequency of AL (58.9%) and

Figure 1. Tissue/organ tropism and identification rate of the amyloid protein per tissue/organ. The tissue/organ distribution of the 19 amyloid types identified in the present study is illustrated in decreasing order of frequency. Among the 26 tissue/organs analyzed herein, the 5 most commonly (67.5% of cases) analyzed anatomic sites were secondary salivary glands (21.0%), heart (17.2%), lung (10.8%), gastrointestinal tract (9.4%), and kidney (9.1%). Fat aspirate/biopsy was not analyzed in this series because this procedure is not as commonly performed in France as in other countries. The diversity of the amyloid types was greater in the kidney and the heart with 10 and 6 different types identified, respectively. The amyloid protein identification rate (IR) is reported for each of the 26 tissues/organs. CNS: central nervous system; GI tract: gastrointestinal tract; PTH: parathyroid hormon.

ATTR (28.4%) being quite similar to that found in our cohort (Table 1).[11] We confirm that AL patients were also a decade younger than ATTR patients (Table 1).[11] For the remaining types, the main difference was represented by the lower proportion of ALECT2, the rarity of which in our cohort being explained by ethnic bias as >92% of ALECT2 patients are Hispanic and particularly Mexican.[3,11] As expected, the present study demonstrates again that IHC/IF alone may lead to misdiagnosis, especially for ATTR and AL.[4,5]

A key finding of our study was the significant differential expression of ApoA1 and ApoA4 between AL, ATTR and AA amyloidosis, suggesting a singular implication of these proteins in the amyloid formation mechanisms.

In conclusion, in addition to its reliability, the several advantages of MS over IHC/IF are now well documented: i) no frozen tissue sample required, ii) very small amounts of material needed, easily obtained from routine biopsy sampling, iii) detection in a single assay of all amyloid types and, iv) determination of the organ tropism for each amyloid protein that can be visualized in a comprehensive map.

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Disclosures

No conflicts of interest to disclose.

Contributions

MCo initiated and supervised the study, performed histological analysis, clinical data collection, prepared samples for MS analysis, performed MS analysis, bioinformatic MS data analysis, and wrote the paper. MG performed histological analysis, clinical data collection, and interpreted MS data analysis. MCa, JD-C performed histological preparations, laser microdissection, and MS analysis. AD, EP, AM, A.F, A.M, J-BG, PR, AH, OL, GG, SG-L, HM, P M, AJ, FB, VP-B, TD and SV provided tissue samples from patients, and carefully read the paper. AP and JG performed statistical analysis. HM and PB carefully read the paper.OB-S provided access to the proteomics facility of Toulouse at IPBS, and carefully read the paper.

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Data-sharing statement
LC-MS/MS data acquired at the Institute of Pharmacology and Structural Biology (CNRS, Toulouse, France) are stored on local servers. They can be made available to investigators upon specific request.

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