SUBTYPING OF AMYLOIDOSIS BY DIRECT PROTEOMIC ANALYSIS OF FIXED BIOPSY SAMPLES

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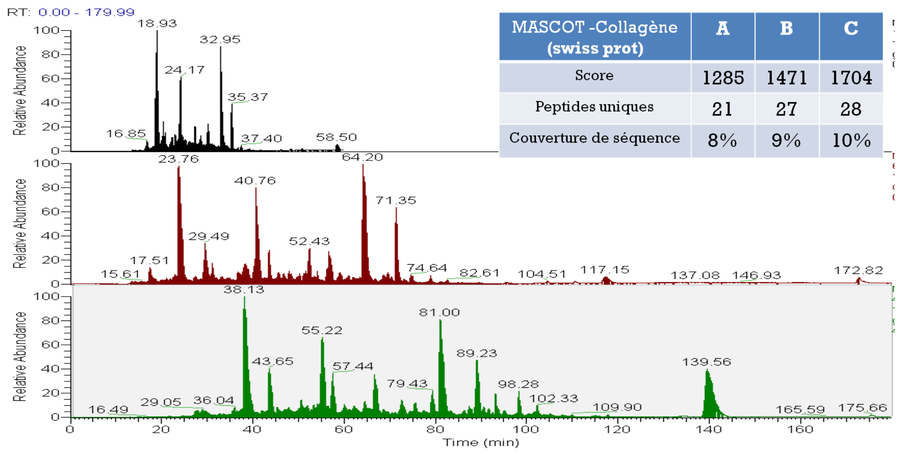
Introduction. Amyloidosis is a disease where specific proteins aggregate in tissues. Different classes of amyloidosis have been reported and their diagnostic relies on the identification of the associated proteins: up to 29 proteins families can be targeted using histochemical approaches. However they can be inconclusive on certain cases, leading to a lack of information about the underlying ethiology. Recent reports have shown that subtyping could be feasible combining laser capture microdissection and mass spectrometry (1). For bottom-up proteomics it is clear that one of the key factors is the completion of enzymatic proteolysis. Ultrasonic treatment could help for this step (2). We have tried to extend the use of ultrasonic treatment to raw biopsy samples in order to avoid the tedious step of LCM and to get closer to the clinical routine application for amyloidosis subtyping.

Methods. Biopsy sample were taken from deseased and control patients. Tissues were immobilized with paraformaldehyde (Bouin or AFA). Tissues and reference proteins were directly digested using an ultrasonic probe. Proteolytic peptide mixtures were analyzed by nanoLC-MS/MS (LTQ-FT Ultra, ThermoFisher) after LC optimization (U3000 Dionex).



MASCOT -Collagène (swiss prot)	A	B	C
Score	1285	1471	1704
Peptides uniques	21	27	28
Couverture de séquence	8%	9%	10%

Figure 1: LC optimization on lung biopsy sample

- nano C18 Acclaim pepMap100 (Dionex) Viper 75µm i.d.x15cm length column, gradient 2% to 40%B in 170 min, buffer A: H2O/AcN/AF 98:2:0.1 (v/v/v)/ buffer B: H2O/Acn/AF 10:90:0.1 (v/v/v)
- Advion nano ESI source, acquisition method: 1 FTMS (Res100000) + top 7 CID LIT MS/MS
- database search using Proteome Discoverer 1.3.0.339 combining Mascot 2.3 and Sequest on UniProt-SwissProt 2011_07 and home made SwissProt/Trembl database containing amyloidosis proteins, 5ppm MS, 0.5Da MS/MS, up to 2 miscleavages, full tryptic peptides, Ox (M) and CAM (C) as partial modifications.
- only consensus proteins identified in triplicate with both search engine with a FDR<1% are kept
- digestion with porcine trypsin (Promega Gold), after reduction (TCEP 2.5mM), alkylation (iodoacetamide 10mM), in ammonium bicarbonate 50mM pH 8.4 using a ultrasonic probe MicroSon XL on a wet ice bath
- tissues slices of 10µm, volume extrapolated using surfaces of slices as measured by anatomopathology
- injected sample were filtered on Proxeon stage tips and reconstituted in the same volume, roughly 0.15 mm3 were injected for each LC (except for the comparison with Laser Capture Microdissection sample where 0.0055 mm3 were injected , 0.3 equivalent slice.

Accession	Amylosis	Description
P02743	non specif	>sp|P02743|SAMP_HUMAN Serum amyloid P-component
P02649	non specif	>sp|P02649|APOE_HUMAN Apolipoprotein E
P02766	ATTR	>sp|P02766|TTHY_HUMAN Transthyretin
P04279	ASeml	>sp|P04279|SEMG1_HUMAN Semenogelin-1
Q9UKY0	APrPsc	>sp|Q9UKY0|PRND_HUMAN Prion-like protein doppel
Q86SH4	APrPsc	>sp|Q86SH4|PRNT_HUMAN Putative testis-specific prion protein
P04156	APrPsc	>sp|P04156|PRIO_HUMAN Major prion protein
P01236	APro	>sp|P01236|PRL_HUMAN Prolactin
Q08431	AMed	>sp|Q08431|MFGM_HUMAN Lactadherin
P61626	ALys	>sp|P61626|LYSC_HUMAN Lysozyme C
P0CG06	AlL	>sp|P0CG06|LAC3_HUMAN Ig lambda-3 chain C regions
P0CG05	AlL	>sp|P0CG05|LAC2_HUMAN Ig lambda-2 chain C regions
P0CG04	AlL	>sp|P0CG04|LAC1_HUMAN Ig lambda-1 chain C regions
P0CF74	AlL	>sp|P0CF74|LAC6_HUMAN Ig lambda-6 chain C region
A0M8Q6	AlL	>sp|A0M8Q6|LAC7_HUMAN Ig lambda-7 chain C region
P01834	Alk	>sp|P01834|IGKC_HUMAN Ig kappa chain C region
O14960	ALect2	>sp|O14960|LECT2_HUMAN Leukocyte cell-derived chemotaxin-2
P02788	ALac	>sp|P02788|TRFL_HUMAN Lactotransferrin
Q15582	AKer	>sp|Q15582|BGH3_HUMAN Transforming growth factor-beta-induced protein ig-h3
P01308	AIns	>sp|P01308|INS_HUMAN Insulin
P10997	AIAPP	>sp|P10997|IAPP_HUMAN Islet amyloid polypeptide
P01871	AHm	>sp|P01871|IGHM_HUMAN Ig mu chain C region
P01857	AHg	>sp|P01857|IGHG1_HUMAN Ig gamma-1 chain C region
P06396	AGel	>sp|P06396-2|GELS_HUMAN Isoform 2 of Gelsolin
P06396	AGel	>sp|P06396|GELS_HUMAN Gelsolin
P02671	AFib	>sp|P02671|FIBA_HUMAN Fibrinogen alpha chain
P01034	ACys	>sp|P01034|CYTC_HUMAN Cystatin-C
P01258	ACal	>sp|P01258|CALC_HUMAN Calcitonin
P05067	ABeta	>sp|P05067|A4_HUMAN Amyloid beta A4 protein
P61769	AB2M	>sp|P61769|B2MG_HUMAN Beta-2-microglobulin
P06727	AApoAIV	>sp|P06727|APOA4_HUMAN Apolipoprotein A-IV
P02652	AApoAII	>sp|P02652|APOA2_HUMAN Apolipoprotein A-II
Q8NCW5	AApoAI	>sp|Q8NCW5-2|AIBP_HUMAN Isoform 2 of Apolipoprotein A-I-binding protein
P02647	AApoAI	>sp|P02647|APOA1_HUMAN Apolipoprotein A-I
P01160	AANF	>sp|P01160|ANF_HUMAN Natriuretic peptides A
P02735	AA	>sp|P02735|SAA_HUMAN Serum amyloid A protein

Table 1: List of proteins associated to amyloidosis that were considered in this study.

Data interpretation. It is a non targeted approach. Only one isoform is listed for each group, and only proteins that are validated and annotated in SwissProt were considered. Abundance of the protein were evaluated according to (5) taking into account the area of the three most intense peptides. Unidentified species were given the global minimum intensity that could be calculated in our experiments. Only biopsies exhibiting a relative abundance >1% and containing Apolipoprotein E and Serum amyloid P component (common to all amyloid deposits) were considered as potential amyloid candidate. Finally amyloidosis were classified according to the relative intensity of protein listed in Table 1.

Results. Application of ultrasonic tryptic treatment on fixed raw tissues allowed us to perform subtyping of amyloidosis. The results were independantly confirmed by immunohistopathology. Using the whole series of antibodies for amyloidosis diagnostic. We have selected some examples from different tissueand pathologies to illustrate our results. The results are visualized with sector diagrams reprensenting the relative intensities of amuloid proteins.

Kidney Results.

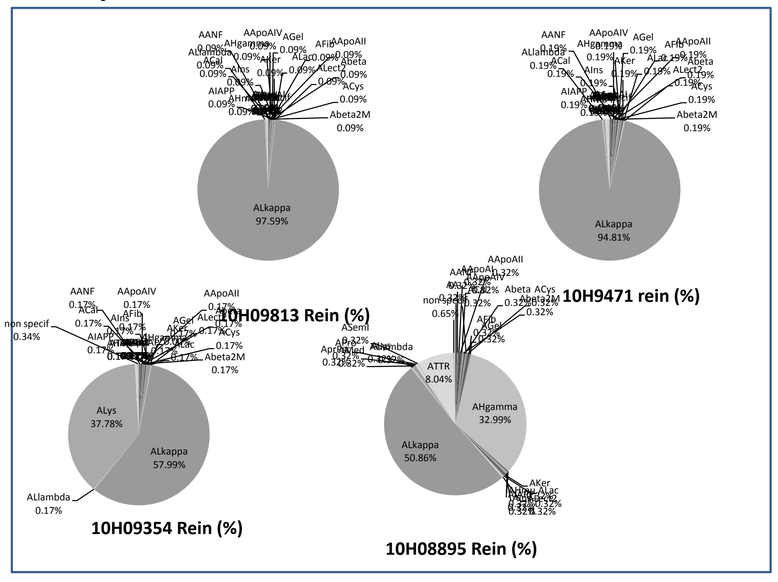
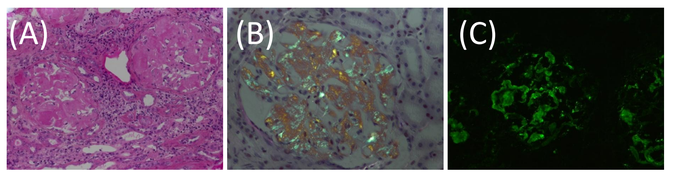


Figure 3: Biopsies of negative controls.

Figure 2: Anatomopathology studies of kidney biopsy for AAL with (A) Hemalun Eosin (HE), (B) Congo red, (C) immunostaining AL

Less than 50% of the tissue were marked for amyloidosis AAL (see C)



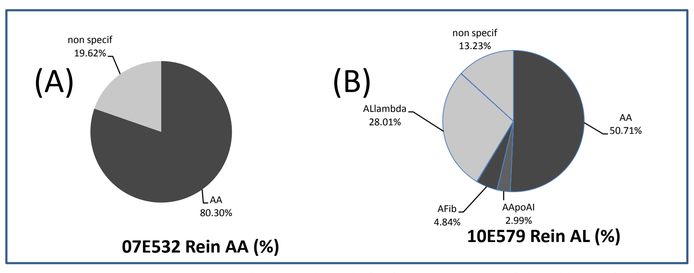


Figure 4: Biopsies of amyloid (A) AA 80% fibrillar, (B) AA 10% fibrillar.

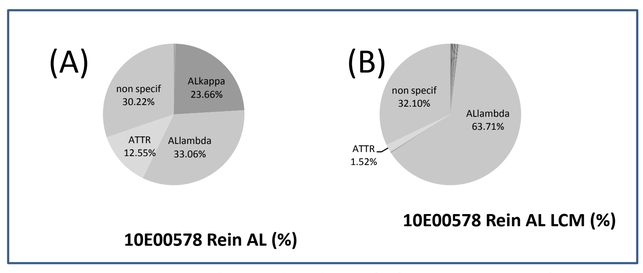


Figure 5: Biopsy of amyloid AL (A) raw tissue (50% , see Figure 2), (B) Laser Capture Microdissected LCM tissue (95%)

No pure negative controls could be assayed because biopies come always from patients and are aimed to identify a pathology. Non amyloid diseased kidney does not present SAMP and ApoE (fibrils biomarkers), even if amyloidogen protein could be detected (Igκ and γ). Their presence could be explained by inflammatory phenomenon or tissue dammage (eg. minor glomerular lesions) . The controls are non amyloid pathologies. As shown in this example when amyloid deposits represents more than 80% of the tissue the diagnostic and the classification are clear (Figure 4 A). When the percentage goes below 50%, amyloid diagnostic is clear but classification is not robust enough (Figures 4B &5A) even if the amyloid type is correct: here we analyzed a raw biopsy without focalising on amyloid fibrils and without slicing. On the opposite LCM treatment on the same sample could offer preparation above 95% where the classification is unambiguous (Figure 5B).

Accessory salivary glands (ASGB) Results.

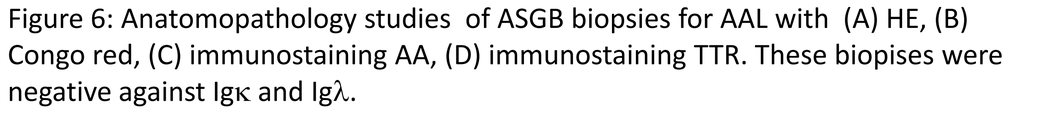


Figure 6: Anatomopathology studies of ASGB biopsies for AAL with (A) HE, (B) Congo red, (C) immunostaining AA, (D) immunostaining TTR. These biopises were negative against Igκ and Igλ.

Figure 7: Biopsies of negative controls. ASGB is enriched in lysozyme, no SAMP or ApoE is present

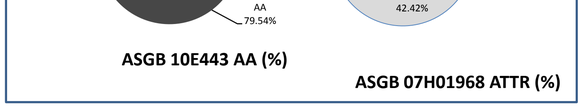


Figure 8: Biopsies of amyloid (A) AA, (B) ATTR.

ASGB are naturally enriched in Lyzosyme C (from saliva). However in the control samples no SAMP and ApoE (amyloid deposits) could be seen. All the biopsies were assayed against the 20 major amyloid antibodies. For the two biopsies in Figures 6 and 8 , the results were negative for ALκ and ALλ, and were positive for (A) AA and (B) ATTR respectively, using both techniques. ApoE and SAMP were also detected unambiguously among the major compounds (Figures 8 A and B).

Conclusion & Perspectives.

This strategy opens the way for a rapid and accurate amyloidosis subtyping directly from raw clinical samples and allows to avoid at the most the laser capture microdissection step which is highly time-consuming. This is of particular interest for classes that could not be distinguished by the classical histochemical analysis. Our next step will be to validate our approach to different amyloidoses and tissues with clinicians.

The whole analysis lasts 1 day for enzymatic treatment and roughly 10 slices are required and lasts 1 day for triplicate LC MS/MS and roughly 1 slice of 10µm is required. In order to increase cohorts for a realistic clinical application we intend to automatize the first step. Miniaturization is required to decrease sample consumption of the first treatment (only 10% of the sample is actually analyzed).

We want to characterize specificaly the isofoms of protein involved. One example is ATTR addressed either by bottom-up (3) or top-down (4) strategies, where the transthyretin isoform can discriminate between senile systemic amyloidosis and familial thranthyretin amyloidosis.

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