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THE USE OF MASS SPECTROMETRY FOR THE DIAGNOSIS OF PLASMA CELL DYSCRASIAS: FOCUS ON LIGHT CHAIN (AL) AMYLOIDOSIS PATTERNS

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INTRODUCTION

The production of monoclonal immunoglobulins (M-proteins) from clonal plasma cells is the specific feature of the plasma cells dyscrasias. In this context, the monoclonal gammopathies represents a group of different conditions that have specific presentation, treatments and outcomes. One of the possible classifications is defined as: (a) protein/low-tumor-burden disease; (b) pre-malignancies or (c) malingnancies[1].

A. the low tumor burden disease are composed by a group of monoclonal gammopathies in which there is a small proliferation of the plasma cells, but the secreted monoclonal proteins itself is able to cause the disease, with deposition or interactions with tissues. In this context, the most important example of a low-tumor-burden disease is light chain (AL) amyloidosis in which a usually 'small dangerous' clone is able to produce the amyloidogenic light chain[2,3].
B. the second group is defined as a pre-malignant disease and it is composed by the monoclonal gammopathy of undetermined significance (MGUS) and by the smoldering multiple myeloma (SMM). In both these conditions, no clinical symptoms are related to the plasma cell clone or the deposition/interaction of the monoclonal protein. However, it was clearly demonstrated and it is now readably acceptable that all cases of multiple myeloma have been preceded by MGUS or SMM[4-6].
C. the specific definition of multiple myeloma was recently updated in the International Myeloma Working Group Consensus paper. In this report, the experts reported specific myeloma defining events and clearly separated the different disease groups[7].

Light chain (AL) amyloidosis

Light chain (AL) amyloidosis is the most common form of systemic amyloidosis in Western countries with an estimated annual incidence of ≈10 new cases per million per year[8]. The amyloid deposits are composed by misfolded monoclonal light chains that are produced by an usually small plasma cell clone (<10% in almost half of the patients)[2]. Any organ could be affected except the brain and the presence and severity of heart involvement is the most important prognostic marker for this disease[9].

The correct diagnosis of AL amyloidosis requires the demonstration of amyloid deposits in tissues, with a correct tissue typing[10], and on the identification of the plasma cell clone producing the amyloidogenic light chain. The identification of the plasma cell clone can be performed either with the bone marrow analysis or by the identification of a monoclonal light chain in the patient's serum and/or urine[11]. The use of all the tests available for the identification and quantification of the monoclonal proteins is also necessary not only for the correct diagnosis and prognostication of survival[12] but also it is required to assess the hematologic response[13-15].

Several methods are used for the identification of monoclonal proteins with different degrees of diagnostic sensitivity. In particular, the currently used in clinical laboratory practice are reported below.

Serum protein electrophoresis (PEL).

Electrophoresis is a method of separating proteins based on their physical properties. Serum is placed on a specific medium, and a charge is applied. The positive or negative charge and the size and shape of the protein are used in differentiating various serum proteins[16]. Monoclonal proteins are visualized as a restricted band in the specific migration pattern. The presence of a non homogeneous distribution of the gamma fraction or a discrete band at electrophoresis results requires the use of immunofixation or immunosubstraction in order to identify the specific M-protein isotype (of both the heavy and the light chain involved). The definition of M-protein by the International Myeloma Working Group reported a serum level >10 g/L and a urine M-protein at PEL of >200 mg/24h[17]. If the M-protein appears less than 30% of the gamma fraction or <5 g/L, the technician will decide to report the presence of M-spike or not. Only few patients with AL amyloidosis could be monitored only with PEL, because the M-protein could not be dosed correctly. Different methods are needed.

Immunofixation (IFE).

Immunofixation is able to detect and type the M-proteins. It has improved sensitivity (with a limit of detection of 100 mg/L) compared to PEL in the identification of M-proteins. The method is based on precipitation. In particular, a specific soluble antigen is brought in contact with the corresponding antibody. The common antisera used for the identification of an M-protein are: IgG, IgA, IgA, total kappa or total lambda. The antisera for IgD and IgE M-proteins are used in the

presence of a M-protein with only kappa or lambda positivity at the first evaluation. The precipitation of the complex antigen antibody occurs and may be visible with naked eye or microscope. As a possible alternative, a penta-specific antiserum could be used as a screening assay for samples with normal electrophoretic patterns. At the Pavia University Hospital a specific approach is used[18]. An home-made high resolution agarose gel electrophoresis (HRAGE), in accordance with the Malmö group, is used[19]. In our laboratory, the HRAGE pattern of the patient is compared with previous analysis for the follow-up determination.

Serum free light chains

The specific free light chain assays use antisera directed against epitopes that are exposed only when the heavy chain is not bound to the specific detectable light chain. The "criptic" sites are involved in the very tight, non-covalent binding of light chains to heavy chains. The specific *Freelite* antisera has a potential 10,000-fold stronger binding avidity for FLC compared to light chains contained within the intact immunoglobulin molecules[20]. For the identification of a monoclonal light chain it is recommended to quantitate both the kappa (that usually exist in the bloodstream as monomers) and the lambda (usually found as dimers). The FLC-ratio is used in order to detect unbalanced light chain synthesis and define the presence of a M-protein. Two different methods are used for the FLC measurement. The FLC measurement is currently used for diagnosis, prognosis and for the definition of response assessment with validated criteria in different plasma cell dyscrasias. All these studies were based on the *Freelite* immunonephelometric assay for FLC using polyclonal antibodies. Few years ago, a novel assay (*N_latex FLC*) based on monoclonal antibodies has been developed[21,22]. This assay is marketed in Europe and currently used in several clinical laboratories. Recently, Mollee et al.[23] and our group[24] compared the *N_latex FLC* and *Freelite* assays in the diagnosis and prognosis of patients with AL amyloidosis and found that the two assays had similar diagnostic sensitivity. In addition, in the manuscript from our group we described that despite a comparable diagnostic performance, the two assays are not interchangeble, i.e. the follow-up for chemotherapy approach should be done with either one. New and specific diagnostic and response criteria should be performed with the *N_Latex FLC* assay[24].

A combination of serum PEL, IFE and FLC measurement with the addition of IFE of the urine allowed the detection of M-proteins in 98.6% of cases[1]. This data was shown in the larger

retrospective study in which patients with different plasma cell proliferative diseases had all the serum and urine tests available. It is important to note that, if urine tests are removed from the diagnostic panel, the diagnostic sensitivity for the detection of M-protein is not reduced for patients with multiple myeloma, smoldering multiple myeloma, Waldenström's macroglobulinemia, palsmocytoma, or POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes). In contrast, the exclusion of urine evaluation reduced significantly the diagnostic ability in cases of AL amyloidosis. Palladini et al. also confirmed these findings[11] and have led the International Myeloma Working Group to recommend a panel of only serum tests (PEL, IFE and FLC) for the screening of monoclonal gammopathies and the addition of urine tests in case of a suspect AL amyloidosis.

AIMS OF TE PROJECT:

The aim of my study is to report the applicability of a matrix assisted laser desorption ionization – time of flight instruments, MALDI-TOF-mass spectrometry method (MASS-FIX), for the detection of monoclonal proteins in serum and urine in the setting of different plasma cells dyscrasias.

Thanks to the observations that reported a suspect for a different posttranslational modification (i.e. glycosylation) path of the light chains of patients affected by AL amyloidosis, two additional studies were performed:

I. In the first part, we showed that a simple immuno-MALDI method could rapidly identify the abnormal LC glycosylation in patients with plasma cell dyscrasias.
II. In the second one, we showed that the glycosylated light chains are present years before the diagnosis of AL amyloidosis is made and should therefore be used clinically for identifying and monitoring patients with presumably asymptomatic plasma cell disorders who are at risk for AL amyloidosis.

In my thesis I will reviewed the results of those studies in order to show the primary role of MASS-FIX for the diagnosis and monitoring of AL amyloidosis patients.

Experimental design 1: The utility of MASS-FIX to detect and monitor monoclonal proteins in the clinic

INTRODUCTION

The approach of hematologists to a patient with monoclonal protein (M-protein) is mainly related by concerns regarding the size of the B-cell clone secreting the M-protein and the concentration of the M-protein itself. However, monoclonal proteins present at low concentration can have significant pathology – as in the case of AL amyloidosis and POEMS syndrome. Currently, monoclonal gammopathies are screened using a combination of the following three serum-based diagnostic tests in order to maximize screening sensitivity: protein electrophoresis (PEL), immunofixation electrophoresis (IFE) and measurement of free light-chain (FLC)[25,26]. As previously reported, it was previously explored by Palladini et al. that the identification of amyloidogenic light chains cannot be based on a single test. It requires the combination of all the three tests in order to obtain the highest diagnostic sensitivity for AL amyloidosis patients[11]. In a screening context in the general population, a panel of experts negated the need for 24-h urine studies for diagnoses other than AL amyloidosis[11,26]. Particularly, serum FLC has been widely used to aid the diagnosis and management of multiple myeloma patients and other plasma cell disorders[1,27,28].

The Mayo clinic researcher recently developed several mass spectrometry (MS) based methods to detect/measure M-proteins, in serum and urine, with a higher analytical sensitivity and specificity[29-33]. In particular, in the setting of AL amyloidosis the Mayo Clinic group in close collaboration with our center, explored the use of the monoclonal immunoglobulin rapid accurate mass measurement (miRAMM) for the identification and quantification of monoclonal light chains from patients with AL amyloidosis[33]. Reducing immunoglobulin disulfide bonds releasing the LC to be analyzed by micro laser capture electrospray ionization quantitative time of flight mass spectrometrer (microLC-ESI-Q-TOF) analysis performed this. In this method serum is enriched for immunoglobulins using different methods (for example with the use of Melon Gel resin), which binds to non-IgG molecules in mild pH conditions. The enriched mixture of immunglobulins are exposed to reserve-phase liquid chromatography and electrospray ionization quantitative TOF mass spectrometric measurement. The presence of an M-protein is defined above a Gaussian distribution of polyclonal light chains. With this method it was shown that the high resolution and high mass measurement accuracy provided by mass spectrometry analyses, eliminates the need for FLC-ratios evaluation in patients with AL amyloidosis[33].

The second proposed method combines nanobody enrichment of immunoglobulins with matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) in order to achieve a more rapid identification of M-proteins. This method utilizes the unique molecular mass signature of the different heavy chain and light chain isotype that composes the M-proteins in combination with nanobody-immunoenrichment to generate information-rich spectra from which M-proteins can be identified, isotyped and quantitated. In addition, the mass-to-charge distribution of the immunoglobulin light chain could be examined for the presence of on over-expressed clone, in a similar manner to PEL. The feasibility of using this MALDI-TOF-MS assay (also called MASS-FIX) to detect M-proteins was demonstrated for both serum and urine[34,35]. The limit of detection of this method is slightly improved comparable to IFE and the analysis time is inferior to the IFE method (less than 1 minute per patient)[35]. In addition, MASS-FIX was shown to be a cost effective M-protein screening method with the same sensitivity of IFE and to be capable of replacing PEL in a screening algorithm.

Aim of the project:

The purpose of this study was to compare serum and urine MASS-FIX to the five standard tests (PEL and IFE of both serum and urine and serum FLC ratios) for the identification of M-proteins in different clinical contexts. No previous studies took into account the diagnostic sensitivity of this method in accordance with the clinical diagnosis. Therefore, we intended to demonstrate that MASS-FIX assay could advance how plasma cell disorders are screened, diagnosed and monitored.

METHODS

Samples from 290 consecutive patients were prospectively collected at the Mayo Clinical laboratories. All those subjects were evaluated at the Mayo Clinic (Rochester, Minnesota, USA) and in all cases the physician had ordered serum and urine PEL, IFE and FLC to assess for monoclonal gammopathy. Only patients who had wasted paired serum and urine samples were accepted. All paired samples were collected between August 2015 and February 2016 and the clinical data were retrospectively collected from patients' reports. Baseline demographics and clinical and other laboratory data were recorded for all subjects. The diagnosis multiple myeloma or any other plasma cell dyscrasia was made in accordance to the published guidelines[7]. In addition, AL amyloidosis patients needs to have a tissue specimens stained positive with Congo red and exhibited green birefringence under polarized light; the typing of the amyloid deposits was with proteomics or immunoistochemical stains[10,36,37]. The Mayo Clinic Institutional Review Board approved this prospective study and it was conducted in accordance with the Declaration of Helsinki.

Laboratory analysis

As per clinical protocol, all the five tests (serum and urine PEL, IFE and serum FLC measurements) had to be performed within 48 hour of the sample collection. The serum PEL assay was performed on the SPIFE 3000 electrophoresis analyzer (Helena Laboratories). The presence of a quantifiable M-spike was defined as a fuzzy band, hypogammaglobulinemia (<5.5 g/L), increased β-fraction (≥16 g/L), or increased α-2 fraction (≥15 g/L). The Hydrasys instrument on Hydragel by Sebia was used for serum IFE in order to assess migration patterns for γ, α, μ, κ, and λ immunoglobulin chains. In any case of samples that contains monoclonal light chains only without a monoclonal heavy chains δ and ε antisera were also tested. The FLC assay used in this study was the Freelite™ (The Binding Site Ltd.). The instrument used for the analysis was a BNII nephelometer (Siemens). In addition to reporting the κ and λ FLC concentration, as previously published the assay reports the FLC κ/λ ratio (diagnostic range 0.26–1.65)[38], and an abnormal FLC result was defined as an abnormal FLC κ/λ ratio. In patients with renal failure, we considered the diagnostic range of 0.37-3.1, as previously reported[39]. Urine samples were concentrated to a maximum of 200X[34,35] to achieve final concentrations of urine protein between 20 and 80 g/L. Urine PEL was performed on agarose gel (REP) and urine IFE on SPIFE IFE 9/15 gels (Helena Laboratories).

MASS-FIX method

The MASS-FIX used with in accordance with the previously reported[34,35]. In particular, immunoglobulins were enriched from serum/urine using camelid-derived nanobodies directed against the heavy-chain (HC) constant domains of IgG, IgA, and IgM or the light-chain (LC) constant domains of κ and λ immunoglobulins (Thermo Fisher Scientific PN: 194288005, 084905, 083305, 289005 and 194320005). Each sample (serum or urine) underwent 5 unique nanobody enrichments. Specific nanobodies against the heavy chain constant domain (IgG, IgA and IgM) or light chain (LC) constant domain for both kappa and lambda were incubated with specific amount of serum (20 uL) and urine (1 mL). After incubation at room temperature for 30 minutes, the supernatant was removed and the beads were washed three times with PBS and twice with water. Finally, samples were eluted with 5% acetic acid containing 50 mmol/L Tris [2-carboxyethyl] phosphine (TCEPT), which has the role to separate LC and HC components[31]. Isolated immunoglobulins were subjected to MALDI-TOF-MS analysis. In particular, samples were spotted into a 96-well microScout polished steel Bruker target plate (Bruker Daltonics) using a sandwich matrix application[40]. The mass analysis was performed in positive ion mode with summation of 500 laser shots using a MALDI-TOF mass spectrometer (Bruker Microflex LT). The +1 and +2 charge states of the LCs and HCs were measured by configuring the mass spectrometer to analyze ions between an m/z of 9000-32000 Daltons. The polyclonal light chains are distributed in multiple charge state (+1 and +2). These appears as overlapping Gaussian distributions corresponding to lambda and kappa, and are identified in the mass spectra of proteins enriched by LC-specific nanobodies as well as in the mass spectra of proteins enriched by heavy chain specific-nanobodies. In contrast to the mass spectra in the healthy adult population, samples with M-proteins have non-Gaussian distributions of light and heavy chains masses with narrower widths and shifted apexes. Four independent reviewers visually inspected resulting mass spectra of each sample in order to detect and isotype any M-proteins present in the patient. All reviewers were blinded to prior laboratory testing results and clinical information at the time of the spectral analysis.

Patients who had abnormal LC mass spectra showing evidence for fragmented LC patterns or suspected for glycosylation were subjected to additional LC-ESI-MS analysis on a Q-TOF mass spectrometer. This analysis was performed using an SCIEX TripleTOF 5600 quadrupole time-of-flight mass spectrometer (Vaughan, ON, Canada) operating in electrospray ionization (ESI) positive mode with a Turbo V dual ion source with an automated calibrant delivery system[29]. Mass spectra converted from m/z accurate molecular mass from the LC-ESI-MS analysis were evaluated for signatures of known post translational modifications[33]. In particular, suspected glycosylated LCs were identified by their increased molecular mass and the presence of multiple peaks associated with

different glycoforms each separated by the molecular mass of one or more monomers of either pentose (132 Da), deoxyhexose (146 Da), hexose (162 Da), hexosamine (191 Da), N-acetylhexosamine (203 Da), or N-acetylneuraminic acid (291 Da).

Data analysis

Descriptive statistical analysis was used for patient demographic, clinical and laboratory data. Differences in diagnostic sensitivity were tested for statistical significance by Fishers' exact test, and confidence intervals were determined using exact binomial distribution. JMP 10.0.0 (SAS Institute) software package was used for all statistical analyses.

RESULTS

The clinical and demographic characteristics of the selected patient cohort are reported in Table 1. The study population was reduced to 257-paired samples (from 290) that had all the five conventional tests available. Samples from all the different plasma cell dyscrasias were collected. In particular, 23 (25%) of 90 multiple myeloma patients were non-previously treated. Of the 65 AL/AH amyloidosis patients, 61 had the diagnosis of systemic AL amyloidosis, 3 patients had localized AL amyloidosis and 1 patient had AH amyloidosis. Of the 61 systemic AL amyloidosis patients, 26 (42%) were newly diagnosed, 39 (64%) had lambda light chain in their amyloid deposits. Organ involvement was assessed according to the amyloidosis criteria[41] and 33 (54%) had heart involvement, 32 (52%) had renal involvement and 16 (26%) had more than 2 organs involved. Fifty-eight patients in cohort had a monoclonal gammopathy of undetermined significance (MGUS) diagnosis. Of the 31 patients that were classified as "other", 19 (61%) had renal failure (defined as a serum creatinine of $\geq$ 2 mg/dL) without a previous history of any plasma cell dyscrasia. None of these subjects were on chronic dialysis at the time of MASS-FIX evaluation. Five patients of the 'other' group were evaluated for a suspected but not-confirmed systemic AL amyloidosis, and 7 patients had non-AL amyloidosis. In particular, 5 patients were diagnosed with wild-type transthyretin amyloidosis (wtATTR), 1 patient mutated-ATTR and 1 patient had leucocyte chemotactic factor 2 amyloidosis (ALECT2). A total of 52 (20%) patients in the whole cohort had an estimated glomerular filtration rate (eGFR) <30 mL/min per 1.73 m^2. In order to avoid any possible interference in the analysis, all patients who received any type of monoclonal antibodies therapy were excluded.

Table 1. Patients' characteristics. Median (IQR range) / Number (%)

	Total (N=257)	MM (N=90)	AL/AH (N=65)	MGUS (N=58)	Other (N=31)	Other PCD (N=13)
Newly diagnosed	54 (21)	23 (25)	26 (40)	-	-	5 (38)
Age, years	65 (58, 73)	66 (60, 72)	62 (56, 68)	69 (61, 79)	63 (59, 75)	63 (49, 78)
Sex, male	161 (62)	53 (59)	44 (67)	37 (64)	23 (74)	4 (30)
Creatinine, mg/dL	1.1 (0.9, 2.1)	1.1 (0.9, 1.5)	1.1 (0.9, 1.7)	1.1 (0.8, 1.8)	2.3 (1.3, 3.9)	0.9 (0.75, 1.45)
eGFR <30 mL/min	50 (19)	14 (15)	8 (12)	10 (17)	14 (45)	1 (7)
Proteinuria, g/24h	0.23 (0.12, 1.12)	0.25 (0.13, 0.57)	0.23 (0.12, 3.41)	0.17 (0.10, 0.31)	0.33 (0.18, 2.17)	0.16 (0.08, 0.43)
Proteinuria ≥3 g/24h	28 (11)	3 (3)	16 (24)	4 (6)	4 (12)	1 (7)

N, number of patients per group; MM, multiple myeloma; AL/AH, systemic or localized AL or AH amyloidosis; MGUS, monoclonal gammopathy of undetermined significance; Other, other diseases group; Other PCD, "other plasma cell dyscrasia" group was composed by: 9 patients with Waldenström's macroglobulinemia, 2 patients with POEMS syndrome and 1 fibrillary glomerulopathy with a monoclonal IgGk and 1 patient with a hypocomplementemic glomerulonephritis with type 2 cryoglobulinemia; eGFR, estimated glomerular filtration rate.

Identification of M-protein in treatment naïve patients by MASS-FIX

We compared the diagnostic sensitivity of MASS-FIX assay in detecting a M-protein with that of PEL, IFE and FLC (Table 2 and Figure 1). We decided to perform the first part of the analysis in a subset of 112 patients with previously untreated disease in order to avoid possible interference of chemotherapy. In this subset, 109 patients' serum samples resulted to have an M-protein when the three serum tests (s-PEL, s-IFE and s-FLC—i.e. an abnormal FLC ratio) were considered; only 96 were positive for M-protein when only s-PEL or s-IFE were used. MASS-FIX of serum identified 95/96 M-proteins that were detected by serum-IFE. The only discordant sample came from an AL amyloidosis patient who had a small monoclonal LC-kappa by s-IFE, FLC-kappa concentration of 122 mg/L (κ/λ-ratio of 116); both u-IFE and u-MASS-FIX were positive for monoclonal LC-kappa.

Overall, other 8 discordant samples were detected in the whole cohort. In particular, serum MASS-FIX detected a M-protein in two patients that were not identified by the routine serum-screening panel, and the s-PEL/IFE/FLC panel identified a M-protein in 6 patients that serum MASS-FIX did not. The two patients that resulted MASS-FIX positive and serum PEL/IFE/FLC negative had a diagnosis of systemic AL amyloidosis and MASS-FIX detected the amyloidogenic LC (it was concordant with the bone marrow clonality and the amyloidogenic protein found by typing). Four patients with systemic AL amyloidosis and two with MGUS had s-IFE and FLC ratio positive and serum MASS-FIX negative. The combination of serum and urine MASS-FIX identified all patients except one. This patient had a slightly elevated FLC κ/λ ratio (1.85) with serum and urine IFE negative (serum creatinine 0.9 mg/dL and proteinuria 0.09 g/24h).

The use of u-PEL/IFE identified M-protein in 74 cases, and urine MASS-FIX alone, detected M-protein in 88 cases (Table 2). MASS-FIX identified 14 samples that resulted uPEL/IFE negative. Among those patients, all except one had M-protein detected with serum studies and it was concordant with MASS-FIX studies. A patient with a newly diagnosed AL amyloidosis was completely discordant: negative by both serum and urine IFE and FLC κ/λ ratio 1.23 with a bone marrow plasma cell infiltration of 5% (lambda restricted). Serum and urine MASS-FIX detected an IgA-lambda M-protein.

Table 2. Diagnostic sensitivity in paired samples of 257 patients (numbers in parenthesis delineate previously untreated patients).

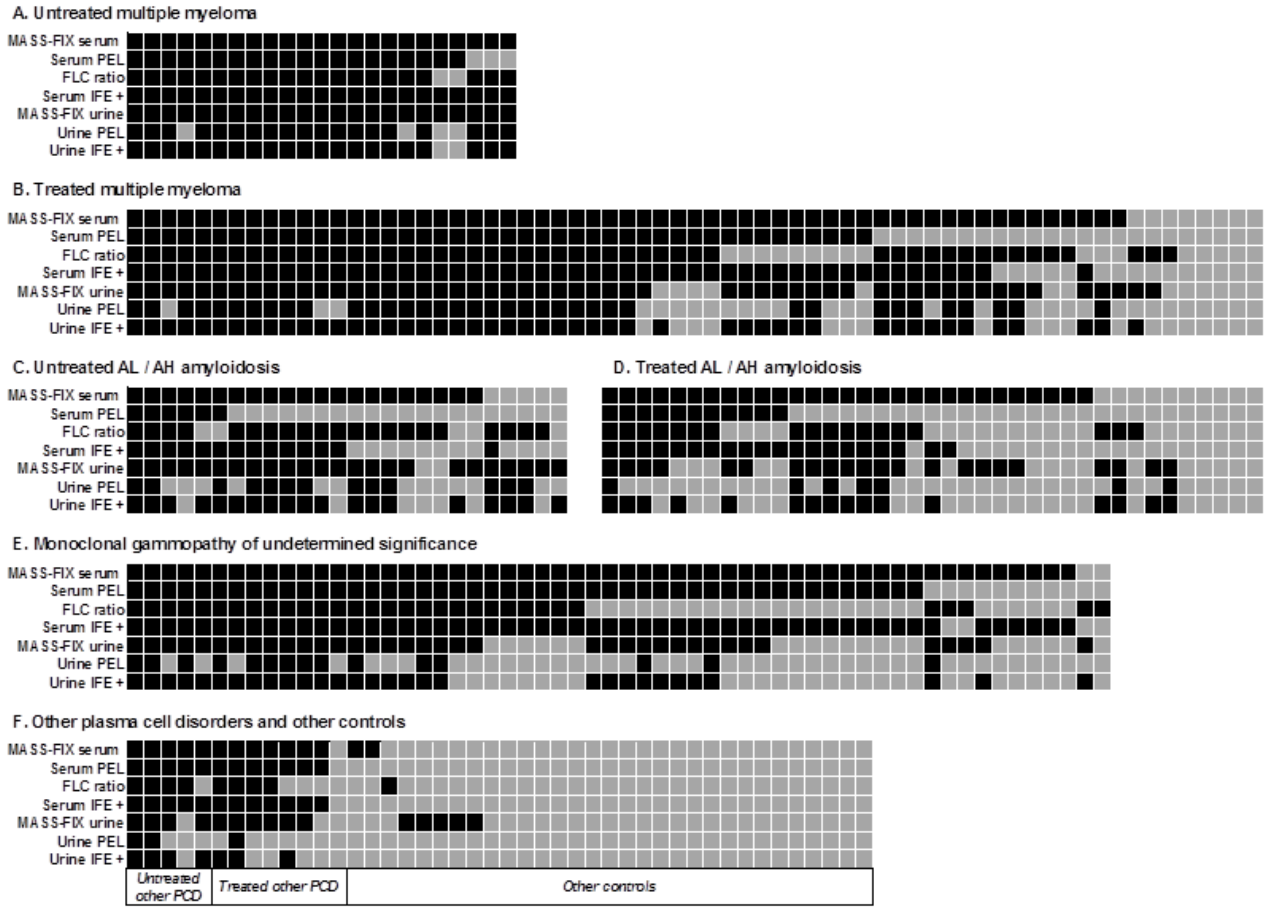
		Serum			Urine			
Diagnosis	**N. of samples analyzed**	**PEL + or IFE + or FLCr +**	**PEL + or IFE +**	**MASS-FIX +**	**PEL + or IFE +**	**MASS-FIX +**	**Any serum & urine tests & FLCr +**	**Any MASS-FIX +**
MM	90 (23)	82 (23)	75 (23)	82 (23)	69 (21)	77 (23)	83 (23)	84 (23)
AL/AH	65 (26)	46 (23)	34 (14)	50 (21)	35 (19)	46 (24)	50 (25)	59 (26)
MGUS	58 (58)	58 (58)	54 (54)	56 (56)	30 (30)	37 (37)	58 (58)	57 (57)
Other PCD	13 (5)	12 (5)	12 (5)	12 (5)	7 (4)	10 (4)	12 (5)	12 (5)
Other	31	1	-	2	-	5	1	7
Total	257 (112)	199 (109)	175 (96)	202 (105)	141 (74)	175 (88)	204 (111)	219 (111)

N, number of patients per group; MM, multiple myeloma; AL/AH, systemic or localized AL or AH amyloidosis; MGUS, monoclonal gammopathy of undetermined significance; Other PCD, other plasma cells dyscrasia group; Other, other diseases group.

PEL, protein electrophoresis; IFE, immunofixation electrophoresis; FLCr +, abnormal FLC κ/λ ratio; MASS-FIX, MALDI time of flight mass spectrometry,

Figure 1. Graphical representation of performance of s-MASS-FIX, s-PEL, FLC-R, s-IFE, u-MASS-FIX, u-PEL, and u-IFE

Each column represents a single patient. A, Untreated multiple myeloma patients. B, Treated multiple myeloma patients. C, Untreated AL/AH amyloidosis patients. D, Treated AL amyloidosis patients. E. MGUS patients. F, Other plasma cell disorders (PCD) and other controls. Modified from Milani et al. *Am J Hematol* 2017.



Identification of M-protein in all patients by MASS-FIX

The screening sensitivity of MASS-FIX was compared with that of PEL and IFE using the entire cohort (N=257), which included samples obtained from both treatment naïve and treated patients (Table 2; Figure 1). M-protein was detected by serum MASS-FIX in 174/175 patients that were found by either s-PEL or s-IFE; a newly diagnosed AL patient previously described was the only exception. In 11 cases the triplet of s-PEL/IFE/FLC found M-protein and MASS-FIX not: in only 1 of these cases s-IFE was positive (small monoclonal light chain kappa). In the other discordant samples an abnormal FLC-κ/λ ratio was the only diagnostic marker. Six patients had systemic AL amyloidosis (4 were the previously described newly diagnosed patients), 2 treated MM, and 3 had light chain-MGUS. Urine-MASS-FIX detected an M-protein in all but 3 cases: one had a previously treated MM (FLC-κ/λ ratio of 3.46), one had a light-chain MGUS (FLC-κ/λ ratio 1.85, described in the newly diagnose group), and one had severe renal failure without any known plasma cell dyscrasia (FLC-κ/λ ratio 3.55, creatinine 5.4 mg/L and eGFR 9 mL/min per 1.73 m^2). Urine MASS-FIX identified 139/141 M-proteins that u-IFE detected; two patients previously treated were the exception (one with MM and one with systemic AL amyloidosis). In both cases, M-protein was confirmed by serum MASS-FIX and was concordant with s-IFE studies.

Overall, MASS-FIX of serum and urine detected an M-protein in 18 patients who were reported to have negative conventional studies. This subgroup is composed of one previously treated MM, 7 "other" patients (3 non-AL amyloidosis, 3 not confirmed AL amyloidosis and 1 renal failure patient), and 9 previously treated systemic AL amyloidosis, 8 of whom were classified as having reached a complete hematologic response after treatment based on serum PEL/IFE/FLC and urine IFE. Notably, three of these systemic AL amyloidosis subjects with negative PEL/IFE/FLC findings had unexplained progressive organ dysfunction. In particular, one patient had a progression of cardiac dysfunction six months after a positive MASS-FIX evaluation and died due to unexplained heart failure. Another patient was evaluated for a progressive worsening of renal dysfunction despite hematologic complete remission. In this case, a bone marrow biopsy detected monotypic lambda plasma cells and serum MASS-FIX detected a monoclonal LC-lambda. The third patient was evaluated after the detection of new hepatic lesions and amyloid deposits were detected by a liver biopsy.

Isotyping by MASS-FIX

The performance of MASS-FIX in isotyping was compared with the IFE gold standard. One hundred and seventy-four serum samples were tested. A perfect agreement was found between the two methods in 157 (90%) samples. In 8 of the 17 discordant serum samples, MASS-FIX and IFE were discordant based on the presence or absence of a biclonal-gammopathy. In fact, an additional smaller M-protein was found by MASS-FIX in 7 cases and s-IFE only in one case. The isotype of the main M-protein was concordant in all samples. Of the remaining 9 discordant samples, there was agreement for the LC involved but not the HC. A similar analysis was performed with 139 urine samples. Both assays were 100% concordant with identified LC's isotype (Table 3). However, in six samples, u-MASS-FIX and u-IFE agreed on the major M-protein but there was disagreement on the presence of additional less-abundant clones.

Table 3. Isotyping monoclonal proteins by IFE and MASS-FIX.

	Concordant Serum IFE – Mass-FIX serum	**Discordant Serum IFE – MASS-FIX serum**
IgGκ / IgGλ	55 / 31	- / 3
IgAκ / IgAλ	14 / 5	1 / -
IgMκ / IgMλ	16 / 9	2 / -
IgDκ / IgDλ	- / 3	
FLCκ / FLCλ	8 / 11	4 / 6
Biclonal	5	1
Total	157	17
	Concordant Urine IFE – MASS-FIX urine	**Discordant Urine IFE – MASS-FIX urine**
FLCκ	83	5
FLCλ	50	1
Total	133	6

Additional analysis on the characteristics of MASS-FIX spectra

Both $[M+2H]^{2+}$ and $[M+1H]^{+}$ regions of each sample's LC spectra were recorded (Table 4). The difference between light-chain m/z of serum and m/z in the urine was tested in all the cases that had an M-protein in both samples. The median value of the absolute difference between (m/z-serum) – (m/z-urine) was 0.3 Daltons (IQR range -2, 3.22 Daltons), in the 157 samples evaluable for this analysis. The mass measurement accuracy of the MALDI-TOF MS instrument is 100 ppm. There is excellent agreement between the m/z of the serum and the urine. No significant difference was seen between the median of the absolute difference between (m/z-serum) – (m/z-urine) according to renal dysfunction: median 1.1 Da in patients with eGFR <30 mL/min per 1.73 m^2 vs. 0.2 Da in patients with eGFR ≥30 mL/min per 1.73 m^2, P=0.347.

A specific analysis of comparison of the different mass spectra was performed. In particular, we analyzed the mass/charge (m/z) distributions of LC spectra for the presence or absence of a mass shifted peak that was distinct from the expected polyclonal LC m/z region (Figure 2A). This was defined as an "atypical" mass spectra and a second round of MASS-FIX analysis was performed in all the samples. Fifty-one patients had atypical spectral patterns with mass shifted LC peaks, which correspond to posttranslational modifications (Figure 2). The clinical diagnosis was collected and a subset analysis was performed that showed a striking. The most frequent diagnosis in this "atypical" group was systemic AL amyloidosis. Thirty-two patients had systemic AL amyloidosis, 7 were affected by MM (1 patients was newly diagnosed), 7 were MGUS and 5 patients had other diseases (2 patients with a suspected but not confirmed diagnosis of AL amyloidosis, 2 patients with renal failure and a previously undetected MGUS, a patient with ALECT2 amyloidosis and a coincident MGUS). Interestingly, 49% of patients with AL amyloidosis had mass shifted patterns as compared to less than 15% of patients with the other conditions (Figure 3). Paired samples of these 51 patients were further analyzed by another high resolution microLC-ESI-Q-TOF MS method, and the fragmentation pattern was consistent with that of glycosylation in 32 of the 51 patients. This included one patient with LC-MGUS suspected, but not proven, to have AL, and another patient with ALECT2 coexisting with LC-MGUS.

Table 4. Light chain mass/charge (m/z) ranges of the detected M-proteins for both [M+2H]2+ and [M+H]+ charge states.

Charge state	**LC type**	**Median m/z (Da) serum** (IQR)	**Median m/z (Da) urine** (IQR)
$[M+2H]^{2+}$	**Kappa**	11789 (11690, 12021)	11793 (11695, 12019)
$[M+H]^{+}$	**Kappa**	23537 (23365, 23990)	23518 (23364, 23962)
$[M+2H]^{2+}$	**Lambda**	11402 (11338, 11556)	11395 (11329, 11584)
$[M+H]^{+}$	**Lambda**	22825 (22715, 23102)	22805 (22695, 23161)

LC, light chain; Da, Daltons; IQR, interquartile range.

Figure 2. Atypical MALDI patterns are seen more commonly in patients with systemic AL amyloidosis. Modified from Milani et al. *Am J Hematol* 2017.

A. Typical mass spectra of a MALDI pattern of a monoclonal protein of a patient affected by multiple myeloma (light chain lambda spectra serum in the upper part of the figure and light chain lambda urine in the lower part of the figure). The insets are focused on the *m/z* regions containing LC ions.
B. Atypical mass spectra of a MALDI pattern of a patient with AL amyloidosis (light chain lambda spectra serum in the upper part of the figure and light chain lambda urine in the lower part of the figure). The insets are focused on the *m/z* regions containing LC ions.
C. Typical mass spectra of a MALDI pattern of a monoclonal protein of a patient affected by multiple myeloma (light chain kappa spectra serum in the upper part of the figure and light chain lambda urine in the lower part of the figure). The insets are focused on the *m/z* regions containing LC ions.
D. Atypical mass spectra of a MALDI pattern of a patient with AL amyloidosis (light chain kappa spectra serum in the upper part of the figure and light chain kappa urine in the lower part of the figure). The insets are focused on the *m/z* regions containing LC ions.

Dotted lines (in all the figures) identified the m/z ranges of both lambda and kappa light chains according to Kohlhagen et al. *Clin Chem* 2016.

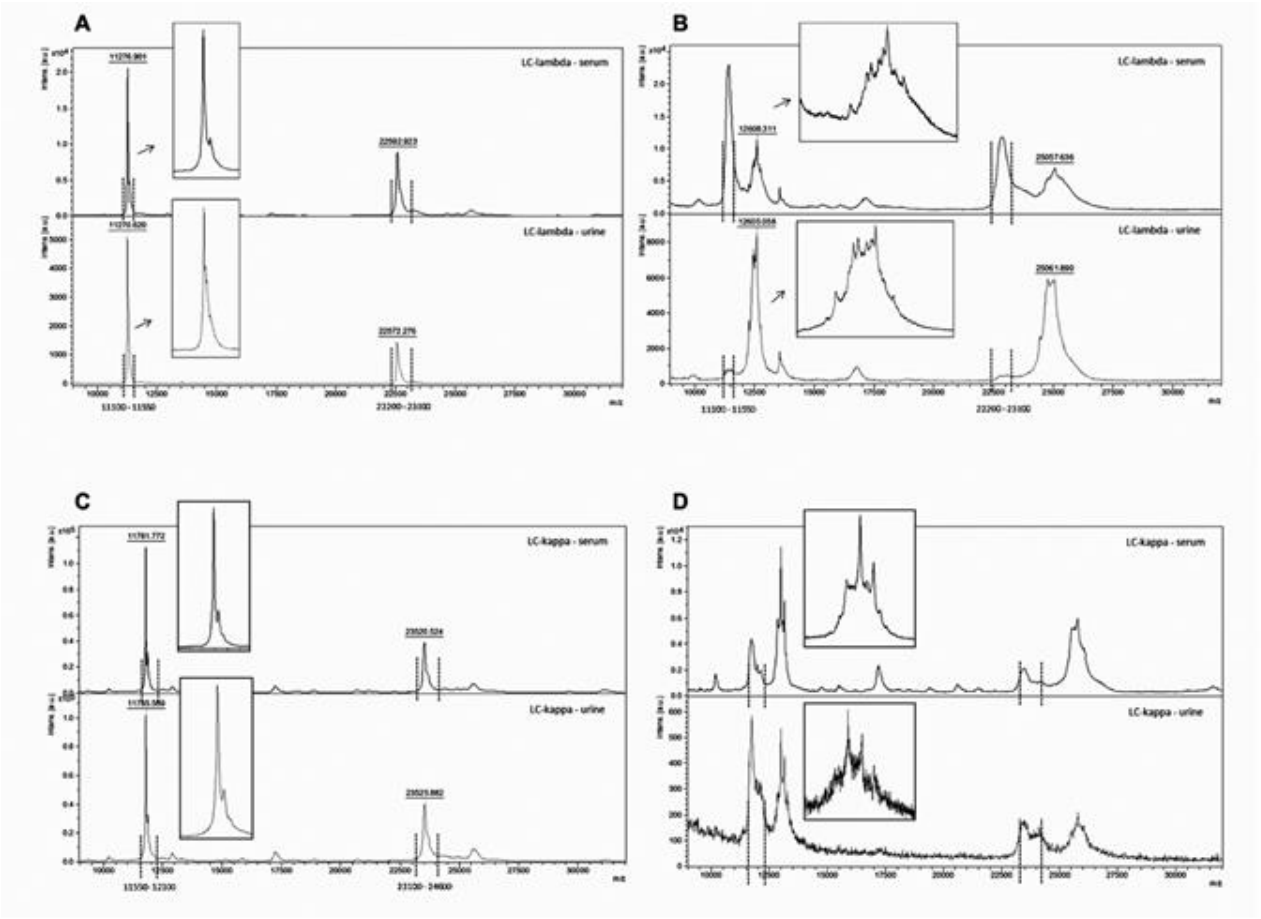
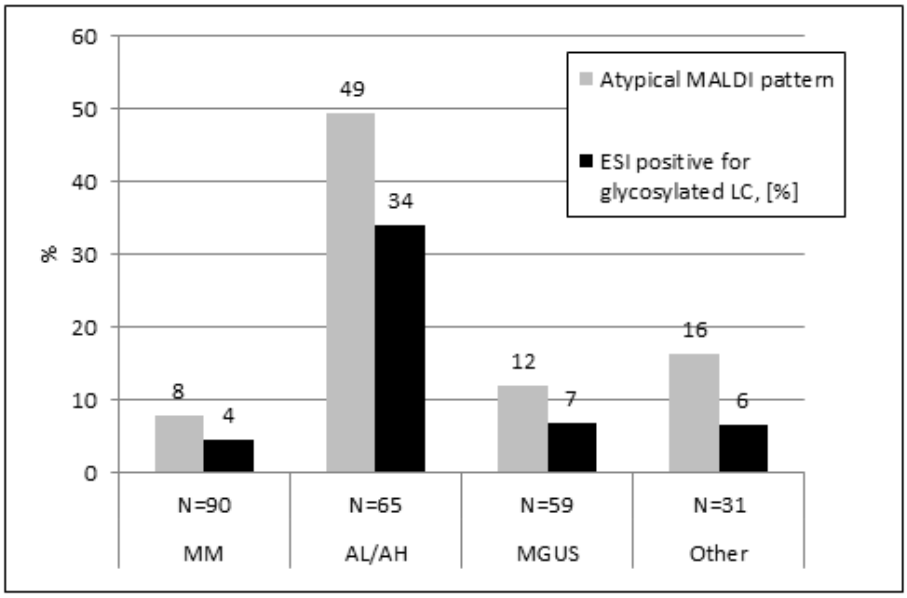


Figure 3. Frequency of atypical MALDI patterns and ESI SCIEX Triple TOF 5600 result by diagnosis. Modified from Milani et al. *Am J Hematol* 2017



DISCUSSION

As previously described by both the Mayo Clinic researcher and by our group, the combination of the triplet IFE, PEL and FLC with the urine studies, had the higher diagnostic sensitivity for monoclonal gammopathies[1,11]. Our study is the first report that evaluates the feasibility of a new screening panel with MASS-FIX in paired serum and urine samples. The use of MASS-FIX could be more informative than standard methodologies that provide M-protein detection, isotype specific kappa to lambda heavy light ratios (HLC), and quantitative information with equivocal or improved analytical characteristics[42]. In this report, MASS-FIX was able to identify the presence of a M-protein in different clinical settings and it showed a diagnostic sensitivity comparable to IFE and serum FLC. In particular, in newly diagnosed patients group, only one was not detected by the combination of serum and urine MASS-FIX. However, MASS-FIX outperformed the currently used screening strategies and was able to detect an M-protein in AL amyloidosis patient at diagnosis that had evidence of the clonal disease only in the bone marrow biopsy. The combination of serum and urine MASS-FIX resulted negative only in 2 out of the 27 newly diagnosed systemic AL amyloidosis patients. In our study, urine MASS-FIX analysis was performed with un-concentrated urine. This is important to note, because it provides time and cost savings over the conventional u-PEL/u-IFE that required a concentrated sample and also leaves ample room for improvement. As previously reported by our group[30], the diagnostic sensitivity of mass spectrometric analysis of LCs using neat urine was similar to IFE using concentrated urine sample.

Serum and urine PEL/IFE and serum FLC measurements are used for evaluating therapy response and guide treatment decisions. In our study, MASS-FIX was able to detect M-protein in an additional 9 patients who were classified as complete response (for systemic AL amyloidosis) or stringent complete response (for MM). Interestingly, 3/8 systemic AL patients who had an M-protein by MASS-FIX method also had a clear organ progression despite being in complete remission as defined by the conventional methods. This is of great clinical relevance, supporting continuation of chemotherapy in patients with a persistent M-protein by MASS-FIX, in order to achieve organ response.

Our study firstly reported data regarding the use of MASS-FIX in patients with reduced renal function. The m/z of the monoclonal light chain in the urine (i.e. the mass of the monoclonal protein) is not altered in presence of reduced glomerular filtration rate as compared to that of the serum, making this

assay a helpful tool for measuring light chains either in the serum or urine in patients with renal disease. It is well recognized the difficulties to know whether patients with renal failure/impairment are strictly in complete response only based on blood and urine studies. In a recent study, we reported that the diagnostic sensitivity of the FLC κ/λ ratio was lower for lambda amyloidgenic FLC in patients with renal failure (81% vs. 60%)[43]. Our data would suggest that renal failure would not be an obstacle for MASS-FIX analysis.

One of the major findings of the first part of my thesis that allow us to move forward with the next steps, was the identification of higher rates of atypical mass spectra among patients with AL amyloidosis by MASS-FIX analysis. In fact, approximately 50% of patients with systemic AL amyloidosis had atypical spectra according to MASS-FIX. Thanks to the subsequent analysis using a high-resolution mass spectrometer with a better mass measurement (LC-ESI-Q-mass spectrometer), we observed that one-third of AL amyloidosis patients had monoclonal light chains that were suspect to be glycosylated. The presence of glycosylation was previously reported in different studies on AL amyloidosis patients' samples[33,44]. These data are too preliminary to delineate sensitivity and specificity for MASS-FIX and/or ESI-TOF-based post-translational modification analysis for predicting AL. However, it is compelling to note that the non-AL patients who had ESI-TOF-defined post-translational modification (the atypical spectra) type patterns included 2 MGUS patients, who had an incomplete evaluation to exclude of AL (i.e. no biopsy of target organ—heart in one and nerve in the other case) and recently diagnosed MM patients who could be at risk for AL amyloidosis over time. Those findings led us moving forward with experimental design 2 and 3.

In conclusion, the first part of my project confirmed previous observations about the high diagnostic ability of MASS-FIX in detection and isotyping M-proteins in both serum and urine. Most importantly, we showed that the performance of the MASS-FIX assay is able to work among the different spectrum of plasma cell disorders and in patients with renal dysfunction.

Additional potential application of this high sensitivity approach could be the definition of a 'minimal residual disease' in patients defined to be in complete response. Of note in our series, among treated patients with AL amyloidosis, MASS-FIX detected minimal residual clonal light chains that standard method (both IFE and FLC) did not. Further studies are warranted to confirm these findings and to assess the role of MASS-FIX in detecting minimal residual disease in comparison with the bone marrow

assessment. As reported in a series of patients evaluated with Euroflow method of bone marrow at the Pavia Center, the presence of residual amyloidogenic non-detectable light chains, could be associated with the persistence of organ damage or un-explained progressions[45]. Another important issue that was not addressed in our study but needs further investigations is the role of MASS-FIX in the detection of therapeutic monoclonal antibodies. In our series we excluded those patients, but MASS-FIX could be able to detect the new drugs and easily exclude those for the analysis of response to therapy.

Experimental design 2: assay to rapidly screen for immunoglobulin light chain glycosylation: a potential path to earlier AL amyloidosis diagnosis for a subset of patients

INTRODUCTION

The amount of clonal light chain alone does not predict AL amyloid formation. Studies have been done looking at primary, secondary and tertiary light chain structures in an attempt to understand why amyloid is formed in some individuals[46]. In other disease states, protein posttranslational modifications (PTM) can also influence protein function[47,48]. Glycan moieties play an integral role in numerous cell-cell and cell-matrix interactions through modulation of adhesion and cell trafficking[49,50]. Altered glycosylation of proteins has been implicated in the pathogenesis of hematological malignancies, often with prognostic implications. However, to date, glycosylation of light chains has been relatively under investigated in AL amyloidosis, largely due to the lack of high throughput procedures to facilitate rapid analysis of glycosylation. Immunoenrichment followed by MALDI-TOF mass spectrometry analysis, a method termed MASS-FIX has been developed and verified as a robust and efficient method to detect and type monoclonal light chains in plasma cell disorders (PCDs)[51,52]. Our previous report (experimental part 1) using MASS-FIX on samples from patients with different plasma cell disorders (PCDs), demonstrated that monoclonal light chains mass distributions from AL amyloidosis patients display different and complex features for their light chains. They have an higher molecular mass than the monoclonal LC, and the PTM have a higher occurrence in AL amyloidosis patients compared to other plasma cells dyscrasias, such as multiple myeloma[52]. As shown in the Experimental 1, MASS-FIX showed that M-protein mass distribution from AL amyloidosis patients often had an additional "atypical" / or "polytypical-like" pattern in addition to the diagnostic monoclonal light chain. These observations were in agreement with our earlier findings using high resolution, high mass measurement accuracy LC-MS that showed that PTM's such as glycosylation and cysteinylation occurred in LC from patients with AL amyloidosis[33].

Aim of the Experimental design 2 was to evaluate the role of MASS-FIX in identifying glycosylated monoclonal LC in patients with amyloidosis. In order to demonstrate our hypothesis we used a deglycosidase PNGase F. Using this approach we analyzed LC from 311 patients with AL amyloidosis

or other plasma cell dyscrasias, providing the most comprehensive mapping of LC glycosylation reported to date[53].

METHODS

Collection of samples:

All samples were obtained with approval of the Mayo Clinic Institutional Review Board. Samples were from two cohorts. Three-hundred and eleven serum or plasma samples were analyzed. The first cohort included 157 patients with AL amyloidosis who had their amyloid protein sequenced by liquid chromatography/tandem mass spectrometry[54]. The second cohort included 154 patients from Experimental design 1, who had either AL amyloidosis (n=32) or another plasma cell dyscrasia: multiple myeloma (n=54), Waldenström's macroglobulinemia (n=8), monoclonal gammopaty of undetermined significance, MGUS (n=57), and other plasma cell dyscrasia[52].

Immunoenrichment method and MALDI-TOF-MS analysis:

Each serum sample was tested five times with immunoenrichment specific for heavy and light chain constant domain following previously described protocol[51,52]. The previously reported approach was used with a sandwich matrix application method[51,52] to spot elutes onto a micro Scout 96-well polished steel target plate (Bruker Daltonics). The spectra for each immune-enrichment (IgG, IgA and IgM and κ and λ) were overlaid and analyzed visually in both $[M+1H]^{1+}$ and $[M+2H]^{2+}$ light chain range using an in-house developed standalone viewer and flexAnalysis software. The mass spectrum acquired for each patient was placed into one of the 2 categories (presence or absence of suspect for PTM) by the reader based on the complex patterns observed.

Deglycosylation protocol:

Deglycosylation was accomplished using peptide N glycosidase F (PNGase F, New England Biolab, Inc.). Briefly, 10 µL of serum immunoenriched by adding to 20 µL agarose beads for either κ or λ specific beads depending on AL isotype and allowed to incubate for 45 minutes at room temperature with mixing. The beads were washed three times with PBS following three times washing with water, and denatured and reduced with 100 µl of 2% sodium dodecyl sulphate (SDS) and 10 mM TCEP in PBS at 56ºC for 30 min with shaking. SDS was removed using a DRS spin column (Thermo Fischer Scientific).

The reduced κ and λ light chains were treated with 1 µl of PNGase F and incubated at 37ºC for 3 hours with shaking. The reactions were stopped by acidifying with 0.1% TFA. The reaction mixture was spotted on the target plate and analyzed by MALDI-TOF-MS following the same procedure described above. In addition, selected samples were analyzed by LC-MS as described below.

LC-MS analysis:

Suspected glycosylated samples were further analyzed by high resolution, high mass measurement accuracy LC-MS using a Thermo Scientific Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany) coupled to a Thermo Ultimate 3000 HPLC system using a ProSwift RP-4H capillary monolithic column. The full scan spectra were acquired in positive mode with the HESI source, scanning 500-3000m/z in the Orbitrap with a resolution setting of 120,000 at 400 m/z. The spectra were deconvoluted into monoisotopic masses using the Extract function in the Thermo Xcalibur Qual Browser software. The difference in mass of κ and λ light chains before and after PNGase F treatment was determined and matched with the calculated masses of theoretical IgG N-glycans. All those experiments were repeated three times.

RESULTS

Distribution of spectral patterns:

One hundred and eighty-nine patients with AL amyloidosis had monoclonal proteins by MASS-FIX. The mass spectra were visually analyzed using an in-house developed standalone software by three independent reviewers and were classified into 2 major categories; suspected glycosylation (n=38), and no suspected glycosylation (n=273). Figure 4 shows the two different categories (upper panel: no glycosylation; lower panel: glycosylation) observed for two λ light chain patients (Figure 4a, 4b) and two κ light chain patients (Figure 4c, 4d). Table 5 summarizes the distribution of suspected glycosylation in patients with AL versus non-AL according to κ and λ light chain isotypes. The suspected glycosylation category consisted of a broad peak that was about 1.0- 2.0 kDa higher than expected for a light chain. Thirty-three percent of the AL (kappa) amyloidosis patients had the suspected glycosylation pattern as compared to only 10.2% of the AL (lambda) patients. Another 122 patients with other types of plasma cell disorders and a positive MASS-FIX were also studied. Overall, the rate of glycosylation among the non-AL patients was only 4.1%; among the kappa non-AL patients, the rate was only 3.7% and among the lambda non-AL patients, the rate was 4.9%.

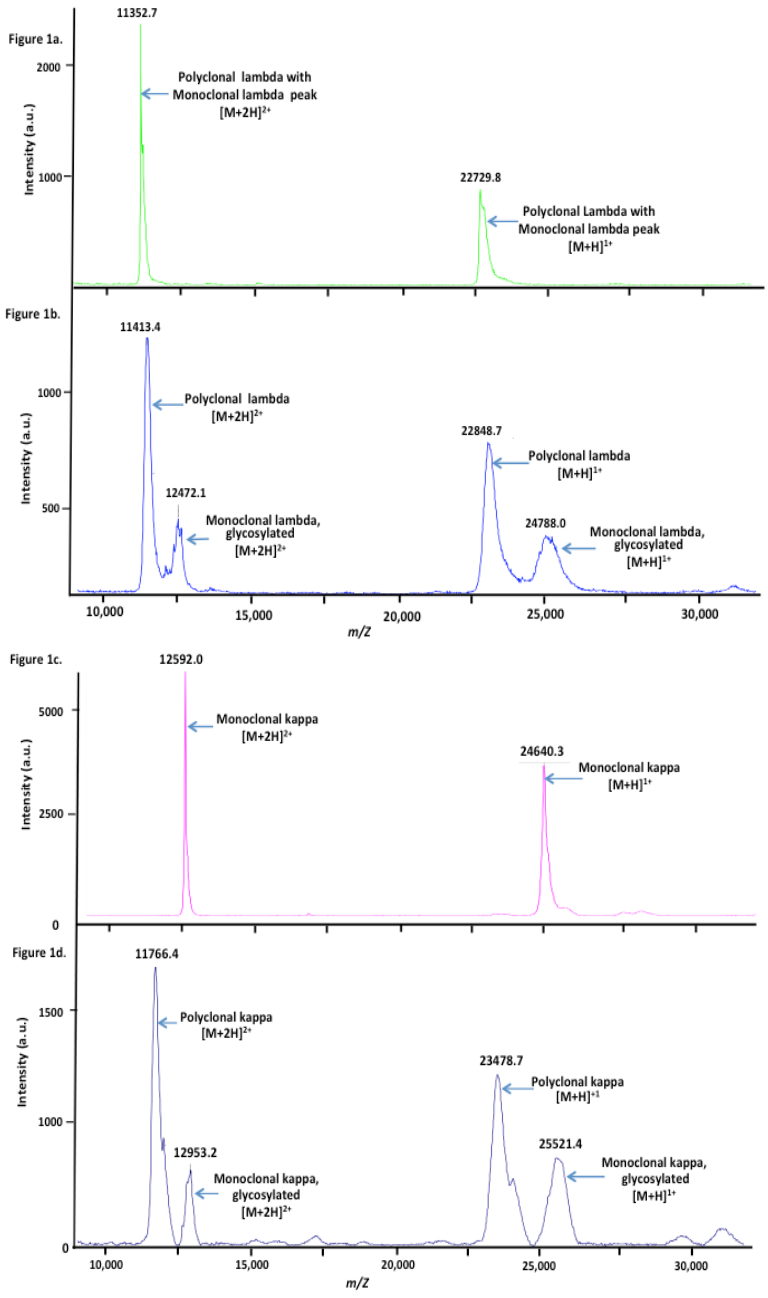
Table 5. Distribution of suspected glycosylation in patients with AL versus non-AL amyloidosis

Type of Samples	**Suspected Glycosylation by MASS-FIX**		
	κ	λ	All
AL amyloidosis, n/N (%)	20/61 (32.8)	13/128 (10.2)	33/189 (17.5)
Non-AL amyloidosis, n/N (%)	3*/81 (3.7)	2**/41 (4.9)	5/122 (4.1)
P-value	0.001	NS	<0.001
Odds ratio	12.68	2.20	4.95

*Diagnoses of these three patients included plasma cell leukemia (n=1) and multiple myeloma (n=2)
** Diagnoses of these two patients were one each of MGUS and multiple myeloma.

Figure 4. MALDI-TOF-MS pattern of non-glycosylated and glycosylated monoclonal light chains. MALDI data was analyzed in microflex analysis software. Modified from Kumar et al. *Leukemia* 2018.

(a) Simple pattern of monoclonal λ peaks in two charge state $[M+2H]^{+2}$ and $[M+H]^{+1}$.
(b) Two peaks in both $[M+2H]^{2+}$ and $[M+H]^{1+}$ charge states representing lighter non-glycosylated form and heavy glycosylated form of monoclonal λ light chain.
(c) Simple pattern of monoclonal κ peaks in two charge state $[M+2H]^{+2}$ and $[M+H]^{+1}$.
(d) Two peaks in both $[M+2H]^{+2}$ and $[M+H]^{+1}$ charge states representing lighter non-glycosylated form and heavy glycosylated form of a monoclonal κ light chain.



Glycosylation analysis in κ and λ light chain by mass spectrometry

A subset of 30 samples (21 κ [18 AL and 3 non-AL] and 9 λ [7 AL and 2 non-AL]) with suspected glycosylation were enriched for κ and λ light chains agarose beads, were immunopurified and treated with the N-deglycosylating enzyme PNGase F. When a shift in spectral position to a peak with narrower lower molecular mass in the 11 to 11.8 m/z mass range in $[M+2H]^{2+}$ and 22 to 23.6 m/z in $[M+1H]^{1+}$ on MALDI-TOF-MS was observed, the light chain was considered to be N-glycosylated (Figure 5a and 5b). All 30 had this mass-shift indicating that there was N-glycosylation.

These same samples were subsequently analyzed using high resolution LC-MS on an Orbitrap Elite mass spectrometer for confirmation and to more accurately characterize the glycan based on the mass shift. All samples suspected of having N-glycosylation following MASS-FIX analysis had monoclonal light chain mass that shifted towards lower molecular weight after PNGase F (Figure 6). This served as a confirmation of the presence of N-glycosylation of these κ and λ light chains. The difference in molecular weights of the κ and λ peaks for light chains before and after PNGase F treatment was matched with molecular weights of known immunoglobulin N-glycans. All 30 had this mass-shift and free glycoforms indicating N-glycosylation. The distribution of glycan groups detected by high-resolution mass spectrometry is shown in Figure 7. The glycan form bisected bisialated biantenary with core fucose group (G2FNSA2) and bisialated biantenary with fucose group (G2FSA2) were observed in most cases, with others having fragments of these forms.

Because 158 patients had their light chain gene sequence resolved by tissue mass spectrometry[54], associations between immunoglobulin light chain usage and the presence of glycosylation were sought. As seen in Figure 8a, 41% of AL patients whose amyloid protein was of the KV1 gene family had glycosylated circulating light chain. The κ light chains derived from KV1-33 and KV1-39 were most represented (Figure 8b). The λ gene family most represented was LV3, with LV3-21 with the highest likelihood (1/9 or 11%) to be glycosylated.

Figure 5. Deglycosylation of monoclonal light chains with PNGase F. Modified from Kumar et al. *Leukemia* 2018.

(a) Monoclonal κ light chain in native form (upper panel), showing shift with reduced molecular weight towards left after PNGase F treatment (lower panel).
(b). Monoclonal λ light chain in native form (upper panel), the glycosylated peak shifted with reduced molecular weight to the left when it was treated with PNGase F (lower panel).

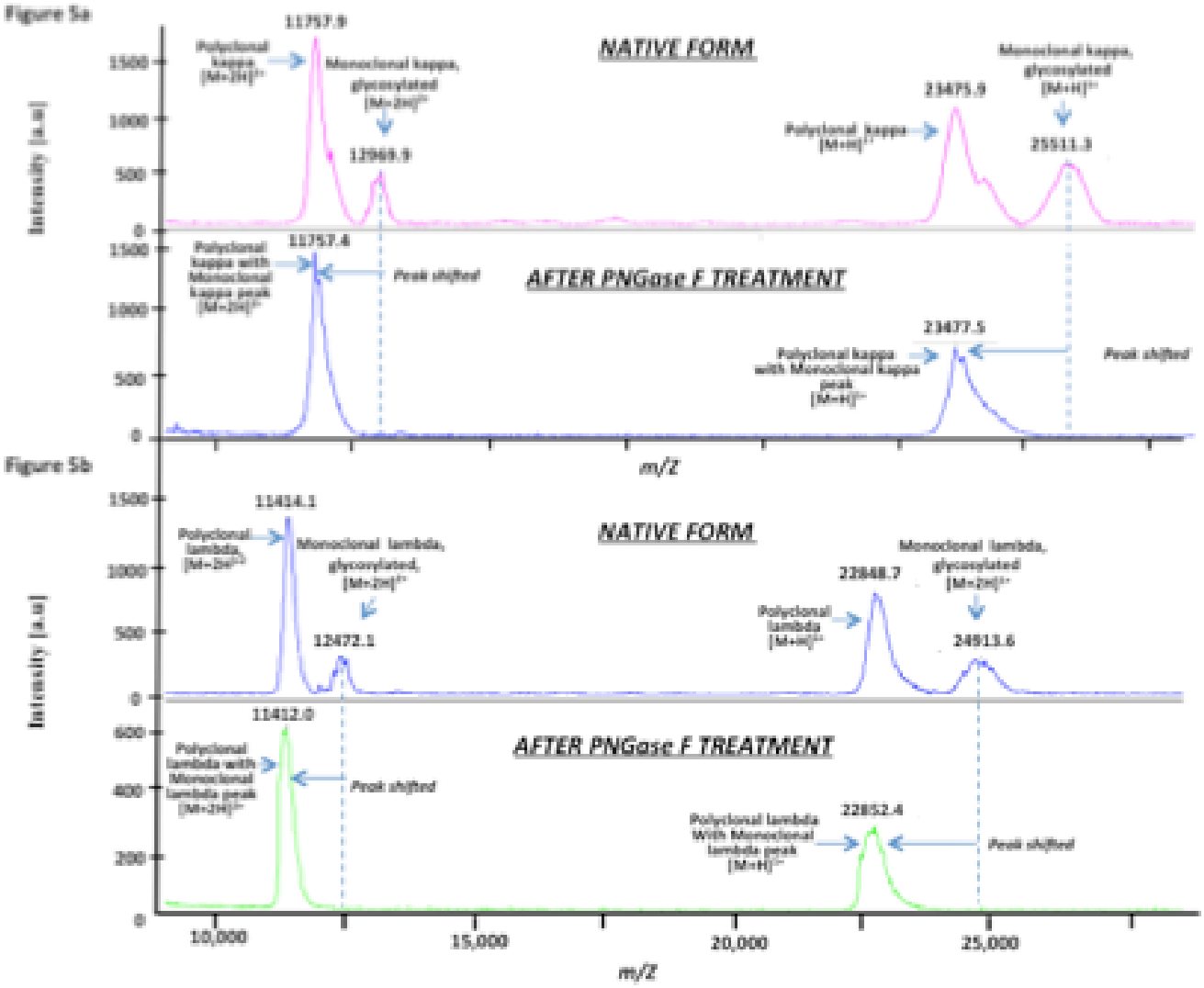


Figure 6. Monoclonal κ light chain glycosylation analysis by LC-ESI on Orbitrap elite. Modified from Kumar et al. *Leukemia* 2018.

Upper panel: monoclonal κ light chain protein showing four N- glycans peaks. Lower panel: After PNGase F treatment, monoclonal κ light chain protein is resolved to a single peak of 23441 Da.

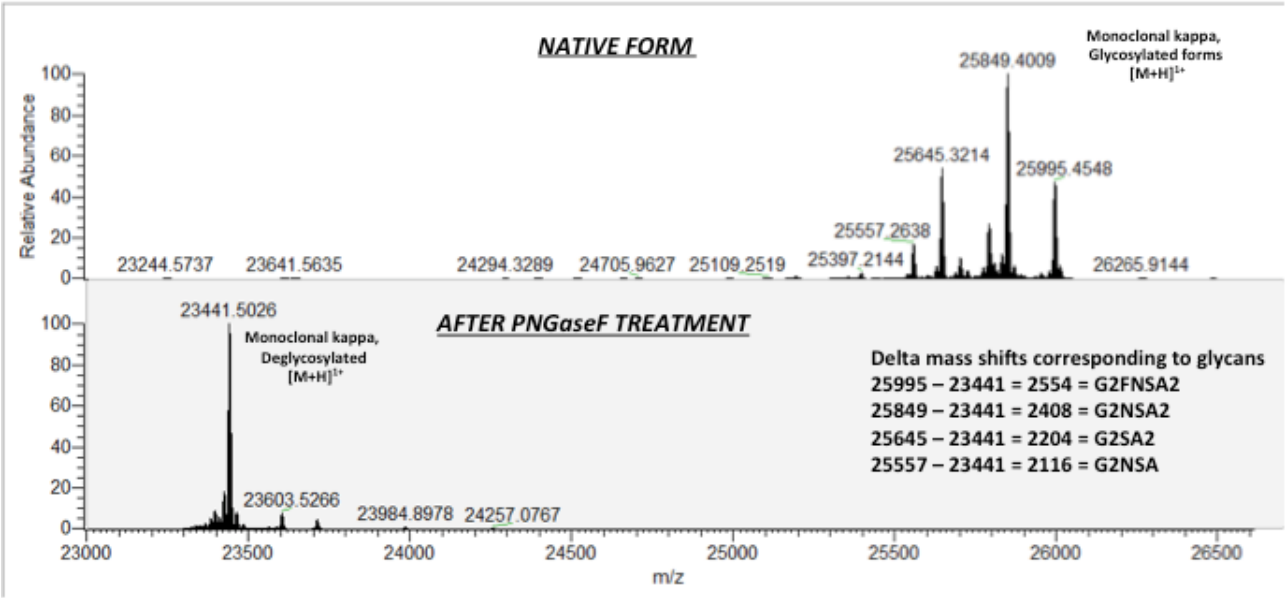


Figure 7. Glycoforms identified by LC-ESI on Orbitrap Elite. Modified from Kumar et al. *Leukemia* 2018.

Samples from 13 κ AL patients and 6 λ AL patients were analyzed, and the frequency of glycan groups observed is plotted. The median number of types of glycan groups was 3.

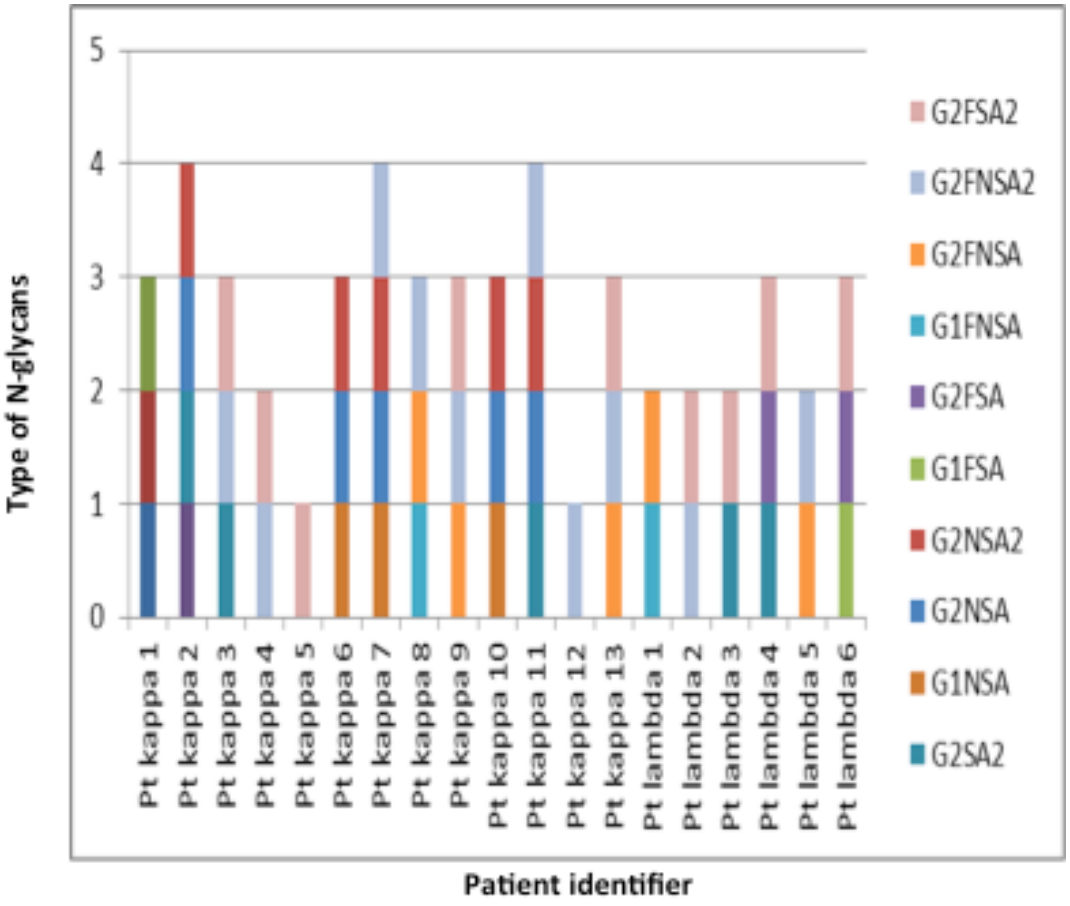


Figure 8. Immunoglobulin light chain gene usage based on presence or absence of light chain glycosylation. Modified from Kumar et al. *Leukemia* 2018.

Gene family and gene usage was determined by bottom up sequencing of amyloid from tissue biopsy.

(a) By gene family: KV1 and KV4 gene families had highest rates of glycosylation.
(b) A closer look at the KV1 gene family: 25% of KV1-33 and 41% of KV1-39 patients' monoclonal serum immunoglobulin light chains were glycosylated.

Figure 8a

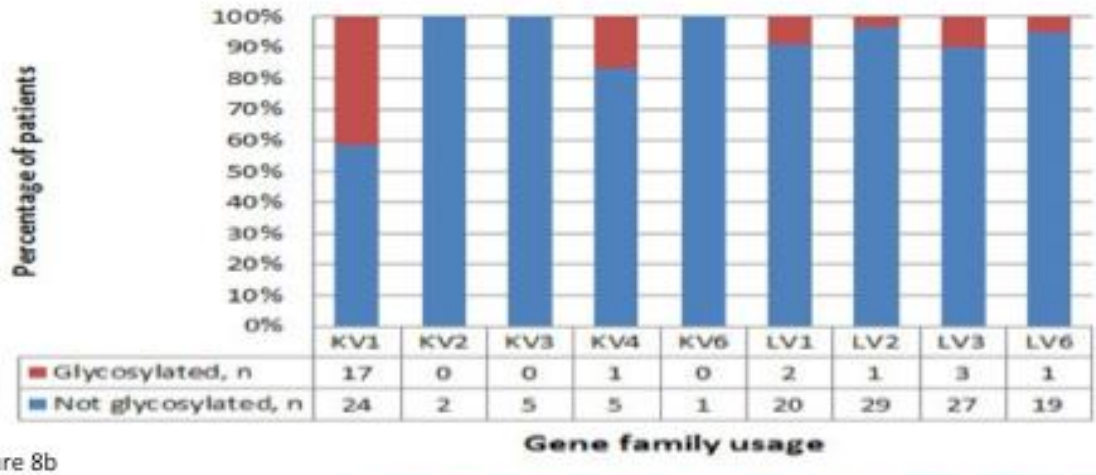
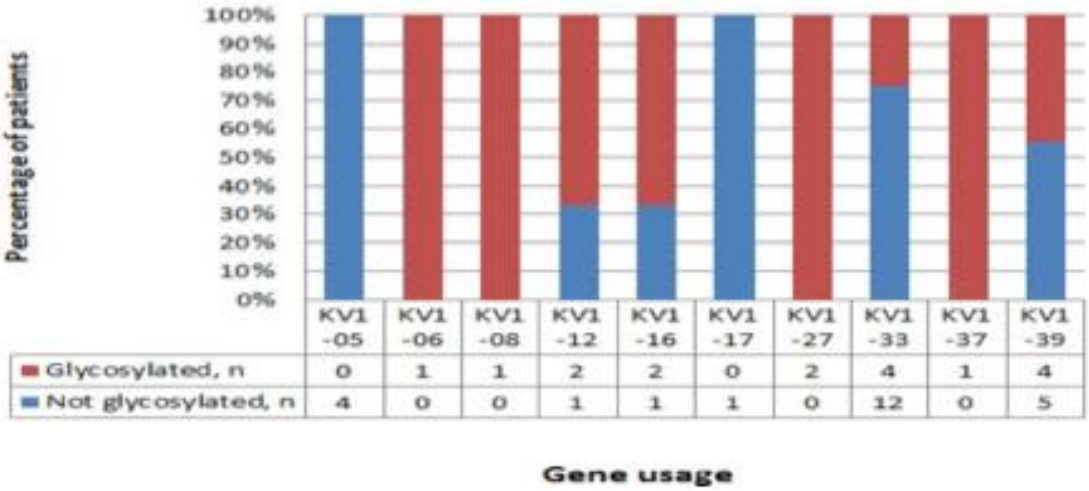


Figure 8b



DISCUSSION

Previous studies on a limited number of patients investigated light chain N-glycosylation in AL amyloidosis[55,56]. Connors et al. reported the presence of N-glycosylation in κ light chains from urine in two AL patients using chromatography and MALDI-TOF mass spectrometry[44]. The use of chromatographic purification of light chains is time consuming, prohibiting high-through-put analyses. In contrast, our study applied a novel immunoenrichment method for easy, rapid light chain characterization with fast analysis using MASS-FIX. The presence of glycosylation patterns (or suspected patterns) seen by MASS-FIX are readily identifiable and have been positively confirmed by PNGase F and high-resolution mass spectrometry LC-MS. Therefore, patterns produced by MASS-FIX can be used to identify N-glycosylation of light chains without needing confirmation by PNGase F treatment and LC-MS. The rapid and easy nature of the MASS-FIX assay allowed for screening a large cohort of patients with AL amyloidosis and estimation of the frequency of N-glycosylation. It was striking to find that a third of the AL κ patients exhibited the N-glycosylation pattern, which was 10-fold more common in than what was seen in non-AL κ patients.

In 2000, Stevens suggested that κ AL light chain proteins had a predisposition of being glycosylated[57]. Eighteen of the 22 potentially glycosylated KV1 light chains reported in the literature to that point were from AL amyloidosis patients despite the fact that no KV1 germline gene encodes for an N-linked glycosylation site (N-x-S/T). This tendency was not apparent with λ light chains of which only 9 of 22 proteins exhibiting potential glycosylation sites were from patients with AL amyloidosis. In our series, the frequency of N-glycosylation among κ light chains from AL amyloidosis patients was approximately three times higher than for λ light chains from AL amyloidosis patients. It may be that somatic hyper mutation affinity maturation in κ light chains results in the presence of higher number of glycosylation sites (N-x-S/T) than λ light chains, making the κ light chains more prone to gain of N-glycosylation.

The question arises as to what role N-glycosylation of light chains may play in AL amyloid formation. It is appreciated that glycans play an important roles in protein structure, function and regulation[58]. As early as 1970, it was recognized that 4% (3/71) of Bence Jones proteins from myeloma patients' urine and 11% (2/18) of light chains from serum myeloma proteins had carbohydrate moieties attached[59]. Sialic acid, N-acetyl-glucsosamine, and N-acetyl-galactosamine, as well as "neutral sugars" were identified. Approximately 15% of immunoglobulin light chains in circulation have been shown to

have oligosaccharides[59] and 25% of serum IgG proteins have been demonstrated to contain carbohydrate in their Fab region[60-62]. In the present study, the most common N-glycans were found to be bisialated biantenary sugars and bisected bisialated biantenary, which supports the previous study that light chains are normally highly sialylated[63,64]. Despite the disparity of frequency of N-glycosylation for κ and λ light chains, the types of N-glycans did not appear different.

Authors have speculated about the role glycosylation of immunoglobulin light chains plays in AL amyloidosis including: (a) increased binding to collagen[65]; (b) reduced catabolism of light chains[65,66]; (c) increased fibril formation[57]. Although there are no data demonstrating how glycosylation of amyloidogenic LC may affect their uptake by cells[67], glycoengineering of the Fc region of therapeutic monoclonal antibodies is a way of optimizing effector-mediated antibody dependent cell mediated cytotoxicity, complement-dependent cytotoxicity, and anti-inflammatory effects[68]. Further studies are warranted to explore the role of glycosylation in pathogenesis of amyloidogenesis.

In conclusion, our findings confirmed N-glycosylation in both κ and λ light chains in serum of large cohort of patients with AL amyloidosis. The major finding of this project is the application of a rapid, cheap and high-throughput method (i.e. MASS-FIX) for the identification N-glycosylation. We have proven that a distinct pattern of a broad multi-peak LC mass outside the normal LC mass distribution seen on MASS-FIX is indeed N-glycosylation. If our observation that N-glycosylation of κ light chains is more common in patients with AL than other PCDs, this assay, which is currently being validated in the clinical laboratory, could provide clinicians with information that could prompt them to have a higher suspicion for AL amyloidosis, potentially leading to earlier diagnosis of AL amyloidosis.

Experimental design 3. MASS-FIX may allow identification of patients at risk for AL amyloidosis before the onset of symptoms.

INTRODUCTION

As previously shown, monoclonal light chains (LC) in AL amyloidosis, especially kappa restricted ones, were initially shown to display unusual patterns that included proteins with higher molecular mass and the presence of additional, high mass peaks. In Experimental design 2, showed deglycosylation experiments and we confirmed this finding with the analysis with high resolution, high mass measurement accuracy LC-MS[33]. In particular, we demonstrated that these patterns are consistent with N-glycosylated forms of the monoclonal LCs and that these were 5 times more common in patients with AL amyloidosis compared to other plasma cells dyscrasias, and up to 13 times more common in kappa restricted patients. It is known that AL amyloidosis is always preceded by an asymptomatic precursor such as monoclonal gammopathy of undetermined significance (MGUS)[69]. This condition could exist years prior to the diagnosis of AL amyloidosis and is always clinically asymptomatic. It is not known if these glycosylated forms are present during the MGUS phase or if they arise closer to AL amyloidosis diagnosis.

Aim of the Experimental design 3 was to show if these glycosylated light chains are present years before the diagnosis of AL amyloidosis is made and should therefore be used clinically for identifying and monitoring patients with presumably asymptomatic plasma cell disorders who are at risk for AL[70].

METHODS

Inclusion criteria for this study are the following: (a) a diagnosis of MGUS or smoldering multiple myeloma (SMM) predating the diagnosis of AL amyloidosis; and (b) a stored serum or plasma sample available at the time of each of the specific diagnoses. The Mayo Clinic Institutional Review Board approved the study. The diagnosis of AL amyloidosis of the patients enrolled was done between April 1982 and June 2013. It was requested the presence of tissue specimens that stained positive with Congo red with apple-green birefringence under polarized light. Amyloid typing was performed by immunohistochemistry or mass spectrometry in accordance with the time of clinical diagnosis. In the cases where amyloid typing was not available, the clinical history of patients was carefully reviewed retaining only those cases with a clinical presentation that allows the confirmation of AL amyloidosis diagnosis. Two cases diagnosed with a non-biopsy confirmation of AL amyloidosis type, a 89 and 90 years old subjects, with indolent cardiac involvement were excluded due to the suspect for other forms of cardiac amyloidosis. A bone marrow biopsy at diagnosis was not always available. Therefore, an clear distinction between smoldering multiple myeloma and MGUS could not always be made and these patients were considered together.

Immuno-enrichment was performed as previously described[51]. The mass spectrum acquired for each patient was placed into one of 2 categories (glycosylated or not) by 4 independent reviewers based on the patterns observed (Figure 9). Statistical analyses was carried out using JMP® 12 (SAS Institute Inc., Cary, NC) statistical software. The Fischer exact test was used to compare categorical variables and the Wilcoxon Rank Sum/Kruskal Wallis for continuous variables.

Figure 9. Mass spectrometry patterns of non-glycosylated and glycosylated monoclonal light chains.
Modified from Kourelis et al. *Am J Hematol* 2018

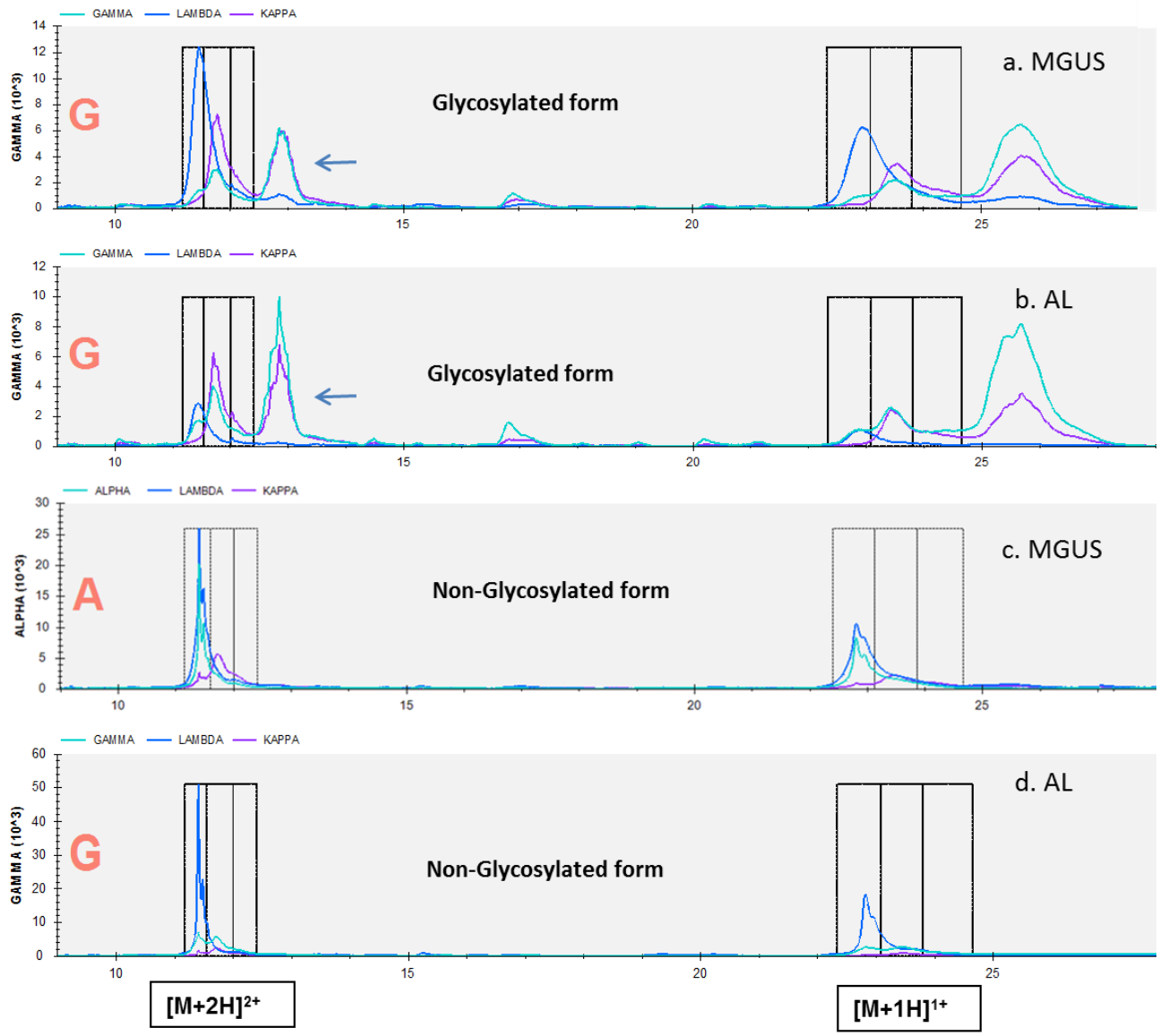


Figure legend. Mass Fix of two patients with a glycosylated IgG kappa (a, b) and a non- glycosylated IgA lambda (c, d) in the MGUS state (a, c) and at the time of diagnosis of light chain amyloidosis (b, d). The left-sided boundary represents the expected mass/charge (m/z) range for lambda light chains, the middle for kappa light chains and the right one the expected m/z range for glycosylated or heavy kappa light chains. Two peaks in the +1 and +2 charge states representing the heavy glycosylated form are noted for the first patient both in the MGUS state (a) (m/z of 25,691 and 12,832, respectively) and in the AL state (b) (m/z of 25,682 and 12,877, respectively) (b). Simple pattern of IgA lambda peaks in the +1 and +2 charge states representing the non-glycosylated forms are noted for the second patient both in the MGUS state (c) (m/z of 22,817 and 11,408, respectively) and at the time of AL amyloidosis diagnosis (d) (m/z of 22,821 and 11,408, respectively).

RESULTS

Table 6 shows the baseline characteristics of the seventy patients that were this part of the study. Atypical MASS-FIX patterns, consistent with glycosylation, were detected in the serum from the time of the amyloidosis diagnosis in 11 patients (16%) of which 6 were kappa (29% of kappa cases) and 5 lambda (10% of lambda cases). The glycosylated form was noted in the smoldering multiple myeloma/MGUS sample in al cases. Patients without glycosylated LCs were more likely to be male (Table 6, P=0.03). The different organs involved were studied. No difference in the incidence of the major organ involved (cardiac or renal) was noted between the glycosylated or non-glycosylated groups. No other differences were noted in the baseline characteristics (Table 6) between the 2 groups. If the presence of absence of amyloid typing was considered, no differences were noted in the two analyzed groups.

Table 6. Baseline characteristics of patients enrolled in Experimental design 3.

Variables (N=70)	**All (N=70)**	**No glycosylation (N=59)**	**Glycosylation (N=11)**
	N(%) or Median (range)		
Sex, male	40 (57%)	37 (62%)	3 (36%)
Age at AL diagnosis	69 (49-90)	67 (49-90)	71 (49-84)
Timeframe from MGUS/SMM/SWM sample to AL sample, months	90 (18-277)	90 (18-277)	71 (22-207)
Amyloid typing performed (MS/IHC)	44 (63%)	38 (64%)	6 (55%)
Lambda restricted	48 (70%)	43 (74%)	6 (55%)
Heavy chain isotype			
IgG	46 (66%)	36 (61%)	10 (90%)
IgA	11 (15%)	10 (17%)	1 (11%)
IgM	9 (11%)	9 (15%)	0
None	2 (3%)	2 (3%)	0
Biclonal	2 (3%)	2 (3%)	0
Cardiac involvement	51 (77%)	43 (78%)	8 (72%)
Renal involvement	38 (59%)	32 (59%)	5 (60%)

AL: immunoglobulin light chain amyloidosis, IHC: immunohistochemistry, MGUS: Monoclonal gammopathy of undetermined significance, SMM: smoldering multiple myeloma, SWM: smoldering Waldenström's macroglobulinemia, MS: tissue mass spectrometry**.**

DISCUSSION

In the last part of my study, we showed that 16% of patients with AL amyloidosis have glycosylated light chains. In addition, we demonstrated that in all cases these glycosylated forms are present years before the diagnosis of AL amyloidosis. This striking information could be used to identify patients at risk for developing AL amyloidosis years before the clinical symptoms occurred. As reported in the Experimental design 2, the respective odds ratios for a diagnosis of AL over another plasma cell dyscrasia if a glycosylated light chain is found by MASS-FIX is 12.7 for kappa and 2.20 for lambda (Table 5). In addition, it has to take into consideration that several different post-translational modifications (including glycosylation) have been described in AL amyloidosis patients[44,66,71]. Lambda-LCs are more frequent in AL amyloidosis compared to multiple myeloma and kappa-AL amyloidosis patients have usually an higher circulating free light chain levels at diagnosis. As previously reported, the presence of glycosylation could be considered a post-translational modification that increases the risk of kappa LCs to form amyloid fibrils[57,72]. Our study further enhanced the clinical utility of MASS-FIX, which is fundamental to detect monoclonal proteins and can easily identify glycated forms.

In conclusion, glycosylated monoclonal proteins are detectable years before the diagnosis of AL amyloidosis. This finding, taken together with our previous report of glycosylation and higher risk for AL amyloidosis, reinforce the importance of identifying glycosylation in patients with clinically asymptomatic plasma cell dyscrasia. Take into consideration that MASS-FIX will probably replace immunofixation in the upcoming decade as a screening tool for monoclonal proteins, patients that are found to have glycosylated light chains should be followed more closely. Those subjects are at a higher risk of progression to AL amyloidosis compared to patients without glycosylated forms.

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