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PhD thesis on

A biomarker-based approach in management of AL amyloidosis: from risk assessment to tailored-treatment strategy

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Dedicated to my Mentors,

to my beloved family,

to my irreplaceable friends

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Abstract

Background: immunoglobulin light chain (AL) amyloidosis is a rare misfolding protein disease caused by the deposition as amyloid fibrils of a light chain (LC) produced by a B-cell clone. In most cases, the B-cell clone resembles the characteristics of a plasma cellular clone, even if more rarely it presents the biological characteristics of a lymphoplasmacytic lymphoma (in the context of an IgM-AL amyloidosis) or a non-lymphoplasmacytic lymphoprolipherative disorder (LPL) (mainly a marginal zone lymphoma [MZL]). The LC may be produced by a B-cell clone in the bone marrow and it can reach every organ (except of the brain) through the blood stream, giving arise of a systemic AL amyloidosis. More rarely, it deposits at the same site of its production, in the context of a localized AL amyloidosis. While localized AL amyloidosis has a good outcome (despite the frequent local progressions) and generally does not need specific treatment, systemic AL amyloidosis is characterized by a dismal outcome (especially when heart is involvement) e requires a chemotherapy targeting the B-cell clone. Organ and clonal biomarkers have a central role in the management of patients with AL amyloidosis and are used for prognostic stratification at diagnosis and for hematologic and organ response after treatment.

Objectives: there is still the need of a biomarker-based approach for the refinement of patients management from the prognostic stratification to the design and tailoring of treatment both at diagnosis and at relapse. Here we present the results of our studies focusing on the use of clonal and organ biomarkers in different times of the clinical history of systemic AL amyloidosis and in rarer forms of this diseases. More precisely we evaluated:

the use of urinary albumin/creatinine ratio (UACR) for diagnosis of renal involvement, prognostic stratification and assessment of renal response after treatment (*Objective 1*);

the effectiveness of a biomarker-based response-driven approach for a sequential treatment strategy of bortezomib-based induction and autologous stem cell transplant (ASCT) (*Objective 2*);

the ability of the cardiac biomarker NT-proBNP to identify early cardiac response after first-line treatment in patients with stage IIIb AL amyloidosis (*Objective 3*);

organ and clonal biomarkers (with a particular focus on cytogenetic aberrations) that identify the patients who could benefit the most from a rescue treatment with lenalidomide and dexamethasone (LDex) (*Objective 4*);

the impact of clonal biomarkers on prognosis in IgM-AL amyloidosis and differences in presentation and outcome according to the clonal B-cell immunophenotype *(Objective 5)*;

the main characteristics of patients with AL amyloidosis and non-lymphoplasmacytic LPD *(Objective 6)*;

biomarkers involved in the clinical history of localized AL amyloidosis and factors affecting local progression of the disease *(Objective 7)*.

Methods: data were collected from the prospectively maintained databases of patients with newly diagnosed AL amyloidosis at the Amyloidosis Research and Treatment Center of Pavia and the Amyloidosis Center of Heidelberg. A satisfactory response after induction with cyclophosphamide, bortezomib and dexamethasone (CyBorD) was defined as achievement of complete response (CR), very good partial response (VGPR) and organ response or partial response (PR) and organ response. Correlation between 24h-proteinuria and UACR at baseline was assessed by Pearson's r test. Multivariable analysis was performed for the identification of prognostic factors. Survival curves were plotted according to Kaplan Meier, and differences in survival were tested for significance with the log-rank test. Landmark analysis was performed to evaluate the benefit in survival of hematologic and organ response excluding early deaths. Deaths occurring in the first 100 days after initiation of CyBorD or ASCT where classified as treatment-related.

Results: *Objective 1.* Five hundred thirty-one patients with newly-diagnosed AL amyloidosis and paired 24h-proteinuria and UACR (on first morning void) were included in the study. A strong linear correlation was found between 24h-proteinuria and UACR at baseline (r=0.90; P<0.001). After a median follow-up of 31 months, 57 (11%) patients required dialysis. A UACR-based renal staging system identified three stages with different dialysis rate at 36 months (I vs. II: 0% vs. 20%, P=0.026; II vs. III: 20% vs. 44%, P<0.001). Achieving a renal response, according to a UACR-based criterion, resulted in lower dialysis rate in both a testing (at 36 months: 0% vs. 17%, P=0.004; n=354) and validation (at 36 months: 0% vs. 31%, P=0.006; n=177) cohorts. *Objective 2.* One hundred thirty-nine patients with newly-diagnosed AL amyloidosis were treated upfront with CyBorD, followed by ASCT only if response was unsatisfactory. Only 1 treatment-related death was observed during induction treatment. After CyBorD, hematologic response rate was 68% (51% ≥VGPR), with 45% satisfactory responses. Transplant was performed in 55 (40%) subjects, and granted an 80% hematologic response rate (65% ≥VGPR). Ten-year survival was 77% and 72% in patients treated with ASCT or CyBorD alone, respectively (P=0.438). Also 6- and 12- month landmark analyses did not show differences in survival. Duration of response was not different in the two groups (60 vs. 49 months; P=0.670). Twenty-one (15%) patients with unsatisfactory response to CyBorD could not undergo ASCT, due to loss of eligibility or refusal, and received rescue chemotherapy, with HR in 38% of cases and 51% 5-year survival. *Objective 3.* Two hundred forty-nine patients with stage IIIb AL amyloidosis were included in the study. P Two-hundred nine patients (84%) died. Median overall survival was 4 months. A hematologic response was observed in 50 (20%) patients (8% ≥VGPR) at 30 days and in 53 (22%) subjects (14% ≥VGPR) at 90 days after starting chemotherapy. Achieving at least a VGPR at 30 and 90 days was associated with a better survival (51 vs. 3 months; P<0.001 and 51 vs. 6 months; P<0.001, respectively). Cardiac response at 90 days was observed in 19 (8%) subjects. Overall survival was significantly better among cardiac

responders (54 vs. 20 months; P<0.001). Cardiac progression was observed in 197 (80%) patients and was associated with a shorter survival (20 vs. 3 months; P<0.001), also in subjects who achieved at least a VGPR at 90 days (50 vs. 20 months; P=0.02). *Objective 4.* Two-hundred and sixty patients with relapsed/refractory AL amyloidosis were treated with LDex. Patients received a median of 2 prior treatment lines (68% had been bortezomib-refractory; 33% had received high-dose melphalan). Median treatment duration was 4 cycles. 3-months hematologic response rate was 31% (18% ≥VGPR). Median follow-up was 56.5 months and median survival and duration of response were 32 and 9 months. Two-year dialysis rate was 15%. Achieving a VGPR or better survival (62 vs. 26 months, P<0.001). Cardiac progression predicted worse survival (22 vs. 40 months, P=0.027), although NT-proBNP increase was frequently observed. Multivariable analysis identified these prognostic factors: NT-proBNP for survival (HR 1.71; P<0.001); gain 1q21 for duration of response (HR 1.68, P=0.014), with a trend for survival (HR 1.47, P=0.084); dFLC(log_{10}) and LC isotype for survival (HR 2.22, P <0.001; HR 1.62, P=0.016) and duration of response (HR 1.88, P<0.001; HR 1.59, P=0.008). 24h-proteinuria (HR 1.10, P=0.004) and eGFR (HR 0.71, P=0.004) were prognostic for renal survival. *Objective 5.* One hundred patients with newly-diagnosed IgM-AL amyloidosis were included in the study. Lymphoid immunophenotype (LPL) was observed in 64% and plasma cellular (PPCN) in 28% patients. LPL exhibited $MYD88^{L265P}$ in 69% of cases, higher prevalence of κ light chain (44% vs. 18%; P=0.028) and higher IgM levels (16.9 vs. 5.7 g/L; P<0.001). PPCN had trends for more heart and renal involvement (75% vs 58%; P=0.160; 64% vs. 45%; P=0.115) and presented t(11;14) and gain 1q21 in 45% and 36% of cases. First-line treatment was rituximab-based in 72% of LPL and 8% of PPCN. Median overall survival and duration of response were 42 and 15 months. No statistically significant differences were observed between LPL and PPCN for survival (47 vs. 78 months; P=0.937) and duration of response (14 vs. 15 months; P=0.271). On multivariable analysis, dFLC(log_{10}) was prognostic for survival (HR 2.51, P<0.001) and

duration of response (HR 2.05, P=0.002). IgM levels showed a trend for shorter duration of response (HR 1.02, P=0.056). Mayo stage was prognostic for only survival (overall P=0.004). Rates of deep hematologic responses (≥VGPR) and IgM-response rates were 32% and 37%. In a 3-months landmark analysis, patients who achieved at least a VGPR had a better survival (97 vs. 16 months, P=0.010). IgM-response also resulted in better survival (not reached vs. 35 months, P=0.020), even if it was poorer in those who did not achieve a concomitant response with dFLC (n=16, 14 months vs. not reached; P=0.009). *Objective 6.* The study comprehends 36 consecutive AL amyloidosis patients with non-lymphoplasmacytic LPD. MZL was the most common (53%) LPD. Amyloidosis was systemic in 21 (58%) and localized in 15 (42%) subjects. Patients with systemic amyloidosis had more advanced Ann Arbor stage (III-IV in 85% vs. 46%). All patients with systemic amyloidosis had a monoclonal component and/or an abnormal free light chain ratio (FLCR). Autoimmune disorders, mostly Sjögren syndrome, were more frequent in localized amyloidosis (53% vs. 5%). Eleven of twelve deaths were due to amyloid progression in systemic amyloidosis. Median survival from diagnosis of systemic amyloidosis was 26.3 months. *Objective 7.* We present 293 patients with immunohistochemically confirmed localized AL amyloidosis. Lung (nodular pulmonary) with 63 patients was the most involved organ. The amyloidogenic LC was λ in 217 cases (κ:λ ratio 1:3). A local B-cell clone was identified in 30% of cases. Sixty-one (21%) had a concomitant autoimmune disorder. A monoclonal component was present in 101 (34%) patients and was more frequent in subjects with an autoimmune disorder (51% vs. 34%; P=0.03). Cigarette smoking was more prevalent in lung localized AL amyloidosis (54% vs. 37%; P=0.018). After a median follow-up of 44 months, 16 patients died and 5- and 10-years localized AL amyloidosis progression-free survival (Local-PFS) were 62% and 44%. Interestingly, Local-PFS was shorter among patients with an identified clonal infiltrate at amyloid deposition site (40 vs. 109 months; P=0.02) and multinuclear giant cells and/or an inflammatory infiltrate resulted in longer Local-PFS

in lung involvement (65 vs. 42 months; P=0.01). However, no differences in Local-PFS were observed in patients with concomitant autoimmune disorders, a monoclonal component and involved organ site.

Conclusions: organ and clonal biomarkers have a key role in management of patients with AL amyloidosis from identification of organ involvement, prognostic stratification, designing first-line treatment strategy and identifying prognostic factors for rescue treatment. UACR is a reliable marker for diagnosis, prognosis, and organ response assessment in renal AL amyloidosis and can reliably replace 24h-proteinuria in clinical trials and individual patients' management. A biomarker-based response-driven sequential approach, offering ASCT to patients who do not attain satisfactory response to upfront CyBorD, is very safe and effective in AL amyloidosis. Achieving an early cardiac response is rare but possible in patients with stage IIIb AL amyloidosis, and is associated with a dramatically longer survival. However, most stage IIIb patients have a rapid cardiac progression which is associated with a dismal outcome. Changes in NT-proBNP remain robust predictors of survival also in patients with advanced cardiac disease. Clonal and organ biomarkers at baseline identify patients with favorable outcome to rescue therapy with LDex, while deep hematologic responses (VGHR) and cardiac progression define prognosis during treatment. Despite clonal heterogeneity, cardiac biomarkers (included in the European Mayo staging system) and dFLC confirmed their prognostic role also in IgM-AL amyloidosis. Systemic AL amyloidosis can be a life-threatening event in LPD. Clinical features, as presence of a monoclonal component and abnormal FLCR, are clues to the diagnosis. Lastly, despite the clinical heterogeneity, the identification of the local B-cell clone seems to be the main factor leading the clinical history of localized AL amyloidosis.

Introduction

Amyloidoses represent a group of protein misfolding disease in which a amyloid precursor protein deposits in organs and tissues as insoluble amyloid fibrils causing a progressive organ damage.[1] The amyloid precursor protein is produced in a specific anatomic site and can be spread through the bloodstream to other organs – as in systemic amyloidoses – or it can deposits locally at the site of production, as it happens in localized amyloidoses.[2] At present, 18 proteins responsible of systemic amyloidosis and 22 causing a localized disease have been identified.[3] The most known and studied amyloid protein precursor in localized amyloidosis is the β-amyloid precursor protein in Alzheimer disease. However, in rare cases, event light chains (LC) produced by a local B-cell clone may deposits locally in tissues in the context of a localized immunoglobulin light chain (AL) amyloidosis. AL amyloidosis is more frequently an acquired systemic amyloidosis and it is the most common form of this disease in the Western countries. However, thanks to novel imaging tools, wild-type transthyretin amyloidosis (ATTRwt) is more easily detected and its incidence is increasing year by year. On the contrary, thanks to the innovation in treatment of rheumatic diseases, the incidence in Western countries of systemic amyloidosis related to chronic phlogosis (AA amyloidosis) is decreasing. Among the hereditary forms of systemic amyloidosis, the most common are caused by point mutation in the transthyretin gene that results in disease phenotypes with heart and peripheral nervous system (PNS) involvement. Less common hereditary systemic amyloidosis are triggered by mutations in the apolipoprotein AI, apolipoprotein AII, apolipoprotein CII, fibrinogen and lysozyme genes.

Despite the symptoms and clinical manifestations of systemic amyloidoses are non-specific and similar, they differ deeply for pathogenesis and molecular mechanisms of disease, clinical history and treatment. Therefore, an unequivocal diagnosis is mandatory and it is generally achieved

through identification of the amyloid precursor protein on tissue biopsy with adequate techniques (i.e. immunoelectron microscopy or proteomic analysis in mass spectrometry).[4-6]

Systemic AL amyloidosis

Epidemiology

Prevalence of systemic AL amyloidosis increases with age and doubles in patients older than 65 year-old when compared with subjects with 35-54 year-old. Median age at diagnosis is 63 years and 55% are males.[7] The main established risk factor for this disease is the presence of a monoclonal component and patients with a monoclonal gammopathy of undetermined significance (MGUS) 8.8 fold higher risk of developing AL amyloidosis.[8] In a large study of patients with MGUS with a long follow-up from diagnosis (up to 50 years), 14 became ill with AL amyloidosis. Systemic AL amyloidosis may also be present concomitantly with multiple myeloma (MM), which is clinically relevant in 10-15% of cases and can be less evident in 38% of subjects.[9] In patients with MM in absence of AL amyloidosis at diagnosis, there is a probability of 1% of developing this form of amyloidosis.[10]

The first study reporting the incidence of systemic AL amyloidosis was conducted in Minnesota (USA), within the *Olmsted County Project*. An incidence of 8.9 million people/year between 1950-1989 and 10.5 million people/year between 1970-1989 was reported.[11] A recent update of this same study revealed an incidence of 12 million people/year in the same region between 1990-2015. In Europe, a similar study was conducted in France and showed an incidence of 12.5 million people/year between 2012-2016.[12] Two studies were done in United Kingdom and Sweden, reporting data from clinical reports and death certificates. In both of these countries an incidence of 3 million new diagnosis of AL amyloidosis per year was reported.[13,14] Lastly, a study conducted

in Queensland (Australia), showed an incidence of 10 million of newly-diagnosis of AL amyloidosis per year between 1999-2013.[15]

Mechanisms of disease

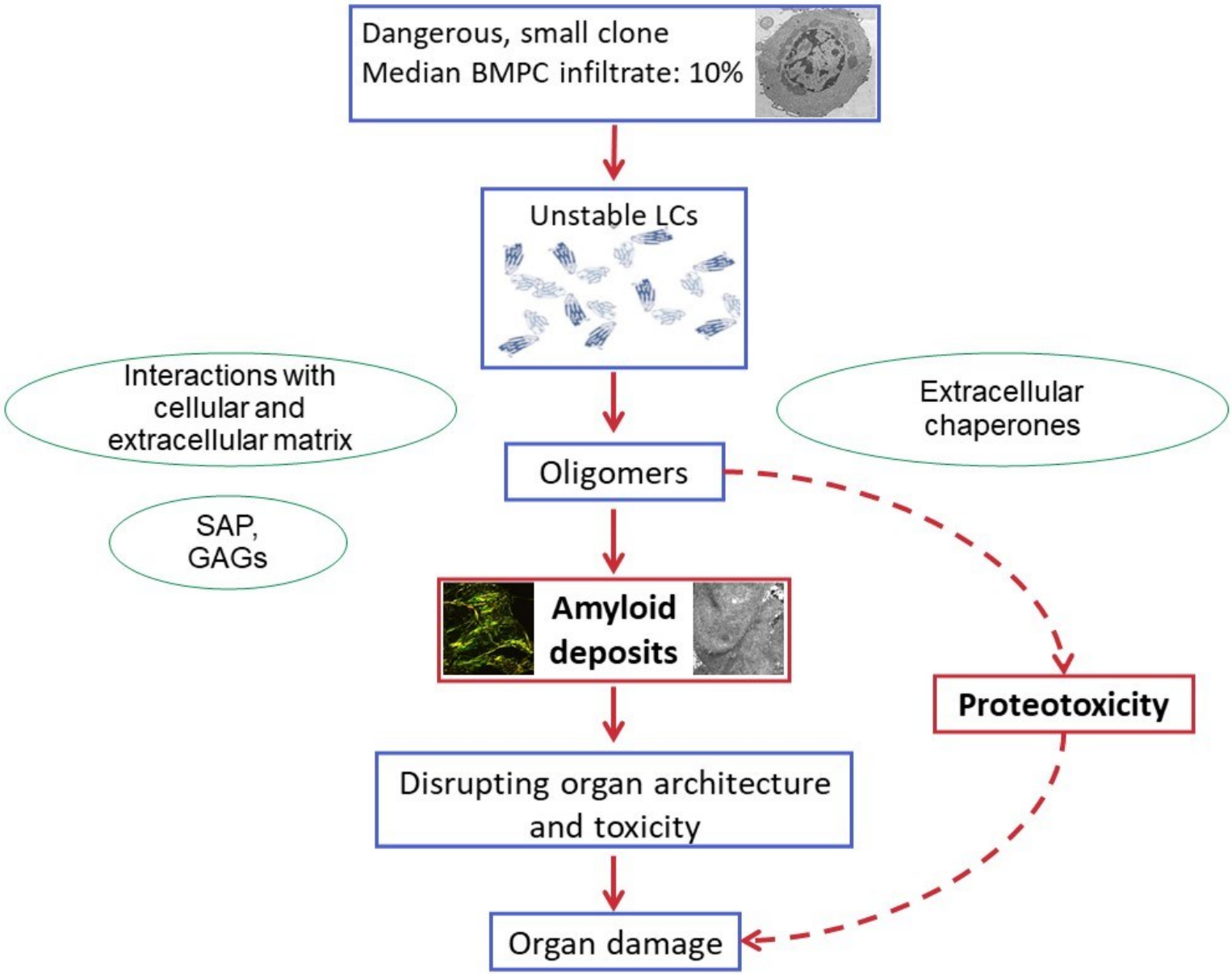
The physiopathology behind AL amyloidosis is summarized in Figure 1. AL amyloidosis is caused by a small B-cell clone, more frequently with the characteristics of a plasma cellular clone with a median bone marrow infiltrate <10%.[16] More rarely, the underlying clone has the phenotype of a lymphoplasmacytic lymphoma or other lymphoproliferative disorders.[17,18]

Differently from MM, the amyloidogenic plasma cells shows a prevalence of LC λ, which is expressed in 75-80% of cases. Besides this difference, the amyloidogenic clone presents phenotypical characteristics and number of chromosomic aberrations similar to the MM. However, its genetic expression profile is far more similar to normal plasma cells.[19] Anyway, in AL amyloidosis plasma cells present peculiar biological features, which play a key role in the therapeutic strategy and on drug effectiveness as a deep dependency on the proteasome system. A full functioning proteasome is indeed necessary to face the proteotoxicity of amyloidogenic free light chains (FLC), which are produced by the plasma cells themselves.[20,21] This explains the effectiveness of proteasome inhibitors – above all bortezomib – in treatment of AL amyloidosis. However, this drug was proven less effective against plasma cells harboring the most common cytogenetic aberration in AL amyloidosis: the translocation t(11;14). Translocation t(11;14) is present in almost 50% of cases and results in worse outcome to bortezomib.[22-25] On the other hand, the exposure to melphalan – both oral in association with dexamethasone (MDex) or high-dose as conditioning therapy in autologous stem cell transplant (ASCT) – is capable to overcome the prognostic adverse effect of this translocation.[26,27] However, MDex itself was proven less effective when plasma cells harbor a different cytogenetic aberration: gain 1q21.[28] Gain 1q21 is

less frequently found in AL amyloidosis than t(11;14) and it is present in less than 20% of cases.[22] High risk cytogenetic aberrations as t(4;14) and del17p are uncommon in AL amyloidosis (less than 10% of cases). Nevertheless, an international study showed a poor prognosis for patients with del17p when its frequency in the B-cell clone is high or when other cytogenetic abnormalities are present.[29] Finally, trisomies are less frequent in AL amyloidosis than in MM and are associated with κ LC restriction, higher bone marrow plasma cellular infiltrate and older age at diagnosis.

Several studies were conducted on LCs in the attempt to define the mechanisms of fibrillation and organ tropism. It was observed that three different immunoglobulin light chain variable region (IGLV) genes - *IGVL2-14*, *IGVL6-45* e *IGVL3-1* - code for the largest part of amyloidogenic λ FLC.[30-32] Moreover, an association between germline *IGLV6-57*, *IGLV1-44* and *IGKV1-33* and renal, cardiac and liver involvement was described, respectively.[33,34] Moreover, it seems that IGLV germline usage is associated to peculiar disease features. It has been found an association with IGVL6-57 and translocation t(11;14), while IGLV2-14 and IGKV1-33 seem to relate with lower and higher serum free light chain concentration, respectively. Recently, the German and Italian researchers managed to define the cryo-EM structures of two λ LC amyloid fibrils from the heart of two patients with AL amyloidosis.[35,36] The results of these studies indicate a role of LC variable region in the mechanisms of misfolding and fibrillogenesis. More interestingly, conserved mutations in the sequence of the two different cardiotoxic LC were identified, suggesting the role of specific highly-conserved motifs in the mechanisms of amyloid formation. Other studies describe different hot spot mutations possibly involved in the first steps of aggregation, with a particular focus on mutations in the interface region of λ LC monomers within the LC dimer, that seem to decrease protein stability and enhance aggregation.[37,38]

Figure 1. Mechanisms of disease in AL amyloidosis.



In 70% of patients, AL amyloidosis is caused by a small plasma cellular clone producing λ light chains. These light chains are thermodynamically unstable and prone to misfolding due to mutations in light chain variable region genes (IGLV). Oligomer formations is favored by interactions between the protein and the components of the extracellular matrix, proteases and metal ions. The oligomers deposits in organs and tissues as fibrils with a β-sheet structures. The fibrils interact with extracellular matrix proteins, like serum amyloid P protein (SAP) – the main constituent of amyloid deposits, which protect them from degradation – and other glycosaminoglycans, which function as a structure promoting the formation of new fibrils. Organ damage is caused both by soluble oligomers and amyloid fibrils in the tissue: the first ones due to mechanisms of direct toxicity, impairing cellular viability; the latter ones disrupting organ architecture, leading to a decrease of organ function, and catalyzing the formation of new oligomers, enhancing this way the mechanisms of toxicity. BMPC, bone marrow plasma cellular infiltrate; GAGs, glycosaminoglycans; SAP serum amyloid P protein.

Major efforts have been made to identify the mechanisms of organ damage caused by AL amyloidosis, especially in heart involvement, which is the most prominent prognostic factor. For a long time, the main hypothesis was based on the mechanic activity of the amyloid deposits, which cause a profound disruption of the heart architecture, leading thus to irreversible heart disfunction. However, in the last two decades, clinical observation and laboratory studies have pointed out a possible role of soluble oligomers in the genesis of cardiac dysfunction. Clinical experience has proved that concentration of N-terminal natriuretic propeptide B type (NT-proBNP) – a cardiac biomarker with a 100% sensitivity in cardiac AL amyloidosis –[39] may decrease concomitantly with amyloidogenic FLCs after chemotherapy with an improvement of cardiac dysfunction and survival.[40] The laboratory data on a direct toxicity of soluble oligomers in cardiac

AL amyloidosis come from experiments on animal models or cellular cultures. First it was observed that the infusion of purified FLCs from the urine of patients with cardiac AL amyloidosis induces a rapid increase of telediastolic pressure in isolated mouse hearts.[41] Further observations were obtained from the experiments in *Caenorhabditis elegans*, a nematode whose pharynx has a rhythmic contractile activity and that has been considered as an analogue of vertebrates heart. In this worm, exposure to cardiotoxic FLCs leaded to a decrease of its pharyngeal pumping rate.[42] Lastly, it was described that the infusion of FLCs of patients with cardiac AL amyloidosis in *zebrafish* heart caused a rapid reduction of cardiac output and survival before the formation of amyloid deposits.[43] In all these experiments, the results obtained with cardiotoxic FLCs were not observed with FLCs of patients with MM or with AL amyloidosis but without cardiac involvement. In another experiment, the exposure of cultures of cardiac cells to FLCs of patients with cardiac AL amyloidosis associated with an increase of oxygen reactive species (ROS) through a p38 mitogen-activated protein kinase (MAPK) mediated mechanisms, leading to cell dysfunction and death.[44,45] Interestingly, regulation of transcription of *NPPB*, the gene encoding natriuretic peptide B type (BNP), is also MAPK mediated. This observation provides the molecular basis of the effectiveness of NT-proBNP in diagnosis, prognostic stratification and organ response assessment in patients with cardiac AL amyloidosis.

Unfortunately, limited information about the mechanisms of LCs mediated damage are available for other organs that may be involved in AL amyloidosis. Kidney involvement is also frequent in this disease and leads to progressive renal failure that may require dialysis and results in impairment of quality of life. In renal AL amyloidosis, amyloid fibrils deposits mainly in the glomeruli, resulting in a disruption of the glomerular membrane causing increased urine protein loss. Some studies suggested a possible role of mesangial cells in mechanisms of renal involvement.[46-48]

Clinical manifestations

Clinical manifestations of AL amyloidosis depend on type of organ involvement (Figure 2) and are in large part non-specific. In fact, the pathognomonic manifestations of AL amyloidosis – i.e. macroglossia and periorbital purpura – are present in no more than 20% of patients at diagnosis. In all other cases, manifestations and symptoms of this disease are similar to other clinical conditions more common in the elderly. Since heart and kidney are the most frequently involved organs in AL amyloidosis (80% and 65% of cases, respectively), heart failure and nephrotic syndrome are the most common clinical manifestations. Heart involvement and its severity is the main factor affecting prognosis and advanced cardiac amyloidosis results in a significant limitation in treatment strategies. Renal involvement does not affect survival, but can result in progressive renal failure, limiting treatment options and quality of life. When renal involvement is diagnosed lately, there is a significant risk of progression to end-stage renal failure requiring dialysis. Liver involvement in AL amyloidosis presents with hepatomegaly with no focal lesions at echography and high indices of cholestasis (mainly alkaline phosphatase). The presence of symmetric and mainly sensitive axonal peripheral neuropathy starting from lower limbs is a sign of PNS involvement. Symptoms of autonomic nervous system involvement are orthostatic hypotension – that can be preceded by “spontaneous resolution” of a clinical history of hypertension –, alterations of gastrointestinal motility and erectile dysfunction in males. These “organ-specific” symptoms can occur along with other non-specific manifestations of AL amyloidosis as fatigue, dysgeusia, anorexia, severe malnutrition up to cachexia. Hemorrhagic manifestations are also frequent in AL amyloidosis and are caused both by small blood vessels frailty and a possible factor X acquired deficiency.

Figure 2. Organ involvement in systemic AL amyloidosis.

Heart:
cardiac failure, dyspnea, peripheral edema, hypotension, syncope, ventricular walls thickening, arrhythmias

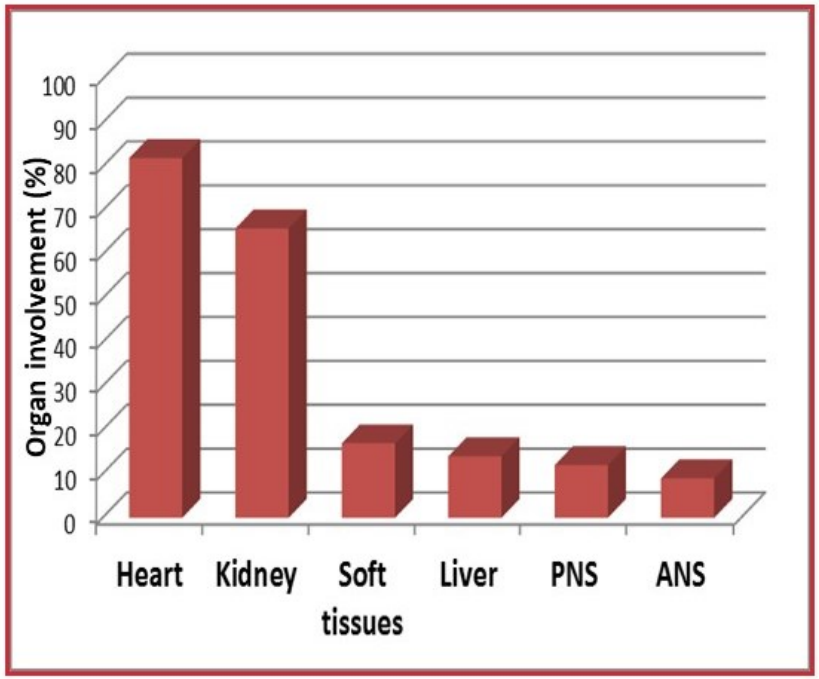
Soft tissues:
macroglossia, periorbital purpura, carpal tunnel syndrome

Kidney:
nephrotic syndrome, renal failure, peripheral edema

Autonomic nervous system:
orthostatic hypotension, altered GI motility, erectile dysfunction in males

Liver:
increase in the indices of cholestasis, hepatomegaly

Peripheral nervous system:
length-dependent axonal polyneuropathy

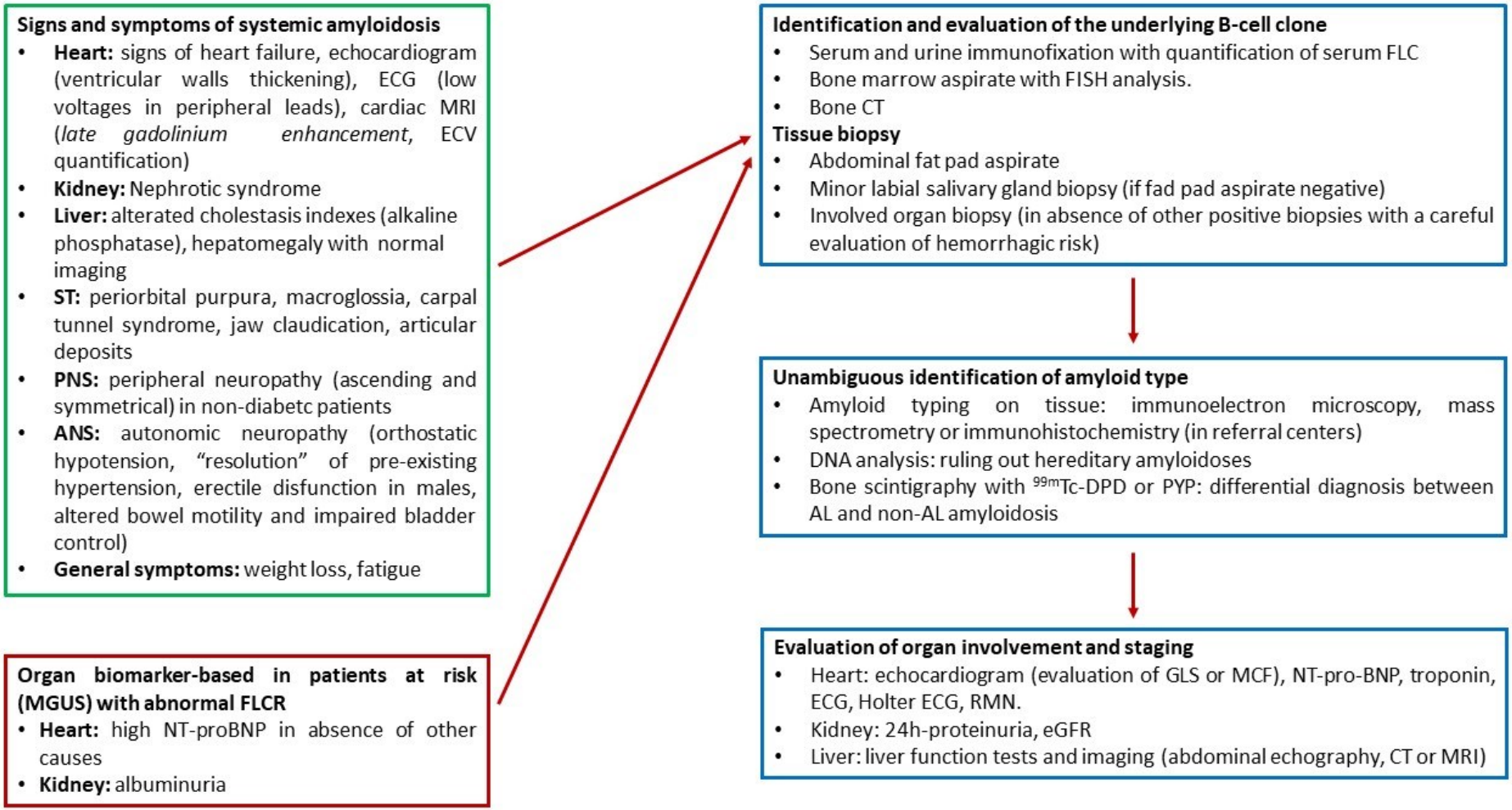


Data observed in 1378 patients with newly-diagnosed AL amyloidosis at the Amyloidosis Research and Treatment Center Pavia. ANS, autonomic nervous system; PNS, peripheral nervous system

Diagnosis

Diagnosis of AL amyloidosis requires the combination of laboratory test, diagnostic imaging techniques, DNA analysis and amyloid typing on tissue biopsy with adequate techniques (Figure 3). Identification of amyloid deposits on tissues is obtained with Congo red staining. This particular dye binds to amyloid fibrils, giving them the characteristic apple green birefringence under polarized light microscopy.[1] With a diagnostic sensitivity of 81%, abdominal fat pad is the most convenient biopsy site for diagnosis of AL amyloidosis, since it is easily sampled (aspiration or biopsy) with low risks for the patients. The fluorescent dye FSB has been recently proven as a sensitive and specific diagnostic tool for identification of amyloid deposits in abdominal fat pad, but it requires the access to fluorescence microscopy.[49] If abdominal fat pad is negative, but the suspicion of AL amyloidosis is still high, the possibility of a second biopsy site should be considered.[4] Minor labial salivary gland biopsy is easily performed and it has a diagnostic sensitivity of 60% in patients with AL amyloidosis and a negative abdominal fat pad aspirate.[50] Involved organ biopsy should be carefully considered in patients with AL amyloidosis because of the higher risk of bleeding. This is particularly relevant for liver biopsy, which presents a high risk of organ rupture in this disease. For this reason, in the selected cases in which a liver biopsy is performed, the transjugular liver biopsy is preferred over the percutaneous approach.

Figure 3. Diagnostic workflow in AL amyloidosis



Suspect of AL amyloidosis rises from symptoms of organ involvement or from biomarkers alterations in patients with a monoclonal gammopathy. Diagnostic imaging is of primary importance in diagnosis of cardiac amyloidosis. Echocardiographic features of advanced cardiac amyloidosis are thickening of ventricular walls, of the interventricular and interatrial septum with "granular sparkling" aspect. Cardiac MRI shows an impaired myocardial kinetic of gadolinium with subendocardial late gadolinium enhancement. Moreover, cardiac MRI allows the measurement of the extracellular volume, which is considered an estimation of the extension of amyloid deposits in the heart. Diagnosis of AL amyloidosis requires the histologic demonstration of amyloid deposits. It is possible to resort to minimally invasive biopsy sites (abdominal fat pad and minor labial salivary gland) or to organ biopsy in selected cases. Amyloid typing, biomarker-based organ staging and evaluation of the underlying plasma cellular clone are required before starting therapy.

ANS, autonomic nervous system; CT, computerized tomography; DPD, 3,3-diphosphono-1,2-propanedicarboxylic acid; ECG, electrocardiogram, ECV, extra cellular volume; eGFR, estimated glomerular filtration rate; FISH, fluorescence in situ hybridization; FLC, free light chains; GLS, global longitudinal strain; MCF, myocardial contraction fraction; MGUS, monoclonal gammopathy of undetermined significance; MRI, magnetic resonance imaging; NT-proBNP, N-terminal natriuretic propeptide B type; PNS, peripheral nervous system; ST, soft tissues

Symptoms and clinical manifestations of AL amyloidosis are frequently shared with other types of systemic amyloidosis (Table 1). Therefore, accurate amyloid typing is mandatory to avoid misdiagnosis. Immunohistochemistry in light microscopy with commercial antibodies is not a reliable technique for the identification of the amyloid precursor protein on tissue biopsy.[51,52] However, this technique has been proven effective for amyloid typing when performed with custom-made antibodies in referral centers.[53] Immunoelectron microscopy with commercial antibodies is a reliable tool for amyloid typing, allowing the correct identification of the amyloid precursor protein in 100% of cases.[4] Proteomic analysis in mass spectrometry on whole tissue biopsy or after *laser capture microdissection* is actually considered the gold standard for amyloid typing.[5,6] DNA analysis is required for the evaluation of hereditary systemic amyloidoses. Scintigraphy with bone tracers, as tecnetium (^{99m}Tc) 3,3-diphosphono-1,2-propanedicarboxylic acid (^{99m}Tc-DPD) is currently used in differential diagnosis between AL and non-AL cardiac amyloidosis. This imaging exam show a strong myocardial uptake of the radiotracer in patients with cardiac transthyretin amyloidosis (ATTR) and no or just mild myocardial uptake in AL amyloidosis.[54] Bone scintigraphy allows a non-biopsy diagnosis of ATTR amyloidosis only in patients without monoclonal components at serum and urine immunofixation.[54] However, in patients with a monoclonal gammopathy, the amyloid typing on tissue biopsy is always mandatory to rule out AL amyloidosis. It is currently under evaluation the role of PET with ^{18}F-florbetapir and ^{18}F-florbetaben – two radiolabeled drugs used for the evaluation of amyloid deposits in the brain in Alzheimer disease – in the identification of heart involvement in AL amyloidosis and for differential diagnosis with ATTR amyloidosis.[55-57] However, further and larger studies are needed to clarify before the use of this diagnostic tool in AL amyloidosis.

Table 1. Common types of systemic amyloidosis

Type of amyloidosis	Amyloid precursor protein	Acquired / hereditary	Organ involvement
Systemic AL	Monoclonal FLC	Acquired	All except CNS
ATTRwt	Transthyretin, wild-type	Acquired	Heart, soft tissues, lung
ATTRv	Transthyretin, variant	Hereditary	Heart, PNS, ANS
AA	Serum amyloid A	Acquired	Kidney, heart, liver, lungs
ApoAI	Apolipoprotein AI	Hereditary	Liver, kidney, testicles, heart
ALECT2	Leukocyte chemotactic factor 2	Acquired	Kidney, mainly

Types of amyloidosis are identified by acronyms composed by the letter "A", which stands for amyloidosis, followed by the abbreviation of the amyloid precursor protein. ANS, autonomic nervous system; CNS, central nervous system; FLC, free light chains; PNS, peripheral nervous system.

A crucial part of the diagnostic workflow in AL amyloidosis is the identification and characterization of the underlying B-cell clone. Considering the usually small size of the plasma cellular clone, identification of the amyloidogenic monoclonal FLC requires the combination of different high-sensitive techniques: capillary serum and urine electrophoresis, serum and urine immunofixation and serum FLC quantification.[58,59] In the last years, novel techniques for the identification of monoclonal components with mass spectrometry have been evaluated.[60] The study of the underlying B-cell clone is completed with bone marrow aspirate or biopsy and FISH analysis to assess the cytogenetic status and the presence of translocation t(11;14) and gain1q21.

Identification of organ involvement in AL amyloidosis is based on the evaluation of organ biomarkers, imaging techniques and clinical manifestations. Organ involvement diagnostic criteria – approved by the International Society of Amyloidosis in 2010 – are reported in Table 2.[61]

Table 2. Organ involvement diagnostic criteria

Organ	Diagnostic criteria
Heart	NT-proBNP >332 ng/L or mLVW >12 mm, in absence of other causes
Kidney	Proteinuria >0.5 g/24h, mainly albuminuria*
Liver	Alkaline phosphatase 1.5 times higher than the upper reference limit Identification of hepatomegaly with imaging (echography, CT o MRI)
Peripheral nervous system Autonomic nervous system	Distal and symmetric sensorimotor peripheral neuropathy Altered bowel motility and bladder continence, orthostatic hypotension, erectile dysfunction in males
Gastrointestinal	Positive biopsy in presence of symptoms
Lung	Positive biopsy in presence of symptoms
Soft tissues	Macroglossia, periorbital purpura, arthropathy, CTS

CT, computerized tomography; CTS, carpal tunnel syndrome; mLVW, mean left ventricular wall thickness at echocardiogram; MRI, magnetic resonance imaging; NT-proBNP, N-terminal natriuretic propeptide B type.

** A recent study from Mayo Clinic proposed urinary albumin/creatine ratio (cut-off: 300 mg/g) for diagnosis of renal involvement in AL amyloidosis.*[62]

Evaluation of cardiac involvement is assessed by different instrumental and imaging techniques. Electrocardiogram generally shows low voltages in peripheral leads. Echocardiography is the cornerstone in diagnosis and evaluation of cardiac amyloidosis. Echocardiographic diagnosis of heart involvement is based on increased left ventricular wall thickness and presence of granular sparkling aspect. Since ejection fraction is normally preserved until late stages of disease, systolic function is accurately assessed by other index as *global longitudinal strain*, *midwall fraction shortening* and *stroke volume index.* These systolic function indices are all altered and prognostic

in cardiac AL amyloidosis.[63-65] Cardiac MRI is also an important tool for diagnosis and evaluation of cardiac amyloidosis and provides information about myocardial tissue characterization. *Late gadolinium enhancement* is a sensitive and specific marker for diagnosis of heart involvement, especially cardiac MRI is performed analyzing T1 mapping and measuring extracellular volume (ECV).[66,67] It has been postulated that ECV calculated by cardiac MRI may represent an estimation of amyloid deposits in the heart.[68] As previously discussed, nuclear medicine imaging plays also a role in the study of cardiac amyloidosis. Scintigraphy with bone tracers is the mainstay for differential diagnosis between AL and non-AL cardiac amyloidosis.

The importance of early diagnosis of AL amyloidosis

Due to its non-specific symptoms, AL amyloidosis is often diagnosed lately and at an advanced stage of disease. A survey showed that diagnosis is made one year later symptoms onset in 40% of cases.[69] This could explain why 30% of patients has an advance and irreversible organ damage at diagnosis. In these cases, prognosis is particularly severe (less than 12 months) and it has not improved in recent years despite the availability of new powerful drugs.[70,71] For this reason, early diagnosis is of utmost importance in AL amyloidosis and treatment should be started when organ involvement is still pre-symptomatic. Organ biomarkers – as NT-proBNP and albuminuria – can be used as screening for AL amyloidosis in patients with known risk factors, as those with MGUS. Furthermore, recent studies suggested that the use of mass spectrometry for the study of the monoclonal LC in serum and urine (i.e. MASS-FIX) allows the identification of possible post-translational modification such as the presence of N-glycosylation, which can be present in patients with MGUS years before the onset of symptoms of amyloidosis. Based on this observation, a closer follow up has been proposed in those patients with MGUS and a suspected glycosylated monoclonal LC.[72,73] In patients with MGUS, NT-proBNP and albuminuria have a

sensitivity of 100% for heart and kidney involvement and allow a pre-symptomatic diagnosis of cardiac or renal AL amyloidosis, which results in a better outcome.[70,74] Cardiac imaging may be also of help for the identification of initial cardiac involvement in pre-symptomatic patients. Particularly, cardiac MRI is able to identify cardiac amyloidosis also in those patients with a mild increase of NT-proBNP.[75]

Prognosis and staging

Survival in AL amyloidosis is mostly determined by presence and severity of heart involvement. Indeed, while patients with advanced cardiac amyloidosis have a dismal prognosis (3-6 months despite novel therapies), those without cardiac involvement have a significant better survival (several years, even in case of no response to first line treatment). Biomarker-based staging systems were studied and refined in other to stratify patients with AL amyloidosis at diagnosis and to allow the identification of those with a more advanced disease. In fact, patients with an advanced organ involvement are particularly fragile and require tailored dose-attenuated treatment in order to improve treatment tolerability and to cope with treatment related toxicity.

The currently used staging system to assess severity of cardiac and renal involvement in AL amyloidosis are reported in Table 3. The first staging system based on cardiac biomarkers (NT-proBNP and cardiac troponins) was proposed by Mayo Clinic researchers in 2004 (Mayo Stage 2004).[76] This staging system was then modified by European researchers, who demonstrated that within the cardiac stage III a NT-proBNP concentration >8500 ng/L identified a particularly frail population of patients with worse outcome (stage IIIb).[77] This latter staging system (European cardiac stage) is mostly used in the clinical practice and in most of clinical trials. Recently, another staging system based on brain natriuretic peptide (BNP) was proposed.[78] The usefulness of this cardiac biomarker was already evaluated in patients with AL amyloidosis and renal failure. As a

matter of fact, NT-proBNP concentration is influenced by renal function and its ability of determine prognosis is impaired in case of renal failure.[79]

Despite severity of heart involvement is the main determinant of early deaths, it seems clear that in the long time the characteristics of the underlying B-cell clone play also a role. It was demonstrated that the presence of a bone marrow plasma cell infiltrate (BMPC) >10% resulted in worse outcome, as the concomitant diagnosis of MM.[80] As already discussed, cytogenetic aberrations affect prognosis and influence treatment effectiveness.[23,26-28] Concentration of the amyloidogenic FLC (expressed as the difference between amyloidogenic/non-amyloidogenic FLC isotype [dFLC]) is also an important prognostic determinant and having a high dFLC at diagnosis results in worse survival. This clonal biomarker (dFLC, cut-off: 180 mg/L), was included in a revised version of Mayo Clinic staging system in 2012 (Mayo Stage 2012).[81] Interestingly, other studies demonstrated that a low dFLC at diagnosis (cut-off: 50 mg/L) identifies a subgroup of patients with a significant better survival, regardless the severity of heart involvement.[82,83]

Stratification of organ damage is also crucial in renal AL amyloidosis, in order to identify patients with higher risk of progression to end-stage renal failure requiring dialysis. A collaborative study between Italian and German researchers allowed the testing and validation of a renal staging system based on 24h-proteinuria and estimated glomerular filtration rate (eGFR).[84] This system significantly identified patients with different risk for end stage renal disease. Recently, Mayo Clinic researchers proposed a renal staging system based on urinary albumin/creatinine ratio (UACR), replacing the 24h-proteinuria (cut-off: 5 g/24h) with this urinary biomarker (cut-off: 3600 mg/g).[62]

Table 3. Staging systems in cardiac and renal AL amyloidosis

Staging system	European cardiac	Mayo Stage 2012	Renal
Markers	NT-proBNP >332 ng/L cTnT >0.035 ng/mL (or cTnI >0.01 ng/mL)	NT-proBNP >1800 ng/L cTnT >0.025 ng/mL dFLC >180 mg/L	Proteinuria >5 g/24h eGFR <50 mL/min
Stages	I. 0 altered markers II. 1 altered marker IIIa. 2 altered markers and NT-proBNP <8500 ng/L IIIb. 2 altered markers and NT-proBNP ≥8500 ng/L	I. 0 altered markers II. 1 altered marker III. 2 altered markers IV. 3 altered markers	I. 0 altered markers II. 1 altered marker III. altered markers
Survival and risk of dialysis*	I. median not reached II. median 67 months IIIa. median 15 months IIIb. median 4 months	I. median not reached II. median 69 months III. median 16 months IV. median 6 months	I. 1% dialysis at 2 years II. 12% dialysis at 2 years III. 48% dialysis at 2 years

*cTn, cardiac troponin; dFLC, difference between amyloidogenic/non-amyloidogenic free light chains; eGFR, estimated glomerular filtration rate; NT-proBNP, N-terminal natriuretic propeptide B type. *Data observed in 1378 patients with newly-diagnosed AL amyloidosis at the Amyloidosis Research and Treatment Center Pavia*

Therapy

Principles of treatment

The coexistence of a B-cell clonal disorders and organ dysfunction – which can be severe and extended to several vital organs – makes AL amyloidosis a real therapeutic challenge for general hematologists. This consideration is confirmed by data showing that survival of patients with AL amyloidosis depends also on clinical expertise in treatment of this rare and complex disease.[85] For this reason, patients should be always referred to referral centers before starting treatment. Therapeutic strategy in AL amyloidosis consist in a treatment targeting the underlying B-cell clone and aims to obtain a rapid and profound reduction of dFLC, avoiding a further progression of organ involvement and improving survival. Since the B-cell clone has the phenotypical characteristics of a plasma cell dyscrasia, most of the therapeutic regimens used in AL amyloidosis are the same of MM. However, the presence of organ involvement makes patients with AL amyloidosis more fragile than those with MM alone. They present a higher mortality risk and are more exposed to treatment related toxicity, especially during the first months after diagnosis. On the other hand, if they manage to survive during the first year – which is the most difficult period of the disease –, patients with AL amyloidosis can benefit from a better long-term survival than those with MM.[86] For these reasons, the therapeutic strategy in AL amyloidosis is risk-adapted and is based on dose-reduced treatment schedules, especially during the first cycles of therapy. Moreover, since the therapeutic aim is the rapid and profound reduction of dFLC, treatment duration should be led by a tight monitoring of treatment effectiveness. Response to therapy should be assessed frequently (at least every two cycles) in order to switch quickly to a second line regimen if a satisfactory response to first line therapy is not observed.

Hematologic and organ response assessment

Hematologic response criteria in AL amyloidosis were identified and validated thanks to an international and multicentric study (Table 4).[87] Four categories of hematologic response were defined: complete remission (CR), very good partial response (VGPR), partial response (PR) e non-response (NR). The better the quality of response, the better is the survival.[88] Later studies identified and validated hematologic response criteria for patients with baseline dFLC <50 mg/L, who were not evaluable according the first hematologic response criteria (Table 4).[82,83] In the last years, other studies proposed new hematologic response criteria based on a profound reduction of dFLC and involved FLC (iFLC). Particularly, two studies reported a significant better survival in patients achieving a dFLC <10 mg/L or an iFLC <20 mg/L after chemotherapy, regardless the achievement of a CR.[89,90] However, another study showed that CR was not outperformed by these FLC-based criteria in terms of benefit on survival and time to next treatment or death.[91] Overall, these FLC-based criteria do not consent an update of current validated criteria of hematologic response in AL – particularly the CR criteria – as they come from single series and they lack external validation.[88] Anyway, major efforts have been made to develop new techniques to evaluate the depth of hematologic response also in those patients who achieved a CR. Mass spectrometry-based tools are able to identify serum and/or urinary monoclonal components even in patients with negative immunofixation and normal FLC ratio.[60] Next generation sequencing (NGS) or flow cytometry on bone marrow aspirate were indagated for the valuation of bone marrow minimal residual disease (MRD) in patients with AL amyloidosis in CR after treatment.[92] A study reported that absence of MRD negativity, assessed by next-generation flow cytometry (NGF), resulted in better 1-year progression free survival.[93] A recent international multicentric study, showed that persistence of MRD assessed by NGF translated in significantly lower rates of cardiac and renal responses.[94]

Biomarker-based cardiac and renal response criteria were also identified and validated (Table 4).[84,87] Cardiac response is defined as a decrease in NT-proBNP from baseline >300 ng/L and >30%. Patients who achieve a cardiac response at 6 months after starting chemotherapy have a significant improvement in survival. Similarly, achieving a renal response – defined as reduction in 24h-proteinuria >30% without a concomitant reduction in eGFR >25% - reduces the risk of progression to dialysis. On the other hand cardiac, and renal progression – defined as increase in NT-proBNP >300 ng/L and >30% and decrease in eGFR <25%, respectively – resulted in worse survival and higher risk of end-stage renal failure.[84,87] Response criteria for other involved organs have been not validated so far and their definition is based on consensus opinion. Particularly, liver response was defined as decrease in alkaline phosphatase >50% from baseline or decrease in liver size radiographically at least 2 cm.[95] The validated criteria of hematologic, cardiac and renal response are currently used in clinical practice and serve as surrogate endpoints in clinical trials.

Table 4. Validated hematologic and organ response criteria

Type of response	**Definition**
Hematologic response	
Complete remission	Negative serum and urine immunofixation and normal FLCR
Very good partial response	dFLC <40 mg/L
Partial response	Decrease in dFLC >50% from baseline
Low-dFLC response	dFLC <10 mg/L (dFLC between 50-20 mg/L at baseline)
Cardiac response	Decrease in NT-proBNP >30% and >300 ng/L from baseline
Renal response	Decrease in 24h-proteinuria >30% without a decrease in eGFR >25% from baseline.

dFLC, difference between amyloidogenic/non-amyloidogenic free light chains; eGFR, estimated glomerular filtration rate; FLCR, free light chain ratio; NT-proBNP, N-terminal natriuretic propeptide B type.

More recently, it has been proposed that depth of organ response should be assessed in a similar way of hematologic response. Four categories for cardiac, renal and liver response have been identified: complete organ response (nadir NT-proBNP ≤400 ng/L; nadir proteinuria ≤0.2 g/24h; nadir alkaline phosphatase ≤2 times institutional lower limit of normal); very good partial organ response (target biomarker reduction >60% from baseline, not meeting complete organ response definition); partial organ response (target biomarker reduction 31-60% from baseline); no response (target biomarker reduction ≤30% from baseline). The first data on this graded organ response showed that the deeper the organ response, the better is the outcome.[96] Lastly, a composite hematologic and organ response (CHOR) model was validated to better identify those patients with a better outcome after treatment. CHOR model was designed using combining scores of 0-3 for hematologic response (0-CR, 1-VGPR, 2-PR, 3-no response) and 0-2 for organ response (0-response in all organs, 1-response in some organs, 2-no organ response). Patients who achieved a CHOR score of 0-3 after treatment have a longer survival compared with those who reached a score of 4-5.[97]

Chemotherapy targeting the underlying B-cell clone

Chemotherapy against the B-cell clone producing the amyloidogenic FLC is the fundament of treatment of AL amyloidosis. As a matter of fact, the innovation in treatment and the development of novel and effective drugs has improved patients survival in the last decades.[98] Treatment choice takes in account several factors, as presence of particular cytogenetic aberrations that can influence treatment effectiveness as t(11;14) and gain1q21. The first results about the impact of cytogenetic aberration on specific treatment outcome were shown by the Heidelberg group. They first report that patients with gain1q21 exposed to oral MDex had lower rate of deep hematologic responses (at least VGPR in 5% vs. 25%) and worse survival (median 12.5

vs. 38.2 months) and shorted duration of response (median 5 vs. 8.5 months).[28] They then provide data about the impact of t(11;14) to first-line treatment with bortezomib. The presence of this cytogenetic aberration was characterized by shorter duration of response (median 3.4 vs. 8.8 months) and survival (median 8.7 vs. 40.7 months) and lower rates of good hematologic responses (at least VGPR in 23% vs. 47%).[23] These data were confirmed also by two independent case series.[24,25] Last, Heidelberg colleagues showed that t(11;14) was a positive prognostic factor in patients treated with ASCT, resulting in higher rates of CR (42% vs. 20%), longer survival (46.1 vs 28.1 months) and duration of response (median not reached vs. 93.7 months).[26] There results were also observed in a study of the Mayo Clinic. [24] Anyway, treatment has to be designed and adjusted according to severity of organ involvement, following a risk-adapted therapeutic strategy. Biomarker-based staging system are valuable tools for patient stratification. Clinical evaluation and laboratory/instrumental exams allow the identification of low-risk, intermediate-risk and high-risk patients. Each of these subgroups require a different and peculiar therapeutic approach (Figure 4).

Low-risk patients represent the 20% of cases of AL amyloidosis. This group of patients is characterized by absence of cardiac amyloidosis or presence of initial heart involvement with good hemodynamic status. These patients are the ideal candidates for ASCT. This procedure has to be considered carefully in patients with AL amyloidosis, because treatment related mortality is higher than in MM. However, when ASCT is performed in specialized centers (more than 4 ASCT in AL amyloidosis per year), the clinical expertise and the refinement of patient selection criteria brought a significant reduction of ASCT related mortality in AL amyloidosis.[85] Eligibility criteria for ASCT are the following: NT-proBNP ≤5000 ng/L, troponin T ≤0.06 ng/mL, ejection fraction >45% at echocardiogram, NYHA class <III, orthostatic systolic blood pressure >100 mmHg, age <65 year-old, *performance status* (*Eastern Cooperative Oncology Group*) ≤2, eGFR >50 mL/min per 1.73 m^2

(unless patient in dialysis) and diffusion capacity of the lungs for carbon monoxide (DLCO) >50%.[99-101] When ASCT is performed in specialized centers and in selected patients, the results of this kind of treatment are outstanding, with a hematologic response rate of 71% (CR in 35-37% of cases) and median overall survival of 7.6 years.[85,102] Moreover, the Boston University researchers reported a long time of remission in patients who achieved a CR (median time to relapse 4.7 years).[103] However, in order to maintain its effectiveness, ASCT should be performed with high dose melphalan (200 mg/m^2). In fact, reduced melphalan dose resulted in lower hematologic response rate – comparable to other treatments with less treatment related toxicity – without a significative reduction of ASCT related mortality.[101] Bortezomib-based regimens – mainly cyclophosphamide, bortezomib and dexamethasone (CyBorD) – can be used as induction treatment before ASCT, especially in patients with a BMPC >10%.[104] This sequential strategy was studied in clinical trials, reporting higher hematologic response rates and better survival when ASCT was preceded by induction therapy.[105-107] The strategy of induction before ASCT in AL amyloidosis is going through a rapid change thanks to the results observed with daratumumab, a fully human IgG1 κ monoclonal antibody directed against CD38 expressed by the plasma cells that represents the first anti-CD38 antibody available in treatment of MM.[108] This antibody acts both as a plasma cells-depleting agent – by engagement of CD38 and Fc-mediated cross-linking apoptosis – and as immuno-modulator – by eliminating immunosuppressive population, while T cell population are increased.[109] Recently, the results of the ongoing phase III ANDROMEDA randomized international clinical trial evaluating the effectiveness of daratumumab combined with CyBorD (daratumumab-CyBorD) vs. CyBorD alone in newly-diagnosed patients (NCT03201965) were published.[110]

Figure 4. Therapeutic strategy in AL amyloidosis

Low-risk patients

ASCT eligible: NT-proBNP <5000 ng/L, cTnT <0.06 ng/mL, age <65 years, PS ≤2, eGFR >50 mL/min per 1.73 m^2 (unless on dialysis), NYHA class <III, EF >45%, sBP >100 mmHg, DLCO >50%

- **Daratumumab-CyBorD:** if available
- **ASCT with high dose melphalan (200 mg/m^2)**
- **Induction with CyBorD:** in patients with BMPC >10% or in those refusing ASCT
- Consolidation with **BDex:** in patients who did not achieve a CR after ASCT

Intermediate risk patients

Stage I-IIIa
Non-eligible for ASCT

- **Daratumumab-CyBorD:** if available
- **MDex, LMDex**: absolute contraindications to ASCT (age >65 years), contraindications to bortezomib (neuropathy), presence of t(11;14)
- **CyBorD**: reversible contraindications to ASCT, renal failure, gain1q21
- **BMDex**: absolute contraindications to ASCT (age >65 years); favorable outcome either in presence of t(11;14) and gain 1q21
- **CLD:** reversible contraindications to ASCT and contraindications to bortezomib

High risk patients

Stage IIIb
NYHA class ≥III
PS=4

Intensive monitoring during therapy:

- Start with reduced doses and escalate if well tolerated
- Sequentially introduce therapeutic agents

Treatment of relapsed or refractory patients

- **First line treatment:** retreatment, if possible
- **Bortezomib or ixazomib:** in bortezomib-naive patients
- **MDex or ASCT:** in alkylating agents-naive patients and in selected cases for ASCT
- **Lenalidomide, pomalidomide, bendamustine:** in bortezomib-refractory patients
- **Daratumumab:** in patients previously exposed to bortezomib and IMiDs

Therapeutic strategy in AL amyloidosis is always risk-adapted and tailored on the severity of organ involvement. ASCT, autologous stem cell transplant; BDex, bortezomib and dexamethasone; BMDex, bortezomib, melphalan and dexamethasone; BMPC, bone marrow plasma cellular infiltrate; CR, complete remission; cTnT, cardiac troponin T; CLD, cyclophosphamide, lenalidomide and dexamethasone; CyBorD, cyclophosphamide, bortezomib and dexamethasone; DLCO, diffusion capacity of the lungs for carbon monoxide; EF, ejection fraction; eGFR, estimated glomerular filtration rate; LMDex, lenalidomide, melphalan and dexamethasone; MDex; melphalan and dexamethasone; NYHA, New York Heart Association; PS, performance status according to the Eastern Cooperative Oncology Group; sBP, systolic blood pressure.

A first safety run-in study conducted on 28 enrolled patients showed high hematologic response rates (96%; at least VGPR in 82% of cases), with a median time to initial and best hematologic response of 9 and 19 days, respectively.[111] These results were further confirmed by the analysis on a larger portion of patients enrolled in the ANDROMEDA study. Daratumumab-CyBorD showed higher rate of hematologic response (92% vs. 77%) as of VGPR/CR (79% vs. 49%) when compared to CyBorD alone. Also cardiac (42% vs. 22%) and renal response rates (54% vs. 27%) were higher in patients treated with daratumumab-CyBorD. Based on these results, daratumumab-CyBorD is currently the most effective treatment option for newly-diagnosed AL amyloidosis and should be considered in all cases if available. Finally, bortezomib-based regimens can be used also after ASCT as consolidation therapy, especially in patients who did not achieve at least a VGPR with ASCT. This therapeutic strategy results in high rates of CR (up to 60%).[112] Moreover, longer duration of response was observed in patients with less than VGPR after ASCT.[113]

Intermediate-risk patients cover the 60% of newly-diagnosed AL amyloidosis. Since they do not fulfill the eligibility criteria for ASCT, this subgroup of patients receive a different type of

chemotherapy. Also in these cases, daratumumab-CyBorD should be considered as fist-line if available. Otherwise, standard chemotherapy for AL amyloidosis may be offered to the patients. Treatment with MDex was considered the standard of care for many years in these cases.[114,115] This treatment regimen is well tolerated and showed high rates of hematologic response 76%, with CR in 31% of cases).[116] A randomized clinical trial failed demonstrating the superiority of ASCT on MDex in terms of hematologic response and survival.[117] However, it should be acknowledged that this study was conducted before the refinement of eligibility criteria for ASCT and study results are probably influenced by a high ASCT related mortality. Nevertheless, study results were confirmed at a landmark analysis excluding early deaths. The role of MDex in ASCT non-eligible patients changed with the introduction of bortezomib, a proteasome inhibitor that showed its effectiveness in relapsed/refractory AL amyloidosis.[118-120] Two retrospective studies demonstrated that the association of bortezomib with alkylating agents and dexamethasone was more effective than MDex or thalidomide-based regimens.[121,122] Cyclophosphamide and melphalan are the alkylating agents that can be associated with bortezomib. CyBorD is a first-line with a hematologic response rate of 60% (CR in 23% of cases). The effectiveness of bortezomib plus melphalan and dexamethasone (BMDex) was evaluated in an international phase III trial and compared with MDex alone in newly-diagnosed AL amyloidosis. The results of this study showed a higher hematologic response rate for BMDex (79% vs. 52%), with higher rate of CR/VGPR (64% vs. 39%) and better survival.[123] On these basis, bortezomib-based regimes are currently considered the standard of care as first-line treatment in intermediate-risk patients who do not present contraindications to bortezomib exposure (e.g. peripheral polyneuropathy and dysautonomia with orthostatic hypotension). If bortezomib is contraindicated, the association of lenalidomide, alkylating agents and dexamethasone – either lenalidomide, melphalan and dexamethasone (LMDex) or cyclophosphamide, lenalidomide and dexamethasone (CLD) – can be considered.[124-127]

The choice of a scheme containing cyclophosphamide or melphalan relies on clinical presentation and can be suggested by cytogenetic aberrations harbored by the B-cell clone. Young patients (age <65 year-old) with potentially reversible contraindications to ASCT are preferably treated with CyBorD. This regimen is also preferable in presence of renal failure, because oral melphalan is less manageable and poorly tolerated in these patients. On the other hand, present data indicate that BMDex could overcome the prognostic impact of the two more common cytogenetic aberrations in AL amyloidosis: t(11;14) and gain 1q21.

High-risk patients represent the remaining 20% of newly-diagnosed AL amyloidosis. This subgroup is characterized by advanced cardiac involvement at diagnosis (cardiac stage IIIb and NYHA class III-IV) and a severe prognosis (median survival 3-7 months). The outcome of these patients remains poor despite the available treatments, including bortezomib-based regimens.[128] Nevertheless, hematologic response can be achieved also in this advanced stage of disease and a rapid reduction in dFLC translates in a significant better survival also in these particular fragile subgroup of patients.[129] Therefore, treatment strategy should be based on dose-adapted regimens in order to mitigate treatment related toxicity, but still ensuring treatment effectiveness. In order to face this therapeutic challenge, high-risk patients should be closely monitored and drug dosages should be modified week by week according to treatment tolerability.[130] Due to its rapid responses, daratumumab may be an effective drug also in patients with advanced cardiac amyloidosis, that require a quick drop in amyloidogenic FLC concentration. It was not possible to explore this hypothesis in the ANDROMEDA trial, since patients with stage IIIb AL amyloidosis were not considered eligible for the study. On these bases, a phase II multicenter study in patients with new-diagnosed stage IIIb amyloidosis has been proposed (NCT04131309) and it is currently ongoing.

Identification of hematologic progression

Hematologic progression criteria in AL amyloidosis were proposed by a consensus panel within the International Society of Amyloidosis.[95] However, there is still a lack of validated hematologic progression criteria that help clinicians to identify the perfect timing to start a new treatment line in relapsed patients. As a matter of fact, an international survey demonstrated that there is not a worldwide agreement on which are the elements that prompt the decision to resume chemotherapy. In this study emerged that this decision was mainly influenced by three factors: baseline dFLC values (35%), disease severity at presentation (24%) and the time between response to frontline therapy and the subsequent rise of FLCs (18%).[131] At present, the international debate on this topic is divided on two different positions: starting rescue treatment as early as dFLC increases and before the worsening of organ dysfunction or delay treatment initiation until relapse is symptomatic.[132,133] The first strategy is supported by the observation that an even slight increase of dFLC (defined as "high risk dFLC progression": a dFLC >20 mg/L, a >20% increase from baseline and a >50% increase from the value at the best response) might precede a cardiac progression by months.[134] The second one is suggested by clinical and socio-economic observations. First, two studies reported that after ASCT relapse may be asymptomatic and that also patients in VGPR may tolerate a mild increase in dFLC.[103,135] Secondly, rescue treatment results in impaired quality of life and represent an important cost for the healthcare system.[136,137] Unfortunately, there are not enough data to definitely conclude on which of these two different approaches is the best in relapsed AL amyloidosis and further international studies are needed to clarify this hot topic in the field.

Treatment of relapsed/refractory patients

If the previous treatment line was effective and well tolerated, the retreatment with the last therapeutic regimen is a possible strategy in relapsed AL amyloidosis. However, this can result in a shorter progression free survival that nevertheless does not translate in worse survival.[138] In selected cases, as those in which first line treatment resulted in improvement of organ dysfunction in transplant-ineligible patients, a delayed ASCT may be considered.[139] Still, a recent collaborative study reported that ASCT at relapse associates with shorter survival and progression free survival if compared to ASCT for consolidation after first line therapy.[140] Besides these considerations, immunomodulatory drugs (IMiDs) represent the backbone of rescue treatment in relapsed/refractory AL amyloidosis.[141] Lenalidomide is second-generation IMiDs which has been proven to overcome resistance to alkylating agents, proteasome inhibitors and thalidomide.[142,143] Moreover, lenalidomide can be combined with alkylating agents (cyclophosphamide or melphalan) or ixazomib – an oral proteasome inhibitor – resulting in higher hematologic response rates but also increased toxicity.[124-127,144] In any case, lendalidomide should be carefully administered in patients with AL amyloidosis. As a matter of fact, the maximum tolerated dose of this drug in AL amyloidosis is 15 mg/day.[142,145] Moreover, lenalidomide is potentially nephrotoxic, particularly in presence of proteinuria and renal failure.[146,147] Lastly, exposure to lenalidomide – as to other IMiDs – is often followed by increase in cardiac biomarkers, specially NT-proBNP.[148] This hampers cardiac response assessment under treatment and rises the suspect of cardiac toxicity. Pomalidomide is a third-generation IMiDs that is effective in extensively pre-treated relapsed/refractory AL amyloidosis, including patients exposed to lenalidomide.[149-151] Hematologic response are rapid and hematologic response rate is 60%.[151] It has to be clear that CR is rare in relapsed/refractory patients treated with IMiDs. However, these drugs are effective and grant long lasting remissions and improved survival.[152] In addition to IMiDs, bendamustine represent another

rescue treatment in AL amyloidosis, especially in combination with rituximab in patients with an IgM clone. [153] Among new-generation proteasome inhibitors, carfilzomib showed promising results with a hematologic response rate of 63% (CR in 12% of cases).[154] However, the observed clinically significant cardiac toxicity (18% of cases) is a limitation to the use of this drug in AL amyloidosis. The oral proteasome inhibitor ixazomib proved its effectiveness in a phase I/II trial in relapsed/refractory AL amyloidosis.[155] A phase III trial evaluating ixazomib and dexamethasone vs. physician's choice in relapsed/refractory AL amyloidosis revealed that this combination was superior to other rescue treatments (lenalidomide and dexamethasone in 57% of cases) in preserving vital organ function, even if did not resulted in higher response rates.[156] Adding lenalidomide to ixazomib and dexamethasone resulted in a highly effective oral rescue regimen in AL amyloidosis with a hematologic response rate of 59% (at least VGPR in 41%).[144,157] Daratumumab has been proven an extremely effective rescue treatment in AL amyloidosis with rapid (within 30 days in 50% of cases) and remarkable hematologic response rates (76%; CR in 36% of cases).[158] The results of a European (NCT02816476) and of an American (NCT02841033) phase II trial of daratumumab in previously treated patients have been recently published, confirming the ability of this drug of inducing rapid hematologic responses (median time to hematologic response was 1 week) with an high rate of high-quality responses (at least VGPR in 47-86%).[159,160] In both these studies, daratumumab was well tolerated and the most common grade ≥3 adverse events were respiratory infections. The Heidelberg group published recently their case series of 168 patients treated with daratumumab (of whom 62 with daratumumab, bortezomib and dexamethasone [DVD]).[161] Hematologic response rate was 64-66% (at least VGPR in 48-55% VGPR). In this study, high NT-proBNP (>8500 ng/L), high dFLC (cut-off:180 mg/L) and harboring gain1q21 were associated with worse outcome. Interestingly, a shorter hematologic progression free survival was observed in presence of glomerular injury (albumin/creatinine ratio; cut-off: 220

mg/mmol). This was interpreted by the Authors as a possible increased renal clearance of daratumumab, impairing its therapeutic effectiveness. More recently, the same group of researchers published a small case series of 44 patients with relapsed/refractory AL amyloidosis treated with daratumumab, lenalidomide and dexamethasone (DRD).[162] Rescue treatment with DRD resulted in impressively high hematologic response rates (84%; at least VGPR in 62% of cases) was observed, although this treatment regimen was accompanied by relevant toxicity (lymphocytopenia in 58% and infectious complication in 55% of cases, respectively). Also in this study high dFLC (cut-off: 180 mg/L) and harboring gain1q21 resulted in shorter hematologic progression free survival.

Supportive treatment and organ transplant

Supportive treatment is a crucial part of the clinic management of patients with AL amyloidosis. A specific aim of supportive therapy is to sustain organ function during therapy and to improve quality of life. Since the heart and the kidney are the most frequently involved organs in AL amyloidosis, treatment of heart failure and nephrotic syndrome plays a central role in patient management. Diuretics and salt restriction have a key role in treatment of fluid overflow. Body weight should be monitored daily and diuretic posology should be modulated according to its variation. A careful attention to avoid a reduction of intravascular volume and cardiac preload should be paid. ACE-inhibitors should be administered carefully and at low dosage because treatment with these drugs can result in severe hypotension, especially in presence of autonomic nervous system involvement (even when it is non-symptomatic).[163] In patients with recurrent arrhythmic syncope pace-maker implantation should be considered, while there is not a clear agreement on the utility of implantable cardioverter defibrillators (ICD). Gabapentin can be useful in management of peripheral polyneuropathy, while octreotide can be effective in controlling

chronic diarrhea in patients with autonomic nervous system involvement. The maintenance of a good nutritional status – which is often impaired in AL amyloidosis – it is very important for quality of life.[164-166] Nutritional support may be needed in presence of weight loss and malnutrition.[167] Recently, bioimpedance vectorial analysis emerged as an useful tool for nutritional assessment in AL amyloidosis.[168]

Heart and kidney transplant can be considered when irreversible end-stage organ failure is documented. In selected, young patients with isolated severe cardiac AL amyloidosis orthotopic heart transplant should be considered, followed by ASCT and aiming to a deep hematologic response to prevent disease recurrence.[169] In other cases, heart transplant can be offered in patients with an irreversible end-stage cardiac failure, despite the achievement of a CR after treatment for AL amyloidosis. Thanks to improvement of treatment, the outcome of patients with cardiac AL amyloidosis after heart transplant it is not significantly different from those with non-amyloid cardiomyopathy.[170] However, it should be kept in mind that treatment with IMiDs in patients with heart transplant is associated with higher risk of transplant rejection.[171] Left-assisted ventricular devices may be used as a bridge to cardiac transplant. Kidney transplant can be considered in patients with end-stage renal failure who achieved a profound hematologic response (at least VGPR). In these selected cases, a long graft survival was observed (78% at 10 years from transplant) and outcome after renal transplant it is not different from patients with diabetic nephropathy.[172]

Systemic IgM AL amyloidosis

Epidemiology and mechanisms of disease

IgM systemic immunoglobulin light chain (IgM-AL) amyloidosis is a rare condition that accounts for 5-7% of all AL amyloidosis cases diagnosed at referral centers.[173-177] This unusual type of AL amyloidosis has been identified as a distinct entity with peculiar clinical presentation.[178]

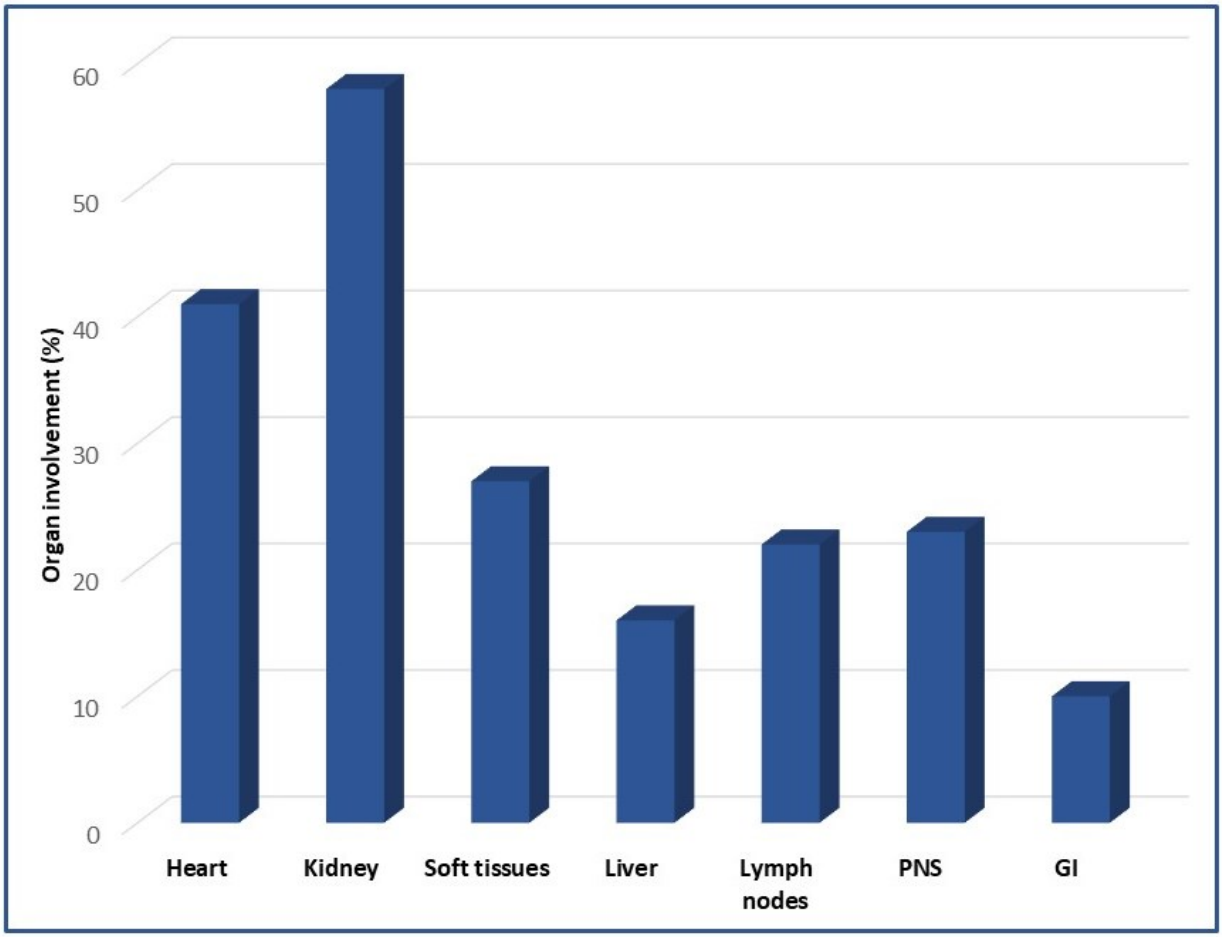
Mechanisms of disease are similar to systemic non-IgM AL amyloidosis. However, the main differences of IgM-AL amyloidosis are based on the peculiar characteristics of the underlying B-cell clone. Some studies have demonstrated the presence of a clonal heterogeneity in IgM-AL amyloidosis. A recent study evaluating the B-cell clone characteristics in this rare form of systemic AL amyloidosis reported that the bone marrow clonal disease shows the characteristics of a lymphoplasmacytic neoplasm (LPL) – which included both lymphoplasmacytic lymphoma and low grade B-cell lymphoma with plasma cellular differentiation – in 63% of cases and a pure plasma cellular neoplasm (PPCN) in 23% of patients.[179] Both LPL and PPCN showed peculiar biological hallmarks that further contribute to highlight the presence of two distinct entities in IgM-AL amyloidosis. The $MYD88^{L265P}$ and CXCR4 mutation are typical of LPL (in 83% and 29% of cases, respectively), while they were not observed in PPCN. On the other hand, the typical cytogenetic abnormalities of AL amyloidosis, as t(11;14), were observed frequently in PPCN, but never in LPL. It is important to remember that identification and characterization of the B-cell clone in IgM-Al amyloidosis is not always possible, resulting in the lack of the identification or characterization of the clone in the 8-14% of cases.[178,179] Compared to non-IgM AL amyloidosis, IgM-AL amyloidosis presents a peculiar IGVL usage. Particularly, LV2-08, LV2-14 and KV4-01 usage was more common in IgM-AL amyloidosis.[180] Interestingly, LV2-14 has been associated with PNS involvement and lower dFLC, which are peculiar characteristics of IgM-AL amyloidosis. [179] LV1-44 and LV6-57 – which are less common in IgM-AL amyloidosis – associated with cardiac involvement and t(11;14),

respectively. Interestingly, both cardiac involvement and t(11;14) are less frequent in IgM-AL amyloidosis compared to non-IgM AL amyloidosis.[70,179]

Clinical manifestations and diagnosis

Clinical manifestations of patients with IgM-AL amyloidosis may differ from those with non-IgM-AL amyloidosis, considering the different pattern of organ involvement. (Figure 5). The most striking differences in clinical presentations are the presence of lymphadenomegaly and polyneuropathy. Lymphadenomegaly is present in 20-30% of cases and it is more frequently caused by amyloid deposition in lymph nodes, rather than the localization of lymphoma. Polyneuropathy is typically long-dependent axonal and presents more frequently with paresthesia, dysesthesia and in more severe and advanced cases with ipo-anesthesia which limits the activities of daily living. In some cases, patients with polyneuropathy and IgM-AL amyloidosis frequently have anti-myelin associated glyco-protein (anti-MAG) antibodies.[181] The diagnostic workflow of IgM-AL amyloidosis is not particularly different from non-IgM AL amyloidosis. Amyloid typing with adequate techniques on tissue biopsy (i.e. abdominal fat pad, minor labial salivary gland or organ biopsy) is mandatory. However, considering the peculiar biological features of the B-cell clone and organ involvement, some additional procedures may be of help. Bone marrow evaluation requires bone marrow biopsy and aspiration and evaluation of the clonal immunophenotype, in order to characterize the B-cell clone as LPL or PPCN. Evaluation of mutational status of CXCR4 and presence of $MYD88^{L265P}$ should be assessed on bone marrow aspirate. A sample of bone marrow aspirate should be also obtained for iFISH analysis and evaluation of cytogenetic aberrations. Total body CT may be useful to evaluate lymph node involvement. A comprehensive neurological evaluation with electroneurography and search of anti-MAG antibodies should be performed in patients with polyneuropathy.[182]

Figure 5. Organ involvement in IgM-AL amyloidosis



Data observed in 644 patients with newly-diagnosed IgM-AL amyloidosis from the main series published so far. Data collected from Milani and Merlini, Best Pract Res Clin Haematol. 2016

It is important to note that the identification of the IgM monoclonal component precedes the diagnosis of IgM-AL amyloidosis in 34% of patients and that it can be diagnosed in patients who have been followed up for an IgM MGUS for more than 2 or 5 years in 14% and 8% of cases.[182] This observation supports the need of a periodical screening for IgM-AL amyloidosis in patients with a known IgM monoclonal component. This should be performed with organ biomarkers (NT-proBNP, troponin, 24h-proteinuria or albuminuria, alkaline phosphatase), with a particular attention on the onset of symptoms of polyneuropathy.

Prognosis and staging

The first information on prognostic factor in IgM-AL amyloidosis come from several single centers case series. Right from the first studies, it became clear that heart involvement and its severity is an important prognostic determinant for survival also in this rarer form of systemic AL amyloidosis.[173,174,176] Other identified prognostic factors that emerged from other single center experience were liver involvement, serum albumin and *performance status* (*Eastern Cooperative Oncology Group*).[174-176] The quest for the identification of prognostic factors was then refined thanks to an European collaborative study involving 250 patients with IgM-AL amyloidosis followed in Italy, France and United Kingdom. In this large and important study, multivariable analysis identified 3 prognostic factors: Mayo stage 2004, liver involvement and PNS involvement.[178] Thanks to these observation, a particular staging system for IgM-AL amyloidosis was proposed and included 4 risk factors: NT-proBNP (cut-off: 332 ng/L), cardiac troponin (troponin T cut-off: 0.035 μg/L and troponin I cut-off: 0.1 μg/L), presence of liver involvement and presence of PNS involvement. This staging system identified 3 stages with different outcome:

- Stage 1: no risk factors (median overall survival [OS] 90 months);
- Stage 2: 1 risk factor (median OS 33 months);
- Stage 3: 2 or more risk factors (median OS 16 months).

It is although important to note that in a more recent study published by Mayo Clinic's researchers, PNS involvement was not prognostic for survival in IgM-AL amyloidosis. In this series of patients Mayo stage 2012 (including cardiac biomarkers and dFLC) and liver involvement were the major determinant of prognosis.[179]

Therapy

Hematologic response assessment

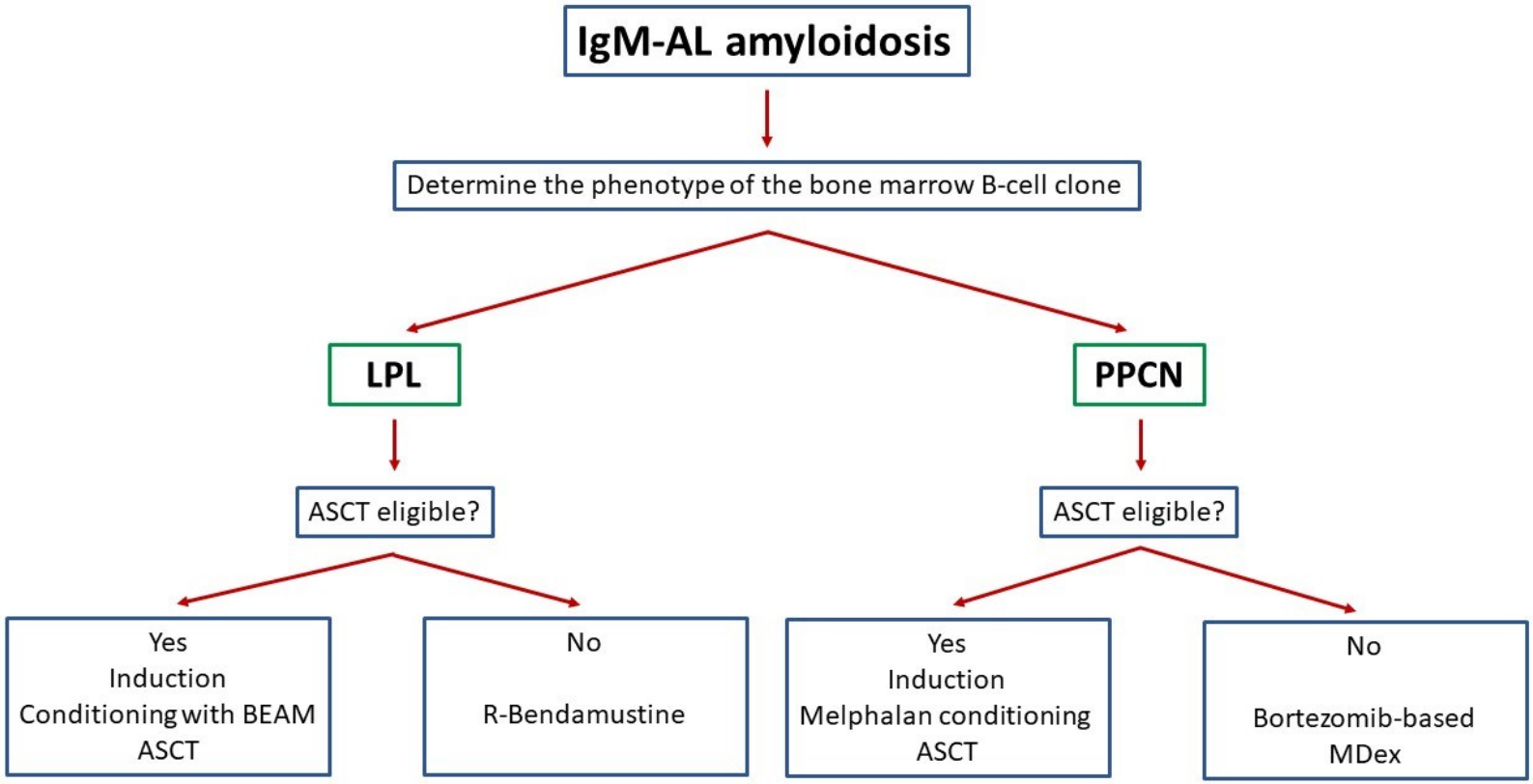
Hematologic response in IgM-AL amyloidosis is assessed by changings in dFLC from baseline as per validated criteria.[87,88] However, the European collaborative study demonstrated that also variations in the concentration of monoclonal IgM can be used for evaluation of hematologic response, as for Waldenström macroglobulinemia (WM).[17] This may be relevant in clinical practice, considering the general lower baseline dFLC in IgM-AL amyloidosis compared to non-IgM AL amyloidosis.

Treatment

The choice of therapeutic strategy in IgM-AL amyloidosis is particularly challenging due to the lack of clinical trials and large retrospective studies on treatment in this rare disease.[17] However, it has been proposed that treatment strategy should be *"B-cell clone phenotype-oriented"* in order to maximize treatment effectiveness and achieve higher rates of deep hematologic response (Figure 6).[183] ASCT remains one of the most effective therapeutic options in selected patients with IgM-AL amyloidosis. The largest study published so far on 38 patients reported high rates of hematologic response (90%; at least VGPR in 76% of cases) with long-lasting progression free survival (75% at 2 years).[184] Type of conditioning treatment should be performed according to the B-cell clone: with melphalan in PPCN and with BEAM (carmustine, etoposide, cytarabine, and melphalan) in LPL. Rituximab-based regimens represent a valuable option in LPL. The effectiveness of a treatment schedule containing rituximab and bortezomib was evaluated in a small group of patients with a hematologic response rate in 78% of cases.[185] However, it should be kept in mind that the presence of PNS involvement may represent a frequent contraindication to bortezomib in this

patients. Treatment with rituximab and bendamustine was also effective, with an high rate of high-quality hematologic response (at least VGPR in 48% of cases).[186] In PPCN, bortezomib-based regimes, without rituximab, may be used even if only few data on the effectiveness of these regimens are available. Another therapeutic option in PPCN is represented by MDex. In 53 patients with IgM-AL amyloidosis within the European collaborative study, treatment with MDex resulted in a hematologic response in 76% of cases (at least VGPR in 26%).[178] Finally, ibrutinib may be considered in LPL as rescue treatment. However, results from a small study indicated that ibrutinib is poorly tolerated in IgM-AL amyloidosis with low hematologic response rate and poor outcome.

Figure 6. Proposed therapeutic strategy in IgM-AL amyloidosis



Therapeutic strategy in IgM-AL amyloidosis should be risk-adapted and tailored according to the B-cell clone phenotype. ASCT; autologous stem cell transplant; BEAM, carmustine, etoposide, cytarabine, and melphalan; LPL, lymphoplasmacytic neoplasm; MDex, melphalan and dexamethasone; PPCN, pure plasma cellular neoplasm ; R-Bendamustine, rituximab and bendamustine. Modified from Wechalekar, et al. Hematol Oncol Clin North Am 2020.

Localized AL amyloidosis

Epidemiology and mechanisms of disease

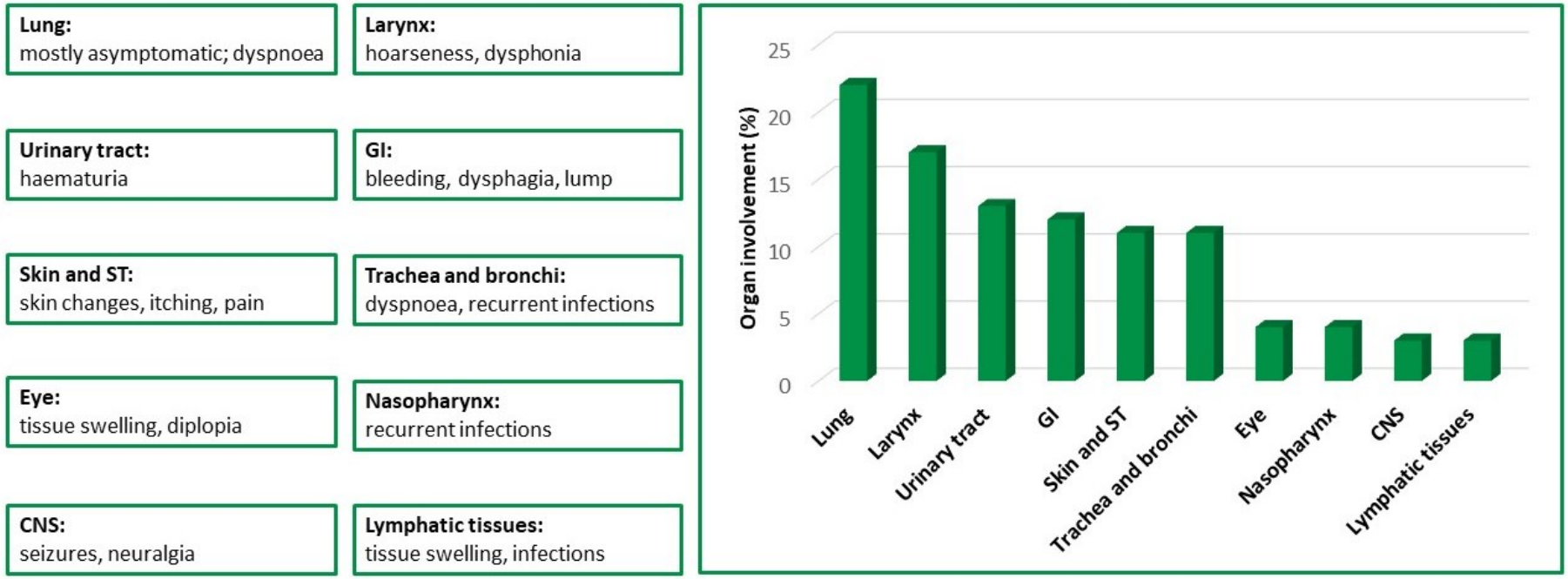
Localized AL amyloidosis is a rare and less studied disease that represents less the 10% of all diagnosis of amyloidosis at international referral centers. In this rare amyloidosis, locally produced LCs (most frequently a κ LC isotype)[187,188] deposit at a single anatomic site, forming one or multiple tumour-like amyloid lesions, called amyloidomas.[2] Our knowledge about localized AL amyloidosis recently improved thanks to the publication of large case series and small but relevant studies on the evaluation of the local B-cell clone.

The local B-cell clone is general interspersed within amyloid fibrils and an important inflammatory infiltrate rich in multinucleated giant cells (MGC). For these reasons, identification of the B-cell clone may be challenging and requires highly sensitive tools, as immunohistochemistry, in situ-hybridization or PCR-based immunoglobulin heavy chain (IGH) and light chain (IGK) gene rearrangement analyses. From current data, it seems that the local-B cell clone is small and does not present the full characteristics of a well-defined malignant disease. The B-cell clone has been characterized as a clonal lymphoplasmacytic infiltrate within the spectrum of marginal zone lymphoma (MZL) in several small studies.[189-192] However, a larger study revealed that the local B-cell clone can be better classified as "localized B-cell neoplasia of undermined significance" in the majority of cases.[193] The etiology of local B-cell clones in this rare disease is currently unknown. Chronic antigen exposure and autoimmune stimulation are putative explanations.[194] This hypothesis is intriguing and could explain the high prevalence of autoimmune disorders in localized AL amyloidosis.[187]

Clinical manifestations and diagnosis

Clinical manifestations and symptoms depend on the localization of amyloidomas (Figure 7).

Figure 7. Organ involvement in localized AL amyloidosis



Data observed in 293 patients with localized AL amyloidosis at the Amyloidosis Center of Heidelberg. CNS, central nervous system; GI, gastrointestinal; ST, soft tissues.

Respiratory and urinary tract and skin are the more often involved anatomical sites in local AL amyloidosis. Frequently, symptoms are caused directly by mass-effect of the growing amyloidoma due to tissue swelling, compression of adjacent structures or obstruction. This mass-effect dependent can be more frequent and important when amyloidomas are localized in small e narrow anatomic sites (e.g. the larynx). Conversely, lung (nodular pulmonary). localized AL amyloidosis is more often asymptomatic and diagnosis may be incidental. Bleeding manifestation are recurrent in urinary tract involvement while recurrent infections are frequently complication of lower airways (tracheobronchial) involvement. Other than the organ dependent symptoms, up to 20% of patients with localized AL amyloidosis have a concomitant autoimmune disorder. Sjögren syndrome is the most frequent autoimmune disorder, particularly association with skin localized amyloidosis.[195] Lastly, a concomitant MGUS can be present in almost 10% of cases and represent a confounding factor for a systemic AL amyloidosis.

As for systemic AL amyloidosis, diagnosis of localized AL amyloidosis requires the identification of amyloid deposits on tissue biopsy and typing of the amyloidogenic precursor with adequate techniques. In the diagnostic workflow, a relevant part is ruling out a systemic amyloidosis. This diagnostic step, that is particularly relevant in presence of MGUS, requires the exclusion of signs of systemic amyloidosis by organ biomarkers (NT-proBNP, proteinuria, alkaline phosphatase) and imaging (mainly echocardiography).

Prognosis and therapy

Localized AL amyloidosis has a generally good prognosis and life expectancy of patients is comparable to general population.[187] However, the natural history of this disease is characterized by frequent local progressions. Local progression can occur in 17-31% of cases,[187,188] and may present with recurrence of the amyloidoma or with progression of amyloid deposition in adjacent

anatomic sites.[196] Local progression may result in further treatment and repeated surgical interventions are often cause of consistent morbidity and quality of life impairment.[188] Progression from localized to systemic AL was observed only in sporadic cases.[187] It has been hypothesized that these rare case of systemic progression are more likely cases in which a paucisymptomatic systemic AL amyloidosis was not correctly identified.[188]

Since patients survival is similar to general population, treatment is not mandatory and should be considered only for symptomatic localizations. Treatment is generally proposed in more than 50-70% of patients and is effective in 50-80% of cases, depending on organ localization.[188] Response to treatment – as local progression – can be assessed according to changes in clinical manifestation, imaging and endoscopic findings. Surgery is the most frequent option and aims to the direct removal of amyloidomas. In laryngeal localized AL amyloidosis, endoscopic surgery with laser CO_2 has been proven effective.[197] There are no evidences that systemic therapy, especially a chemotherapy, may be effective. Radiotherapy has proven effective in small case series and may be considered in selected cases.[198,199]

Objective of the thesis

In this thesis we show the result of our main projects assessing biomarker-based approach in the management of patients with AL amyloidosis. The presented data were mostly collected at the Amyloidosis Research and Treatment Center of Pavia. However, three of the seven presented works were designed and developed thanks to a collaboration with the Amyloidosis Center of Heidelberg. The first part of this work depicts an hypothetical clinical journey in systemic AL amyloidosis, from the assessment of severity of organ involvement to designing the first-line treatment strategy, jumping forward to the choice of a rescue treatment for relapsed/refractory patients. In all of these steps through the clinical history of this rare disease, we show that evaluation of both organ and clonal biomarkers have a central role in the decision-making process and for a tailored-treatment approach. Summarizing our first four projects aimed to:

- evaluate the use of UACR for diagnosis of renal involvement, prognostic stratification and assessment of renal response after treatment **(Objective 1)**;
- assess the effectiveness of a biomarker-based response-driven approach for a sequential treatment strategy of bortezomib-based induction and ASCT in patients with AL amyloidosis **(Objective 2)**;
- indagate the ability of cardiac biomarkers (NT-proBNP) to identify early cardiac response after first-line treatment in patients with stage IIIb AL amyloidosis **(Objective 3)**;
- identify the organ and clonal biomarkers – with a particular focus on cytogenetic aberrations – that could identify the patients who could benefit the most from a rescue treatment with lenalidomide and dexamethasone **(Objective 4)**.

In the second part of the thesis, we moved to other rarer forms of AL amyloidosis, as IgM-AL amyloidosis, AL amyloidosis caused by non-lymphoplasmacytic lymphoprolipherative disorders (LPD) and localized AL amyloidosis. Our attempt was to investigate how clonal and organ

biomarkers help in the characterization of this less frequent diseases and provide us information useful to clinical management. More in detail, the last three projects included in the thesis aimed to:

- evaluate the impact of clonal biomarkers on prognosis in IgM-AL amyloidosis and differences in presentation and outcome according to the clonal B-cell immunophenotype **(Objective 5)**;
- identify the main characteristics of patients with AL amyloidosis and non-lymphoplasmacytic LPD **(Objective 6)**;
- investigate the biomarkers involved in the clinical history of localized AL amyloidosis and factors affecting local progression of the disease **(Objective 7)**.

Objective 1: the use of UACR for diagnosis of renal involvement, prognostic stratification and assessment of renal response after treatment in AL amyloidosis

Renal involvement is frequent in AL amyloidosis, being present in approximately two thirds of patients[200] and although it does not have a major impact on patients' survival, it can result in end-stage renal failure, hampering quality of life and limiting access to treatment and interfering with the interpretation of key clinical chemistry tests.[79,201] 24h-proteinuria is currently required for diagnosis, staging, and response assessment in AL amyloidosis.[84] However, 24h-urine collection is a cumbersome procedure that can cause discomfort to patients and may introduce a preanalytical error.[202,203] Moreover, urine total protein quantification presents analytical limitations compared to albumin measurement.[204] Urinary albumin/creatinine ratio (UACR) represents a valid alternative to estimate urine protein loss in various chronic kidney diseases.[205-208] In the last years, the use of this biomarker in AL amyloidosis was largely discussed and recently the Mayo Clinic group proposed UACR cut-offs for identification of renal involvement, prognostication of renal

survival at diagnosis and definition of renal response.[62] However, in this large study (575 patients included) UACR and 24h-proteinuria samples were collected on the same day only in a half of cases and paired samples at diagnosis were available only in 155 patients (of whom 109 with renal involvement). In the Mayo Clinic study, UACR sample was collected on a random spot urine. More importantly, their study did not include a validation set and UACR-based response criteria were not evaluated on renal outcome. We present our data from a large prospective study on 531 patients with newly-diagnosed AL amyloidosis and paired 24h-proteinuria and UACR (first morning void) samples at baseline and at response assessment in order to define and validate the possibility to replace the 24h-urine collection with a simpler test in the diagnosis, staging, and response assessment of renal AL amyloidosis.

Objective 2: the effectiveness of a biomarker-based response-driven approach for a sequential treatment strategy of bortezomib-based induction and ASCT in patients with AL amyloidosis

ASCT is very effective in AL amyloidosis and the refinement of eligibility criteria led to a significant decrease in transplant-related mortality decreased. In this setting, deep hematologic responses are frequent, with almost 70% of patients achieving at least a VGPR after ASCT.[85,99,102,209] This progress was accompanied by advancements in nontransplant chemotherapy.[89,210,211] Bortezomib is usually offered to subjects who are not eligible for ASCT.[212] However, bortezomib can also be used after ASCT to improve quality of response and extend survival[112,113] and before ASCT, aiming at decreasing plasma cell and light chain burden.[104] This latter approach was evaluated in three clinical trials, confirming that this sequential strategy can result in deeper hematologic responses and longer survival than ASCT alone.[105-107] In particular, bortezomib-based induction can result in deep hematologic responses and organ improvement, considered a satisfactory end point in AL amyloidosis, before ASCT.[100,200] At our center, transplant-eligible patients are treated upfront with

CyBorD and they proceed to ASCT only in case of an unsatisfactory response, evaluated with clonal (dFLC) and organ biomarkers. We report the outcome of 139 consecutive subjects treated according to this sequential biomarker-based response-driven approach.

Objective 3: the ability of NT-proBNP to identify early cardiac response after first-line treatment in patients with stage IIIb AL amyloidosis

Despite the innovation in treatment in AL amyloidosis, patients with an advanced cardiac disease defined as an European cardiac stage IIIb have still a dismal outcome with median OS of 4 months. These patients are considered as high risk and treatment strategy is based on dose-reduced treatment regimens in order to mitigate treatment related toxicity.[212] However, despite the severe prognosis, patients who achieved a rapid and profound reduction of dFLC after chemotherapy benefit from a better survival. This finding pushes forward the research for novel drugs that may be able to grant an extremely rapid a profound decrease in dFLC. Recent studies conducted in vitro and animal models demonstrated that amyloidogenic FLCs have a cardiotoxic activity and directly contribute to cardiac disfunction in AL amyloidosis.[41-45] Therefore, it is reasonable that reductions in dFLC after treatment can be also associated with cardiac responses assessed with NT-proBNP. This is particularly interesting, considering that a possible etiological link between amyloidogenic FLCs and NT-proBNP through activation of the MAPK38 has been proposed in cardiac AL amyloidosis. However, the possibility of an early cardiac response and its impact on outcome have not been evaluated yet in these patients. We tried to respond to this unanswered question presenting data from 249 patients with newly-diagnosed stage IIIb cardiac amyloidosis. Particularly we aimed to evaluate the ability of NT-proBNP to identify early cardiac responses that could translate in an improvement of outcome also in these patients with an extremely severe cardiac amyloidosis.

Objective 4: organ and clonal biomarkers identifying patients with relapsed/refractory AL amyloidosis who could benefit the most from a rescue treatment with lenalidomide and dexamethasone

Lenalidomide and dexamethasone (LDex) is considered a standard treatment for relapsed/refractory AL amyloidosis. The effectiveness of this regimen was first documented in two small clinical trials, even if the maximum tolerated dose of lenalidomide was only 15 mg/day.[142,145] Later, three retrospective studies with less than 100 patients each have further confirmed the efficacy of LDex as rescue treatment, with a hematologic response in 41-61% of cases.[143,147,213] However, treatment with LDex is still a field of open issues like frequent hematologic and non-hematologic toxicities that often require dose reduction and treatment discontinuation. Nephrotoxicity[146,147] and increase of cardiac biomarkers (mainly NT-proBNP) represent further challenges in patient management.[148] Considering this tolerability issues related to LDex, it is important to identify patients who could benefit the most from this rescue treatment. This is particularly relevant, considering that lenalidomide has been recently coupled with new and powerful drugs as daratumumab and ixazomib. Cytogenetic aberrations, as t(11;14) and gain1q21, emerged as another driver of prognosis in AL amyloidosis, especially according to treatment strategy and iFISH results are generally considered in the decision of first-line treatment. However, only few information are available on the impact of t(11;14), gain1q21 and other cytogenetic aberrations on LDex effectiveness. We present the data from 260 patients with relapsed/refractory AL amyloidosis, with a median follow-up of 56 months and cytogenetic data in more than 70% of cases. We tried to evaluate clonal and organ biomarkers, with a peculiar focus on cytogenetic aberrations, that might identify patients with a better outcome after LDex and predict toxicity.

Objective 5: impact of clonal biomarkers on prognosis in IgM-AL amyloidosis and differences in presentation and outcome according to the clonal B-cell immunophenotype

IgM-AL amyloidosis is a rare (5-7% of all AL amyloidosis cases diagnosed at referral centers) a distinct clinical entity, characterized by lower rates of heart involvement, while lymph nodal, soft tissue, lung and PNS involvement are more common.[178] Several studies evaluated the biomarkers and clinical features affecting OS in this rarer form of systemic AL amyloidosis.[173-176,178,179] Even if there is still an open discussion on the impact of liver and PNS involvement, it appears clear that heart involvement and its severity has a major role on prognosis even in IgM-AL amyloidosis. Moreover, it is likely that also soluble clonal biomarkers, as dFLC and IgM levels, may affect the outcome. The study of clonal biomarkers in IgM-AL amyloidosis is particularly relevant, considering the heterogeneity of the B-cell clone that can resemble the characteristics of LPL or PPCN.[179] As recently reported, $MYD88^{L256P}$ mutation was found more frequently in LPL, similarly to what is observed in WM. On the other hand, the cytogenetics aberrations typical of AL amyloidosis – as translocation t(11;14) and gain1q21 – were found mainly in PPCN. These biological differences are potentially relevant for a possible B-cell clone phenotype-oriented treatment strategy, as it has been recently proposed.[183] This targeted ad tailored-treatment approach could result in better hematologic response rate in IgM-AL amyloidosis. Interestingly, the hematologic response assessment is also an open topic in this rare disease. It is largely assessed evaluating changings in dFLC after treatment, as per validated response criteria in AL amyloidosis, but it can also be evaluated also with IgM levels according to WM response criteria.[17] Since this was the result of a consensus, it is not clear which of this two biomarkers can identify patients with a better outcome after treatment. Finally, another open topic in IgM-AL amyloidosis is the evaluation of factors affecting hematologic progression. Since it is has been hypothesized that clonal markers are responsible of long term outcome in AL amyloidosis, it is likely that they

have an impact also in disease progression. We tried to answer to these open question presenting data from 100 patients with newly-diagnosed IgM-AL amyloidosis. We concentrated first on an extensive characterization of the B-cell clone, with a peculiar focus on identification of clonal prognostic factors – especially dFLC and IgM – for OS, hematologic progression and hematologic response. Second, we reported the outcome of different treatment strategies and put them into perspective with the few published results, providing further data for a B-cell clone phenotype-oriented treatment strategy in PPCN and LPL.

Objective 6: clinical characteristics of patients with AL amyloidosis and non-lymphoplasmacytic LPD

In the great majority of cases, systemic AL amyloidosis is caused by an underlying plasma cell clone that genetically resembles MGUS or MM.[19,214,215] In 5-7% of patients, systemic AL amyloidosis is associated with an IgM producing clone, with characteristics of MGUS or WM, that harbor the $MYD88^{L265P}$ mutation in almost three fourths of cases.[17,216] Rarely, systemic and localized AL amyloidosis can be associated with non-lymphoplasmacytic LPD, requiring a distinct, sometimes challenging diagnostic and therapeutic approach. Differently from IgM-AL amyloidosis, this rare association has not been systematically studied so far. Only few, small case series reported an association between MZL of Mucosa Associated Lymphoid Tissue (MALT) and localized AL amyloidosis, mainly with nodular pulmonary involvement.[189,190,217] Recently, German investigators published a series of 29 patients with AL amyloidosis and localized B cell neoplasia, mostly with a MZL-immunophenotype: only 5 patients had systemic lymphoma and 2 were classified as systemic AL amyloidosis.[193] We report the clinical presentation and outcome of 36 patients with non-lymphoplasmacytic LPD and AL amyloidosis. Particular attention was given to the identification of

biomarkers that may help in differential diagnosis between systemic and localized AL amyloidosis in patients with non-lymphoplasmacytic LPD.

Objective 7: biomarkers involved in the clinical history of localized AL amyloidosis and factors affecting local progression of the disease

Our knowledge on localized AL amyloidosis has improved in the past 5 years thanks to description of two large case series. The National Amyloidosis Center (NAC) described for the first time the natural history of this disease in 606 patients.[187] Bladder, larynx and skin emerged as the most commonly involved organs. Furthermore, patients with localized AL amyloidosis may also present concomitant autoimmune disorders, especially Sjögren syndrome, lymphoproliferative diseases, mainly MZL and MGUS.[189,190,195,218] Recently, a report from the Mayo Clinic's group added valuable information about response to therapy and local progression from 413 cases of localized AL amyloidosis.[188] Although life expectancy in this disease was comparable to the general population, the clinical history was characterized by frequent local progressions requiring further treatment. Importantly, repeated surgical interventions were often cause of consistent morbidity and quality of life impairment. Currently, it is not clear if some peculiar characteristics of this complex and heterogeneous disease may affect the prognosis in localized AL amyloidosis, particularly local progression. Moreover, few data are available on local cellular infiltrate and B-cell clone at amyloid deposition site and its role in natural history of localized AL amyloidosis has not been studied so far.[193] We present the results of a comprehensive study conducted on a large series of 293 consecutive patients with verified localized AL amyloidosis. We coupled an extensive characterization of clinical features with detailed pathology data from tissue biopsy. Finally, we described treatment and outcome and studied local and systemic factors that may affect local progression for the first time.

Materials and Methods

Study population

Data were collected from the prospectively maintained databases of patients with newly diagnosed AL amyloidosis at the Amyloidosis Research and Treatment Center of Pavia and the Amyloidosis Center of Heidelberg. More in detail, data were collected in Pavia for the following studies:

- the use of UACR for diagnosis of renal involvement, prognostic stratification and assessment of renal response after treatment in AL amyloidosis;
- the effectiveness of a biomarker-based response-driven approach for a sequential treatment strategy of bortezomib-based induction and ASCT in patients with AL amyloidosis;
- the ability of NT-proBNP to identify early cardiac response after first-line treatment in patients with stage IIIb AL amyloidosis;
- clinical characteristics of patients with AL amyloidosis and non-lymphoplasmacytic LPD.

Data were collected at the Amyloidosis Center of Heidelberg for the following projects:

- organ and clonal biomarkers identifying patients with relapsed/refractory AL amyloidosis who could benefit the most from a rescue treatment with lenalidomide and dexamethasone;
- impact of clonal biomarkers on prognosis in IgM-AL amyloidosis and differences in presentation and outcome according to the clonal B-cell immunophenotype;
- biomarkers involved in the clinical history of localized AL amyloidosis and factors affecting local progression of the disease.

All patients gave written informed consent for their clinical data to be used in retrospective studies in accordance with the Declaration of Helsinki.

Collection of urine samples at the Amyloidosis Research and Treatment Center of Pavia

From October 2013 both 24h-proteinuria and UACR were systemically evaluated in all patients with newly diagnosed AL amyloidosis at the Amyloidosis Research and Treatment Center of Pavia. Patients were asked to bring at first evaluation and at each follow-up visit the 24h-urine collection as well as the first urine morning void. All subjects received oral and written instructions on appropriate 24h collection.

Diagnosis of AL amyloidosis

Histologic diagnosis and amyloid typing

Diagnosis of amyloidosis was confirmed in all cases by Congo red staining on tissue biopsy. Amyloidogenic light chain isotype was identified on tissue by immunohistochemistry with custom made antibodies, immunoelectron microscopy or proteomic analysis in mass spectrometry.[4,6,53] In patients with localized AL amyloidosis, reports from the Pathology Unit were systematically reviewed for data regarding sample biopsy size and cellular infiltrate at amyloid deposition site.

Diagnosis of organ involvement

Organ involvement was defined according to current criteria.[61] In patients with IgM-AL amyloidosis, the presence of hepatomegaly, splenomegaly and lymphadenopathy were assessed by physical examination and imaging techniques. Severity of cardiac and renal involvement at diagnosis were evaluated with European cardiac staging system and renal staging system, respectively. In patients with localized AL amyloidosis, a systemic disease was ruled out by clonal and organ biomarkers in all cases and echocardiogram and fat pad aspirate in selected ones. A multifocal involvement of localized AL amyloidosis was defined as the presence of multiple amyloid lesions in the absence of systemic involvement.

Evaluation of the underling B-cell clone

iFISH evaluation was performed after plasma cell purification by auto-magnetic-activated cell sorting with CD138 immunobeads with commercial 2-color probe sets according to the manufacturer's instructions (Kreatech, Amsterdam, The Netherlands, and MetaSystems, Altlussheim, Germany). The tested panel included IgH translocations t(11;14), t(4;14), and t(14;16) as well as probes for detecting numerical changes of the loci 1q21, 5p15, 5q35, 8p21, 9q34, 13q14, 15q22, 17p13, and 19q13. As in previous analyses, t(4;14), t(14;16), and deletion 17p13 were classified as high-risk aberrations in analogy to MM.[219] Gains of 5p15/5q35, 9q34, and 15q22—whenever 2 of 3 were present—were categorized as hyperdiploidy according to the score by Wuilleme et al.[220] In patients with IgM-AL amyloidosis, the characterization of the B-cell clone was performed combining bone marrow histology, multiparameter flow cytometry, $MYD88^{L256P}$ and iFISH data, allowing a correct identification of LPL or PPCN in 92% of cases. In patients with localized AL amyloidosis, the local B-cell clone was searched on tissue biopsy by immunohistochemistry, in situ-hybridization or PCR-based immunoglobulin heavy chain (IGH) and light chain (IGK) gene rearrangement analyses.

Treatment strategy and schedule

Sequential strategy of bortezomib induction followed by ASCT

From 2009 upfront therapy with CyBorD – with bortezomib 1.3 mg/m^2 and dexamethasone 40 mg weekly – was offered to all patients with newly-diagnosed AL amyloidosis at the Amyloidosis Research and Treatment Center of Pavia who were eligible for ASCT. Eligibility required age <65 years, NT-proBNP <5000 ng/L, eGFR >50 mL/min (unless dyalisis), NYHA <III, *performance status* (*Eastern Cooperative Oncology Group*) ≤2, ejection fraction >45%. Hematologic and organ response response were assessed every two CyBorD cycles and 3 months after ASCT, according to

validated criteria. Subjects attaining ≥PR after 2 CyBorD cycles continued chemotherapy until response plateaued, for up to 6 cycles. Patients who did not achieve satisfactory response after CyBorD proceeded to ASCT (melphalan 200 mg/m^2), if still eligible. Satisfactory response was defined as CR, VGPR plus organ response, or PR plus organ response.

Treatment with LDex in relapsed/refractory AL amyloidosis

Patients received lenalidomide (days 1-21) and dexamethasone (days 1, 8, 15 and 22) in 28-days cycles. Lenalidomide dose was adjusted according to clinical status and renal function. Every patient received thrombosis prophylaxis with acetylsalicylic acid (100 mg/day) or with low-molecular-weight heparin in case of history of thrombosis. Duration of treatment was decided according to treatment effectiveness and tolerability.

Response assessment in systemic AL amyloidosis

Hematologic response

Hematologic response was evaluated by intent-to treat according to the current validated criteria of the International Society of Amyloidosis (ISA).[87,88] In patients with dFLC at diagnosis between 20-50 mg/L, hematologic response was assessed according to recent proposed low-dFLC criteria. A very good hematologic response (VGHR) was defined as the achievement of CR, VGPR or low-dFLC partial response. In patients with IgM-AL amyloidosis, hematologic response was also evaluated with changes of IgM levels from baseline after chemotherapy, according to WM response criteria. More precisely, CR was defined as normal IgM levels and absence of IgM monoclonal protein at immunofixation, VGPR as reduction in IgM levels >90% and PR as reduction of IgM levels >50%.[221] Hematologic response was evaluated at 3 and 6 months after treatment initiation. In patients with stage IIIb cardiac AL amyloidosis, hematologic response was evaluated at 30 and 90 days after

starting therapy in order to detect early responses. In subjects treated with the sequential treatment approach, hematologic response was reported at the end of induction therapy and at 3 months after ASCT.

Organ response and progression

Organ response and progression were defined according to current validated criteria. More in detail, cardiac response was defined reduction in NT-proBNP >30% and >300 ng/L from baseline.[87] Renal response was identified by reduction in 24h-proteinuria >30%, in absence a worsening in eGFR >25%.[84] Conversely, cardiac and progression were defined as increase in NT-proBNP >30% and >300 ng/L and reduction in eGFR >25% from baseline, respectively. In patients with stage IIIb cardiac AL amyloidosis, depth of cardiac response was graded according to novel proposed criteria for graded cardiac response:

- complete cardiac response (cardiac CR): nadir NT-proBNP ≤400 ng/L;
- very good partial cardiac response (cardiac VGPR): NT-proBNP reduction >60% from baseline, not meeting complete organ response definition;
- partial cardiac response (cardiac PR): target biomarker reduction 31-60% from baseline.[96]

Organ response and progression were evaluated at 3 and 6 months after treatment initiation. Cardiac response was assessed earlier (at 30 and 90 days after starting therapy) in patients with stage IIIb cardiac AL amyloidosis. In subjects treated with the sequential treatment approach, organ response was reported at the end of induction therapy and at 3 months after ASCT.

Combined cardiac and hematologic response model

In patients with stage IIIb cardiac amyloidosis, a combined hematologic and cardiac and hematologic response (CHCR) model was tested for the identification of subjects with better outcome after treatment. CHCR was built referring to the proposed and validated CHOR model.[97]

More precisely, it was designed using combining scores of 0-3 for hematologic response (0-CR, 1-VGPR, 2-PR, 3-no response) and 0-1 for cardiac response (0-cardiac response, 1-no cardiac response).

Local progression in localized AL amyloidosis

Follow-up of amyloidomas was performed by clinical, radiographic and endoscopic examination. Local progression was defined according to changes in symptoms and/or size of the amyloidomas and was calculated from diagnosis. Particularly, changes in size of the amyloidomas at imaging were evaluated for progression in asymptomatic patients. Progression to systemic AL amyloidosis was defined by detection of the amyloidogenic FLCs in the serum and/or urine, onset of another involved organ site with detection of the FLCs in abdominal fat pad or organ biopsy.

Statistical analysis

Correlation between 24h-proteinuria and UACR and UACR-based renal staging system and response

Correlation between 24h-proteinuria and UACR at baseline was assessed by Pearson's r test. We tested in the overall population The UACR cut-offs identified by the Mayo Clinic investigators for identification of renal involvement (300 mg/g, corresponding to the 24h-proteinuria cut-off of 0.5 g/24h), renal staging (3600 mg/g, corresponding to the 24h-proteinuria cut-off of 5 g/24h) and renal response (decrease in UACR from baseline >30%, corresponding to a decrease in 24h-proteinuria >30%) were tested in the overall population.[62] The study population was further divided in a testing (354 patients) and in an internal validation cohort (177 patients) to explore and validate the benefit of the proposed UACR-based renal response criterion – i.e. reduction in UACR from baseline >30% in absence of a reduction in eGFR >25% at 6 months – on renal survival.

Patients were randomly assigned to one of these cohorts with a rate of 2:1. Only patients evaluable for UACR-based renal response – defined as a baseline UACR >300 mg/g – were included in this analysis.

Descriptive statistical analysis

Associations between categorical variables were tested using the Chi-squared test, Kruskal-Wallis tests were used to test for a difference in continuous variables. Fisher's exact test and Mann-Whitney test were used to assess differences in nominal and continuous variables between testing and validation cohort for evaluation of UACR-based renal response and between LPL and PCCN in IgM-AL amyloidosis. Median and interquartile range (IQR) were reported for continuous variables.

Survival analysis

Overall survival, progression free survival (reported as hematologic event-free survival [hemEFS] or time to next treatment or death [TNTD]) and renal survival (RS) were calculated from diagnosis or from treatment initiation in case of relapsed refractory AL amyloidosis treated with LDex. Renal survival was defined as time from diagnosis of AL amyloidosis – or LDex initiation – to dialysis or last contact. Patients who died before progression to dialysis were censored at time of death. Since there are no validated progression criteria for AL amyloidosis, time to disease progression is not uniformly evaluated between different Centers. In Pavia, this is assessed by TNTD. Time to next treatment or death was defined as time from diagnosis of AL amyloidosis – or LDex initiation – to next treatment line, death or last contact. In Heidelberg, hemEFS is used for evaluation of time free from disease progression. Hematologic-event free survival is defined as time from diagnosis of AL amyloidosis – or LDex initiation – to hematologic relapse or progression, change of treatment, death or last contact. Survival curves were plotted according to Kaplan Meier, and differences in survival were tested for significance with the log-rank test. Deaths occurring in the

first 100 days after initiation of CyBorD or ASCT where classified as treatment-related. Landmark analysis was performed to evaluate the benefit in survival of hematologic and organ response excluding early deaths.

Multivariable analysis models

Multivariate (cause-specific) Cox hazard regression models and logistic regression analysis were used for the identification of prognostic baseline factors for survival and VGHR in patients with IgM-AL amyloidosis and in relapsed/refractory AL amyloidosis treated with LDex.

Multivariable analysis and logistic regression analysis in relapsed/refractory AL amyloidosis treated with LDex

Factors included in the model were age (as standard variable), LC isotype (to evaluate differences between λ and κ clones), dFLC, NT-proBNP and eGFR and 24h-proteinuria (important established prognostic biomarkers), t(11;14), gain1q21 and high risk cytogenetics (as relevant cytogenetic aberrations), starting dose of lenalidomide (to evaluate whether higher doses resulted in better outcome), previous ASCT (to assess the impact of the most effective treatment before RD) and year of RD initiation (to investigate possible changings in RD administration and/or availability of novel rescue treatments over time). Patients in dialysis were included for the identification of prognostic factors for OS, hemEFS and VGHR and were excluded for evaluation of RS.

Multivariable analysis in IgM-AL amyloidosis

We included in the model age (as a standard variable), LC isotype and clonal phenotype (to assess differences between λ and κ clones and LPL and PPCN), dFLC, IgM levels, European cardiac staging system and treatment status before diagnosis of IgM-AL amyloidosis (to investigate whether previous treatment history of the underlying B-cell clone affected prognosis).

Statistical imputation and missing data

In the project involving patients with relapsed/refractory AL amyloidosis treated with LDex, statistical imputation has been performed for the covariates used in the multivariate models for the endpoints OS, hemEFS and RS separately using multiple imputations by chained equations.[222] In addition to a selected set of covariates that are expected to be associated with the variable to be imputed, the respective survival endpoint itself has been included in the imputation model via the baseline hazard function and the event indicator.[223] The results of the 100 imputed data sets have been pooled using Rubin's rules.[224] Replacement of missing data of European cardiac and renal staging system in patients with IgM-AL amyloidosis or relapsed/refractory AL amyloidosis treated with LDex was performed based on expert knowledge thanks of an extensive evaluation of organ biomarkers throughout the whole clinical history and considering the severity of organ involvement at diagnosis in case of relapsed/refractory AL amyloidosis. European cardiac stage was inserted in 20 patients with IgM-AL amyloidosis and 107 with relapsed/refractory AL amyloidosis treated with LDex, respectively. Renal staging system was inserted in 51 cases of relapsed/refractory AL amyloidosis treated with LDex.

Software for statistical analysis

Two different software for statistical analysis were used at the Amyloidosis Research and Treatment Center of Pavia and at the Amyloidosis Center of Heidelberg. In Pavia, MedCalc Statistical Software version 14.12.0 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014) was used for computation. At the Amyloidosis Center of Heidelberg, calculations were performed using the statistical software environment R (version 4.0.1), together with the R packages survival (version 3.2-3), mice (version 3.9.0) and multcomp (version 1.4-13).

Results

Objective 1: the use of UACR for diagnosis of renal involvement, prognostic stratification and assessment of renal response after treatment in AL amyloidosis

From October 2013 to December 2018, 531 consecutive patients, newly-diagnosed at the Amyloidosis Research and Treatment Center of Pavia with AL amyloidosis were included in the study. Patients' characteristics are reported in Table 5.

Table 5. Characteristics of 531 patients with newly-diagnosed AL amyloidosis and 24h-proteinuria and UACR evaluation

Variables	**Overall population 531 patients N (%) – median (IQR)**
Age, years	66 (57-73)
Sex, male	304 (57)
Organ involvement Heart / Kidney / Liver / ST / PNS / ANS	393 (74) / 328 (62) / 46 (9) / 92 (17) / 35 (7) / 26 (5)
Mayo Stage I / II / IIIa / IIIb	N=482 71 (15) / 185 (38) / 132 (27) / 94 (20)
Renal staging I / II / III	236 (44) / 221 (42) / 74 (14)
Proteinuria, g/24h	1.30 (0.24-5.68)
eGFR, mL/min x 1.73 m^2	60 (35-85)
UACR, mg/g	1185 (89.8-5990)
Monoclonal component IgG / IgA / IgM / IgD / FLC	184 (35) / 60 (11) / 28 (6) / 3 (1) / 251 (47)
LC isotype Kappa / Lambda	112 (21) / 419 (79)
dFLC, mg/L	170 (66-438)
dFLC <50 mg/L	104 (20)
BMPC, %	10 (7-15)
Treatment CyBorD / BMDex / BDex / MDex / IMiDs Rituximab / other*	274 (52) / 92 (17) / 22 (4) / 64 (12) / 28 (5) 20 (3) / 10 (2)

ANS, autonomic nervous system involvement; BDex, bortezomib and dexamethasone; BMDex, bortezomib, melphalan and dexamethasone; BMPC, bone marrow plasma cells; CyBorD, cyclophosphamide, bortezomib and dexamethasone; dFLC, difference between involved and uninvolved free light chains; eGFR, estimated glomerular filtration rate; FLC, free light chain; IMiDs,

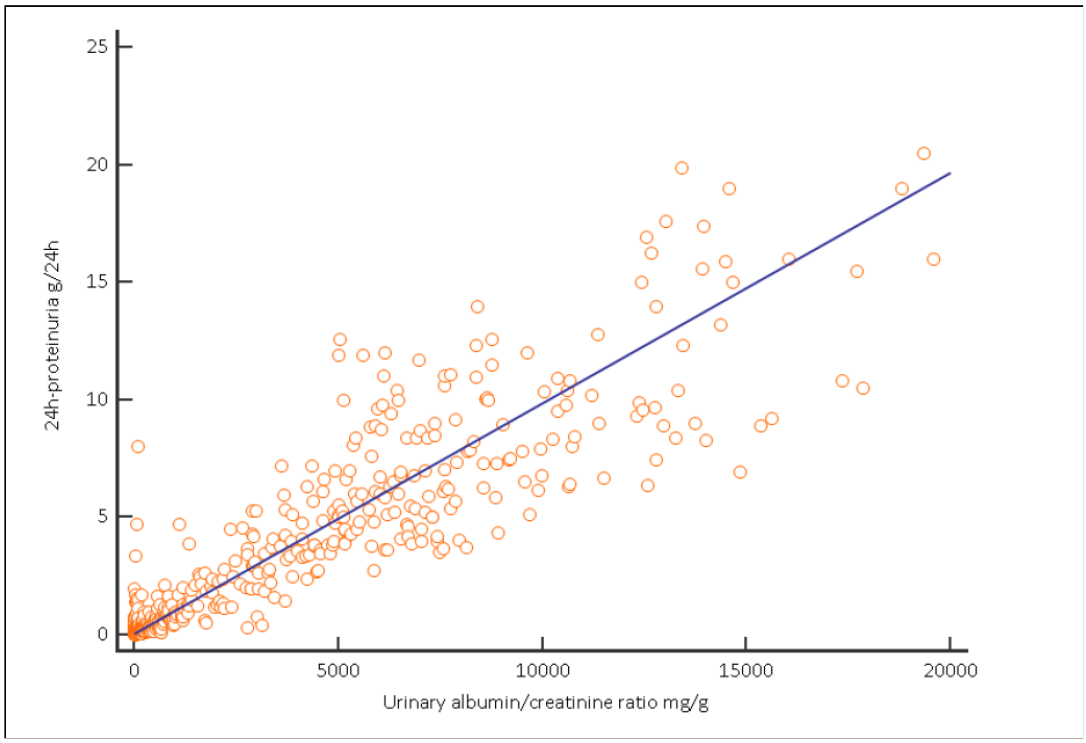
immunomodulatory drugs; LC, light chains; MDex, melphalan and dexamethasone; PNS, peripheral nervous system; ST, soft tissues; UACR, urinary albumin to creatinine ratio

**High dose dexamethasone in 8, autologous stem cell transplant in 1 and daratumumab, bortezomib and dexamethasone in 1 case respectively.*

Renal involvement, defined as a baseline 24h-proteinuria >0.5 g/24h, was present in 328 patients (62%). In patients with renal AL amyloidosis, renal stage, based on 24h-proteinuria and eGFR, was I in 93 (28%), II in 161 (49%) and III in 74 (23%) patients, respectively. In the overall population, baseline median 24h-proteinuria and UACR were 1.30 g/24h (IQR: 0.24-5.68) and 1185 mg/g (IQR: 90-5990), respectively. No statistically significant differences were observed between the internal testing and validation cohorts.

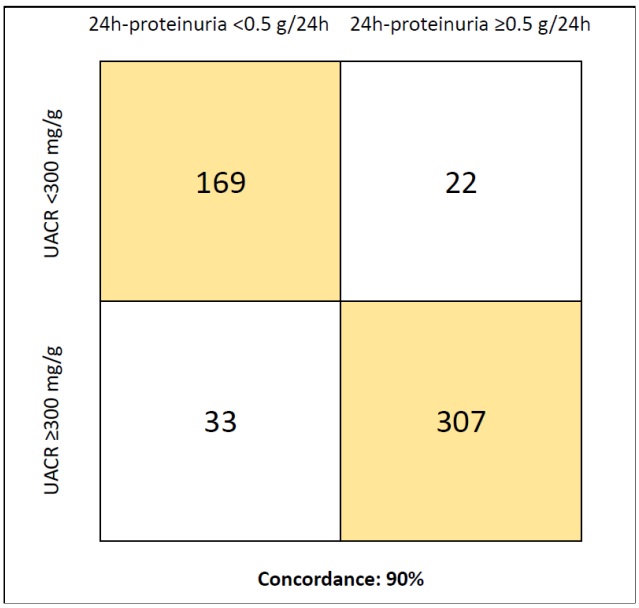
In the overall population, there was a strong linear correlation between 24h-proteinuria and UACR at baseline, with a correlation coefficient r of 0.90 (95% CI: 0.89-0.92; P<0.001; Figure 8).

Figure 8. Correlation between 24h-proteinuria and UACR



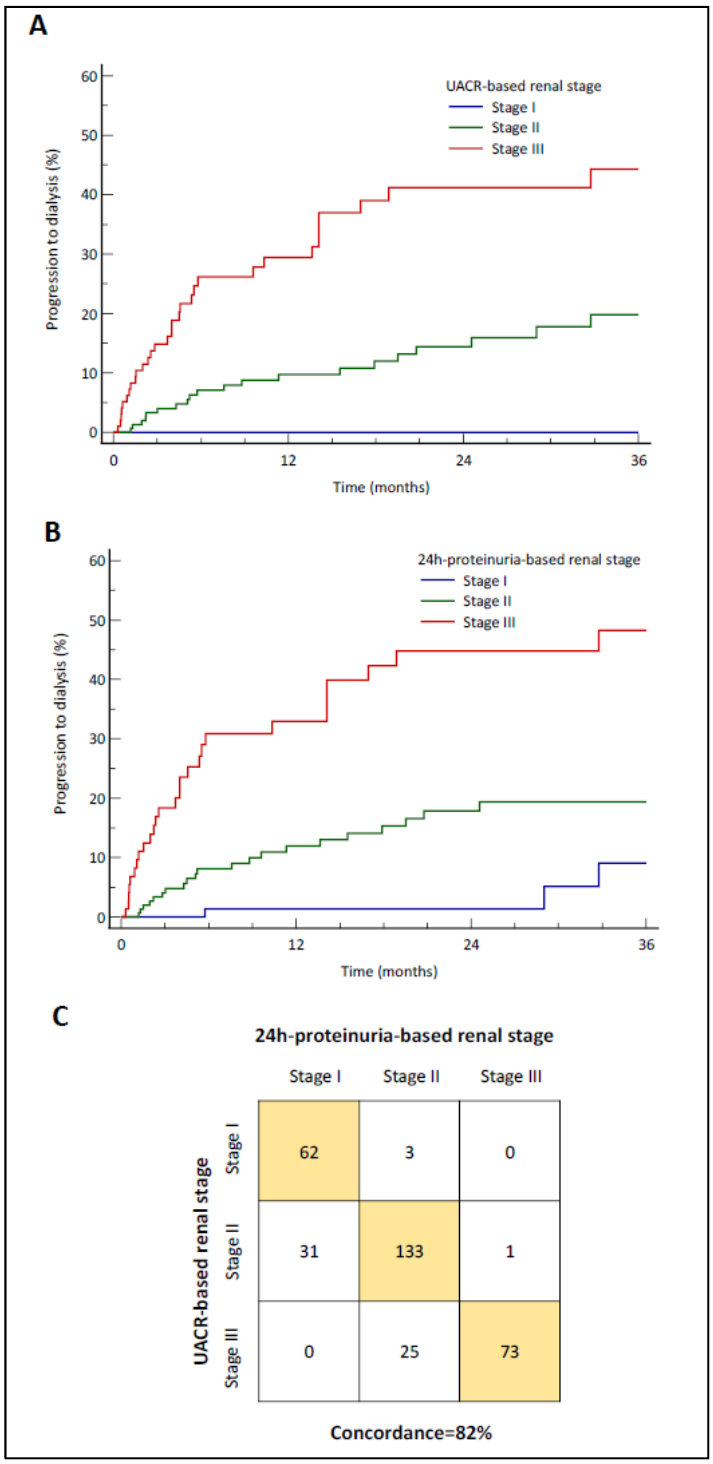
The Mayo Clinic UACR cut-off (300 mg/g) classified as having renal involvement 340 (64%) patients, with a 90% concordance (95% CI: 87-92%) with the 24h-proteinuria cut-off (Figure 9).

Figure 9. Concordance in identification of renal involvement between 24h-proteinuria and UACR



After a median follow-up of living patients of 31 months, 57 (11%) had a progression of renal dysfunction to end stage renal failure requiring dialysis. We explored whether a UACR-based renal staging system (replacing the 24h-proteinuria of 5 g/24h cut-off with the UACR cut-off of 3600 mg/g) was able to stratify patients with renal involvement in three stages with statistically significant different risk of progression to dialysis. Of 328 patients with renal involvement, 65 (20%) were stage I, 165 (50%) stage II and 98 (30%) stage III. Rate of dialysis at 36 months was significantly lower in stage I patients compared to stage II (0% vs. 20%; P=0.026) and in stage II subjects compared to stage III (20% vs. 44%; P<0.001). No stage I patient required dialysis during the study period (Figure 10A). According to the validated 24h-proteinuria-based renal staging system, of the same 328 patients with renal involvement, 93 (28%) were stage I, 161 (49%) stage II, and 74 (23%) stage III (Figure 10B).

Figure 10. UACR-based compared to 24h-proteinuria-based for renal staging system



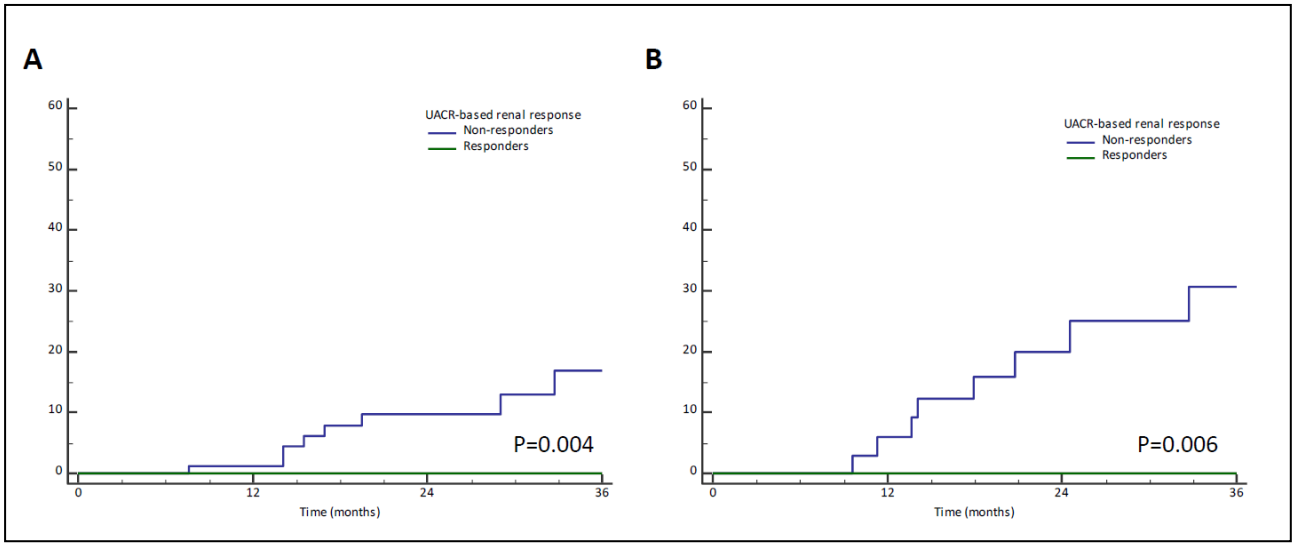
Renal staging system based on UACR (cut-off 3600 mg/g) and eGFR (cut-off 50 mL/min) (A); renal staging system based on 24h-proteinuria (cut-off 5 g/24h) and eGFR (cut-off 50 mL/min): rate of dialysis at 36 months was 10% in stage I, 20% in stage II and 49% in stage III (B); concordance

between renal staging system based on 24h-proteinuria (cut-off 5 g/24h) and that one based on UACR (cut-off 3600 mg/g) (C)

Three stage I patients progressed to end stage renal failure. The 24h-proteinuria-based and UACR-based renal staging systems were concordant in 82% (95% CI 77%-86%) of cases (Figure 10C). Interestingly, 3 of the 31 subjects who were re-classified as renal stage II from renal stage I according to the UACR-based renal stage required dialysis initiation.

A UACR-based renal response defined as a reduction of UACR >30% from baseline at 6 months in the absence of a concomitant reduction of eGFR >25%, was observed in 58/220 (26%) evaluable patients. We evaluated whether this UACR renal response criterion was able to predict renal survival in the testing (N=354 patients) and in the validation cohort (N=177 patients). Details about first-line treatment are reported in Table 5. UACR-based renal response was observed in 41/153 (27%) evaluable patients in the testing cohort and in 17/67 (25%) evaluable cases in the validation cohort. The 6-months landmark analysis showed that patients who achieved a renal response according to this UACR-based criterion had a statistically significant lower dialysis-rate at 36 months both in the testing (0% vs. 17%; P=0.004; Figure 11A) and in the validation cohort (0% vs. 31%; P=0.006; Figure 11B). Concordance between UACR-based renal response and 24h-proteinuria-based renal response was 85% (95% CI 79-89%). Comparison of renal response rate according to 24h-proteinuria and UACR-based criteria and rate of dialysis in renal responders is reported in Table 6. Four patients who resulted as renal responders according to the 24-proteinuria criterion progressed to end-stage renal failure. Importantly, these subjects did not achieve a UACR-based renal response. No patient who achieved a UACR-based renal response progressed to end-stage renal failure requiring dialysis, both in the testing and in the validation cohort.

Figure 11. Impact of UACR-based renal response at 6 months on renal survival



UACR-based renal response was defined as reduction in UACR >30% from baseline, in absence of a concomitant decrease in eGFR >25%. UACR-based renal response in the testing cohort (A). UACR-based renal response in the validation cohort (B). Analysis was performed with a 6-months landmark.

Table 6. Renal response according to 24h-proteinuria and UACR-based criteria

Variables	**24h-proteinuria-based criteria 222 evaluable patients**	**UACR-based criteria 219 evaluable patients**
Renal response rate at 6 months	32% (95% CI 26-38%)	26% (95% CI 21-33%)
Rate of dialysis at 36 months from diagnosis in renal responders	7% (95% CI 2-13%)	0% (95% CI 0-5%)

UACR, urinary albumin to creatinine ratio

Objective 2: the effectiveness of a biomarker-based response-driven approach for a sequential treatment strategy of bortezomib-based induction and ASCT in patients with AL amyloidosis

Between 2009 and 2018, 139 consecutive patients (Table 7) were included, representing 15% of all of our patients. They received a median of 4 cycles of CyBorD (range, 2-6 cycles). Twenty subjects (14%) experienced grade 3-4 adverse events (fluid retention in 7 patients, cytopenia in 5 patients, infection in 3 patients, and acute renal failure, deep venous thrombosis, angina, hypokalemia, and neuropathy in 1 patient each). One patient (cardiac stage IIIa) died suddenly within 100 days. Overall hematologic response was 68% (95 patients), with 26 (19%) CRs and 45 (32%) VGPRs. Cardiac and renal responses were observed in 13 of 43 (30%) and in 31 of 100 (31%) evaluable cases, respectively. Overall, 63 patients (45%) achieved a satisfactory response. None of them was transplanted at relapse. Sixteen subjects (11%) did not respond satisfactorily but did not proceed to ASCT because of organ progression. Five additional subjects (4%) with an unsatisfactory response refused to proceed to ASCT. None of these 21 patients received ASCT at relapse.

Table 7. Characteristics of 139 patients with newly diagnosed AL amyloidosis eligible to ASCT

Variables	**Overall population 139 patients N (%) – median (IQR)**
Age, years	56 (50-61)
Sex, male	77 (55)
Organ involvement Heart / Kidney / Liver / ST / ANS / PNS	66 (47) / 104 (75) / 16 (11) / 19 (14) / 6 (4) / 9 (6)
More than 2 organs involved	15 (12)
Cardiac stage I / II / IIIa / IIIb	62 (45) / 71 (51) / 3 (22) / 0 (0)
NT-proBNP, ng/L	360 (133-840)
Renal stage I / II / III	70 (51) / 69 (49) / 0 (0)
Proteinuria, g/24h	4.80 (0.67-7.00)
eGFR, mL/min per 1.72 m^2	84 (64->90)
dFLC, mg/L	100 (42-318)
dFLC <50 mg/L	42 (30)
BMPC, %	10 (6-15)

ANS, autonomic nervous system; BMPC, bone marrow plasma cells; dFLC, difference between involved and uninvolved free light chains; eGFR, estimated glomerular filtration rate; NT-proBNP, N-terminal natriuretic pro-peptide type B; PNS, peripheral nervous system; ST, soft tissues.

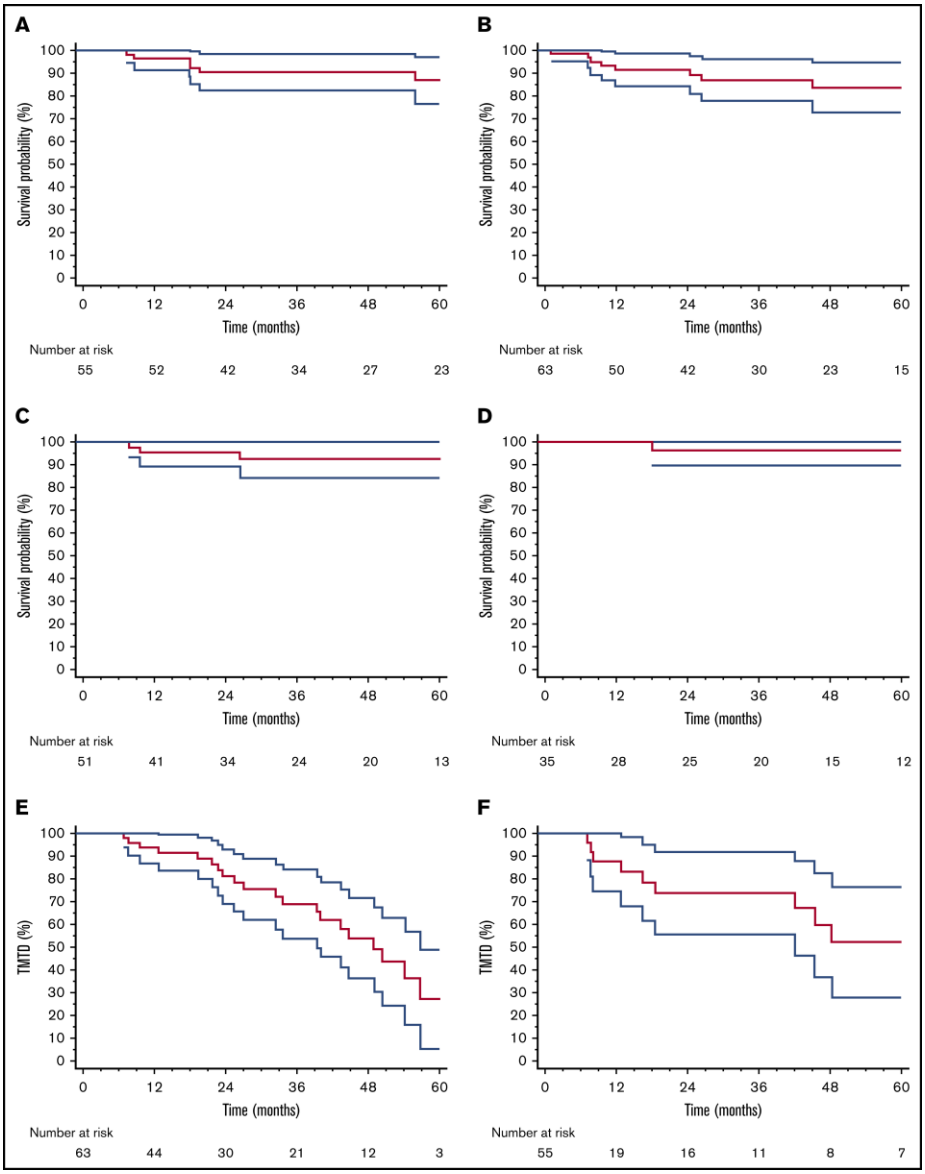
The remaining 55 subjects (40%) underwent ASCT after a median of 5 months from CyBorD initiation. Thirteen had achieved a VGPR after CyBorD and 6 had attained a PR, whereas the remaining were nonresponders. Grade 3-4 adverse events were infection in 10 patients (18%), renal insufficiency in 3 patients (5%), heart failure in 3 patients (5%), and cytopenia, syncope, and deep venous thrombosis in 2 (4%) cases each. No patient died within 100 days. Overall hematologic response rate was 80%, with 21 (38%) CRs and 15 (27%) VGPRs. Cardiac or renal response was observed in 2 of 9 (22%) and 17 of 37 (46%) evaluable patients, respectively.

In the overall cohort, hematologic response rate after CyBorD, with or without ASCT, was 76%, with 47 (34%) CRs and 40 (29%) VGPRs. Cardiac response was achieved in 15 of 43 (35%) patients, and renal response was achieved in 48 of 100 evaluable subjects (48%). Among the 21 patients with unsatisfactory response after CyBorD who did not proceed to ASCT, 12 were rescued with lenalidomide, 4 were rescued with daratumumab, and 5 were rescued with melphalan/dexamethasone. Overall, 8 patients (38%) attained hematologic response, with 2 CRs (10%) and 3 VGPRs (14%). Cardiac response was observed in 1 of 6 patients, and renal response was achieved in 2 of 8 evaluable patients.

After a median follow-up of living patients of 48 months, 27 subjects died, and OS was 80% at 5 years. Five-year OS was 86% in patients who proceeded to ASCT and 84% in subjects who satisfactorily responded to CyBorD (Figure 12A-B; P=0.438). Survival also was not different between the 2 groups in 6- and 12-month landmark analyses accounting for different treatment durations or when considering only patients who achieved a VGPR or better (5-year OS 92% vs 96% with CyBorD alone or followed by ASCT, P=0.425; Figure 12C-D). Likewise, TNTD was not different between patients treated with CyBorD alone and those who also received ASCT (median, 49 vs 60 months, P=0.670; Figure 12E-F). This was confirmed when the analysis was limited to patients in CR (median, 54 months vs not reached; P=0.692). In patients who did not proceed to

ASCT despite failing to satisfactorily respond to CyBorD, OS was 51% at 5 years (P<0.001 vs other groups).

Figure 12. OS and TNTD in patients treated with CyBorD and ASCT and in those who received CyBorD alone



OS in patients treated with CyBorD and ASCT (A). OS in patients with a satisfactory response to CyBorD (B). Six-month landmark plotted OS in patients treated with CyBorD (C) or with CyBorD and ASCT (D) who achieved a VGPR or better. Six-month landmark plotted TNTD in patients who received only CyBorD (E) or CyBorD and ASCT (F). Figure from Basset, et al. Blood Advances 2021

Objective 3: the ability of NT-proBNP to identify early cardiac response after first-line treatment in patients with stage IIIb AL amyloidosis

Two hundred forty-nine patients with stage IIIb AL amyloidosis were identified from the prospectively maintained database of the Amyloidosis Research and Treatment Center of Pavia. Patients' characteristics are reported in Table 8.

Table 8. Characteristics of 249 patients with stage IIIb AL amyloidosis

Variables	**Overall population 249 patients N (%) – mean (IQR)**
Age, years	68 (60-74)
Sex, male	145 (58)
Organ involvement (other than heart) Kidney / Liver / ST / GI / ANS / PNS	 129 (52) / 43 (17) / 51 (20) / 6 (2) / 21 (8) / 23 (9)
Number of involved organs	2 (1-3)
Isolated heart involvement	74 (30)
NYHA class: I / II / III / IV	33 (13) / 64 (26) / 139 (56) / 13 (5)
PS-ECOG: 0 / 1 / 2 / 3 / 4	3 (1) / 59 (24) / 103 (41) / 80 (32) / 4 (2)
NT-proBNP, ng/L	17089 (12179-25014)
Troponin I, ng/mL	0.265 (0.169-0.500)
eGFR, mL/min x 1.73m^2	43 (27-61)
Renal stage: I / II / III	108 (43) / 111 (45) / 26 (10)
Intact MC IgM	121 (49) 7 (3)
LC isotype Kappa / lambda	 53 (21) / 196 (79)
dFLC, mg/L	259 (142-543)
dFLC <50 mg/L	13 (5)
dFLC >180 mg/L	167 (67)
BMPC, %	12 (8-20)
Treatment Bortezomib / MDex / IMiDs / Rituximab	 118 (47) / 95 (38) / 28 (11) / 5 (1)

ANS, autonomic nervous system involvement; BMPC, bone marrow plasma cells; dFLC, difference between involved and uninvolved free light chains; eGFR, estimated glomerular filtration rate; GI, gastrointestinal; IMiDs, immunomodulatory drugs; LC, light chain; MC, monoclonal component; MDex, melphalan and dexamethasone; NYHA, New York Heart Association; PNS, peripheral nervous system; PS-ECOG, performance status according to the Eastern Cooperative Oncology Group; ST, soft tissues

Kidney was involved in 129 (52%) patients, while in 74 (30%) heart was the only involved organ. Median NT-proBNP and troponin I were 17089 ng/L (IQR: 12179-25014 ng/L) and 0.265 ng/mL (IQR: 0.169-0.500 ng/mL), respectively. NYHA class and PS-ECOG were than 2 in 152 (61%) and 84 (34%) cases. Median BMPC was slightly higher than observed in systemic AL amyloidosis (12%; IQR: 8-20%).[16] Similarly, the percentage of patients with low dFLC at diagnosis (dFLC cut-off: 50 mg/L) seems to be also lower that previous reported data in AL amyloidosis.[82,83]

After a median follow-up of living patients of 52 months, 219 (84%) patients died and median overall survival was 4 months. Patients received a bortezomib-based regimen in 118 (47%) cases (CyBorD in 29% and BMDex in 18% of patients). MDex was administered in 95 patients (38%), of whom 65 were treated before 2011 (i.e. the approbation year of bortezomib in newly-diagnosed AL amyloidosis). Data on hematologic and cardiac response are summarized in Table 9. Hematologic response was observed in 50 (21%) patients (9% at least VGPR) at 30 days and in 53 (23%) subjects (15% at least VGPR) at 90 days after starting chemotherapy. A 30 days- and 90 days-landmark analysis showed that Achieving at least a VGPR at 30 and 90 days after starting chemotherapy was associated with a better overall survival (51 vs. 3 months; P<0.001 and 51 vs. 6 months; P<0.001, respectively). Cardiac response at 90 days was observed in 19 (8%) subjects. The impact of cardiac response on survival was evaluated with a 90-months landmark analysis.

Table 9. Hematologic and cardiac response in stage IIIb AL amyloidosis

Response	Response at 30 days N (%)	Response at 90 days N (%)
Any hematologic response*	50 (21)	53 (23)
CR / VGPR / PR	6 (3) / 14 (6) / 30 (12)	11 (5) / 23 (10) / 19 (8)
At least VGPR	20 (9)	34 (15)
Cardiac response†	Not evaluated	19 (8)
CR / VGPR / PR		0 (0) / 10 (4) / 9 (4)
CHCR‡		
Score 0-2	Not evaluated	37 (16)
Score 3-4		196 (84)

CCHR, composite cardiac and hematologic response; CR, complete response; PR, partial response; VGPR very good partial response.

**235 patients were evaluable for hematologic response at 30 and 90 days from starting treatment*

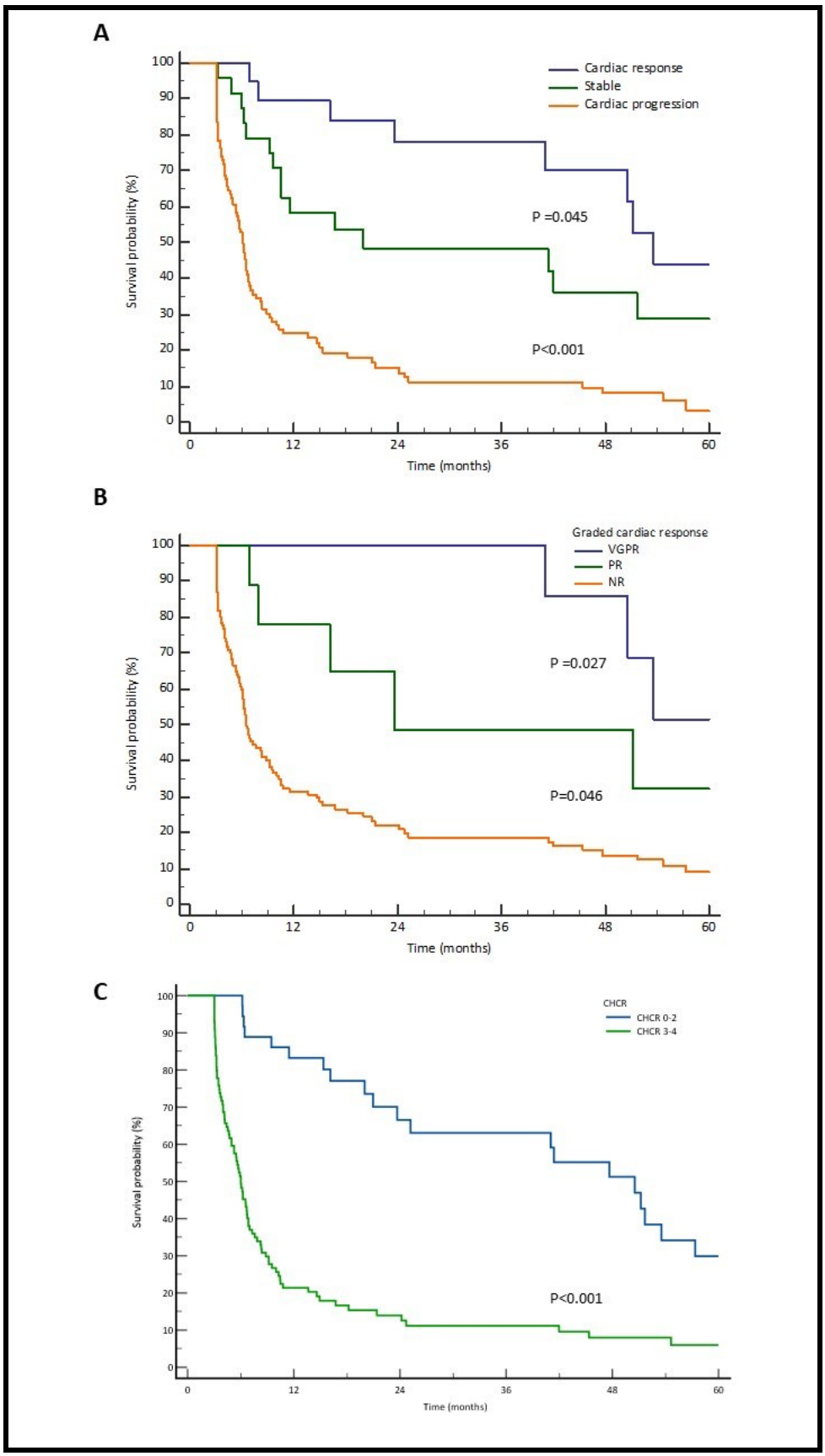
†236 patients were evaluable for cardiac response at 30 days from initiation of chemotherapy

‡233 patients were evaluable for hematologic and cardiac response at 90 day from treatment initiation

Overall survival was significantly better among cardiac responders compared to those with stable cardiac disease and those that had a cardiac progression (median OS 54, 20 and 6 months, respectively; Figure 13A). According to the proposed criteria for graded cardiac response, no patients achieved a cardiac CR, 10 a cardiac VGPR and 9 a cardiac PR. Response data at 90 months showed that the deeper the cardiac response is, the better is the benefit in survival (Figure 13B). We also evaluated the ability of a CHCR model in 233 evaluable patients to identify subjects with a better outcome after 90 months of starting treatment. A CHCR score 0-2 was observed in 37 (16%) evaluable patients, while it was 3-4 in the remaining 196 (84%) cases. Patients with a CCHR score

of 0-2 had a better outcome compared to those with a score of 3-4 (median OS 51 vs. 6 months; P<0.002; Figure 13C). Cardiac progression was observed in 197 (80%) patients and was associated with a shorter survival (20 vs. 3 months; P<0.001), also in subjects who achieved at least a VGPR at 90 days (50 vs. 20 months; P=0.02). Univariable analysis identified baseline dFLC >500 mg/L (HR 1.94, 95% CI 1.43-2.63, P<0.001) and troponin I >0.5 ng/mL (HR 1.50, 95% CI 1.10-2.05, P=0.01) as prognostic factors for survival. Patients with either of these biomarkers above the cutoff had a worse prognosis (6 vs. 3 months; P<0.001). Having a dFLC >500 mg/L was associated with lower rates of high-quality hematologic response at 90 days (at least VGPR in 5% vs. 18%; P=0.002). However, there was no significant difference in the rate of cardiac response in patients with dFLC (25% vs. 20%; P=0.687) or troponin I (19% vs. 20%; P=0.796) above or below the cutoffs

Figure 13. 90-months landmark analysis evaluating the impact of cardiac response in stage IIIb AL amyloidosis



Overall survival in patients with cardiac response (median OS 54 months), cardiac stable disease (median OS 20 months) and cardiac progression (median OS 6 months) at 90 months (A). Overall survival in patients with cardiac VGPR (median OS 92 months), cardiac PR (median OS 24 months) and cardiac NR (median OS 6 months) at 90 days (B). Overall survival in patients with isolated organ involvement with a CHCR score of 0-2 (median OS 51 months) and 3-4 (median OS 6 months) at 90 days (C).

Objective 4: organ and clonal biomarkers identifying patients with relapsed/refractory AL amyloidosis who could benefit the most from a rescue treatment with lenalidomide and dexamethasone

Two-hundred sixty patients with relapsed/refractory AL amyloidosis were treated with LDex as a rescue treatment (Table 10). Median time from diagnosis to treatment with LDex was 17 months (IQR: 8-36). Patients received a median of 2 (IQR: 1-2) previous treatments (up to 6 in 4 cases), including ASCT in 87 (33%) cases. One-hundred and six (41%) patients were bortezomib-refractory. Twenty-five (10%) patients were already on dialysis at LDex initiation. Translocation t(11;14) was observed in 103/193 (53%), gain 1q21 in 40/193 (21%) and high risk cytogenetic in 17/193 (9%) cases respectively. Median duration of LDex was 4 cycles (IQR: 2-7) and 18 (8%) patients received at least 12 cycles (up to 38 cycles in 1 case).

Adverse events were observed in 198 of 260 (76%) patients and resulted in treatment discontinuation in 57 (22%) and lenalidomide dose reduction in 42 (16%) cases (Table 11).

After a median follow-up of 56.5 months, 229 (88%) had a progression-defining event and 167 (64%) had died. Median hemEFS and OS were 9 and 32 months, respectively. Thirty-one (12%) patients progressed to end stage renal failure requiring dialysis. Rate of progression to dialysis at 1- and 2-years from LDex initiation was 9% and 15% respectively. Six patients in renal stage 1 (all with kidney involvement) progressed to dialysis after a median of 12 months (IQR: 11-16).

Hematologic response rates at 3 and 6 months are reported in Table 12. The 3-months landmark analysis showed that achieving a VGHR at 3 months resulted in better OS (62 vs. 26 months, $P<0.001$; Figure 14A). A benefit in OS was observed also in those who achieved a VGHR at 6 months (Figure 14B).

Table 10. Characteristics of 260 patients with relapsed/refractory AL amyloidosis treated with LDex

Variables	Overall population 260 patients N (%) – mean (IQR)
Sex, male	163 (63)
Age, years	60 (54-68)
Intact monoclonal component / Monoclonal FLCs	138 (53) / 122 (47)
LC isotype Kappa / Lambda	 60 (23) / 200 (77)
Underlying clonal disease MGCS / SMM / MM	 69 (26) / 163 (63) / 28 (11)
dFLC, mg/L Missing data	123 (60-293) 13 (5)
dFLC >180 mg/L Missing data	90 (36) 13 (5)
dFLC <50 mg/L Missing data	51 (21) 13 (5)
Time to LDex, months	17 (8-36)
Year of LDex initiation Before 01/01/2014 / After 01/01/2014	 125 (48) / 135 (52)
Previous treatment lines	2 (1-2)
Pre-treatment strategies Bortezomib / ASCT / IMiDs	 177 (68) / 87 (33) / 18 (7)
Refractory to bortezomib	106 (41)
Lenalidomide starting dose, mg/die 25 mg/die / 15 mg/die / 10 mg/die / 5 mg/die Missing data	15 (1-15) 17 (7) / 136 (55) / 66 (27) / 29 (12) 12 (5)
Dexamethasone starting dose, mg Dexamethasone 40 mg Missing data	20 (8-20) 6 (3) 44 (17)
Number of cycles Missing data	4 (2-7) 26 (10)
Organ involvement Heart / Kidney / Liver / Soft tissues / PNS / ANS	 182 (70) / 144 (55) / 42 (16) / 108 (42) / 56 (22) / 47 (18)
Number of involved organs 1 / 2 / ≥3	 56 (22) / 82 (32) / 122 (47)
NT-proBNP, ng/L Missing data	1746 (413-5776) 34 (13)
NT-proBNP >8500 ng/L Missing data	39 (17) 34 (13)
Mayo staging* I / II / IIIa / IIIb Missing data	 35 (21) / 60 (36) / 52 (31) / 21 (13) 92 (35)
Proteinuria, g/24h†	1.57 (0.2-5.6)

Missing data	70 (27)
eGFR, mL/min x 1.73 $m^{2‡}$ Missing data	70 (45-94) 19 (7)
eGFR <50 mL/min x 1.73 m^2	46 (18)
Renal staging[||] I / II / III Missing data	 138 (64) / 57 (27) / 19 (9) 46 (18)
Dialysis at LDex initiation	25 (10)
iFISH t(11;14) / gain1q21° / High risk[¶] / Hyperdiploidy del8p21	193 (74) 103 (53) / 40 (21) / 17 (9) / 33 (13) 7 (4)

ANS, autonomic nervous system; ASCT, autologous stem cell transplant; dFLC, difference between involved and uninvolved free light chains; FLCs, free light chains; iFISH, fluorescence in situ hybridization; IMiDs, immunomodulatory drugs; LDex, lenalidomide and dexamethasone; MGCS, monoclonal gammopathy of clinical significance; MM, multiple myeloma; SMM, smouldering multiple myeloma.

**Mayo staging was imputed in 107 patients with relapsed/refractory AL amyloidosis. According to non-imputed data 10 patients were in stage I, 23 in stage II, 19 in stage IIIa and 9 in stage IIIb.*

†24h-proteinuria was not available in 25 patients in dialysis at LDex initiation due to anuria.

‡Patients in dialysis at LDex initiation were not considered for the evaluation of median eGFR.

[||]Renal staging was imputed in 51 with relapsed/refractory AL amyloidosis. According to non-imputed data 99 patients were in stage I, 49 in stage II and 15 in stage III. Patients in dialysis at LDex initiation were not evaluable for renal staging.

°In two of these cases 1q21 amplification was observed.

¶High risk cytogenetic was defined as either presence of del17, t(4;14) or t(14;16).

Table 11. Adverse events in 260 patients with relapsed/refractory AL amyloidosis treated with LDex

Adverse events	Any grade N (%)	Grade 3-4 N (%)
Cytopenia	**101 (39)**	**21 (8)**
Lymphocytopenia	34 (13)	4 (1)
Neutropenia	27 (10)	8 (3)
Thrombocytopenia	20 (8)	2 (1)
Anemia	11 (4)	5 (2)
Leucopenia	5 (2)	0 (0)
Pancytopenia	4 (1)	2 (1)
Infections	**77 (30)**	**18 (7)**
Infections nos	28 (11)	2 (1)
Lung infections	15 (6)	4 (1)
Airways infections	10 (4)	0 (0)
Abdominal infections	5 (2)	4 (1)
Soft tissues infections	5 (2)	2 (1)
Urinary tract infections	5 (2)	0 (0)
Sepsis	4 (1)	4 (1)
Conjunctivitis	1 (<1)	0 (0)
Otitis	1 (<1)	0 (0)
Meningitis	1 (<1)	1 (<1)
Endocarditis	1 (<1)	1 (<1)
Sinusitis	1 (<1)	0 (0)
GI toxicity	**57 (22)**	**1 (<1)**
Diarrhea	28 (11)	0 (0)
Constipation	17 (7)	1 (<1)
Nausea and/or vomiting	8 (3)	0 (0)
Dyspepsia	3 (1)	0 (0)
Duodenal ulceration	1 (<1)	0 (0)
Cardiac toxicity	**54 (21)**	**34 (12)**
Heart failure	31 (12)	17 (7)
Hypotension	17 (7)	13 (5)
Cardiac arrhythmias	6 (2)	4 (1)
Renal toxicity	**26 (10)**	**15 (6)**
Acute kidney injury	6 (2)	6 (2)
Chronic kidney failure	20 (8)	9 (3)
Skin and mucosal toxicity	**23 (9)**	**4 (1)**
Skin rash	21 ()	4 (1)
Mucositis	2 (1)	0 (0)
Dexamethasone toxicity	**15 (6)**	**0 (0)**
Insomnia	9 (3)	0 (0)
Poor tolerability	3 (1)	0 (0)
Hiccups	1 (<1)	0 (0)
Hoarseness	1 (<1)	0 (0)
Palpitations	1 (<1)	0 (0)
CNS and PNS toxicity	**26 (10)**	**6 (2)**
Dizziness	10 (4)	2 (1)

Polyneuropathy	9 (3)	1 (<1)
Depression	4 (1)	0 (0)
Seizures	1 (<1)	1 (<1)
Optic nerve neuritis	1 (<1)	1 (<1)
Encephalopathy	1 (<1)	1 (<1)
Thromboembolic event	**8 (3)**	**2 (1)**
Deep venous thrombosis	4 (1)	0 (0)
Pulmonary embolism	1 (<1)	1 (<1)
Superficial venous thrombosis	1 (<1)	0 (0)
Atrial thrombosis	1 (<1)	1 (<1)
Ictus	1 (<1)	0 (0)
Bleeding	**12 (5)**	**2 (1)**
Bleeding nos	5 (2)	0 (0)
GI bleeding	4 (1)	2 (1)
Conjunctival bleeding	1 (<1)	0 (0)
Periorbital bleeding	1 (<1)	0 (0)
Skin bleeding	1 (<1)	0 (0)

CNS, central nervous system; GI, gastrointestinal; LDex, lenalidomide and dexamethasone; nos, not otherwise specified; PNS, peripheral nervous system.

Table 12. Hematologic response rate at 3 and 6 months after LDex initiation

Response	**Response at 3 months** **N° of evaluable patients=197**	**Response at 6 months** **N° of evaluable patients=201**
Any hematologic response	62 (31)	62 (31)
VGHR	36 (18)	40 (20)
CR / VGPR / Low-dFLC PR*	8 (4) / 25 (12) / 3 (2)	11 (5) / 29 (15) / 0 (0)
PR	26 (13)	22 (11)

Data were reported as N (%)

CR, complete response; dFLC, difference between involved and uninvolved free light chains; PR, partial response; VGHR very good hematologic response; VGPR, very good partial response.

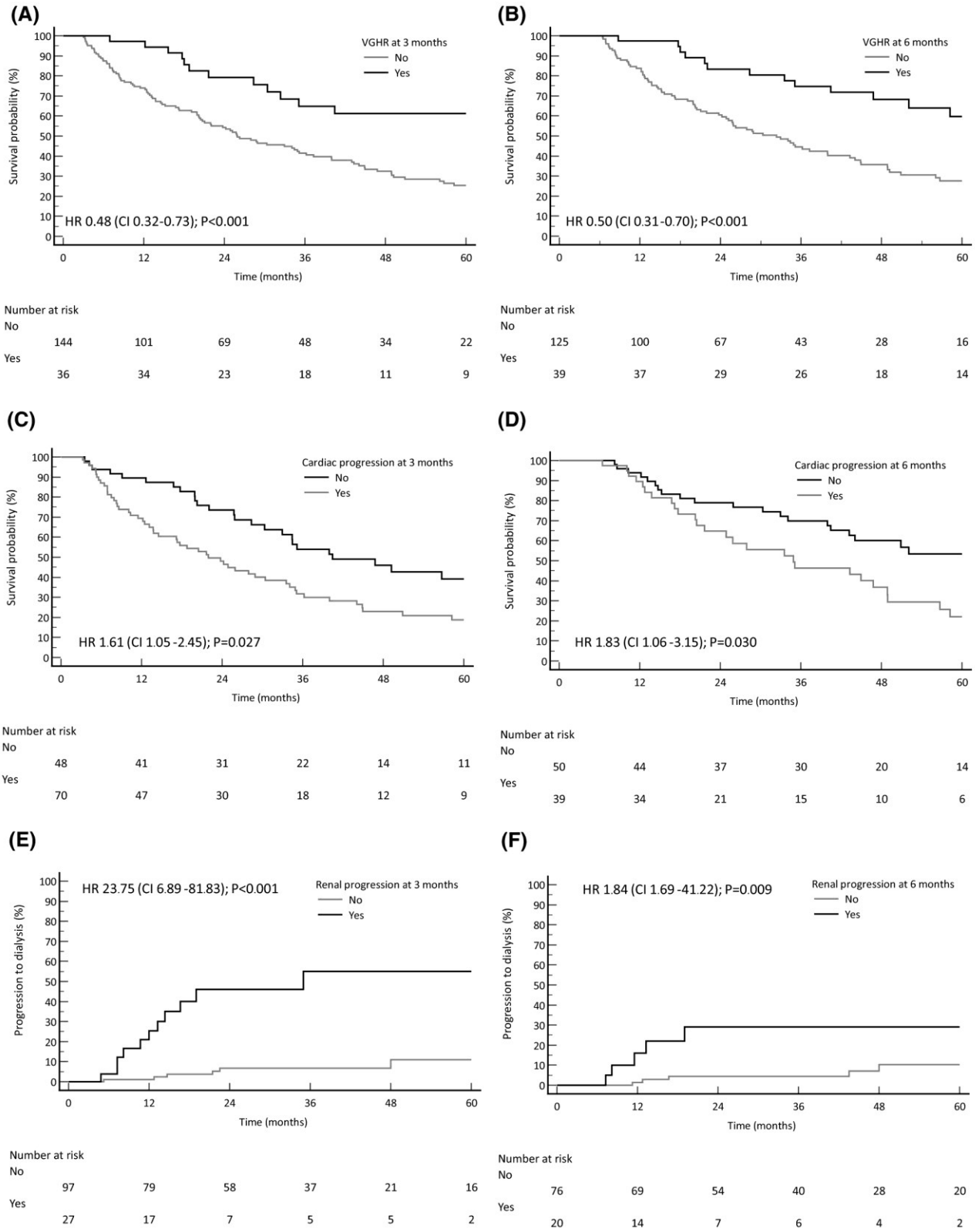
**Twenty-two patients evaluable for response at 3 months had a dFLC between 20-50 mg/L before starting LDex: 3 achieved a low-dFLC PR and 1 a CR. Among those evaluable for response at 6 months, 23 subjects had a dFLC at LDex initiation between 20-50 mg/L: only 1 patient achieved a CR.*

NT-proBNP increased in 101/122 (83%) evaluable patients after 3 months of LDex, both with and without cardiac amyloidosis. The current NT-proBNP-based cardiac progression criteria were reached in 73/122 (60%) cases and resulted in worse OS (22 vs. 40 months, P=0.027; Figure 14C). Similar results were observed when cardiac progression occurred at 6 months (40/90 [44%] cases; Figure 14D).

A worsening in eGFR was observed in 90/131 (69%) evaluable subjects after 3 months of therapy, regardless of whether renal amyloidosis was present or not. A decrease in eGFR >25% - as per current renal progression criteria – was observed in 30/131 (23%) cases and resulted in shorter RS (35 months vs. not reached, P<0.001; Figure 14E). Renal progression at 6 months also resulted in poorer RS (22/99 [22%] cases; Figure 14F).

Multivariable analysis with statistical imputation for OS and hemEFS included age, clonal and organ biomarkers and information about treatment (Table 13). Gain1q21 resulted as a negative prognostic factor for hemEFS (HR 1.68, 95% CI 1.12-2.53, P=0.014), along with high dFLC(log10) (HR 1.88, 95% CI 1.47-2.39, P<0.001) and LC λ isotype (HR 1.59, 95% CI 1.14-2.23, P=0.008). Gain 1q21 was the only cytogenetic aberration with a trend to statistical significance for an effect on OS (HR 1.47, 95% CI 0.95-2.28, P=0.084). Other predictors of OS were high dFLC(log10) (HR 2.22, 95% CI 1.62-3.03, P<0.001), LC λ isotype (HR 1.62, 95% CI 1.10-2.39, P=0.016) and high NT-proBNP(log10) (HR 1.71, 95% CI 1.27-2.31, P<0.001). Year of LDex initiation was associated with a benefit in OS (HR 0.94, 95% CI 0.89-0.99, P=0.014), but slightly worse hemEFS (HR 1.06, 95% CI 1.01-1.11, P=0.012). These results are partially illustrated by Kaplan-Meier plots in Figure 15. Combination of 1q status and dFLC at treatment initiation (cut-off: 180 mg/L) identified patients who could benefit more from LDex (Figure 16AB).

Figure 14. The impact of hematologic response and organ progression at 3 and 6 months from starting LDex.



3-months landmark analysis evaluating the impact of 3-months VGHR on OS (median OS 62 vs. 26 months) (A). 6-months landmark evaluating VGHR at 6 months with respect to OS (median OS 71 vs. 32 months) (B). 3-months landmark analysis shows that cardiac progression at 3 months results in worse OS (median OS 22 vs. 40 months) (C). 6-months landmark for cardiac progression at 6 months with respect to OS (median OS 35 vs. 60 months) (D). 3-months landmark analysis assessing the effect of renal progression at 3 months on RS (median RS 35 months vs. not reached). One-year and 2-year dialysis rate was 25% and 46% for patients with renal progression and 1% and 7% for patients with no renal progression (E). 6 months landmark evaluating renal progression at 6 months with respect to RS. One-year and 2-year dialysis rate was 16% and 29% for patients with renal progression and 1% and 4% for patients with no renal progression (F). From Basset, et al. BJH 2021

Multivariable analysis with statistical imputation for predictors of RS was also performed, adjusting eGFR and 24h-proteinuria for starting dose of lenalidomide, NT-proBNP and dFLC concentration (Table IV). This analysis revealed higher proteinuria (HR 1.10, 95% CI 1.03-1.16, P=0.004) and lower eGFR (HR 0.71, 95% CI 0.57-0.88, P=0.004) as the only statistically significant prognostic factors for RS (for complete case analysis see Supplemental Table II). When proteinuria and eGFR were combined in the validated renal staging system at LDex initiation, three different groups of patients with significantly different risk of progression to dialysis were identified (Figure 17).

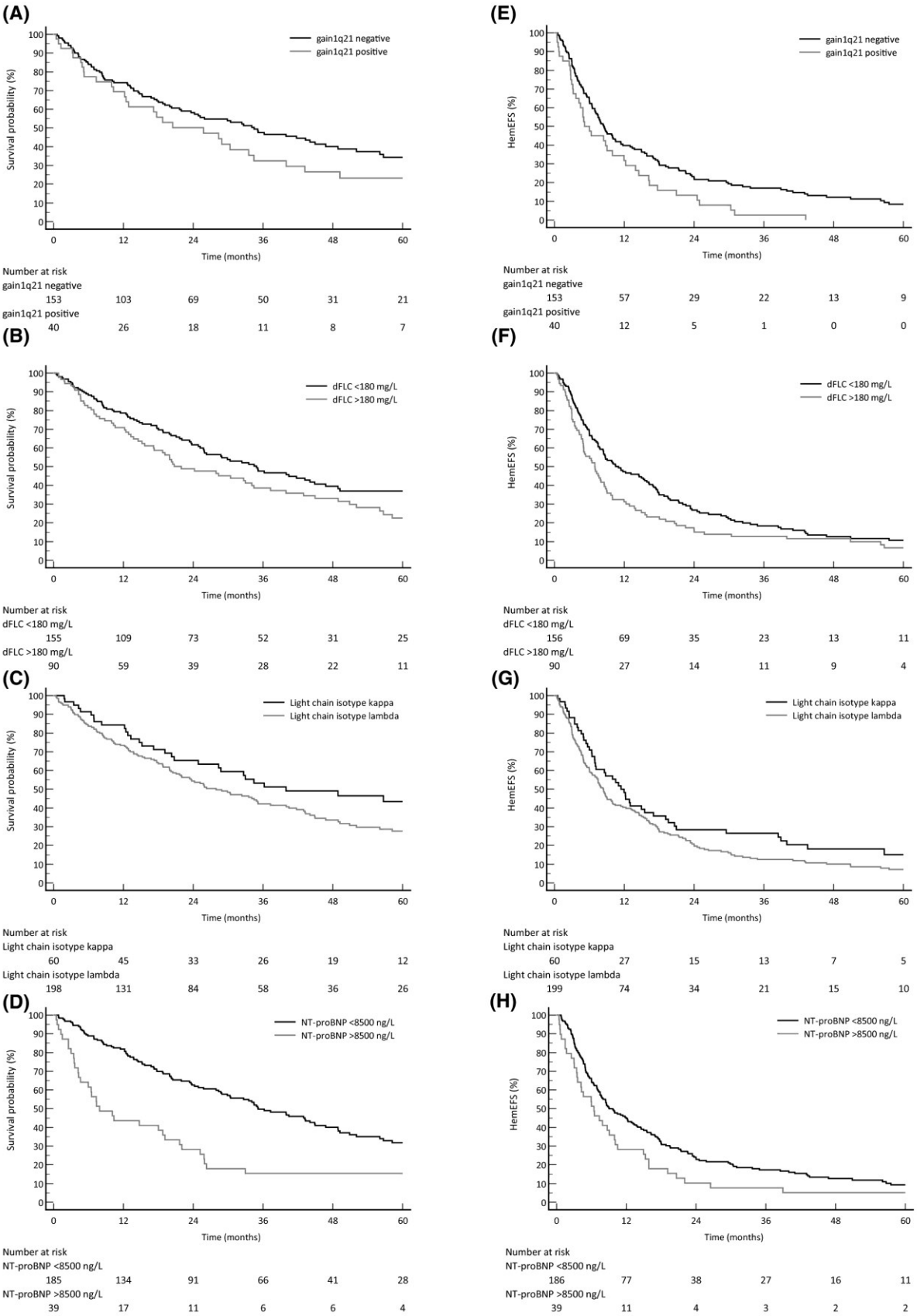
Complete case multivariable analysis was performed for 3-months VGHR. Three-months VGHR was predicted by dFLC(log10) (OR 0.11, 95% CI 0.02-0.40, P=0.002). Interestingly, harboring high risk cytogenetics or t(11;14) was associated with higher chances of achieving VGHR at 3 months (Table 13).

Table 13. Multivariable analysis for OS, hemEFS, 3-months VGHR and RS in relapsed/refractory AL amyloidosis treated with LDex

Variables	OS, n=260			hemEFS, n=260			VGHR, n=132			RS, n=235		
	HR	95% CI	P	HR	95% CI	P	OR	95% CI	P	HR	95% CI	P
Age at LDex, years (HR for changes of 10 years)	1.03	0.86-1.25	0.716	0.96	0.82-1.13	0.632	0.97	0.92-1.03	0.339	1.02	0.98-1.06	0.400
Light chain isotype Lambda vs. kappa	1.62	1.10-2.39	0.016	1.59	1.13-2.24	0.008	1.36	0.37-6.13	0.666	1.01	0.39-2.62	0.991
dFLC (log_{10}), mg/L	2.22	1.62-3.03	<0.001	1.88	1.47-2.39	<0.001	0.11	0.02-0.40	0.002	1.20	0.65-2.23	0.568
t(11;14), yes	0.91	0.61-1.35	0.717	0.89	0.63-1.27	0.528	4.78	1.42-19.50	0.017	-	-	-
Gain1q21, yes	1.47	0.95-2.28	0.084	1.68	1.11-2.53	0.014	0.70	0.15-2.72	0.620	-	-	-
High risk iFISH, yes	0.80	0.42-1.53	0.501	0.69	0.39-1.20	0.188	6.40	1.13-39.29	0.037	-	-	-
NT-proBNP (log_{10}), ng/L	1.71	1.27-2.31	<0.001	1.17	0.92-1.49	0.194	0.84	0.38-1.82	0.649	1.73	0.83-3.59	0.159
eGFR, mL/min (HR for changes of 10 mL/min)	1.00	0.99-1.01	0.903	0.98	0.92-1.04	0.449	1.01	0.99-1.03	0.504	0.71	0.57-0.88	0.004
Proteinuria, g/24h	-	-	-	-	-	-	-	-	-	1.10	1.04-1.16	0.004
Starting dose of lenalidomide, mg/day	0.89	0.72-1.01	0.281	0.93	0.77-1.44	0.461	1.09	0.94-1.27	0.256	1.04	0.94-1.15	0.469
Pre-treatment with ASCT, yes	0.85	0.59-1.24	0.407	1.05	0.76-1.44	0.770	1.28	0.40-4.12	0.674	-	-	-
Year of LDex initiation	0.94	0.89-0.99	0.014	1.06	1.01-1.11	0.012	-	-	-	-	-	-

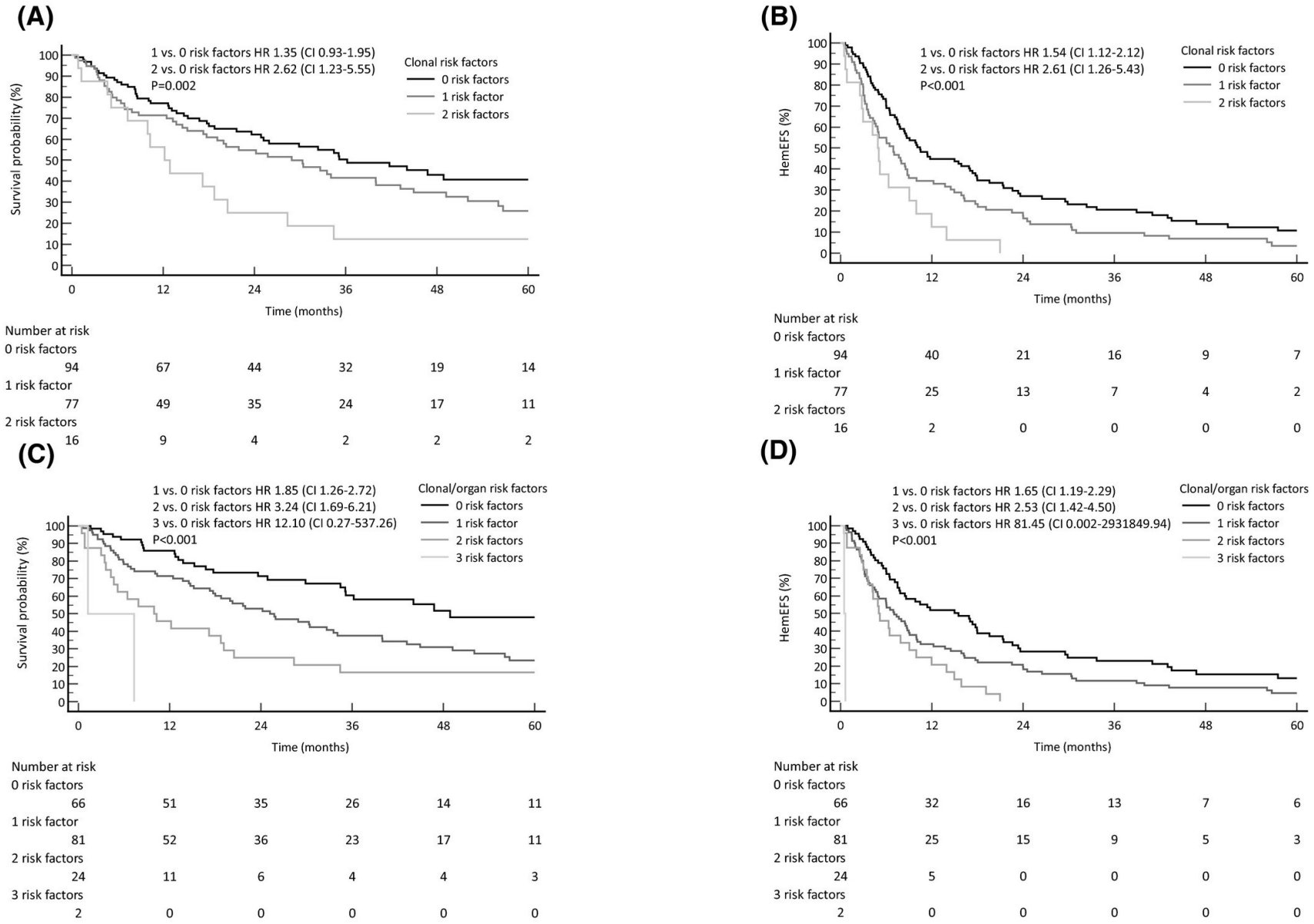
CI, confidence interval; dFLC, difference between involved and uninvolved free light chains; hemEFS, hematologic event-free survival; HR, hazard ratio; iFISH, fluorescence in situ hybridization; OR, odds ratio; LDex, lenalidomide and dexamethasone; RS, renal survival; VGHR, very good hematologic response. Number of events was 166 for OS, 229 for hemEFS and 56 for RS.

Figure 15. Prognostic factors for OS and hemEFS in patients with relapsed/refractory AL amyloidosis treated with LDex.



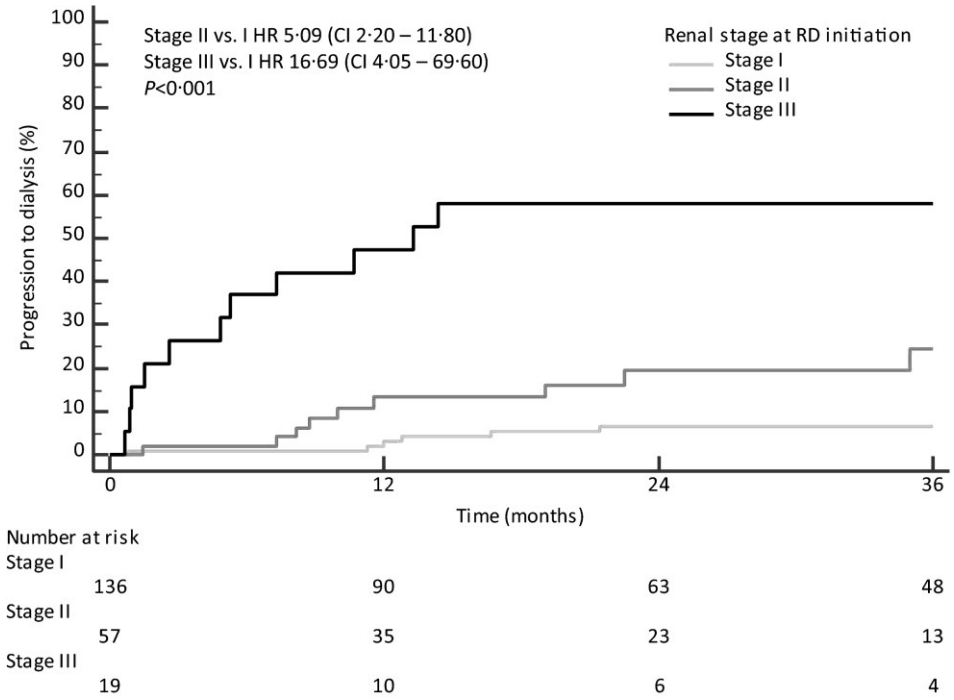
OS in patients harboring gain1q21 (median OS 26 vs. 34 months) (A). OS according to dFLC cut-off 180 mg/L (median OS 22 vs. 35 months) (B). OS in patients with light chain isotype κ or λ (median OS 40 vs. 29 months) (C). OS according to NT-proBNP cut-off 8500 ng/L (median OS 8 vs. 35 months) (D). HemEFS in patients with gain1q21 (median hemEFS 5 vs. 9 months) (E). HemEFS according to dFLC cut-off 180 mg/L (median hemEFS 11 vs. 7 months) (F). HemEFS in patients with light chain isotype κ or λ (median hemEFS 12 vs. 8 months) (G). HemEFS according to NT-proBNP cut-off 8500 ng/L (median hemEFS 9 vs. 6 months) (H). The dFLC cut-off of 180 mg/L and the NT-proBNP cut-off of 8500 ng/L were used for Kaplan Meier analysis since they were already established as prognostic in AL amyloidosis. Survival and hemEFS were calculated from time of LDex initiation. From Basset, et al. BJH 2021

Figure 16. Combination of clonal risk factors identify patients with a worse outcome to LDex.



Combination of clonal and organ risk factors identify patients with a worse outcome to LDex. OS in patients with no clonal risk factors (median OS 36 months), one clonal risk factor (median OS 29 months) and two clonal risk factors (median OS 12 months) (none vs. one risk factor: P = 0·121; one vs. two risk factors: P = 0·019) (A). HemEFS in patients no clonal risk factors (median hemEFS 10 months), one clonal risk factor (median hemEFS 7 months) and two clonal risk factors (median hemEFS 5 months) (none vs. one risk factor: P = 0·006; one vs. two risk factors: P = 0·051) (B). Clonal risk factors: gain 1q21 and dFLC >180 mg/l. OS in patients with no clonal/organ risk factors (median OS 49 months), one clonal/organ risk factor (median OS 25 months), two clonal/organ risk factors (median OS 10 months) and three clonal/organ risk factors (median OS 1 month) (none vs. one risk factor: P = 0·004; one vs. two risk factors: P = 0·023; two vs. three risk factors: P = 0·141) (C). HemEFS in patients no clonal/organ risk factors (median hemEFS 16 months), one clonal/organ risk factor (median hemEFS 7 months), two clonal/organ risk factors (median hemEFS 5 months) and three clonal/organ risk factors (median hemEFS 0·5 months) (none vs. one risk factor: P = 0·003; one vs. two risk factors: P = 0·061; two vs. three risk factors P < 0·001) (D). Clonal/organ risk factors: gain1q21 and dFLC >180 mg/l, NT-proBNP >8500 ng/l. The dFLC cut-off of 180 mg/l and the NT-proBNP cut-off of 8500 ng/l were used for Kaplan–Meier analysis as they were already established as prognostic in AL amyloidosis Survival and hemEFS were calculated from time of LDex initiation. From Basset, et al. BJH 2021

Figure 17. Progression to dialysis according to renal staging at LDex initiation.



Rate of dialysis at 3 years was 7% for Stage I, 24% for Stage II and 89% for Stage III (Stage I vs. Stage II, P < 0·001; Stage II vs. Stage III, P = 0·002). From Basset, et al. BJH 2021

Objective 5: impact of clonal biomarkers on prognosis in IgM-AL amyloidosis and differences in presentation and outcome according to the clonal B-cell immunophenotype

Patients' characteristics are reported in Table 14. Patients presented with PPCN in 28% and LPL in 64% of cases, respectively. Clonal characterization was not possible in 8% of cases. Median age at diagnosis was 66 years (IQR: 60-74) and 73% were males. Heart was involved in 62% and kidney in 50% of cases. Lymphadenopathy was present in 43% of patients and amyloid was identified in lymph nodes in 22% of cases. The most commonly amyloidogenic FLC isotype was λ. Median dFLC was 82 mg/L (IQR: 32-222) and was <20 mg/L in 16% of cases, respectively. Median IgM concentration was 13.4 g/L (IQR: 5.8-22.8). The IgM monoclonal component was detected before diagnosis of AL amyloidosis in 44% of cases. In those 44 patients median time from monoclonal component identification to AL diagnosis was 33 months (IQR: 12-98). Median time from symptoms onset to diagnosis of amyloidosis was 8 months (IQR: 4-19).

Patients with PPCN showed a trend for higher rates of cardiac (75% vs. 58%) and renal involvement (64% vs. 45%) even if no statistical differences was observed for NT-proBNP concentration, 24h-proteinuria and eGFR between these two groups. Patients with LPL were more frequently males (81% vs. 54%; P=0.010), with a higher prevalence of κ LC isotype (44% vs. 18%; P=0.019), higher levels of IgM (median 16.9 vs. 5.7 g/L; P<0.001) and more extensive bone marrow disease (median 20% vs. 10%; P=0.008). However, no statistically significant difference in dFLC was observed (median 87 vs. 61 mg/L; P=0.396). Patients with LPL also had more frequently an IgM monoclonal component detected before diagnosis of amyloidosis (52% vs. 22%; P=0.011), with a longer time from monoclonal component identification to diagnosis (median 46 vs. 6.5 months; P<0.001). However, time from onset of symptoms to diagnosis of amyloidosis was not different compared to PPCN (median 9 vs. 8 months; P=0.833).

Table 14. Baseline characteristics of 100 patients with newly-diagnosed IgM-AL amyloidosis.

Variables	Overall population 100 patients N (%) – mean (IQR)	PPCN 28 patients N (%) – mean (IQR)	LPL 64 patients N (%) – mean (IQR)
Age, years	66 (60-74)	66 (61-71)	66 (60-75)
Sex, male	73 (73)	15 (54)	52 (81)
Organ involvement Heart / Kidney / Liver ST / Lymph nodes / GI Lung / PNS / ANS	 62 (62) / 50 (50) / 17 (17) 28 (28)/ 22 (22) / 26 (26) 5 (5) / 21 (21) / 15 (15)	 21 (75) / 18 (64) / 3 (11) 9 (32) / 5 (18) / 7 (25) 0 (0) / 5 (18) / 2 (7)	 37 (58) / 29 (45) / 12 (19) 18 (28) / 13 (20) / 16 (25) 3 (5) / 14 (22) / 13 (20)
Number of organs 1 2 3 or more	 24 (24) 36 (36) 40 (40)	 8 (29) 9 (32) 8 (29)	 15 (23) 23 (36) 26 (41)
NT-proBNP, ng/L Missing data	1276 (471-2948) 19 (19)	1594 (462-2539) 12 (19)	1171 (438-3384) 4 (14)
European Mayo stage* I / II / IIIa / IIIb Missing data	 28 (29) / 44 (45) / 18 (18) / 8 (8) 2 (2)	 7 (26) / 13 (48) / 5 (19) / 2 (7) 1 (4)	 20 (32) / 26 (41) / 12 (19) / 5 (8) 1 (2)
Albumin, g/L Missing data	37.2 (29.1-41) 11 (11)	32.3 (27.5-40) 2 (7)	37.9 (31.1-41) 8 (13)
Proteinuria, g/24h Missing data	0.84 (0.13-5.31) 35 (35)	2.06 (0.34-6.52) 10 (36)	0.38 (0.11-3.54) 21 (33)
eGFR, mL/min x 1.73 m^2 Missing data	71 (53-91) 11 (11)	62 (48-87) 2 (7)	74 (56-92) 6 (9)
Renal stage I / II / III Missing data	 42 (63) / 17 (25) / 8 (12) 33 (33)	 10 (56) / 4 (22) / 4 (22) 10 (36)	 30 (67) / 13 (29) / 2 (4) 19 (30)
Lymphadenopathy	43 (43)	9 (32)	29 (45)

Hepatomegaly / splenomegaly	16 (16) / 13 (13)	3 (11) / 0 (0)	12 (19) / 11 (17)
Kappa : lambda	37 (37) : 63 (63)	5 (18) : 23 (82)	28 (44) : 36 (56)
IgM, (median, range / IQR) g/L Missing data	13.4 (0.5-57.8 / 5.8-22.8) 4 (4)	5.7 (0.5-37.4 / 2.4-13.7) 2 (7)	16.9 (1.7-57.8 / 7.4-28.5) 2 (3)
IgG, (median, range / IQR) g/L Missing data	5.7 (0.03-17 / 2.8-8) 20 (20)	3.8 (0.2-12.7 / 1.8-6.8) 6 (21)	5.9 (0.03-13 / 3.8-8.3) 13 (20)
IgA, (median, range / IQR) g/L Missing data	1.1 (0.05-10.8 / 0.6-1.7) 25 (25)	0.9 (0.08-2.1 / 0.6-1.4) 6 (21)	1.1 (0.05-10.8 / 0.6-1.7) 18 (28)
dFLC, (median, range / IQR) mg/L Missing data	82 (1-3741 / 32-222) 7 (7)	61 (1-1413 / 32-142) 0 (0)	87 (1-3741 / 35-355) 5 (8)
dFLC <50 mg/L Missing data	36 (39) 7 (7)	13 (46) 0 (0)	22 (37) 5 (8)
dFLC >180 mg/L Missing data	27 (29) 7 (7)	6 (21) 0 (0)	20 (34) 5 (8)
Clonal infiltrate, (median, range / IQR) % Missing data	10 (0-80 / 5-25) 26 (26)	10 (3-30 / 5-10) 2 (7)	20 (0-80 / 9-40) 21 (33)

ANS, autonomic nervous system; dFLC, difference between amyloidogenic and non-amyloidogenic free light chain; eGFR, estimated glomerular filtration rate; GI, gastrointestinal involvement; LPL, lymphoid clone; ST, soft tissues; PPCN, plasma cellular clone; PNS, peripheral nervous system.

**European Mayo stage data were imputed in 20 patients. According to non-imputed data 18 patients were in stage 1, 39 in stage 2, 14 in stage 3a and 7 in stage 3b (see Supplement for more details)*

MYD88^{L265P} mutation was observed in 27 of 44 (61%) tested patients, all with LPL. Chromosomal aberrations detected by iFISH were present in 80% of subjects (24/30), including 12 IgH rearrangements - t(11;14) in 5 patients – in 12 cases, del13q14 in 7, gain1q21 in 4 and del17 and hyperdiploidy in 1 subject each. Notably, t(11;14) and gain1q21 were observed exclusively in patients with PPCN.

Treatment history is resumed in Table 15. Sixteen patients received a treatment for the underlying clonal disease before diagnosis of AL amyloidosis. This was more frequent in LPL (22% vs. 4%; P=0.033). In 7 cases, patients received up to two treatment lines before diagnosis of AL amyloidosis. First line treatment for AL amyloidosis was rituximab-based in 49 patients, more often in LPL than in PPCN (72% vs. 7%). Eight patients underwent ASCT. At least 2 different lines of treatment for AL amyloidosis were administered in 44% of cases. Rescue treatment with rituximab-based regimens was less frequent (18%), but still more often in LPL than in PPCN (23% vs. 7%). Non-rituximab-based treatments were used in 36% of patients, without significant difference according to B-cell clonal phenotype.

After a median follow-up of 45 months, 53 patients died. Median OS and hemEFS were 42 and 15 months respectively (Figure 18AB). No differences in OS and hemEFS were observed between LPL and PPCN (Figure 18CD). Only 5 patients required dialysis during the study period and 12- and 36-months dialysis rate were 3% and 7%.

Hematologic response was evaluable at 3 months in 47 patients according to validate criteria for AL amyloidosis and in 62 with WM response criteria (Table 16). Notably, in 21 cases (10 with missing dFLC data and 11 with dFLC <20 mg/L at diagnosis) hematologic response was evaluable only by WM criteria. Overall, 68 patients were evaluable either with AL amyloidosis or WM criteria and hematologic response rate was 41%. A VGHR was observed in 32%, with a trend for lower VGHR rate in LPL compared to PPCN (24% vs. 44%; P=0.197).

Table 15. Treatment history in IgM-AL amyloidosis

Treatment	Overall population 100 patients N (%)	PPCN 28 patients N (%)	LPL 64 patients N (%)
Therapy before diagnosis of IgM-AL amyloidosis			
Treated before diagnosis of amyloidosis	16 (16)	1 (4)	14 (22)
1 line of treatment	9 (9)	0 (0)	9 (14)
2 lines of treatment	7 (7)	1 (4)	5 (8)
Rituximab-based treatment	12 (11)	0 (0)	11 (17)
R-Bendamustine	8 (8)	0 (0)	8 (12)
Other*	4 (4)	0 (0)	3 (6)
Non-rituximab-based treatment	6 (6)	1 (4)	4 (6)
Alkylators-based	5 (5)	0 (0)	4 (6)
Bortezomib-based	1 (1)	1 (4)	0 (0)
First-line therapy after diagnosis of IgM AL amyloidosis			
Rituximab-based treatment	49 (49)	2 (8)	46 (72)
R-Bendamustine	35 (35)	0 (0)	35 (55)
R-Bortezomib	9 (9)	1 (4)	7 (11)
Other†	5 (5)	1 (4)	4 (5)
Non-rituximab-based treatment	35 (35)	21 (75)	12 (19)
Bortezomib-based	15 (15)	11 (39)	3 (5)
Alkylators-based	10 (10)	4 (14)	5 (8)
ASCT‡	8 (8)	5 (19)	2 (3)
Lenalidomide-based	2 (2)	1 (4)	1 (4)
Ibrutinib	1 (1)	0 (0)	1 (2)
No treatment°	16 (16)	5 (18)	7 (11)
Rescue treatment after diagnosis of IgM-AL amyloidosis			
Rescued with rituximab-based treatment	18 (18)	2 (7)	15 (23)
R-Bendamustine	11 (11)	2 (7)	8 (12)
R-Bortezomib	3 (3)	0 (0)	3 (5)

Other[||]	4 (4)	0 (0)	4 (6)
Rescued with non-rituximab-based treatment Bortezomib-based Ibrutinib Alkylator-based Lenalidomide-based ASCT	36 (36) 6 (6) 10 (10) 11 (11) 6 (6) 3 (3)	10 (35) 0 (0) 0 (0) 7 (25) 3 (11) 0 (0)	26 (40) 6 (9) 10 (16) 4(6) 3 (5) 3 (5)
N° of treatment lines 1 / 2 / 3 or more	42 (42) / 26 (26) / 16 (16)	13 (46) / 6 (21) / 4 (14)	26 (41) / 19 (30) / 12 (19)

ASCT, autologous stem cell transplant; BEAM, carmustine, etoposide, cytarabine, and melphalan; CAD, cyclophosphamide, doxorubicin, dexamethasone; LPL, lymphoid clone; MP, melphalan and prednisone; PPCN, plasma cellular clone; R-BDex, rituximab, bortezomib and dexametasone; R-CHOP, rituximab, cyclophosphamide, hydroxydaunorubicin, vincristine and prednisone; R-LDex, rituximab, lenalidomide and dexamethasone

**Other: rituximab alone, rituximab and cyclophosphamide, rituximab and leukeran and R-CHOP in 1 case each.*

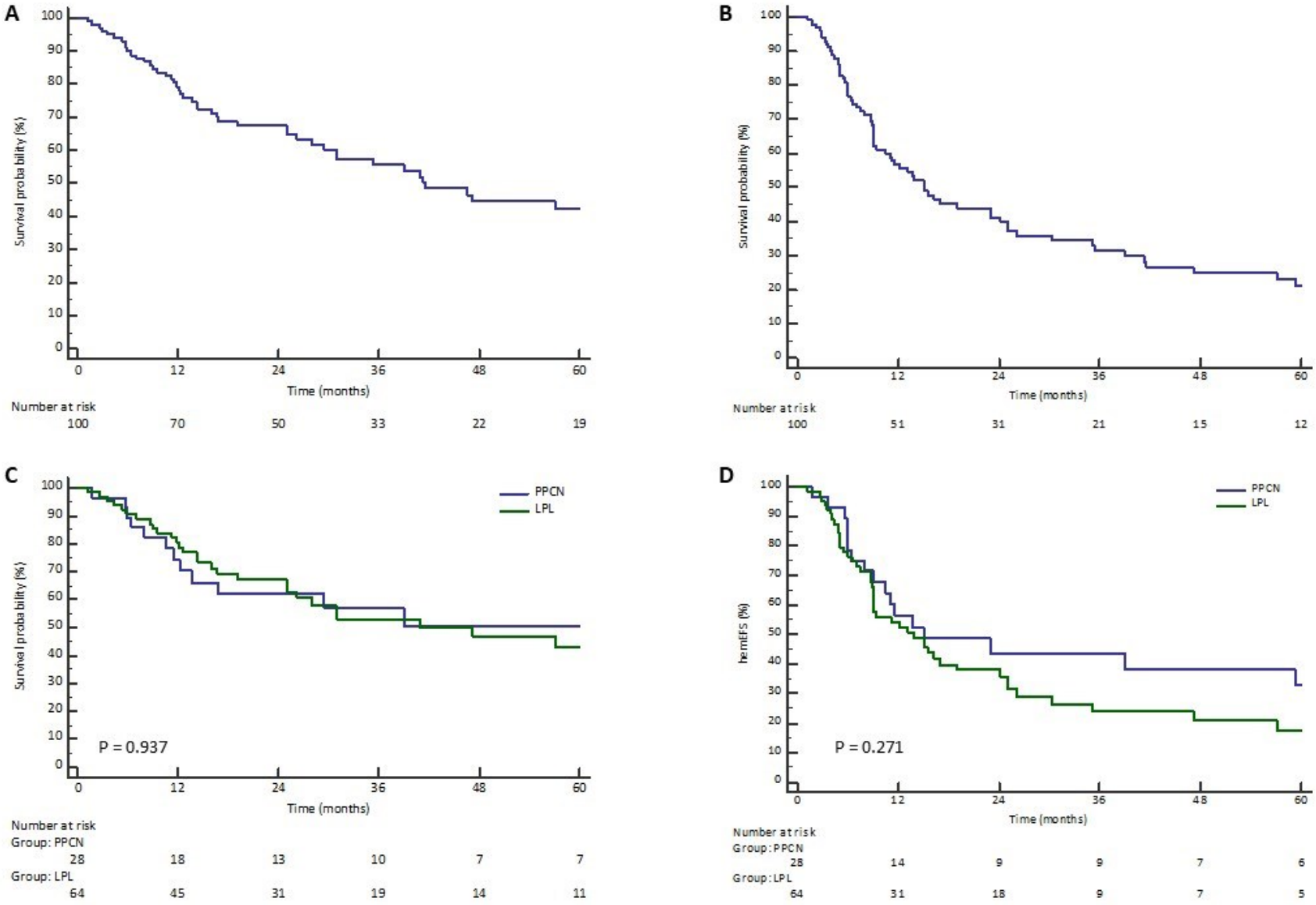
†Other: 2 rituximab monotherapy, 1 R-CHOP, 1 R-LDex, 1 R-Methotrexate.

‡The induction treatment was high dose dexamethasone in 2 patients and MP, R-BDex and rituximab monotherapy in 1 case each. Stem cell chemo-mobilization was performed with CAD in 4 cases and conditioning with BEAM regime in 1 patient with lymphC.

°Three patients who did not receive treatment for AL amyloidosis were previously treated for lymphoma.

||Other: rituximab, ixazomib and dexamethasone, rituximab and prednisone, rituximab, cyclophosphamide and fludarabine and rituximab alone in 1 case each.

Figure 18. Outcome of patients with IgM-AL amyloidosis.



Overall survival: median 42 months (A). Hematologic progression free survival: median 15 months (B). Overall survival in LPL (median 47 months) and PPCN (median 78 months) (C). HemEFS in LPL (median 14 months) and PPCN (median 15 months) (D).

Table 16. Hematologic response after 3 months from first-line treatment for IgM-AL amyloidosis

Hematologic response	AL amyloidosis criteria* Evaluable = 47 pts. N (%)	WM criteria Evaluable = 62 pts. N (%)	AL amyloidosis or WM criteria† Evaluable = 68 pts. N (%)
Any HR	21 (45)	23 (37)	28 (41)
PR	6 (13)	21 (34)	12 (18)
Low-dFLC PR	3 (6)	-	3 (4)
VGPR	11 (23)	1 (2)	12 (18)
CR	1 (2)	1 (2)	1 (2)
VGHR	15 (32)	n.a.	n.a.

CR, complete response; dFLC, difference between involved and uninvolved free light chains; HR, hematologic response; ISA, International Society of Amyloidosis; n.a., not applicable; PR, partial response; pts., patients; VGPR, very good partial response; VGHR, very good hematologic response; WM, Waldenström macroglobulinemia.

**Of these 47 patients, 38 (81%) were evaluable according to the standard ISA criteria and 9 (19%) according to low-dFLC criteria.*

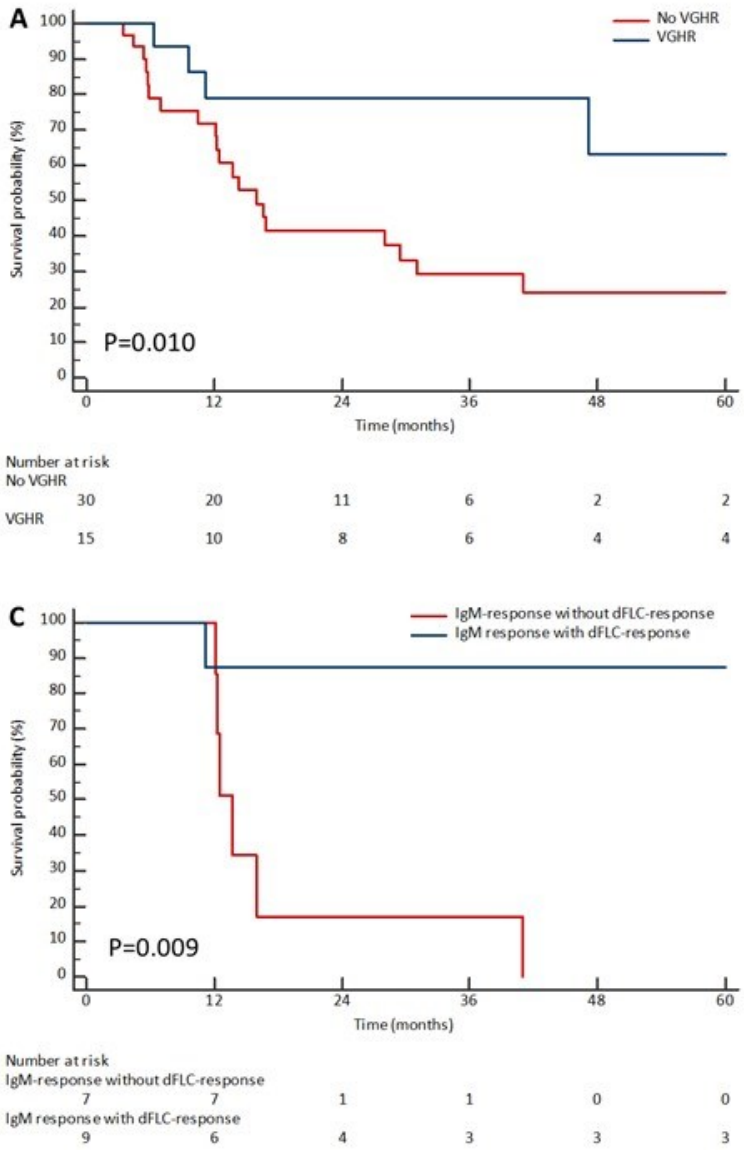
†Response was evaluated with WM criteria only in patients who were not evaluable with ISA/low-dFLC criteria

Hematologic response according to AL amyloidosis and WM response criteria was not concordant in 40% of cases (n=43). Particularly, among 16 patients with an IgM response 7 had no dFLC response. Hematologic response rate at 6 months was 51% in 67 patients evaluable either with AL amyloidosis and WM criteria.

Cardiac response was observed in 18% (n=28) of cases at 6 months and in 36% (n=22) at 12 months, while cardiac progression occurred in 39% of patients at 6 months and 41% at 12 months. Renal response rate at 6 and 12 months was 27% (n=22) and 22% (n=18) respectively, whereas renal progression was observed in 23% of cases at 6 months and 44% at 12 months.

Landmark analyses were performed starting at the time of response assessment after 3 months from diagnosis of AL amyloidosis (n=47). A significant benefit in OS (median 97 vs. 16 months, P=0.010) was observed in patients who achieved a VGHR according to AL amyloidosis criteria at that time (Figure 19A). An IgM response also resulted in better OS (median OS not reached vs. 35 months, P=0.020; Figure 19B). None of the 2 patients who achieved an IgM reduction >90% died. It was not possible to statistically evaluate the benefit of IgM-response in the subgroup of patients with a baseline dFLC<20 mg/L due to the small size (n=10). However, no obvious differences in OS were observed between responders (n=4) and non-responders (n=6). Interestingly, patients who achieved an IgM response but not a dFLC response had a shorter OS compared to those with a response according to both criteria (n=16, median 14 months vs. not reached; P=0.009; Figure 19C).

Figure 19. 3-months landmark analysis for OS according to hematologic response in IgM-AL amyloidosis



Achieving a VGHR resulted in a better OS (A). IgM-response resulted in longer OS (B). Patients with IgM-response but not a dFLC response had a worse OS (C). The analysis was performed with a 3-months landmark.

On univariable analysis, baseline dFLC(log_{10}) was prognostic for both OS (HR 2.48, 95% CI 1.60-3.83, P<0.001) and hemEFS (HR 1.94, 95% CI 1.34-2.81, P<0.001). Higher IgM levels showed a trend for worse OS (HR 1.02, 95% CI 1.00-1.04, P=0.068) and resulted in significantly worse hemEFS (HR 1.03, 95% CI 1.01-1.05, P=0.002). On the other hand, liver (HR 1.14, 95% CI 0.59-2.21, P=0.698) and PNS involvement (HR 0.87, 95% CI 0.45-1.67, P=0.676) did not seem to affect OS. A multivariable analysis was performed to identify prognostic factors for OS and hemEFS. Multivariable model included age, light chain isotype, B-cell clonal phenotype, dFLC and IgM concentration at baseline, European Mayo staging and treatment status (Table 17). The complete case model included 83 patients for OS and hemEFS. European Mayo Stage was prognostic for OS (P=0.004), but not for hemEFS (P=0.140). Baseline dFLC(log_{10}) was prognostic for both OS (HR 2.51, 95% CI 1.47-4.28, P<0.001) and hemEFS (HR 2.04, 95% CI 1.31-3.19 P=0.002). IgM concentration showed only a trend for hemEFS (HR 1.02, 95% CI 1.00-1.05, P=0.056). A separate univariable analysis for OS and hemEFS was performed for LPL patients. Interestingly, this analysis showed that, differently from overall population, higher IgM concentration at diagnosis resulted in shorter OS (HR 1.04, 95% CI 1.02-1.07 P=0.001), while only a trend toward statistical significance for dFLC on hemEFS was observed (HR 1.44, 95% CI 0.94-2.20 =0.097).

Finally, we evaluated hematologic response (at least PR) at 3 months and OS and hemEFS according to different treatment strategies both in treatment-naïve and relapsed/refractory patients (Table 18). In the bortezomib-based regimens group, PPCN patients achieved more frequently a response than LPL (80% vs. 17%; P=0.048). No differences in hematologic response

rate between LPL and PPCN were observed among other treatment groups. Patients treated with ASCT showed a long OS (median 120 months) and hemEFS (median 53 months). This seemed particularly evident in LPL subjects (median OS and hemEFS not reached), while in PPCN patients median OS and hemEFS were 9 and 5 months, respectively (P value for OS=0.060; P value for hemEFS=0.022). Rituximab-based regimens, bortezomib and alkylator-based therapies resulted in a median OS of 35-40 months and a median hemEFS between 10-15 months. Patients treated with lenalidomide presented a median OS and hemEFS of 19 and 7 months, respectively. Ibrutinib resulted in short OS (median 9 months) and hemEFS (median 2 months).

Table 17. Multivariate analysis for OS and hemEFS in IgM-AL amyloidosis

Variables	OS			hemEFS		
	HR	95% CI	P	HR	95% CI	P
Age, years (HR for changes of 10 years)	1.01	0.97-1.06	0.520	1.01	0.97-1.04	0.780
dFLC (log_{10}), mg/dL	2.51	1.47-4.28	<0.001	2.05	1.32-3.19	0.002
IgM, g/L	1.01	0.99-1.03	0.425	1.02	1.00-1.05	0.056
Clonal immunophenotype LPL vs. PPCN	0.88	0.37-2.10	0.772	0.95	0.47-1.92	0.879
Amyloidogenic LC isotype Kappa vs. lambda	0.64	0.29-1.42	0.272	0.96	0.50-1.83	0.900
European Mayo stage II vs. I IIIa vs. I IIIb vs. I	 2.75 3.73 14.75	 1.08-7.01 1.24-11.18 3.53-61.51	 0.034 0.019 <0.001	 0.83 1.24 3.22	 0.42-1.65 0.57-2.71 1.08-9.60	 0.589 0.583 0.036
Treatment status Pre-treated vs. treatment-naïve	1.04	0.44-2.44	0.935	1.38	0.68-2.81	0.379

CI, confidence intervals; dFLC, difference between involved and uninvolved free light chains; HR, Hazard ratio; LPL, lymphoid clone; PPCN, plasma cellular clone.

Table 18. Different treatment strategies in IgM AL amyloidosis: data from present study and in previously published case series.

Treatment	N° of pts.	Previously treated pts.	Any HR / VGHR*	Median follow-up	PFS†	OS‡
R-Bortezomib					Median PFS	1-year OS
Palladini, et al. 2011	10	4	78% / n.a.	13 months	n.a.	90%
Present study	12	4	33% / 17%	41 months	11 months	70%
R-Bendamustine					1-year / 3- year PFS	1-year / 3-year OS
Manwani, et al. 2019	27	5	59% / 48%	18 months	88% / 79%	65% / 56%
Present study	45	15	44% / 12%	41 months	47% / 28%	74% / 56%
ASCT					2-year PFS	2-year OS
Sidiqui, et al. 2019	38	22	92% / 76%	n.a.	75%	90%
Present study	11	4	43% / 43%	112 months	54%	73%
Bortezomib-based					Median PFS	2-year OS
Sanchchithanantam, et al. 2016	8	n.a.	57% / 42%	n.a.	Not reached	88%
Present study	21	8	53% / 41%	25 months	13 months	69%
Alkylating agents					Median PFS	2-year OS
Sanchchithanantam, et al. 2016	53	n.a.	70% / 26%	n.a.	8 months	49%
Present study	18	9	17% / 8%	60 months	15 months	65%
Lenalidomide-based					Median PFS	Median OS
Present study	8	6	66% / 0%	78 months	7 months	18 months
Ibrutinib					Median PFS	Median OS
Pika, et al. 2018	8	8	25% / 12%	6 months	3 months	9 months
Present study	11	11	22% / 11%	25 months	4 months	10 months

ASCT; autologous stem cell transplant; HR, hematologic response; OS, overall survival; PFS, progression-free survival; pts. patients; R-Bendamustine, rituximab and bendamustine; R-bortezomib, rituximab and bortezomib; VGHR, very good hematologic response

**Any HR/VGHR assessment: hematologic response was assessed after 2 cycles in Palladini's work, after a median of 5 cycles in Manwani's study and at 100-days from ASCT in Sidiqui's one; in the present study we report 3-months response rate.*

†PFS was evaluated as time to next treatment in Manwani's study and as in Sanchchithanantam's one.

Objective 6: clinical characteristics of patients with AL amyloidosis and non-lymphoplasmacytic LPD

A total of 36 patients with AL amyloidosis and a non-lymphoplasmacytic LPD were identified. Twenty-one (58%) patients had systemic AL amyloidosis, and 15 (42%) had localized AL amyloidosis. They represented 2% and 5% of all patients with systemic and localized AL amyloidosis referred to our center in the study period, respectively. Patient characteristics are reported in Table 19. Nineteen (53%) patients were diagnosed with MZL, and 11 (58%) of these cases were extranodal. Autoimmune disorders were more frequent in patients with localized amyloidosis (53% vs 5%; P=0.001). Sjögren syndrome was the most common autoimmune disease (6 of 9 patients). In the overall cohort, patients with systemic amyloidosis were more likely to have advanced Ann Arbor stage than subjects with localized amyloidosis (85% vs 46%, respectively; P=0.006). A serum and/or urinary monoclonal component and/or an abnormal free light chain ratio were present in all patients with systemic AL amyloidosis and in 6 subjects (54%) with localized amyloid deposits (P=0.002).

Table 19. Characteristics of 36 patients with AL amyloidosis and non-lymphoplasmacytic lymphoproliferative disorders

Variables	Systemic AL amyloidosis 21 patients N (%) – mean (IQR)	Localized AL amyloidosis 15 patients N (%) – mean (IQR)
Age, years	63 (63-69)	70 (64-74)
Male sex	15 (71)	9 (60)
Marginal zone lymphoma (MZL) Extranodal / Nodal / Disseminated / NOS	9 (43) 4 (19) / 0 (0) / 1 (5) / 4 (19)	10 (67) 7 (46) / 1 (7) / 1 (7) / 1 (7)
Non-marginal zone lymphoma Low grade B cell lymphoma NOS DBLCL / CLL-SLL / Other diagnosis*	12 (57) 5 (24) 2 (10) / 2 (10) / 3 (13)	5 (33) 2 (13) 2 (13) / 1 (7) / 0 (0)
Organ involvement in systemic AL amyloidosis Heart / Kidney / Liver / Soft tissues / ANS / PNS	12 (57) / 8 (38) / 1 (5) / 4 (19) / 3 (13) / 3 (13)	n. a.
Site of localized AL amyloidosis Nodular pulmonary / Lymph nodes Skin / Tracheobronchial / Bladder / nodular GI	n. a.	7 (46) / 3 (10) 2 (13) / 1 (7) / 1 (7) / 1 (7)
NT-proBNP, ng/L	1113 (558-6979)	152.28 (47-265)
Proteinuria, g/24h	1.21 (0.10-5.00)	0.07 (0.06-0.09)
Cardiac Stage I / II / IIIa / IIIb	8 (32) / 11 (44) / 4 (16) / 2 (8)	n. a.
Renal stage I / II / III	15 (60) / 8 (32) / 2 (8)	n. a.
Ann Arbor Stage I / II / III / IV	2 (10) / 1 (5) / 1 (5) / 17 (80)	8 (53) / 0 (0)/ 2 (13) / 5 (34)
MC at serum and/or urine immunofixation	20 (95)	8 (53)
Kappa : Lambda	4 (20) : 16 (80)	4 (50) : 4 (50)
Monoclonal component IgGλ / IgGκ / IgAλ / IgAκ IgMλ / IgMκ / FLC λ / FLC κ	 1 (5) / 6 (28) / 1 (5) / 1 (5) 2 (10) / 2 (10) / 5 (24) / 0 (0)	 1 (7) / 3 (20) / 0 (0) / 0 (0) 2 (13) / 0 (0) / 1 (7) / 1 (7)
Free light chain only : complete MC	6 (29) : 14 (67)	2 (25) : 6 (75)
Positive fat pad aspirate (Congo red)	17 (80)	0 (0)
Abnormal FLCR	15 (71)	5 (33)
dFLC, mg/L	105 (31-415)	2 (0-14)
dFLC <50, mg/L	8 (38)	14 (93)
B symptoms	3 (13)	1 (7)
Autoimmune disorder	1 (5)	8 (53)
HCV / HBV infection	2 (10) / 3 (13)	0 (0) / 3 (20)
Treatment for lymphoma R-CHOP / R-CVP / Radiotherapy Alkylating agents / Other treatment†	14 (66) 7 (33) / 2 (10) / 0 (0) 3 (13) / 2 (10)	10 (67) 5 (34) / 1 (7) / 2 (13) 0 (0) / 2 (13)

ANS, autonomic nervous system; CLL-SLL, chronic lymphocytic leukemia-small lymphocytic lymphoma; DLBCL, Diffuse Large B Cell Lymphoma; FLCR, free light chain ratio; GI, gastrointestinal; MC, monoclonal component; NOS, not otherwise specified; PNS, peripheral nervous system; R-CHOP, rituximab, cyclophosphamide, adriamycin, vincristine, prednisone; R-CVP, rituximab, cyclophosphamide, vincristine, prednisone.

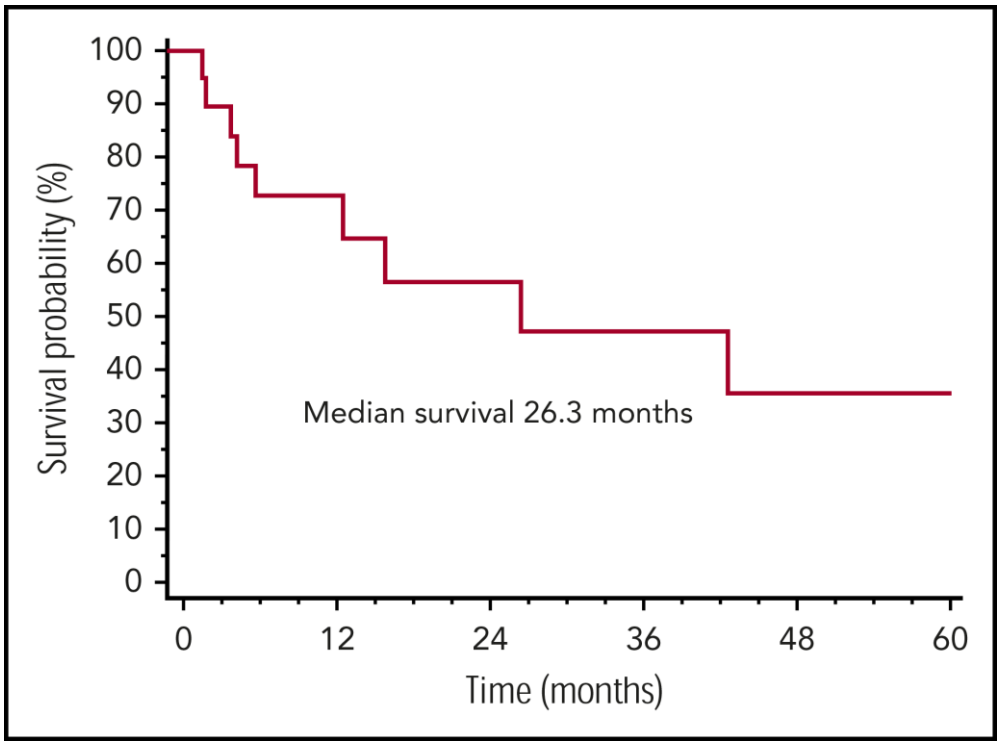
**Other diagnosis: 1 follicular lymphoma, 1 hairy cell leukemia and 1 Hodgkin lymphoma.*

†Other treatment: 1 fludarabine and cyclophosphamide and rituximab (FCR) and 1 cladribine in patients with systemic AL amyloidosis and 1 rituximab in monotherapy, 1 chlorambucil in patients with localized AL amyloidosis

In 14 of 21 patients with systemic AL amyloidosis, the diagnosis of lymphoma preceded the clinical manifestations of amyloidosis by a median of 32 months (IQR: 7-74). The diagnosis of systemic amyloidosis was established after a median of 64 months (IQR: 40-84) from the diagnosis of lymphoma, with a median diagnostic delay of 24 months (IQR: 11-33) from the onset of symptoms of amyloidosis, despite 16 (64%) of these subjects having a monoclonal component detected at the time of the lymphoma diagnosis. In the remaining 7 cases, lymphoma was diagnosed during the investigations for systemic amyloidosis.

Overall, 24 (64%) patients received therapy for LPD, before the diagnosis of AL amyloidosis. Sixteen (44%) were treated with a rituximab-based regimen, and after a median of 5 cycles (range, 2-8 cycles), 17 (74%) subjects achieved a response (complete in 13 [57%]) for lymphoma. Treatment of systemic amyloidosis was bortezomib based in 11 patients, rituximab based in 7, and oral melphalan-dexamethasone based in 3. Eleven patients (52%) achieved a hematologic response that was complete in 2 subjects and very good partial response in 6. The median follow-up of living patients was 16 months. Overall, 12 patients with systemic amyloidosis died, 11 due to progression of amyloidosis and 1 because of an unrelated cause (gastric cancer). None of the patients with systemic amyloidosis died of progressive lymphoma. The median survival from the diagnosis of systemic amyloidosis was 26 months (Figure 20).

Figure 20. Overall survival in systemic AL amyloidosis and nonlymphoplasmacytic LPDs



Overall survival from diagnosis of AL amyloidosis of 21 patients with systemic amyloidosis and nonlymphoplasmacytic LPDs. Figure from Basset, et al. Blood 2021

Objective 7: biomarkers involved in the clinical history of localized AL amyloidosis and factors affecting local progression of the disease.

Two hundred ninety-three patients with localized AL amyloidosis were identified from the prospective maintained database of the Amyloidosis Center of Heidelberg. Patients characteristics are reported in Table 20 and detailed information on organ involvement are summarized in Table 21. Lung (nodular pulmonary) was the most commonly involved organ. Patients with lung localized AL amyloidosis were older at diagnosis (68 vs. 55-year-old; P<0.001), had more frequently multifocal involvement (62% vs. 40%; P=0.026) and presented a high prevalence of smokers (54% vs. 37%; P=0.014). A female preponderance was observed among patients with skin, soft tissues, eye and central nervous system (CNS) localized AL amyloidosis.

Median length of the histological material from tissue biopsy or surgical resection was 1.2 cm (range 0.1-14.5 cm). Larger samples were obtained from patients with lung localized AL amyloidosis (median length 2.5 cm vs. 1 cm; P<0.001), while smaller specimens came from patients with lower airways localized AL amyloidosis (median length 0.6 cm vs. 1.5 cm; P=0.001). An amyloidogenic LC λ was identified by amyloid typing on tissue in most cases with a κ:λ ratio of 1:3. Interestingly, we observed an organ site specific variation of κ:λ ratio though patients with different organ involvement (Table 21). A particular predominance of LC λ (i.e. >75%) was observed in urinary tract, gastrointestinal (GI) tract, skin and soft tissues localized AL amyloidosis. The prevalence of LC λ was even higher in eye and CNS localized AL amyloidosis (92% and 99% respectively). Information about the cellular infiltrate was available in 154 (53%) samples, of which 30% were obtained from patients with lung localized AL amyloidosis. An inflammatory infiltrate was present in 123 (80%) cases and multinucleated giant cells (MGC) were identified in 91 (59%) samples. A lymphoplasmacytic infiltrate was found in 76 (49%), and clonality was identified in 46

(30%, composed by plasma cells in 27 and by B-cells in 19). In patients with an identified local clone, the LC restriction of the clone always matched the amyloidogenic LC.

Table 20. Baseline characteristics of 293 patients with localized AL amyloidosis

Variables	**Overall population 293 patients N (%) - median (IQR)**
Sex, male	145 (49)
Age, years	58 (47-78)
Organ involvement Lung (nodular pulmonary) / Larynx / Urinary tract GI / Skin and ST / Lower airways (tracheobronchial) Eye / Nasopharynx / CNS / Lymphatic tissue / Other*	 63 (22) / 51 (17) / 37 (13) 35 (12) / 31 (11) / 31 (11) 12 (4) / 12 (4) / 9 (3) / 8 (3) / 4 (1)
Multifocal involvement	130 (44)
Smokers	116 (40)
First evaluation in Heidelberg within 12 months from diagnosis	210 (73)
NT-proBNP, ng/L Missing data	83 (50-168) 9 (3)
NT-proBNP >332 ng/L	40 (14)
Proteinuria g/24h[†] Missing data	0.1 (0.10-0.11) 61 (21)
Proteinuria >0.5 g/24h	5 (2)
eGFR, mL/min x 1.73 m^2 Missing data	89 (77-99) 1 (<1)
eGFR <30 mL/min x 1.73 m^2	1 (<1)
Alkaline phosphatase (concentration/u.r.l. ratio) Missing data	0.6 (0.5-0.8) 3 (<1)
Alkaline phosphatase concentration/u.r.l. ratio >1.5	2 (<1)
Monoclonal protein[‡] IgG / IgA / IgM / LC	63 (22) 47 (16) / 3 (2) / 15 (5) / 5 (2)
LC isotype of MC matching with aLC	39 (13)
FLC ratio Missing data	1.10 (0.85-1.46) 5 (2)
Abnormal FLC ratio Missing data	63 (22) 5 (2)
Abnormal FLC ratio matching with aLC Missing data	28 (10) 5 (2)
MC and/or abnormal FLC ratio	101 (34)

LC isotype of MC and/or abnormal FLC ratio matching with aLC	53 (18)
Clonal infiltrate at amyloid deposition site (n=154) Missing data	46 (30) 139 (47)
aLC isotype Kappa : lambda	76 (28) : 217 (74)
Concomitant lymphoma[¶]	7 (2)
Concomitant multiple myeloma[#]	5 (2)
Autoimmune disorders Sjögren syndrome / Autoimmune thyroiditis Rheumatoid arthritis / Psoriasis / SLE / CREST ITP / Other**	61 (21) 18 (7) / 17 (6) 7 (2) / 7 (2) / 2 (1) / 2 (1) 2 (1) / 6 (2)
ANA titer ≥1:640 Missing data	40 (21) 98 (33)

aLC, amyloidogenic light chain; ANA CNS, central nervous system; eGFR, estimate glomerular filtration rate; FLC, free light chain; GI, gastrointestinal; IQR, interquartile range; ITP, idiopathic thrombocytopenic purpura; LC, light chain; MC, monoclonal component; NOS; not otherwise specified; NT-proBNP, N-terminal pro-brain natriuretic peptide; SLE, systemic lupus erythematosus; ST, soft tissues; u.r.l., upper reference limit

**Other: 2 bone involvement (cervical and thoracic vertebra), 1 parotis, 1 perineural amyloidoma*

[†]All the patients without a 24-proteinuria had a urinary albumin to creatinine ratio, that was normal (u.r.l. 30 mg/mmol)

[‡]Three patients had biclonal and 1 a triclonal gammopathy

[¶]Ann Arbor staging: 3 patients in stage I (1 in stage IE), 1 in stage IIIE and 1 in stage IV

[#]Durie-Salmon staging: 3 patients in stage IA and 1 in stage IIIA

***Other: contact urticaria, rheumatic polymyalgia, Reinke edema, primary biliar cholangitis, collagenosis NOS, vasculitis NOS and rheumatic disease NOS in 1 case each*

Table 21. Organ involvement in 293 patients with localized AL amyloidosis

Type of organ involvement	Sex male (N - %)	Age (years)	Multifocal involvement (N - %)*	aLC kappa : lambda (N - %)	MC and/or abnormal FLCR (N - %)	MC and/or abnormal FLCR matching aLC (N - %)	AD (N - %)	Local progression (N - %)	Local-PFS 1 year / 5 years (%)
Respiratory tract, 157 pts	**81 (52)**	**60 (18-82)**	**79 (50)**	**50 (32) : 107 (68)**	**59 (37)**	**32 (20)**	**35 (22)**	**45 (29)**	**89 / 66**
Lung, 63 pts.	37 (59)	68 (35-80)	39 (62)	16 (25) : 47 (75)	29 (46)	17 (27)	21 (33)	15 (23)	95 / 64
Larynx, 51 pts.	23 (45)	51 (19-80)	16 (31)	20 (39) : 31 (61)	14 (27)	7 (14)	9 (18)	19 (37)	78 / 52
Lower airways, 31 pts	14 (45)	55 (26-78)	24 (77)	8 (26) : 23 (74)	13 (42)	7 (23)	4 (13)	8 (26)	94 / 80
Nasopharynx, 12 pts	7 (58)	49 (18-82)	-	6 (50) : 6 (50)	3 (25)	1 (8)	1 (8)	3 (25)	87 / 73
Urinary tract, 37 pts	**21 (58)**	**58 (25-83)**	**12 (32)**	**7 (19) : 30 (81)**	**10 (27)**	**5 (13)**	**6 (16)**	**11 (30)**	**70 / 59**
Bladder, 28 pts	14 (50)	58 (38-83)	10 (36)	5 (18) : 23 (82)	6 (21)	4 (13)	6 (21)	6 (21)	-
Ureter, 5 pts	4 (80)	65 (56-74)	1 (20)	1 (20) : 4 (80)	2 (20)	-	-	4 (80)	-
Urethra, 3 pts	3 (100)	47 (25-57)	1 (33)	1 (33) : 2 (67)	2 (67)	1 (33)	-	1 (33)	-
Kidney interstitium, 1 pt	Female	72	-	Lambda	-	-	-	-	-
GI tract, 35 pts	**24 (69)**	**59 (38-80)**	**16 (46)**	**6 (17) : 29 (83)**	**8 (23)**	**2 (6)**	**3 (9)**	**7 (20)**	**89 / 71**
Bowel, 25 pts	17 (68)	62 (43-78)	11 (44)	3 (12) : 22 (88)	5 (20)	1 (4)	1 (4)	4 (16)	-
Stomach, 5 pts	3 (60)	51 (38-68)	4 (80)	2 (40) : 3 (60)	2 (20)	1 (20)	1 (20)	-	-
Oral mucosa, 3 pts	2 (67)	55 (49-80)	1 (33)	All lambda	1 (25)	-	1 (33)	2 (50)	-
Tongue, 2 pts	2 (100)	52 (40-63)	-	1 (50) : 1 (50)	-	-	-	1 (50)	-
Skin and ST, 31 pts	**11 (35)**	**57 (28-82)**	**15 (48)**	**5 (16) : 26 (84)**	**13 (42)**	**8 (26)**	**14 (45)**	**14 (45)**	**82 / 52**
Skin, 23 pts	8 (35)	58 (28-82)	12 (52)	3 (13) : 20 (87)	9 (29)	5 (22)	11 (48)	10 (43)	-
Soft tissues, 6 pts	3 (50)	45 (38-80)	2 (33)	1 (17) : 5 (83)	4 (67)	3 (50)	2 (33)	3 (50)	-
Breast, 2 pts	All females	52 (39-65)	1 (50)	1 (50) : 1 (50)	-	-	1 (50)	1 (50)	-
Eye, 12 pts	**2 (17)**	**51 (27-64)**	**1 (8)**	**1 (8) : 11 (92)**	**3 (25)**	**-**	**2 (17)**	**4 (33)**	**90 / 52**
Conjunctiva, 9 pts	1 (11)	51 (27-59)	1 (11)	1 (11) : 8 (89)	2 (17)	-	1 (11)	2 (22)	-
Eyelid, 2 pts	1 (50)	57 (51-64)	-	All lambda	1 (50)	-	1 (50)	1 (50)	-
Orbit, 1 pt	Female	36	-	Lambda	-	-	-	Progressed	-
CNS, 9 pts	**All females**	**48 (37-61)**	**-**	**1 (1) : 8 (99)**	**3 (33)**	**3 (33)**	**-**	**6 (67)**	**89 / 41**
Brain, 7 pts	All females	48 (37-61)	-	All lambda	3 (42)	3 (42)	-	5 (71)	-
Gasser ganglion, 2 pts	All females	46 (44-48)	-	1 (50) : 1 (50)	-	-	-	1 (50)	-
Lymphatic tissue, 8 pts	**4 (50)**	**56 (23-72)**	**6 (75)**	**5 (62) : 3 (38)**	**3 (38)**	**2 (25)**	**1 (13)**	**2 (25)**	**100 / 76**
Lymph nodes, 6 pts	3 (50)	65 (33-72)	All	4 (67) : 2 (33)	2 (33)	1 (17)	1 (17)	1 (20)	-
Tonsils, 1 pt	Male	43	-	Kappa	1	1	-	-	-
Adenoids, 1 pt	Female	23	-	lambda	-	-	-	Progressed	-

AD, autoimmune disorders; aLC, amyloidogenic light chain; GI, gastrointestinal; LC, light chain; Local-PFS, local progression free survival; pts, patients; ST, soft tissues.

**In 9 (3%) patients, multiple amyloid deposits extended through more than one organ. In 7 cases, amyloid was disseminated through the respiratory tract. In one case localized AL amyloidosis presented as nodular amyloidomas in the skin (skin and soft tissues involvement) and in the oral mucosa (GI tract involvement). In another patient, amyloid deposits were found in nasopharynx (respiratory involvement) and in a lateral cervical lymph node (lymphatic tissue involvement).*

In 5 biopsies a MZL was diagnosed, while in 8 the clonal infiltrate was characterized as plasma cellular differentiated B-cell neoplasia. A clonal infiltrate was more frequently identified in samples from patients with skin and soft tissues involvement (64% vs. 26%; P=0.01). A significant correlation was found between biopsy length and identification of a clonal infiltrate (OR 1.21, 95% CI 1.02-1.45; P=0.02). In the 9 patients with a skin and soft tissues lo and a documented clonal infiltrate at site of amyloid deposition, median biopsy length was 3 cm (range 0.7-9 cm), 5 of them presented with a concomitant autoimmune disorder (Sjögren syndrome in 2 cases), 6 had an ANA titer ≥1:640 and 3 had a concomitant monoclonal component and/or abnormal FLC ratio matching the amyloidogenic LC.

Overall, 101 (34%) patients had a monoclonal component and/or an abnormal FLC ratio, matching the LC isotype of the amyloidogenic LC identified in the tissue specimen in 53 (18%) cases (Table 21). Five patients had a concomitant MM and 3 received chemotherapy. Seven (2%) patients had a of B-cell lymphoma, that was a MZL in 4 and MALT lymphoma in 3 cases, respectively. A systemic lymphoma disease was present in 4 patients. The presence of the lymphomatous infiltrate at the site of amyloid deposition was demonstrated in 5 of 7 cases.

A concomitant autoimmune disorder was present in 61 (21%) patients and was more frequent in skin localized AL amyloidosis (45% vs. 18%; P=0.001). Sjögren syndrome was most common and present in 18 (6%) patients (8 with lung and 6 with skin and soft tissues localized AL amyloidosis), followed by autoimmune thyroiditis in 17 (6%). Among the 195 patients tested for ANA, 40 (21%) presented an ANA titer ≥1:640, even if a clear autoimmune disorder was not identified in 17 of these patients. In patients with an ANA titer ≥1:640, organ involvement was skin and soft tissues in 16 (40%) and lung in 9 (23%) cases, respectively. A monoclonal component and/or an abnormal FLC ratio was present in 31 patients with a concomitant autoimmune disorders and, overall, was more commonly found in these cases (51% vs. 34%; P=0.03). In subjects with an ANA titer ≥1:640, a concomitant monoclonal and/or an abnormal FLC ratio was observed in 19 (48%) cases. Finally, Moreover, hypergammaglobulinemia was observed in 30 (10%) cases.

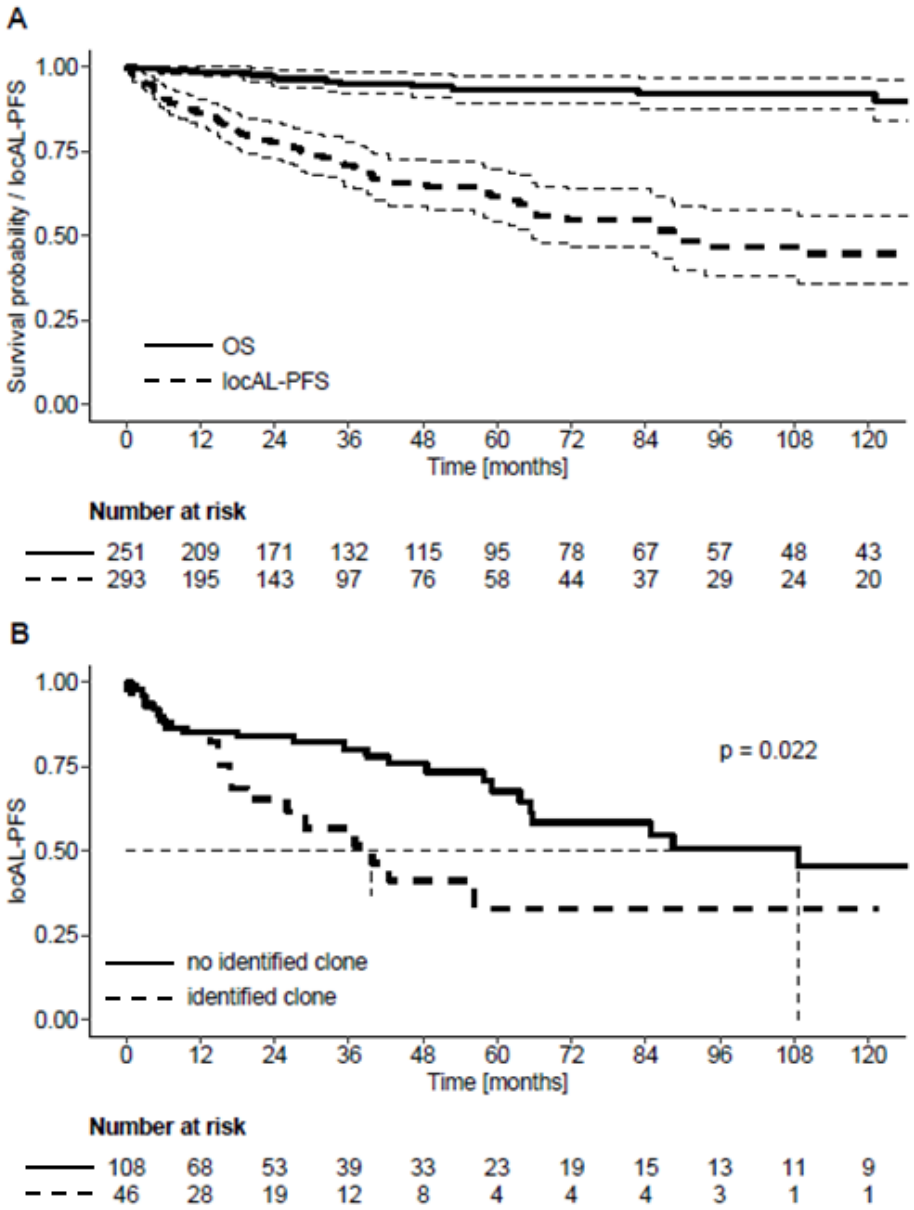
After a median follow-up of 44 months, 16 patients died (9 with lung, 4 with lower airways and 3 with nasopharynx involvement). Ten of these subjects were also smokers. Median OS at 5- and 10-years was 94% and 92% (Figure 21A). OS was poorer in patients with lung involvement (5-years OS 79% vs. 97%; P<0.001). Death was attributed to localized AL amyloidosis in only one case. Not-amyloid related causes of death were neoplasia (2 patients), chronic-obstructive pulmonary disease exacerbation (2 patients), sepsis (1 patient) and cerebral haemorrhage (1 patient). Cause of death was unknown in the remaining 9 subjects. One of these patients died 22 months after progression to systemic AL amyloidosis.

Progression to systemic AL was observed in 3 patients (2 with lung and 1 with nasopharynx involvement) after 24, 46 and 241 months from diagnosis of localized AL amyloidosis. In these subjects, the detection of a monoclonal component matching the amyloidogenic LC isotype preceded the new involved organ site. New involved organ site was thyroid and GI tract in one case each of lung localized AL amyloidosis and lymph nodes in a patient with nasopharynx

involvement. However, no patient had a progression to systemic AL amyloidosis with heart, kidney or liver involvement.

During the follow-up, 91 (31%) patients had a local progression and median Local-PFS was 88 months, with 62% and 44% patients without a local progression at 5 and 10 from diagnosis (Figure 21A). Organ-site specific disease outcomes from diagnosis are summarized in Table 21. No difference in Local-PFS was observed between patients with κ or λ LC isotype (88 vs. 86 months; P=0.63). In patients with lung, larynx, urinary tract, GI tract and skin localized AL amyloidosis (i.e. the most frequently involved organs), 5-year Local-PFS was similar with no significant differences in median time to local progression (overall median Local-PFS 85 months; P=0.34). In CNS localized AL amyloidosis, 5-years locAL-PFS was particularly low (41%). When this small group was compared with the overall population, significance was slightly missed (40 vs. 88, P=0.07). No difference in Local-PFS was found in patients with multifocal involvement (85 vs. 138 months; P=0.99), concomitant autoimmune disorders (138 vs. 88 months; P=0.28) or ANA titer ≥1:640 (138 vs. 88 months; P=0.67), as in those with a monoclonal component and/or an abnormal FLC ratio with the same LC isotype as the amyloidogenic LC (88 vs. 65 months; P=0.78, Supplemental Figure 2B). Notably, a shorter Local-PFS was observed among patients with an identified clonal infiltrate at amyloid deposition site (40 vs. 109 months; P=0.022, Figure 21B), and was similar in those with a plasma cellular or a lymphoid clone (40 vs. 43 months; P=0.89). Presence of MGC and/or inflammatory infiltrate showed no significant difference (85 vs. 43 months; P=0.37). However, a better Local-PFS was found in lung localized AL amyloidosis patients in which MGC and/or an inflammatory infiltrate (65 vs. 42 months; P=0.01) were identified. Importantly, presence of an identified local plasma cell or B-cell clone again showed a shorter Local-PFS (42 vs. 65 months; P=0.02).

Figure 21. Clinical history of localized AL amyloidosis and factors affecting local progression.



Overall survival and Local-PFS from diagnosis of localized AL amyloidosis. Projected overall survival at 10 years was 92%, while median Local-PFS after diagnosis of amyloidosis was 88 months. Overall survival data were missing in 42 patients (A). Local-PFS from diagnosis in 154 patients with a characterized cellular infiltrate at amyloid deposition site: patients with an identified clone have a shorter Local-PFS (B). Figure from Basset, et al. AJH 2020

Discussion

The present studies extensively evaluated the role of clonal and organ biomarkers in different aspects of AL amyloidosis and aimed to show the effectiveness of a biomarker-based approach from diagnosis and staging of organ involvement to the more complex field of a tailored-treatment strategy.

Our first objective was evaluating the possibility to use UACR instead of 24h-proteinuria for diagnosis of renal involvement, prognostication of renal outcome at diagnosis and renal response assessment after chemotherapy. The rationale of this study lies on the convenience of a renal marker that is independent of 24h-urine collection. Patients with AL amyloidosis have often to travel to national referral centers for diagnosis and response to therapy assessment that requires frequent evaluations (every 1-3 months). In this setting, the 24h-urine collection is uncomfortable and exposed to a potential preanalytical error, due to an incorrect collection. We observed a remarkably good concordance between UACR and 24h-proteinuria at baseline (Pearson coefficient r: 0.90; $P<0.001$). This was in agreement with the results observed by Mayo Clinic. We then tested the UACR cutoffs identified by Mayo Clinic investigator in our case series, giving the possibility of validating them in an independent and larger population. First we found that the proposed diagnostic cutoff (300 mg/g) can identify renal involvement with a 90% concordance with the 24h-proteinuria cutoff (0.5 g/24h). Second, replacing 24h-proteinuria (5 g/24h) with UACR (3600 mg/g) in the renal staging systems it was possible to discriminate between 3 different groups of patients with significantly different renal survival and risk of progression to end-stage renal failure requiring dialysis. Notably, when the two renal staging systems were compared, patients who reclassified from stage I to stage II according to the UACR-based one eventually progressed to dialysis. This probably indicates that the this staging system might even improve the discriminating

ability of the standard staging system, possibly because of lower probability of preanalytical error. Importantly, in the our study UACR was measured on the first morning void, that is considered more reliable than random spot urine samples for the assessment of microalbuminuria.[225] Finally, for the first time we showed prospectively that UACR-based renal response, defined as a UACR reduction >30% at 6 months from baseline without a worsening of eGFR >25%, can predict renal outcomes. This analysis was not conducted by Mayo Clinic investigator in their paper. Therefore, we provided an internal validation. We observed that UACR renal responders had a significantly longer renal survival both in the testing and in the validation cohort. Similarly to what observed for renal staging, patients who achieved a renal response assessed with 24h-proteinuria and that eventually progressed to dialysis were reclassified as non-responders according to the UACR-based renal response criterion. This further indicates a better discriminating ability of UACR in the identification of patients with worse renal outcome, even in renal response assessment.

Our second objective was assessing the effectiveness of a biomarker-based response-driven approach for a sequential treatment strategy of bortezomib-based induction and ASCT in newly-diagnosed AL amyloidosis. Based on our data, this sequential treatment approach was highly effective, with a VGPR/CR rate of 63%. Overall organ response rate was 35% for cardiac response and 48% for renal response. It is interesting to note that our data on deep hematologic responses are comparable to those observed in the HOVON 104 trial (VGPR/CR in 50% of cases by intention-to-treat). According to our results, this sequential approach resulted in sparing ASCT in 45% of subjects. Importantly, OS (exceeding 10 years in >70% of patients) and duration of response and duration of response (median 4.5 years) were not different between patients who achieved satisfactory response after CyBorD alone or after CyBorD followed by ASCT. Moreover, the duration of response observed in our study in patients with a satisfactory response was similar to

that reported by the Boston University group with ASCT (4.3 years).[103] This means that even induction with CyBorD alone can result in long-lasting responses if a deep hematologic response is achieved. It is noteworthy to note that in this study 16 (11%), who did not satisfactorily respond to CyBorD progressed and lost eligibility for ASCT. It is not possible to exclude that in these cases upfront ASCT would have resulted in a better outcome. Right now, there are limited data of prognostic factors able to predict outcome before first-line CyBorD. However, it is clear that iFISH evaluation may play a role identifying identify subjects who are less likely to respond to CyBorD. For example, the presence of t(11;14) results in less frequent and less profound responses in patients exposed to CyBorD, resulting consequently in shorter survival.[23] In these cases high dose melphalan can result in significantly better outcome.[26]

Our third objective was evaluating the possibility of cardiac responses based on variation of NT-proBNP after treatment in patients with stage IIIb AL amyloidosis. In agreement with previous studies, we observed that in these cases achieving an early and deep hematologic responses resulted in better survival, with a median OS of 4.25 years. We observed that cardiac responses are rare (8% of cases) but possible. Importantly, patients who achieved a cardiac response after 90 days from starting therapy had a median OS exceeding 4 years. Survival was even longer (median 7.6 years) in patients with a deep cardiologic response (cardiac VGPR) as for new proposed graded cardiac response criteria. A worse outcome. Conversely, cardiac progression resulted in shorter survival even in those patients achieving a VGPR at 90 days. The use of a CHCR model nicely show the combination of deep hematologic responses and cardiac responses in these patients. Particularly, a long survival (median 4.3 years) was observed in those with a CHCR score of 0-2 after 90 days of treatment. Taking in count the way the CHCR score is calculated, patients in this group had achieved at least a PR with cardiac response. Looking at the composition of this group,

it seems that CHCR score 0-2 nicely resembles our definition of satisfactory response in the sequential approach of induction and ASCT. These results further highlight the need of a treatment that could grant rapid and profound hematologic responses that can translate in as quick cardiac responses. The results from precedent published studies, especially those from ANDROMEDA trial,[110] suggest that dararatumumab can represent a valuable treatment option also in these patients with a severe cardiac involvement. Actually, the effect of daratumumab is under evaluation in newly-diagnosed stage IIIb AL amyloidosis inside of a currently ongoing phase II multicenter clinical trial (NCT04131309).

Our fourth objective was evaluating whether clonal and organ biomarkers can identify patients with relapsed/refractory AL amyloidosis who can benefit the most from a treatment with LDex. We observed a relatively low hematologic response rate (31%) after 3 months of treatment with an OS of 32 months. However, this data were comparable to those observed in a pooled analysis of three clinical trials evaluating effectiveness of IMiDs (lenalidomide and pomalidomide) in AL amyloidosis (hematologic response rate: 39%; OS: 36 months). On multivariable analysis, NT-proBNP before LDex initiation was confirmed as a powerful prognostic factor.[147,213] Three identified clonal prognostic factors were identified: high dFLC at LDex initiation, gain1q21 and λ LC isotype. The adverse prognostic role of gain 1q21 was already described in patients with MM treated with LDex,[226] while in AL amyloidosis it represent a negative prognostic factor in subjects exposed to oral melphalan and dexamethasone[28] and, more, recently, to daratumumab.[161] We showed for the first time that gain1q21 resulted in shorter OS and hemEFS in patients with relapsed/refractory AL amyloidosis treated with LDex. Translocation (11;14) was not associated with better survival although these patients were more likely to achieve VGHR after 3 months of treatment. Interestingly, the same observation was made in patients with high risk iFISH. We

showed that patients presenting these clonal prognostic factors and NT-proBNP at LDex initiation are capable to identify patients with worse outcome. Regarding treatment toxicity, multivariable analysis revealed that 24h-proteinuria and eGFR were the only prognostic factors for renal survival. These results further highlight the issue of nephrotoxicity of lenalidomide in AL amyloidosis, especially in presence of renal failure and proteinuria.[146] Lastly, we observed that even if follow-up with this cardiac biomarker is hampered by the frequent increase of its concentration during treatment with IMiDs, cardiac progression – defined as increase of NT-proBNP >30% and >300 ng/L from baseline – at 3 and 6 months from treatment initiation still predicts survival. Therefore, signs of early cardiac progression should be evaluated carefully and are clinically meaningful. The role of lenalidomide in AL amyloidosis is animatedly discussed, especially after the advent of novel and powerful drugs as daratumumab and ixazomib. However, both this drugs can be used in combination with LDex. Ixazomib, lenalidomide and dexamethasone is a powerful oral triplet in AL amyloidosis, with an hematologic response rate of 59% (VGHR in 41%).[144,157] Regarding the possibility of treatment with DRD, one rationale of this combination is the synergic activity of lenalidomide, enhancing the expression of CD38 on the cellular membrane of MM plasma cells.[227] In relapsed/refractory AL amyloidosis, treatment with DRD resulted high rate of hematologic responses (VGHR in 65%) and long-lasting responses (median hemEFS 17.3 months). Interestingly, gain 1q21 resulted again in shorter hemEFS and lower VHGR rate.[228] Giving the better hematologic response rate, lenalidomide combinations, especially those with proteasome inhibitors and daratumumab, seems particularly appealing. However, it should be kept in mind that triple regimens are characterized by increased treatment-related toxicity and mortality, especially in frail AL amyloidosis patients.[124,229]

After these four studies, we tried to evaluate the use of biomarker for characterization, prognostic stratification and response assessment in rarer forms of amyloidosis, i.e. that represent less than 10% of patients with amyloidosis at international referral centers: IgM-AL amyloidosis, AL amyloidosis with a non-lymphoplasmacytic LPD and localized AL amyloidosis.

Our fifth objective was exploring the role of clonal biomarkers in IgM-AL amyloidosis. Our study represents one of the largest single-center case series of patients with systemic IgM-AL amyloidosis with a median follow-up of almost 4 years. Our study confirmed most of the previous observations on IgM-AL amyloidosis. This rare disease is characterized by a higher prevalence of κ iFLC isotype, with a κ:λ ratio usually of 1:2 (1:1.7 in our series), and a lower dFLC (median 81 mg/L in our study). However, our series of patients reports the highest rate of cardiac involvement (62%), which is still lower than within the overall AL patient population.[230] We first searched for differences in clinical presentation between LPL and PPCN. Sidana and co-workers already showed that LPL and PPCN presented different molecular and genetic characteristics.[179] Importantly, the cytogenetic profile of PPCN was similar to those of non-IgM-AL amyloidosis. We confirmed and extended these observations. Translocation t(11;14) and gain1q21, that are typical cytogenetic aberrations in AL amyloidosis and, more generally, in plasma cell dyscrasias, were observed only in PPCN. Moreover, these patients presented with a higher prevalence of λ LC isotype (82% vs. 56%). Organ involvement seemed also different in PPCN and more similar to non-IgM-AL amyloidosis with a trend for more frequent cardiac and renal involvement. On the other hand, LPL seemed to resemble the classic and well-known characteristics of IgM-AL amyloidosis. Mutation $MYD88^{L265P}$ was observed only in this group, in 69% of analyzed cases. Heart was involved in 58% of cases, κ:λ ratio was 1:1.3, IgM levels were higher and bone marrow clonal infiltrate more extensive. Patients with LPL were more likely to have a long history of hematologic disease, presenting with an

identified IgM monoclonal component several years (median 3.8 years) before diagnosis of AL amyloidosis in almost half of cases. Hematologists should be aware that AL amyloidosis may be a rare but life-threatening event in patients with IgM-MGUS or IgM-related lymphoproliferative disorders. As a consequence, organ biomarkers (e.g. NT-proBNP and albuminuria) should be part of the standard clinical follow-up, along with evaluation of signs distal symmetrical neuropathy and cholestasis in order to be able to diagnose AL amyloidosis at a pre-symptomatic stage. Multivariable analysis allowed the identification of prognostic factors for OS and hemEFS. As previously observed, B-cell clone phenotype and previous treatment before diagnosis of AL amyloidosis did not affect prognosis.[179] European Mayo staging and dFLC were the only prognostic factors for OS, while only dFLC was clearly prognostic for hemEFS. The prognostic role of IgM concentration in IgM-AL amyloidosis at diagnosis is less clear. However, it seems that higher IgM levels result in higher risk of progression, even if they are not associated with a greater risk of mortality. The possible explanation behind this observation could be that IgM levels are just a marker of clonal disease, while FLCs are the effectors of organ dysfunction, also by direct toxicity.[45] However, it is interesting to point out that in LPL patients IgM concentration was associated with worse OS and hemEFS at univariable analysis. This nicely fits with the observations made in WM and IgM-MGUS.[8,231]

Within IgM-AL amyloidosis it is widely accepted to assess hematologic response both with dFLC and IgM concentration.[17,178] However, our results showed that a profound dFLC reduction after treatment translated in significantly longer OS (median 8 years for patients in VGHR). Thus, early VGHR according to AL amyloidosis response criteria should be the therapeutic goal as in non-IgM-AL amyloidosis. This is supported also by the shorter OS observed in IgM-responders lacking a concomitant dFLC-response, further highlighting that the profound reduction of the amyloidogenic precursor is required to improve patient outcome. Nevertheless, IgM-response also translated in

better OS, making reasonable to use IgM levels for response assessment in those isolated cases in which hematologic response cannot be evaluated with dFLC (i.e. subjects with a baseline dFLC<20 mg/L). However, in all other cases dFLC should be preferred as clonal marker for response assessment.

Finally, described the outcome observed with the most commonly used therapies. Rituximab-based regimens, as rituximab and bortezomib and rituxiamab and bendamustine, represent a reasonable option in LPL patients. This is in line with the results of two different retrospective studies (Table 18).[185,186] Non-rituximab-based strategies were more often used as first-line treatment in PPCN, although they were also frequently offered to relapsed/refractory patients with LPL, probably due to the previous extensive exposure to rituximab. Bortezomib-based regimens showed a high rate of deep hematologic responses, particularly in treatment-naïve patients (VGHR >50%). Higher response rate was observed in PPCN than in LPL (80% vs. 17%). However, bortezomib-based regimens were also offered in LPL as rescue treatment in most of cases. It should be considered that PNS involvement may represent a limitation to bortezomib exposure in IgM-AL amyloidosis. Only a minority of patients were eligible to ASCT. However, patients treated with ASCT presented a long OS (median 10 years) and hemEFS (median >4 years), confirming the effectiveness of high dose therapy, as already shown in two single-center series,[174,232] and most recently in a larger retrospective study.[184] Interestingly, the benefit on OS and hemEFS was particularly evident in LPL. Oral alkylating agents did not seem an appealing option in these patients, although this is partially in contrast with the observations from two different studies reporting data on melphalan and dexamethasone.[176,178] Finally, we had the chance to extend the follow up and to increase the number of the Heidelberg case series exposed to ibrutinib,[233] confirming that this therapy results in low response rates and short OS and hemEFS.

Our sixth objective was characterizing AL amyloidosis with non-lymphoplasmacytic LPD, with a focus on biomarkers that may help in the identification of systemic forms of amyloidosis. To the best of our knowledge, our work represents the largest clinical series of patients with AL amyloidosis and non-lymphoplasmacytic LPD published so far. Among all the non-lymphoplasmacytic LPD, MZL is the most common one associated with AL amyloidosis. However, other types of LPD can also underlie light chain amyloid deposition. From our observation, it seems that patients with more advanced stage lymphomas were more likely to have systemic amyloidosis. On the other hand, presence of autoimmune disorders (particularly Sjögren syndrome), appeared to be more typical of localized disease. Distinguish from these two forms of diseases is critical considering that systemic AL amyloidosis portended a poor outcome, being the leading cause of death in our series. Notably, a significant diagnostic delay was observed in this group of patients, even in those followed by hematologists for the underlying lymphoma. Earlier diagnosis could improve the outcome of these patients, and the presence of a monoclonal component or an abnormal FLC ratio can be clues to the diagnosis of systemic AL amyloidosis in patients with LPD.

Lastly, our seventh objective was exploring biomarkers in natural history of localized AL amyloidosis, focusing our attention in factors that may affect local progression. Our findings confirm and extend data from previous studies. A concomitant autoimmune disorder and a monoclonal component and/or an abnormal FLC ratio are particularly common in these patients. However, differently from other case series, we observed a prevalence of the λ LC isotype with a κ:λ ratio of 1:3. This is comparable to systemic AL amyloidosis and nicely fits with the evidence for a higher inclination of λ LC to form amyloid fibrils. We also observed an organ type-specific variation of the κ:λ ratio. For example, almost all patients with CNS involvement presented with LC

λ, while in lymph nodes localized AL amyloidosis LC κ was more frequent. These observations confirm and extend previous findings from small case series of CNS and lymph node localized AL amyloidosis.[218,234] Interestingly, the preferential manifestation of either λ or κ in different tissues and organ sites has also been described in systemic AL amyloidosis.[30,235] Moreover, data from Mayo Clinic suggest that also in localized AL amyloidosis IGVL usage may play a role in organ involvement, as in systemic AL amyloidosis.[33] For the first time, we evaluate factors that might affect local progression. In the entire cohort, local progression seems to be not affected by anatomical site, amyloidogenic LC isotype and presence of autoimmune system dysregulation (either as a concomitant autoimmune disorder or an ANA titer >1:640). The presence of a monoclonal component and/or an abnormal FLC ratio did not seem to influence local progression, even in case of a match of the LC with the amyloidogenic LC deposited in tissue. Interestingly, the only factor affecting local progression was the identification of a local B-cell clone, resulting in shorter local-PFS, without difference between clonal plasma cells or B lymphocytes. Due to paucicellularity and a concomitant inflammatory response, detecting the local B-cell clone is a difficult task. Immunohistochemistry may not be sensitive enough to detect the clone in most cases, while more sophisticated approaches for clonality assessment, as in situ hybridization and molecular pathological analyses, are more effective.[2,193,218] Since these latter techniques were not systematically used in our study, it is possible that we identified only cases with more extended B-cell clones, while cases with more subtle infiltrations could have been missed. In any case, it is reasonable to think that a local B-cell clone is always present at amyloid deposition site, even if it cannot be identified. It is important to remember that several studies demonstrated that the local B-cell clone resembles the characteristics of a MZL. However, in our case series, only 5 patients with MZL or MALT presented with a B-cell lymphoma infiltrate at the site of amyloid deposition. Therefore, it seems that a concomitant lymphoma is rarely a cause localized AL amyloidosis. It is

likely that the local B-cell clone can be currently better classified as “localized B-cell neoplasia of undermined significance” in the majority of cases, as reported by a recent study.[193] Thus, like in systemic AL amyloidosis, the B-cell clone is usually small and has not the full characteristics of a well-defined malignant disease. However, it is probably that other unstudied biological characteristics of the local B-cell clone can affect the natural history of localized AL amyloidosis. Finally, some of our results suggest that other characteristics of the local cellular infiltrate may have a role in local progression. More precisely, our data from samples of patients with lung localized AL amyloidosis showed a possible protective role of the inflammatory infiltrate and MGC against local progression. Indeed, it has been hypothesized that these cells may interact with amyloid deposits.[2]

All of these studies present some limitations that are mostly related to their retrospective nature. However, prospective studies are limited by the rarity of the disease and this is particular relevant for rarer forms of AL amyloidosis as IgM-AL amyloidosis and localized AL amyloidosis. The retrospective data collection may have been resulted in an underestimation of adverse events to treatment either in patients treated with the sequential approach of induction with CyBorD and ASCT and in those with treated with LDex. Cytogenetic evaluation with iFISH was not available in all cases. Moreover, iFISH was performed mostly at diagnosis, resulting in a possible underestimation of prevalence of gain1q21 in patients with a relapsed/refractory disease.[214] Finally, in patients with localized AL amyloidosis, clonality assessment of the infiltrate was performed with different techniques with different sensitivity, resulting in a possible underestimation of detected local B-cell clones.

Conclusions and future perspectives

In conclusion, all the present studies showed the central role that biomarkers have in the management of patients with AL amyloidosis. Organ biomarkers are crucial for diagnosis of organ involvement, prognostic stratification and organ response both at diagnosis and in relapsed/refractory disease. We prospectively validated the use of UACR as a biomarker for the identification and staging of renal involvement in AL amyloidosis, and demonstrated that renal response assessment with UACR consistently predicts renal outcomes. Our results combined with those from the Mayo Clinic study allow replacement of 24h-proteinuria with UACR in individual patients' management and in the design of clinical trials.

Organ and clonal biomarkers can be effectively used also for a tailored-treatment strategy in AL amyloidosis in order to identify patients that can benefit the most from treatment targeting the B-cell clone. This is suggested by three different studies here presented. We showed that a sequential, biomarker-based and offering ASCT to patients failing to achieve satisfactory response to upfront CyBorD is very safe and effective in AL amyloidosis. Clonal biomarkers as gain1q21 status, dFLC and LC isotype identified patients with relapsed/refractory AL amyloidosis that benefit more from treatment with LDex. Cardiac and renal biomarkers are useful in treatment management, distinguishing patients more fragile at LDex initiation, in whom treatment with lenalidomide should be considered with attention, and detecting early organ progression. The heterogeneity of B-cell clone in IgM-AL amyloidosis, even if it not plays a role on prognosis, leads the therapeutic strategy following a B-cell clonal phenotype-based approach. Therefore, the characterization of the B-cell clone by immunohistochemistry on bone marrow biopsy, flow cytometry on bone marrow aspirate, iFISH analysis and other molecular analysis (e.g. evaluation of MYD88 mutation state) are of utmost importance in this rarer form of systemic AL amyloidosis. However, given the rarity of IgM-AL amyloidosis, large international multicentric studies are

needed to investigated whether this therapeutic approach may result in better hematologic response rates, OS and duration of response.

The characterization of the B-cell clone can be also important in other rarer forms of AL amyloidosis. In localized AL amyloidosis, for instance, the clonality assessment of the cellular infiltrate at amyloid deposition site with sensitive techniques will improve our ability of identifying the local B-cell clone. This would allow us to extend the study of the biology of the B-cell clone producing amyloidogenic LC to localized AL amyloidosis and would definitely contribute to shed some light on the pathophysiology of this rare amyloidosis.

In the foreseeable future, the availability of new powerful drugs targeting the B-cell clone will probably reshape the clinical history of AL amyloidosis. Particularly, daratumumab is already changing the way patients are treated worldwide. Daratumumab-CyBorD is an highly effective first-line treatment that grants an high rate of deep hematologic responses and organ responses.[110] This will probably change the way ASCT is offered to our patients. Daratumumab will be also modify the role of IMiDs, as lenalidomide, in relapsed/refractory combinations and may be a new powerful therapeutic option in IgM-AL amyloidosis with PPCN. Most importantly, the rapid reduction of dFLC with daratumumab makes this drug particularly appealing for patients with stage IIIb AL amyloidosis, in which a rapid decrease in dFLC with therapy can translate in improvement of cardiac disfunction. We showed here that NT-proBNP is able to detect early cardiac responses also in stage IIIb patients. Moreover, patients with a profound reduction in this biomarker may enjoy a long survival.

Anyway, there is still room for improvement in the quest for organ biomarkers in systemic AL amyloidosis. For example, there is still a lack of validated criteria for liver responses based on survival. The evaluation of PNS involvement is cumbersome and it is often based on neurological examination and nerve function tests. More recently, nerve MRI has been proposed for the study

of PNS involvement in AL amyloidosis.[236] It is likely that for both PNS and liver involvement, the combination of soluble and imaging biomarkers could help the characterization and prognostication of organ involvement. Importantly, our group is currently involved in a prospective study evaluating the role of soluble biomarkers (dFLC, NT-proBNP and troponin) and imaging tools (echocardiogram, cardiac MRI and PET with ^{18F}Florbetaben) in cardiac AL amyloidosis at baseline and at response (NCT04392960). The results of this study are eagerly awaited in order to evaluate the possibility of a better comprehension of the best use soluble and imaging biomarkers in cardiac amyloidosis.

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Scientific production arisen from this thesis

Peer-reviewed publications

1. Basset M, Defrancesco I, Milani P, Nuvolone M, Rattotti S, Foli A, Mangiacavalli S, Varettoni M, Benvenuti P, Cartia CS, Paulli M, Merlini G, Arcaini L, Palladini G. Non-lymphoplasmacytic lymphomas underlying light chain amyloidosis: distinct clinical features and outcome. Blood. 2019 Nov 12. Blood. 2020 Jan 23;135(4):293-296
2. Basset M, Hummedah K, Kimmich C, Veelken K, Dittrich T, Brandelik S, Kreuter M, Hassel J, Bosch N, Stuhlmann-Laeisz C, Blank N, Müller-Tidow C, Röcken C, Hegenbart U, Schönland S. Localized immunoglobulin light chain amyloidosis: Novel insights including prognostic factors for local progression. Am J Hematol. 2020 Jun 29 [Online ahead of print]
3. Basset M, Nuvolone M, Palladini G, Merlini G. Novel challenges in the management of immunoglobulin light chain amyloidosis: from the bench to the bedside. Expert Rev Hematol. 2020 Sep;13(9):1003-1015
4. Basset M, Milani P, Nuvolone M, Benigna F, Rodigari L, Foli A, Merlini G, Palladini G. Sequential response-driven bortezomib-based therapy followed by autologous stem cell transplant in AL amyloidosis. Blood Adv. 2020 Sep 8;4(17):4175-4179
5. Basset M, Kimmich CR, Schreck N, Krzykalla J, Dittrich T, Veelken K, Goldschmidt H, Seckinger A, Hose D, Jauch A, Müller-Tidow C, Benner A, Hegenbart U, Schönland SO. Lenalidomide and dexamethasone in relapsed/refractory immunoglobulin light chain (AL) amyloidosis: results from a large cohort of patients with long follow-up. Br J Haematol. 2021 Oct;195(2):230-243
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renal AL amyloidosis: results from a large cohort of patients. Clin Chem Lab Med. 2022 Jan 13;60(3):386-393

Abstracts at international meetings

1. Light Chain Amyloidosis and Non-Hodgkin's Lymphomas: A Peculiar Association with Distinct Clinical Features and Outcome; poster presentation at 60° ASH annual meeting and exposition, December 1-4 2018
2. Sequential response-driven bortezomib-based therapy followed by autologous stem cell transplant in AL amyloidosis; poster presentation at 61° ASH annual meeting and exposition, December 7-10 2019*
3. Early cardiac response is possible in patients with immunoglobulin light chain amyloidosis and is associated with prolonged survival; poster at the XVII International Symposium on Amyloidosis, September 14-18 2020 (online event)
4. Gain 1q21 is an adverse prognostic factor in patients with relapsed/refractory AL amyloidosis treated with lenalidomide and dexamethasone" poster presentation at the 62° ASH annual meeting and exposition, December 5-8 2020*

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"Man überschätzt wohl leicht das eigene Wirken und Tun in seiner Wichtigkeit gegenüber dem, was man nur durch andere geworden ist." (Translation: it might easily happen to overestimate your own working and doing in their importance compared to what you have become only through others).

Dietrich Bonhoeffer

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