Flow cytometry for fast screening and automated risk assessment in systemic light-chain amyloidosis

Running title: Flow cytometry for AL amyloidosis

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ABSTRACT

Early diagnosis and risk stratification are key to improve outcomes in light-chain (AL) amyloidosis. Here, we used multidimensional-flow-cytometry (MFC) to characterize bone marrow (BM) plasma cells (PCs) from a series of 166 patients including newly-diagnosed AL amyloidosis (N=94), MGUS (N=20) and multiple myeloma (MM, N=52) vs. healthy adults (N=30). MFC detected clonality in virtually all AL amyloidosis (99%) patients. Furthermore, we developed an automated risk-stratification system based on BMPCs features, with independent prognostic impact on progression-free and overall survival of AL amyloidosis patients (hazard ratio: ≥2.9;*P*≤.03). Simultaneous assessment of the clonal PCs immunophenotypic protein expression profile and the BM cellular composition, mapped AL amyloidosis in the crossroad between MGUS and MM; however, lack of homogenously-positive CD56 expression, reduction of B-cell precursors and a predominantly-clonal PC compartment in the absence of an MM-like tumor PC expansion, emerged as hallmarks of AL amyloidosis (ROC-AUC=0.74;*P*<.001), and might potentially be used as biomarkers for the identification of MGUS and MM patients, who are candidates for monitoring pre-symptomatic organ damage related to AL amyloidosis. Altogether, this study addressed the need for consensus on how to use flow cytometry in AL amyloidosis, and proposes a standardized MFC-based automated risk classification ready for implementation in clinical practice.

INTRODUCTION

Systemic amyloidosis is caused by misfolding and extracellular deposition of circulating proteins as amyloid fibrils leading to progressive organ damage. Thus, targeting the earlier steps of the amyloid pathogenic process would facilitate the highest therapeutic efficacy by preventing the propagation of abnormal protein folding and its aggregation that precede tissue deposition and organ damage.(1) Light chain (AL) amyloidosis is the most common form of systemic amyloidosis, which is caused by a small plasma cell (PC) clone that produces misfolded light-chains that might target virtually every organ except the brain.(2)

Survival of patients with AL amyloidosis depends on the extent of organ involvement (mainly cardiac damage), response to treatment and the PC burden/biology.(1) Cardiac involvement is the leading cause of death, and early diagnosis is critical to anticipate irreversible end-organ damage.(3) Thus, biomarkers that help identifying patients with (both intact immunoglobulin and light-chain) monoclonal gammopathy of undetermined significance (MGUS) or multiple myeloma (MM) at greater risk of developing AL amyloidosis, are highly valuable to monitor pre-symptomatic organ damage (e.g. NT-proBNP and albuminuria for cardiac and renal involvement, respectively).(4,5) Unfortunately, no tumor clone feature or phenotype has been identified so far that could anticipate this risk; in fact, the low incidence of AL amyloidosis and its low tumor burden, often masked by a polyclonal PC background, account for limited information on the tumor PC biology, particularly when compared to MM.(6)

Final diagnosis of AL amyloidosis typically relies on histology and it is often difficult to achieve. Because of the small size of the PC clone in AL amyloidosis, identification of the M-component requires at least immunofixation of both serum and urine, plus serum free light-chain (sFLC) measurements. Of note, around 15% of patients with AL amyloidosis present with significant organ involvement but a normal sFLC ratio, and additional tests are required in these patients for the identification of

the amyloidogenic PC clone.(7) Thus, new high-sensitive tools for the detection of very small clones have been proposed, such as mass spectrometry (8,9) and next-generation flow (NGF) cytometry.(10),(11) In recent years, flow cytometry immunophenotyping has proven to be a valuable technique in the differential diagnosis of some monoclonal gammopathies (12,13) and to predict outcome in MM, based on the extent of PC clonality in the BM.(14,15) Sporadic studies based on small patient series and/or earlier generations of the technique have pointed out the potential clinical utility of flow cytometry in AL amyloidosis (16,17), but no consensus exists on how to use and interpret flow cytometry results in these settings.(11)

Here, we used multidimensional flow cytometry (MFC) and the EuroFlow NGF antibody panel to characterize a large series of patients with newly-diagnosed AL amyloidosis vs MGUS and MM, as well as age-matched healthy adults. Our results support the use of flow cytometry-based automated risk-stratification in AL amyloidosis, mapped the disease in the crossroad between MGUS and myeloma, and identified immunophenotypic markers significantly associated with AL amyloidosis that could potentially be used as red-flags for monitoring of pre-symptomatic organ damage in MGUS and MM patients at risk of developing an associated AL amyloidosis.

SUBJECTS AND METHODS

Patients. A total of 94 patients with confirmed new diagnosis of AL amyloidosis based on the presence of amyloid-related systemic syndrome, positive amyloid tissue staining with Congo red, restricted LC deposition, and evidence of PC clonality, were studied with a median follow-up of 15 months (range: 1-40 months). The number of events for progression and for death were 14 and 30, respectively. Nine of the 94 cases were diagnosed with AL amyloidosis plus MM based on the presence of ≥20% BMPCs by cytomorphology. Patients' demographics and clinical characteristics are described in Table 1. Organ involvement was defined according to the updated consensus criteria for amyloid-related organ involvement (18,19). In parallel, 30 healthy adults of similar age, 20 MGUS and 52 newly-diagnosed MM patients were also included in the study for comparison with AL amyloidosis. Overall, a total of 196 bone marrow (BM) samples were collected after informed consent was given, in accordance with the local ethics committee guidelines and the Declaration of Helsinki.

Tumor PC immunophenotyping. NGF-based antibody combinations were used for the characterization of BM samples of healthy adults, and for baseline assessment of clonality in patients with MGUS, as well as newly-diagnosed AL amyloidosis and MM.(10) Briefly, the EuroFlow lyse-wash-and-stain standard sample preparation protocol (adjusted to 10^6 nucleated cells in MGUS, AL amyloidosis and MM) together with the optimized 2-tube 8-color EuroFlow NGF antibody panel, for accurate identification of BM PCs and discrimination between phenotypically aberrant and normal PCs -tube 1: CD138-BV421, CD27-BV510, CD38-FITC, CD56-PE, CD45-PerCPCy5.5, CD19-PECy7, CD117-APC, CD81-APCH7; and, tube 2: CD138-BV421, CD27-BV510, CD38-FITC, CD56-PE, CD45-PerCPCy5.5, CD19-PECy7, cyKAPPA-APC, cyLAMBDA-APCH7-, were used. The two-tube strategy allows detection of clonality with specific confirmation of light-chain restriction on phenotypically aberrant PCs, identified either by antigen under-expression (CD19, CD27, CD38, CD45, CD81)

and/or antigen over-expression (CD56, CD117, CD138). Tube 2 was not stained in BM samples from patients with newly-diagnosed MM due to the high-level infiltration by clonal PCs. In a subset of patients with AL amyloidosis (N = 38) and in all cases with MM (N = 52), additional staining for the following five 8-color monoclonal antibody combinations (BV421, BV510, FITC, PE, PerCPCy5.5, PECy7, APC, APCH7) were performed: 1) CD138, CD24, CD38, SLAMF7, CD45, CD19, CD229, CD20; 2) CD138, CD9, CD38, CD21, CD45, CD19, CD63, CD10; 3) CD138, HLADR, CD38, CD53, CD45, CD19, CD200, CD268; 4) CD138, CD49d, CD38, CD74, CD45, CD19, CD274, CD69; 5) CD138, CD196, CD38, CD184, CD45, CD19, CD31, CD43. Data acquisition was performed in a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) using the FACSDiva 6.1 software (BD). Data analysis was performed using the Infinicyt software (Cytognos SL, Salamanca, Spain).

Profiling of the BM cellular composition. The first 8-color antibody combination of the NGF panel described above was used for enumeration of $CD38^{-}$ ($CD117^{+}$, $CD38^{-}$, $CD45^{+}$, SSC^{lo}), $CD38^{+}$ ($CD117^{+}$, $CD38^{+}$, $CD45^{+}$, SSC^{lo}), erythroid ($CD117^{+}$, $CD38^{-/dim}$, $CD45^{-/dim}$, SSC^{lo}) and myeloid ($CD117^{+}$, $CD38^{+}$, $CD45^{dim}$, SSC^{hi}) hematopoietic progenitors, basophils ($CD38^{+}$, $CD81^{-}$, $CD45^{dim}$), eosinophils ($CD45^{bright}$, $CD81^{bright}$, SSC^{hi}), monocytes ($CD45^{+}$, $CD38^{+}$, $CD81^{+}$, SSC^{int}), mast cells ($CD117^{bright}$, $CD45^{dim}$), neutrophils ($CD45^{dim}$, $CD81^{-}$, SSC^{hi}), erythroblasts ($CD45^{-}$, $CD38^{-}$, SSC^{lo}), TNK- plus NK-cells ($CD45^{+}$, $CD56^{+}$, $CD19^{-}$, SSC^{lo}) and other remaining non-T/NK T-lymphocytes ($CD45^{+}$, $CD56^{-}$, $CD19^{-}$, SSC^{lo}), as well as B-cells and their subsets of B-cell precursors ($CD19^{+}$, $CD45^{dim}$, $CD38^{bright}$, $CD27^{-}$), naïve ($CD19^{+}$, $CD45^{+}$, $CD38^{-/dim}$, $CD27^{-}$) and memory ($CD19^{+}$, $CD45^{+}$, $CD38^{-/dim}$, $CD27^{+}$) B-lymphocytes; such data were used to generate individual patient' BM immune profiles for each case analyzed (N = 196 BM samples).

Automated immunophenotypic characterization of patients with AL amyloidosis. Automated immunophenotyping for patient's classification and prediction of outcome was performed by comparing the percentage of BM PCs plus the relative percentage of clonal and normal PCs within the whole BM PC compartment for each patient with AL amyloidosis vs a database previously developed that contained information on the same three parameters from a total of 1,774 patients, including 497 MGUS and 1,227 newly-diagnosed MM enrolled in the GEM2000 (N = 486) plus GEM2005MENOS65 (N = 330) protocols for transplant-eligible cases, and the GEM2005MENOS65 (N=239) plus GEM2010MAS65 (N=222) protocols for transplant-ineligible patients.(15) Using EuroFlow software tools, principal component analysis (PCA) based on those three parameters was performed and graphically displayed via the automatic population separator (APS; PCA1 vs. PCA2) representation of Infinicyt. Based on the APS view defined through direct comparison of MGUS and symptomatic MM patients, two clear cut groups of patients defined by 1.5 standard deviation borders emerged, corresponding to MGUS and MM cases, respectively; each AL amyloidosis patient was then represented by a dot in such bi-dimensional (PC1 vs PC2) APS representation of the patient profiles. Finally, each patient with AL amyloidosis plotted against the dataset, was classified as MGUS-like, intermediate or MM-like. In parallel, automated discrimination of AL amyloidosis patients displaying different immunophenotypic protein expression profiles (iPEP) of clonal PCs was performed using the t-Distributed Stochastic Neighbor Embedding (t-SNE) projection available in the Infinicyt software. Comparison between the iPEP of clonal PCs from patients with AL amyloidosis vs MGUS and MM cases, was performed using the canonical-correlation analysis (CCA) tool and graphical representation of the Infinicyt software. The BM cellular composition in healthy adults, MGUS, AL amyloidosis and MM patients was compared and the distance among individual cases evaluated using the 2-dimensional minimum spanning tree (MST) force-directed classification and graphical

representation available in the Infinicyt software, based on Euclidean distances and the Boruvka algorithm.

Statistical analyses. Progression-free survival (PFS) was measured from time of diagnosis to hematological and/or organ progression or death from any cause; overall survival (OS) was defined as time from diagnosis to death from any cause.(20) Survival was analyzed by the Kaplan–Meier method, and differences between curves were tested for statistical significance with the (two-sided) log-rank test. A multivariate Cox proportional hazard model was developed to explore the independent effect on PFS and OS of well-known prognostic factors in AL amyloidosis: patients' age, number of organs involved, Mayo (2012) staging, the percentage of BM PCs based on cytomorphology, and treatment with autologous stem cell transplantation (ASCT).(21) Receiver Operating Curve (ROC) analysis was used to assess the accuracy of the scoring model developed with immunophenotypic parameters with significantly different prevalence in AL amyloidosis vs MGUS and MM. The χ^2 or Mann-Whitney U and the Kruskal-Wallis non-parametric tests were used to evaluate the statistical significance of differences observed between two and more groups, respectively. The SPSS software (version 20.0; IBM, Chicago, IL,) were used for all statistical analyses.

RESULTS

Assessment of clonality in AL amyloidosis using MFC. Clonal PCs were detected in BM samples from 93 of the 94 (99%) patients with AL amyloidosis (median, 0.8%; range, 0% - 21%), whereas a (serum and/or urine) M-component by electrophoresis or immunofixation and an abnormal serum free-light chain ratio were detected in 80.5%, 93% and 75% of patients, respectively (Table 1). Overall, only 4 patients had undetectable M-component by any of the three methods (4%), and clonal PCs were detectable by MFC in all 4 cases (range, 0.004% - 0.22%). Morphological assessment of PC infiltration in BM aspirates of AL amyloidosis patients' (median, 8%; range, 0% - 60%), showed ≤1% PCs in 9% of cases, ≤5% in 33% of patients and 55% had <10% BM PCs.

The median number of PCs by MFC was 0.9% and the percentage of clonal and normal PCs within the BM PC compartment was of 93% and 7%, respectively. Firstly, we validated thresholds of total BM PCs (i.e. <1% vs ≥1%), clonal PCs (i.e. <2.5% vs ≥2.5%) and normal PCs within the BM PC compartment (i.e. ≤5% vs >5%), that had been previously shown to be of prognostic value (Supplementary Figure 1).(16,17) Subsequently, we used the frequency of BM PCs plus the percentage of clonal and normal PCs within the whole BM PC compartment, to investigate the prognostic impact of MFC-based automated population separator (APS) classification, by comparing the overall distribution for the above defined three PC-associated parameters in patients with AL amyloidosis vs MGUS and newly-diagnosed MM (Figure 1A). Of the 93 patients in whom clonality by MFC was detected, 6 displayed an MM-like signature, 38 an MGUS-like signature, whereas the remaining 49 cases had a signature intermediate between the MGUS and MM reference datasets. Except for the frequency of renal involvement, patients with AL amyloidosis stratified according to this APS classification showed similar baseline clinical features (Supplementary Table 1), but significantly different survival (Figure 1B-C). Thus, patients with AL amyloidosis classified as MM-like displayed significantly higher rates of early mortality (median PFS

and OS of 1 month), whereas cases classified as MGUS-like displayed the best outcome (median PFS and OS not reached), significantly superior to that of patients with intermediate-AL amyloidosis (median of 15 and 25 months for PFS and OS, respectively). Multivariate analysis of baseline prognostic factors for survival, including the MFC-APS profile, patients' age, number of organs involved, Mayo 2012 staging, the percentage of BM PCs based on cytomorphology and eligibility for ASCT, showed that the intermediate plus MM-like profiles had an independent adverse effect on patients' PFS (hazard ratio: 2.9; *P* = .01) and OS (hazard ratio: 3.0; *P* = .03) (Table 2).

iPEP of clonal PCs in AL amyloidosis vs MGUS and MM. After establishing the utility of flow cytometry immunophenotyping to assess clonality and predict survival in AL amyloidosis, we subsequently investigated if the same combination of markers could identify phenotypic signatures associated with different outcomes. We used the percentages of surface expression for CD19, CD27, CD45, CD56, CD81 and CD117, whereas for CD38 and CD138, patients were classified into low vs bright staining according to the mean fluorescence intensity observed for these markers in clonal PCs vs other nucleated cells. Thus, t-Distributed Stochastic Neighbor Embedding (t-SNE) defined by the iPEP of clonal PCs (Supplementary Figure 2A) based on all possible combinations of markers (i.e. CD19, CD27, CD38, CD45, CD56, CD81, CD117, CD138), revealed two major patient clusters defined by opposite patterns of expression for CD45 (*P* = .02), CD56 (*P* < .001) and CD138 (*P* = .002): cases with higher expression of CD45, CD56 and CD138 ($CD45^{+ve}CD56^{+ve}CD138^{hi}$, N = 50; coded green in Figure 2B) vs patients with lower reactivity for CD45, CD56 and CD138 ($CD45^{-ve}CD56^{-ve}CD138^{lo}$, N = 43; coded brown in Supplementary Figure 2B). Interestingly, patients with a $CD45^{+ve}CD56^{+ve}CD138^{hi}$ iPEP displayed a significantly prolonged PFS vs cases with a $CD45^{-ve}CD56^{-ve}CD138^{lo}$ iPEP (median PFS of 26 vs 10 months, respectively; *P* = .03), as well as a trend for prolonged OS (Supplementary Figure 2C-D).

The higher frequency of AL patients without homogeneously positive CD56 expression (56 of 93 cases, 60%) when compared to other monoclonal gammopathies, led us to investigate whether flow cytometry immunophenotyping could contribute to the differential diagnosis between AL amyloidosis and MGUS/MM based on the tumor PC antigen expression profile. Interestingly, canonical-correlation analysis (CCA) of tumor iPEP showed only partial overlap between AL amyloidosis vs MGUS and MM tumor PC (Figures 2A and 2B, respectively), as patients with newly-diagnosed AL amyloidosis displayed an iPEP between MGUS and MM (Figure 2C and Supplementary Table 2). Thus, progressively higher percentages of cases with a $CD38^{lo}$, $CD45^{-ve}$, $CD81^{-ve}$ and $CD138^{lo}$ iPEP were observed from MGUS to AL amyloidosis and MM. By contrast, patients with AL amyloidosis displayed a significantly lower reactivity for CD56 vs both MGUS and MM (mean of 55% vs 79% $CD56^{+ve}$ clonal PCs in AL amyloidosis vs MGUS/MM, respectively; $P \leq .03$), which explains why in both CCA projections, most MGUS and MM patients grouped together with $CD56^{+ve}$ AL patients (Figures 2A-B). The emergence of clear immunophenotypic differences across monoclonal gammopathies led us to further investigate other markers that could be differentially expressed in AL amyloidosis (N = 38) vs MM (N = 52). For this purpose, a comprehensive screening for another 20 markers including maturation-associated cell membrane proteins (CD10, CD20, CD21, CD24, HLADR), tetraspanins (CD9, CD53, CD63) and other adhesion molecules (CD31, CD49d), chemokine receptors (CD196, BAFF-R, CXCR4), immune regulatory receptors (CD43, CD69, CD200) and immunotherapy targets (CD74, CD229, SLAMF7, PD-L1) was performed, showing that CD20, CD43, CD53 and CD63 were significantly upregulated in BM clonal PC from AL amyloidosis vs MM patients (Supplementary Figure 3A-D). Furthermore, immune therapeutic targets such as CD38 and SLAMF7 were also significantly overexpressed in patients with AL amyloidosis; by contrast, no significant differences were noted in the mean fluorescence intensity of CD138 or PD-L1, and CD229 was significantly downregulated in patients with AL amyloidosis (Supplementary Figure 4).

Bone marrow cellular composition in AL amyloidosis vs MGUS and MM. Since some of the markers found to be differentially expressed in AL amyloidosis vs MM corresponded to immune regulatory receptors, further characterization of the BM cellular composition was performed aimed at investigating whether patients with AL amyloidosis, MGUS and MM displayed different profiles. Based on the same 8-color MoAb combination described above to assess clonality, up to 16 cell populations and 18 parameters were systematically assessed in all studied cases (Figure 3A). Subsequently, we investigated the degree of similarity and divergence across patients with AL amyloidosis, MGUS and active MM, based on a minimum spanning tree (MST) model built upon the 18 phenotypic parameters. Interestingly, the three disease categories converged into a (unique) specific node when integrated into the natural diversity of age-matched healthy adults (Figure 3B). Of note, in the MST representation, AL amyloidosis cases plotted between MGUS and MM patients, with a more detailed analysis of the BM cellular composition revealing that for 6/18 immunophenotypic parameters (i.e. erythroid hematopoietic progenitors, neutrophils, monocytes, TNK- plus NK-cells and other remaining non-T/NK T-lymphocytes, PCs) AL amyloidosis patients were closer to MGUS; for another 6/18 parameters (i.e. $CD38^-$ and $CD38^+$ hematopoietic progenitors, eosinophils, erythroblasts, naïve and memory B-lymphocytes) AL amyloidosis patients were between MGUS and MM; whereas for the remaining 6 immune parameters (i.e. myeloid progenitors, basophils, mast cells, B-cell precursors, normal and clonal PCs) (Figure 3C) AL amyloidosis patients were closer to MM. Thus, MFC-based analysis of the tumor microenvironment mapped AL amyloidosis in between MGUS and MM. Of note, while the BM cellular composition in AL amyloidosis showed progressively more differences from elderly healthy adults, MGUS and MM (4, 7 and 12 significantly different phenotypic parameters, respectively) (Figure 3D), the percentage of B-cell precursors was consistently lower in patients with AL amyloidosis than in elderly healthy adults, MGUS and MM (P = .004; Supplementary Table 3).

Immunophenotypic hallmarks of AL amyloidosis. Simultaneous iPEP of tumor PCs and monitoring of the BM cellular composition unveiled that the lack of homogeneously positive CD56 expression, a reduction of B-cell precursors and a predominantly-clonal PC compartment, in the absence of an MM-like tumor PC expansion, were significantly more frequent in AL amyloidosis vs MGUS and MM. Thus, based on optimal cut-off values to discriminate between AL amyloidosis vs MGUS and MM, we built a scoring model based on the presence of <100% $CD56^{+ve}$ clonal PCs, <0.1% B-cell precursors, >80% clonal PCs within total BM PCs and <2% BM PCs, were each of the above 4 variables was assigned a score of 1; subsequently, patients were ranked according to their individual score. Overall, a significant (P < .001) association was found between a progressively higher score (score range, 1 - 4) and the diagnosis of AL amyloidosis (14%, 63%, 64% and 95%, respectively) vs MGUS (25%, 10%, 8%, 5%, respectively) or MM (61%, 27%, 28%, 0%, respectively) (Table 3). Thus, a t-SNE projection based on these four phenotypic parameters displayed selective clustering of patients with AL amyloidosis vs MGUS and MM (Figure 4A), with a 74% accurate classification based on ROC analysis (AUC of 0.74; 95% CI = 0.66 - 0.82; P < .001) of the performance of the scoring model (Figure 4B).

DISCUSSION

Despite important advances have been achieved, diagnosis of AL amyloidosis and prognostic stratification of the disease remains a challenge in a significant fraction of patients. In recent years, flow cytometry immunophenotyping of BM PCs in AL amyloidosis had emerged as a potential complementary diagnostic tool (16,17,22–24); however, as it has occurred in MM, disturbing levels of variability on the results due to the use of highly heterogeneous and non-standardized methodological approaches, exists.(25) Here, we followed standard EuroFlow protocols to study BM samples from a large series of patients with AL amyloidosis, and developed an automated classification algorithm that identified patients with significantly different outcomes, despite showing similar organ involvement and Mayo staging. Importantly, this MFC-based automated risk classification emerged as an independent prognostic factor in AL amyloidosis, and is ready for multicentric validation and implementation in routine clinical practice.(10)

Currently, there is limited data on the potential contribution of the underlying PC clone and its features for prognostication in AL amyloidosis. In addition to the PC percentage by cytomorphology(26) and iFISH abnormalities (27,28), only the sFLC load has consistently been shown to have an impact on patient survival.(29) However, abnormal PCs in AL amyloidosis are difficult to identify by morphology due to their small number, minor morphological alterations, and co-existence with normal BM PCs.(30) In turn, the typically low tumor burden in AL amyloidosis together with the co-existence of normal PCs in the patients' BM also hamper accurate assessment of cytogenetic abnormalities by iFISH, even when conventional PC-enrichment magnetic procedures are used; thus, according to Muchtar et al in a large patient series, accurate iFISH analysis of tumor PCs in AL amyloidosis would only be possible in two-thirds of patients.(28) Most likely, the co-existence of clonal and normal PCs also accounts for those 15% cases with a normal FLC ratio, identified among almost 1,000 patients diagnosed with AL amyloidosis in Pavia (Italy) (7), and the 14% of >1,000 newly-diagnosed amyloidosis patients with dFLC<5 mg/dL reported by Sidana et al.(31)

Here, we confirm and extend on previous findings based on small patient series (6,16) and demonstrate that MFC allows fast, accurate and sensitive demonstration of clonality in virtually every patient with AL amyloidosis, including those with undetectable M-protein. In addition, we validated different phenotypic thresholds that have been previously reported to be of prognostic relevance in AL amyloidosis (16,17) and propose a new automated computerized algorithm based on simultaneous assessment of tumor burden and the degree of clonality within the BM PC compartment, that identifies three AL amyloidosis patient subgroups (i.e. MGUS-like, intermediate and MM-like) with a similar frequency of cardiac involvement and distribution for the Mayo staging system, but significantly different survival rates, pointing out the independent prognostic value of BM PC clonality vs other conventional prognostic factors, including cardiac biomarkers.(32) However, it should be noted that the present series is skewed towards more aggressive disease stages (e.g.: only 5% of patients have Mayo stage I), and further analyses in series with higher frequencies of patients with Mayo 2012 stage I are warranted to validate our results. Another limitation of this study is the relatively low number of events for PFS (n=39) or OS (n=30), and further analyses in larger series and with longer follow-up are also warranted to confirm the independent prognostic value of MFC immunophenotyping.

The availability of software tools for automated analysis of immunophenotypic NGF data generated with the EuroFlow protocols used here (10), together with the large databases for unbiased/objective patient classification (33), allows for easy implementation in diagnostic laboratories worldwide of standardized flow cytometry approaches to asses clonality and predict outcome in AL amyloidosis. Thus, this study addresses the need for consensus on how to use flow cytometry in AL amyloidosis (e.g. amount of cells, which markers, gating strategies) (11) and overcomes the usage of different arbitrary thresholds of PC clonality to risk stratify patients.(16,17) This may be particularly relevant in diseases with low tumor burden such as AL amyloidosis, in

which different phenotypic protocols or even hemodilution may impact on the percentage of PCs detected by flow cytometry.(6,16,17)

The immunophenotypic characterization of tumor PCs has shown to be of prognostic value in newly-diagnosed MM patients (34,35) but, thus far, no data has been reported in AL amyloidosis. Here, NGF was used for unbiased/objective identification of clusters of patients with AL amyloidosis based on their distinct iPEP, our results showing that cases with a $CD45^{-ve}CD56^{-ve}CD138^{lo}$ phenotype have an inferior survival. These findings differ from those recently reported in MM (35), where expression of CD45 is associated with an inferior outcome and CD56 was found to be the only marker without prognostic value.

Driven by these observations and the surprisingly high number of patients that showed lack of homogeneous positivity for CD56, we also explored whether flow cytometry could help distinguishing AL amyloidosis from other monoclonal gammopathies. It has been hypothesized that AL amyloidosis, MGUS and MM are the same disease entity at the cellular level, with AL amyloidosis just being a clonal PC disorder with an “unlucky protein”.(36) Thus, genetic studies performed in patients with AL amyloidosis have shown different frequencies but not different type of cytogenetic abnormalities in the former vs MM.(27,28,37–39) Genome-wide association studies have also provided evidence for common genetic susceptibility to AL amyloidosis and MM.(40) By contrast, here we demonstrate that on phenotypic grounds, AL amyloidosis is on the crossroad between MGUS and MM, particularly for markers such as CD38, CD45, CD81 and CD138. Namely, the density of CD38 (as well as other potential immunotherapy targets such as CD138 and SLAMF7) expressed on the surface of tumor PCs, was significantly higher in AL amyloidosis than in MM. These findings suggest that anti-CD38 monoclonal antibody therapy might be highly effective in AL amyloidosis, as recently reported.(41) By contrast, lack of homogenously-positive CD56 expression emerges as a hallmark of AL amyloidosis vs. both MGUS and MM. In addition, other markers such as CD20, CD43, CD53 and CD63 were also found to be

significantly upregulated in AL amyloidosis vs MM. While the role of the CD53 and CD63 tetraspanins remains to be determined (42), increased CD20 expression likely reflects the higher frequency of t(11;14) in AL amyloidosis vs MM (27,28,36,37,43); interestingly, CD43 appears to be important for immune function and it has been identified as an adverse prognostic factor in diffuse large B-cell lymphoma.(44,45)

Recently, Muchtar et al have demonstrated the prognostic value of the balance between monotypic vs polytypic PCs, and suggested that this could be partially related to an impaired immune surveillance, and thus potentially associated with a poorer patient outcome.(17) Since the enumeration of all different cell types is a standard procedure in flow cytometry diagnostics of hematological samples, herein we took full advantage of the multidimensionality of flow cytometry as well as the expression in other BM cells of those markers used to assess PC clonality, to unravel the tumor microenvironment in AL amyloidosis vs MGUS and MM. In line with our observations based on the iPEP of clonal PCs, profiling of the BM cellular composition also mapped AL amyloidosis in the crossroad between MGUS and MM. Thus, patients with AL amyloidosis displayed a clonal/polyclonal PC distribution similar to MM, but with overall BM PC infiltration levels closer to those of MGUS patients. Most importantly, patients with AL amyloidosis showed a marked reduction of B-cell precursors when compared to age-matched healthy adults, MGUS and MM patients; thus, the combination of these four BM features emerged as a hallmark of AL amyloidosis. Interestingly, patients with AL amyloidosis and MM displayed significantly further reduction of B-cell precursors when compared to the rest of AL cases (0.001% vs 0.05%, respectively; $P < .001$).

Disease recognition and early diagnosis are key steps to improve outcome in AL amyloidosis, a disease in which early mortality remains high, particularly in transplant-ineligible patients.(46) Here we demonstrate the value of MFC for fast diagnostic screening of BM PC clonality in AL amyloidosis and simultaneous automated patient risk-stratification, based on the BM tumor burden and PC phenotype. In addition, our results also provide new immunophenotypic markers (i.e.

lack homogenously-positive CD56 expression, reduced numbers of BM B-cell precursors, a predominantly-clonal PC compartment in the absence of a significant tumor PC expansion in the BM) for the identification of patients with monoclonal gammopathies that are candidates for monitoring of pre-symptomatic organ damage related to AL amyloidosis.

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COMPETING INTERESTS

All authors declared no conflicts of interest.

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Figure legends.

Figure 1. Outcome of patients with newly-diagnosed AL amyloidosis classified according to the automated population separator (APS) immunophenotypic profile of BM clonal PCs. (A) Patients plotted in the overlapping area between monoclonal gammopathy of undetermined significance (MGUS) and newly-diagnosed multiple myeloma (MM) (N = 49, 53%), were classified as intermediate AL amyloidosis cases; otherwise they were classified as MGUS-like (N = 38, 41%), or assigned to the MM reference group (N = 6, 6%); this classification showed significantly different progression-free (PFS) **(B)** and overall survival (OS) **(C)** for the above three Al amyloidosis patient subgroups.

Figure 2. Immunophenotypic protein expression profile (iPEP) of monoclonal gammopathies. In the canonical-correlation analysis (CCA) graphical view, every patient is represented by a single dot and disease reference groups by 1 (dashed lines) and 2 (solid lines) standard deviation curves. **(A)** and **(B)** display CCA between subjects with monoclonal gammopathy of undetermined significance (MGUS, in green; N = 20) vs patients with newly-diagnosed light-chain amyloidosis (AL, in blue; N = 93) and between the later vs patients with newly-diagnosed multiple myeloma (MM, in red; N = 52), respectively. **(C)** Graphical representation of the expression of markers used for assessment of PC clonality in subjects with MGUS vs patients with AL and MM (described in detail in Supplementary Table 2). The dynamics of expression from MGUS to MM are depicted by the geometric shape graded from green (MGUS) to red (MM), whereas the relative proximity (determined by comparing the median values of antigen expression described in Supplementary Table 2) of AL amyloidosis to MGUS or MM is depicted by the blue bar: the closer to MGUS the closer to the left; the closer to MM the closer to the right. The vertical black bar represents the mean value (half-distance) between MGUS and MM for each marker. Markers with statistically significant differences are underlined.

Figure 3. Landscape of the bone marrow cellular composition in elderly healthy adults and patients with monoclonal gammopathies. (A) Illustrative automated population separator (APS) plot based on principal component analysis of the tumor microenvironment of a patient with newly-diagnosed light-chain amyloidosis (AL). **(B)** 2-dimensional minimum spanning tree (MST) plot defined by the distribution of 18 phenotype-based cell parameters in the bone marrow (BM) of elderly healthy adults (HA, N = 29; each represented by a single grey dot) and subjects with monoclonal gammopathy of undetermined significance (MGUS, N = 20), patients with newly-diagnosed AL amyloidosis (N = 93) and active multiple myeloma (MM, N = 52) (grouped into blue, green and red clusters, respectively). **(C)** Graphical representation of the similitude of each cell population in AL amyloidosis vs. MGUS or MM. The vertical bar represents the mean value (half-distance) between MGUS and MM for each cell population. **(D)** Bone marrow cell populations decreased or increased (statistical significance is depicted by grades of blue and red, respectively) in patients with newly-diagnosed AL amyloidosis vs HA, MGUS and MM (described in detail in Supplementary Table 3). We used the Mann–Whitney U test to evaluate the statistical significance of differences observed between AL amyloidosis vs each of the other three groups. Overall, MFC-based analysis of the BM cellular composition mapped AL amyloidosis in between MGUS and MM; for example, when compared to MGUS, patients with AL amyloidosis showed significantly higher percentages of $CD38^{-ve}$ and $CD38^{+ve}$ $CD117^{+ve}$ hematopoietic progenitors as well as higher total and clonal PC numbers, whereas the opposite pattern was found when patients with AL amyloidosis were compared to MM cases. Conversely, the percentage of normal PCs was significantly decreased in AL amyloidosis vs MGUS but increased when compared to MM.

Figure 4. Differential diagnosis between AL amyloidosis vs MGUS and MM based on phenotypic parameters. (A) t-Distributed Stochastic Neighbor Embedding (t-SNE)

projection of patients with AL amyloidosis (N = 93), MGUS (N = 20) and MM (N = 52) represented by blue, green and red circles, respectively, based on the percentage of $CD56^{+ve}$ clonal plasma cells (PC)s, B-cell precursors, clonal PCs within total bone marrow (BM) PCs, as well as PCs from all nucleated BM cells. **(B)** Receiver operating characteristic (ROC) curve generated using the scoring model described in Table 3.

Table 1. Demographics, clinical-biological characteristics and PC clonality status at baseline in newly-diagnosed patients with AL amyloidosis (N = 94).

Patient feature	Distribution
Age (years)*	67 (41 – 86)
% of males	61%
N. of organs involved*	2 (1 – 4)
Cardiac involvement (%)	53%
Renal involvement (%)	52%
Peripheral neuropathy (%)	11%
Liver involvement (%)	14%
Gastrointestinal involvement (%)	24%
2012 Mayo stage (%)	
I	5%
II	33%
III	26%
IV	36%
Isotype (%)	
Free Kappa	10%
Free Lambda	31%
IgG-Kappa	12%
IgG-Lambda	35%
IgA-Kappa	1%
IgA-Lambda	11%
M-component by protein electrophoresis (%)	80.5%
M-component by immunofixation (%)	93%
Abnormal serum free light-chain ratio (%)	75%
dFLC (mg/L)*	171 (0.2 - 18891)
% PCs in BM aspirates by morphology*	8 (0 – 60)
Clonality by multidimensional flow cytometry, (%)	99%
First-line treatment (%)	
ASCT	15%
Bortezomib-based	56%
Melphalan-based	14%
Immunomodulatory drugs-based	1%
No treatment	4%
Unknown	10%

Results expressed as percentage of cases or as *median (range). PCs: plasma cells; dFLC: difference between the involved and uninvolved serum free-light chains; ASCT: autologous stem cell transplantation

Table 2. Multivariate analyses of prognostic factors for progression-free survival (PFS) and overall survival (OS) in AL amyloidosis patients.

	PFS		OS	
	HR (95% CI)	***P***	**HR (95% CI)**	***P***
2012 Mayo stage IV	2.9 (1.4 – 6.0)	.005	3.1 (1.3 – 7.4)	.009
≥20% PCs by morphology	1.5 (0.5 – 4.4)	.48	2.3 (0.8 – 7.3)	.14
Intermediate- or MM-like APS profiles	2.9 (1.3 - 6.6)	.01	3.0 (1.1 - 8.4)	.03

PCs: plasma cells; APS: automated population separator; MM: multiple myeloma

Table 3. Scoring model to predict for the diagnosis of AL amyloidosis vs monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM), based on the following phenotypic parameters (each assigned a score value of 1): <100% $CD56^{+ve}$ clonal PCs, <0.1% B-cell precursors, <2% BM PCs and >80% clonal PCs within all BM PCs. Patients were ranked according to the overall score for the four phenotypic parameters detected by multidimensional flow cytometry.

Score (N. of patients)	AL amyloidosis (N = 93)	MGUS (N = 20)	MM (N = 52)
1 (N = 36)	5 (14%)	9 (25%)	22 (61%)
2 (N = 49)	31 (63%)	5 (10%)	13 (27%)
3 (N = 60)	38 (64%)	5 (8%)	17 (28%)
4 (N = 20)	19 (95%)	1 (5%)	0 (0%)