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ORIGINAL RESEARCH

Reactive Vasodilation Predicts Mortality in Primary Systemic Light-Chain Amyloidosis

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RATIONALE: Cardiac involvement and hypotension dominate the prognosis of light-chain amyloidosis (AL). Evidence suggests that there is also peripheral vascular involvement in AL but its prognostic significance is unknown.

OBJECTIVE: To evaluate vascular dysfunction in patients with AL as a potential future area of intervention, we assessed the prognostic utility of flow-mediated dilatation (FMD), a marker of vascular reactivity, which is augmented under conditions of hypotension and autonomic dysfunction.

METHODS AND RESULTS: We prospectively evaluated 115 newly diagnosed untreated AL patients in whom FMD was measured. FMD in AL patients was significantly higher than age-, sex- and risk factors–matched controls (4.0% versus 2.32%; P=0.006) and comparable with control groups at lower cardiovascular risk (P>0.1). Amyloidosis patients presented increased plasma and exhaled markers of the NO pathway while their FMD significantly correlated with augmented sustained vasodilatation after sympathetic stimulation. Increased FMD (≥4.5%) was associated with early mortality (hazard ratio, 4.36; 95% CI, 1.41–13.5; P=0.010) and worse survival (hazard ratio, 2.11; 95% CI, 1.17–3.82; P=0.013), even after adjustment for Mayo stage, nerve involvement and low systolic blood pressure. This finding was confirmed in a temporal validation AL cohort (n=55; hazard ratio, 4.2; 95% CI, 1.45–12.3; P=0.008). FMD provided significant reclassification value over the best prognostic model (continuous Net Reclassification Index, 0.61; P=0.001). Finally, better hematologic response was associated with lower posttreatment FMD.

CONCLUSIONS: FMD is relatively increased in AL and independently associated with inferior survival with substantial reclassification value. Reactive vasodilation merits further investigation as a novel risk biomarker in AL.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: autonomic nervous system ■ brachial artery ■ case-control studies ■ immunoglobulin light-chain amyloidosis ■ mortality ■ ultrasonography ■ vasodilation

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Light-chain amyloidosis (AL) is a systemic lethal disease caused by the accumulation of amyloid fibrils in various tissues and organs and with special predilection for the cardiovascular system.[1] Cardiac amyloid involvement and resulting dysfunction is the main cause of death in patients with immunoglobulin AL. In addition to cardiac dysfunction,

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Novelty and Significance

What Is Known?

- Cardiac involvement, hypotension, and autonomic dysfunction are associated with poor prognosis in immunoglobulin light-chain amyloidosis (AL).
- Flow-mediated dilatation (FMD) of the brachial artery—a well established noninvasive marker of vascular reactivity—is augmented in conditions characterized by hypotension or autonomic dysfunction.
- The clinical significance of vascular reactivity in AL is unknown.

What New Information Does This Article Contribute?

- Vascular reactivity in AL is relatively increased when compared with matched controls.
- Vascular reactivity determined by FMD is an independent predictor of all-cause mortality in AL.
- Increased FMD improves risk stratification over established markers of adverse outcome in AL, including cardiac involvement, cardiobiomarkers, and hypotension.
- Increased vascular reactivity in AL patients closely correlates with vascular autonomic dysfunction.

In a cohort study, we explored the clinical value of FMD as an established marker of vascular reactivity in AL patients. This study shows for the first time that reactive vasodilatation is augmented in AL, is associated with vascular autonomic dysfunction, independently predicts mortality, and stratifies patients into correct risk categories over established risk factors of this disease including cardiac involvement, cardiobiomarkers, and hypotension. Overall, these findings provide proof of concept on the clinical importance of assessing peripheral reactive vasodilation in AL, thus setting the stage for further investigation of its utility as a biomarker in clinical practice. Finally, this study opens the field for the investigation of peripheral reactive vasodilation in diseases characterized by hypotension and autonomic dysfunction.

Nonstandard Abbreviations and Acronyms

AL	light-chain amyloidosis
BP	blood pressure
CPT	cold pressure test
CVD	cardiovascular disease
FMD	flow-mediated dilatation
HR	hazard ratio
hs TnT	high-sensitive Troponin T
NRI	Net Reclassification Index
NT-proBNP	N-terminal pro-B-type natriuretic peptide
NTY	3-nitrotyrosine

evidence suggests that there is also vascular involvement[2] in AL; however, its prognostic significance is unknown. Notably, the presence of low blood pressure (BP)—a common manifestation of AL disease and possibly indicative of vascular impairment—identifies a group of patients with poor outcome.[3] The mechanisms of low BP in AL remain elusive; low cardiac output and low oncotic pressure due to hypoalbuminemia have been implicated. However, autonomic dysfunction due to disease-related nerve involvement that is manifested as vascular sympathetic denervation and a generalized dysfunction in the autonomic control of vascular reactivity[4] may also be implicated. Simultaneously, with the presence of autonomic dysfunction, hypotension is often evident in AL possibly because of compensatory mechanisms under investigation.[4] This suggests that more sensitive hemodynamic markers than resting arterial BP measurement may provide prognostic information at earlier stages of the disease and possibly allow more accurate risk stratification. Flow-mediated dilatation (FMD) is a well-validated noninvasively acquired research tool and marker of vascular reactivity, which is decreased in conditions of endothelial dysfunction mainly because of low bioavailability of NO[5–8] but augmented in conditions associated with hypotension and sympathetic deactivation such as vasovagal syncope and liver cirrhosis.[9–12] Interestingly, sympathetic nerve stimulation decreases FMD,[13] whereas sympathetic attenuation increases FMD.[14] We hypothesized that FMD as an estimate of vascular reactivity could be an objective marker of abnormal reactive vasodilation in patients with AL and aimed to provide proof of concept for this hypothesis. Furthermore, we aimed to explore the prognostic and risk reclassification value of FMD as a possible novel biomarker in AL.

METHODS

The data that support the findings of this study are available from the corresponding authors on reasonable request.

Population and Design

From 2008 to 2014, 115 consecutive, newly diagnosed, treatment-naive patients with systemic AL were prospectively included in this study (prospective derivation cohort). All patients had biopsy-proven AL and were diagnosed and followed in the Department of Clinical Therapeutics, Alexandra Hospital, Athens, Greece. Consensus criteria were used for the definition and assessment of organ involvement.[15] In the

derivation cohort, AL patients were followed for all-cause mortality. Events were confirmed by inspection of medical records or death certificate. To maximize diagnostic accuracy, in all patients, a strict institutional protocol regarding diagnosis, initial evaluation, treatment, and follow-up was applied.[16,17]

To assess differences in vascular characteristics attributed to the disease, AL patients from the prospective derivation cohort were matched with control subjects for age, sex, cardiovascular disease (CVD), risk factors, and coronary artery disease, aiming to minimize bias from factors known to affect FMD.[18–20] We applied a hierarchical matching process, in a 1:k (maximum k, 7) ratio by using a greedy matching algorithm of the nearest neighbor for age and sex.[21] Sex was matched exactly while the caliper for age enabled matching within a range of 2 years. Subsequently, estimated glomerular filtration ratio stage, coronary artery disease, diabetes mellitus, smoking, and hyperlipidemia were matched in a hierarchical order as described in Methods in the Online Data Supplement. This method resulted in almost perfect match for age, sex, and estimated glomerular filtration ratio, and close simulation for smoking, diabetes mellitus, and coronary artery disease as depicted in Table 1. To assess the impact of CVD risk factors on differences in vascular function between cases and controls irrespective of age and sex, we additionally performed a triple 1:1 matching among (1) AL patients, (2) age- and sex-matched healthy controls without any cardiovascular risk factors or disease (ideal cardiovascular risk profile), and (3) controls matched for age, sex, and risk factors. Accordingly, 23 triplets of subjects with the same age and sex were derived. In addition, a group of young (<40 years) healthy adults with ideal cardiovascular risk profile were recruited as positive controls. All control subjects were retrospectively recruited from an FMD

Table 1. Characteristics of the Amyloidosis Population and of the Age-, Sex-, and Risk Factor–Matched Population

Parameters	AL (n=115)	Matched (n=115)	*P* Value
Age, y	64.4±10.2	64.3±10.1	0.938
Sex (male), n (%)	62 (53.9)	62 (53.9)	0.999
Diabetes mellitus, n (%)	14 (12.2)	21 (18.3)	0.189
Hyperlipidemia, n (%)	50 (43.5)	69 (60.0)	0.009
Smoking, n (%)	18 (15.7)	23 (20.0)	0.371
Arterial hypertension, n (%)*	45 (39.1)	68 (59.1)	0.002
eGFR stage, n (%)			0.288
Stage 1	36 (31.3)	26 (22.6)	
Stage 2	27 (23.5)	33 (28.7)	
Stage 3A	18 (15.7)	15 (13.0)	
Stage 3B	13 (11.3)	5 (4.35)	
Stage 4	8 (6.96)	6 (5.22)	
Stage 5	13 (11.3)	6 (5.22)	
History of coronary artery disease, n (%)	12 (10.4)	14 (12.2)	0.659
SBP, mm Hg	123.7±22.5	132.0±20.9	0.005
Orthostatic hypotension, n (%)	37 (32.2)	0 (0)	…
DBP, mm Hg	72.2±10.2	75.0±10.3	0.041
Baseline brachial diameter, mm/m^2	2.22±0.41	2.31±0.40	0.101
Baseline flow stimulus, cm/s	7.46±4.85	7.58±6.18	0.876
Postocclusion hyperemic flow stimulus, cm/s	20.7±11	23.2±12.6	0.121
Shear stress, dyn/cm^2	5.35±3.7	5.04±4.19	0.560
FMD (%)	4.00 (1.92–6.06)	2.32 (0.961–4.55)	0.004† (0.006)‡
Allometrically scaled FMD, %	1.78±1.53	0.99±2.68	0.005
Presence of any carotid plaque, n (%)	57 (49.6)	63 (54.8)	0.428
Presence of femoral plaque, n (%)	45 (39.1)	39 (33.9)	0.238
Presence of any plaque, n (%)	69 (60)	68 (59.1)	0.957
AL-specific treatment, n (%)			
Alkylator/steroid-based regimens, n (%)	17 (14.8)		
Proteasome inhibitors or lenalidomide-based regimens, n (%)	98 (85.2)		

All continuous and dichotomous variables are described as mean±SD (except FMD where median [interquartile range] is provided) and n (%), respectively. *P* values are derived from independent samples *t* test and χ^2 test for nominal variables. AL indicates light-chain amyloidosis; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration ratio; FMD, flow-mediated dilatation; and SBP, systolic blood pressure.

*History of hypertension before diagnosis of AL.

†*P* value corresponds to the comparison of FMD values after logarithmic transformation or inverse rank normalization.

‡Baseline brachial artery diameter is normalized for body surface area.

registry of 1071 subjects examined in our laboratory with various prevalence of traditional risk factors, renal dysfunction, and CVD as described in Online Table I. Although these subjects were retrospectively included in the study, FMD registry was recruited during the same period (2008–2018) of the prospective AL cohorts, using the same methods for documentation of descriptive characteristics and vascular assessment.

To explore mechanisms and to validate our prospective survival results, we recruited 4 additional AL populations: (1) from 2016 to 2017, 26 consecutive AL patients and 30 healthy control subjects with normal lung functional tests were recruited for exhaled NO and FMD assessment (exhaled NO cohort); (2) from March to November 2018, 19 consecutive AL patients and 10 young healthy volunteers were recruited for assessment of brachial artery reactivity by cold pressor test (CPT) and of FMD (CPT cohort); (3) a prospective validation AL cohort of 55 newly diagnosed untreated patients who were consecutively recruited from 2015 to 2018 from our Department and followed for all-cause mortality; (4) aiming to estimate associations between hematologic response and post-AL treatment effect FMD changes, we further analyzed data on 15 patients from the derivation cohort (recruited between December 2007 and December 2009) with available FMD measurements at baseline (before treatment initiation) and 6 to 12 months post-treatment (prospective observational cohort) as described in Methods in the Online Data Supplement. Exclusion criteria of the study are depicted in Methods in the Online Data Supplement. A flowchart illustrating enrolled cohorts and recruitment procedures in our study is provided in Figure 1.

This was a prospective noninterventional study, approved by the Institutional Review Board of the Department of Clinical Therapeutics, National and Kapodistrian University of Athens (Local Ethics Committee Approval reference No. 6.12.09.2008), and conducted in full compliance with Health Insurance Portability and Accountability Act and the principles of Good Clinical Practice and the Declaration of Helsinki. All patients gave written informed consent before their participation in the study.

FMD and Carotid and Femoral Ultrasound

A detailed description of the vascular studies is provided in the Online Data Supplement. In both prospective cohorts, during initial evaluation visit and before initiation of any chemotherapy or steroids, a series of vascular studies were performed. Reactive vasodilatation was assessed by FMD. FMD was measured using high-resolution ultrasonography (14.0-MHz multifrequency linear array probe, Vivid 7 Pro; General Electric Healthcare, Milwaukee, WI). In the derivation cohort, FMD was measured in the right brachial artery of each subject offline on ECG R wave by acquiring an average of 3-diameter measurements at baseline and the maximum diameter from a series of continuous manual measurements taken every 15 seconds after cuff deflation (post-5 minutes of ischemic inflation at the level of antebrachium) for a time period of 90 seconds. FMD was calculated as described previously.[22] In the validation, exhaled NO, and CPT cohorts, brachial artery was continuously acquired by ECG gating on R wave for 30 seconds at baseline and for 120 seconds after cuff deflation. Diameter was measured offline using dedicated automatic border detection software (Medical Imaging Applications LLC) as described previously.[23] Aiming to use a second approach to correct FMD results for baseline brachial artery diameter, we also calculated an allometrically scaled FMD that takes into consideration more accurately differences in baseline diameter of brachial artery.[24] Nitrate-dependent vasodilation was not measured, for ethical reasons, to avoid nitroglycerin-induced serious adverse reactions in this group of patients with high prevalence of orthostatic hypotension (32.2%; Table 1). Most importantly, as explained in detail in the Online Data Supplement, autonomic and cardiac dysfunction, which are commonly met in patients with AL (40% and 67% in our population, respectively), may exacerbate serious and unpredictable adverse effects by nitroglycerin. In the derivation cohort, high-resolution ultrasound was performed in the carotid and femoral arteries using similar equipment to that for FMD measurement. The presence and number of peripheral plaques were assessed as described previously.[25,26]

Additional Assessment of Underlying Mechanisms Associated With Increased FMD in AL

Because of the lack of validated animal models and unacceptably high risk of adverse reactions from performing nitroglycerin-induced vasodilation in patients with AL, implementation of direct experimental investigation of increased FMD in AL patients was not feasible. Instead, we focused on the following approaches: (1) we evaluated brachial artery diameter responses to sympathetic stimulation induced by the CPT cohort and (2) we measured markers of NO bioavailability and associated nitrosative stress because FMD is known to be at least partly mediated by NO bioavailability[7,8] (prospective and exhaled NO cohort). Detailed rationale and methods used for this approach are provided in the Online Data Supplement.

Cold Pressure Test

Brachial artery diameter reaction to CPT was used as a validated method to measure vascular response during and after sympathetic stimulation in humans.[27]

Circulating Markers of NO Pathway Measurements

Plasma levels of nitrites (Cayman Nitrate/Nitrite Colorimetric Assay Kit 780001) as an index of total NO availability,[28] cyclic GMP (cGMP; Direct cGMP ELISA kit: ADI-900-013; Enzo Life Sciences) as a critical downstream molecule in the signaling cascade of NO in vascular smooth muscle cells,[29] and 3-nitrotyrosine (NTY; 3-Nitrotyrosine ELISA Kit: No. K4158-100; BioVision) as an index of nitrosative stress[30] were measured.

Exhaled Alveolar NO

To further explore possible associations between NO bioavailability and increased FMD, exhaled alveolar NO (MasterScreen Body, Jaeger, Germany) was measured as an indicator of the endogenously produced NO levels in the pulmonary vasculature and as a second marker of basal total NO release from vasculature.[31]

Statistical Methods

Normal distribution of all continuous variables was tested with the parametric test Shapiro-Wilk and graphically assessed by histograms and P-P plots. Differences between controls and

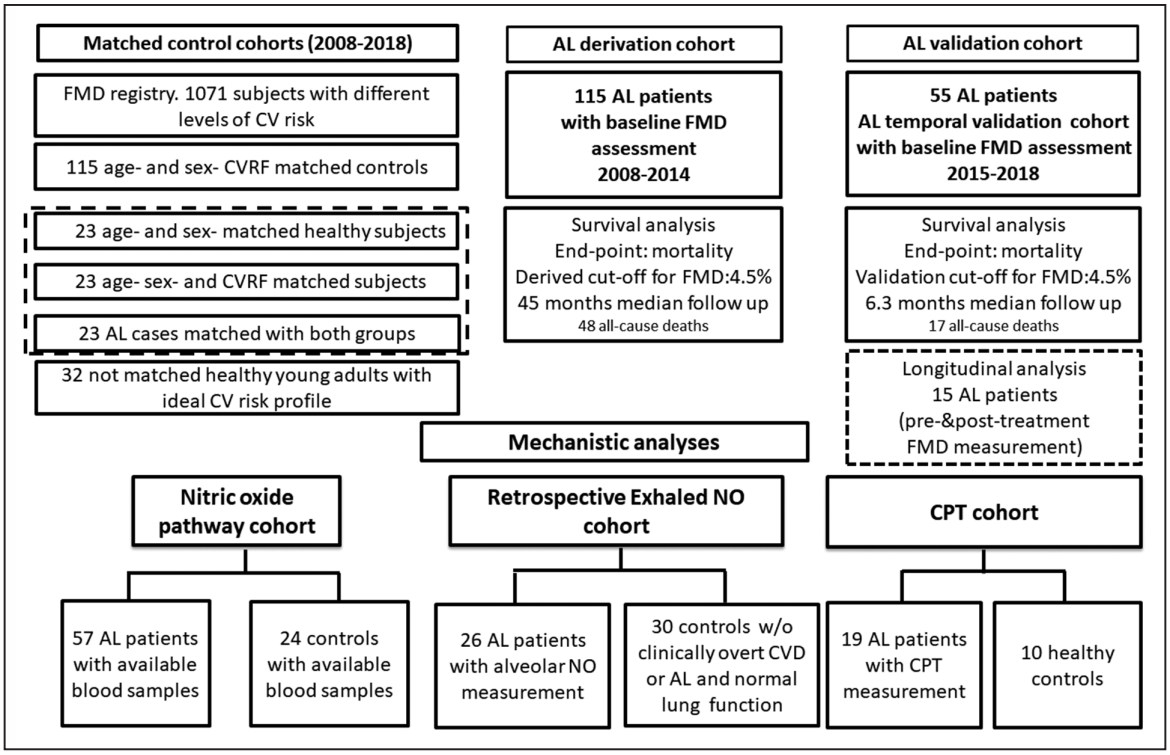

Figure 1. Flowchart of study design.
AL indicates light-chain amyloidosis; CPT, cold pressure test; CV, cardiovascular; CVD, cardiovascular disease; CVRF, cardiovascular risk factor; and FMD, flow-mediated dilatation.

cases were assessed by independent samples *t* test or 1-way ANOVA with post hoc comparisons corrected by the Dunnett method for continuous variables and χ^2 test for nominal variables. FMD did not follow the normal distribution in histograms and quantile-quantile plots and was tested after natural log transformation or inverse rank normalization. For FMD (in logarithmic scale), we used linear regression analysis to further compare the 2 groups after adjusting for potential confounders: (1) traditional risk factors, that is, systolic and diastolic BP, hypertension, hyperlipidemia, smoking, and diabetes mellitus; (2) baseline brachial artery diameter and postocclusion hyperemic flow stimulus; and (3) the presence of peripheral plaque or coronary artery disease.

Cox proportional-hazards models were used to examine the association between a series of baseline predictors and the main end points (ie, early mortality at 6 months and overall mortality) of the study. We tested all available predictors of survival in AL as proposed in medical literature,[3] as well as variables with biological plausibility (age and sex) in univariate Cox regression models (Statistical Methods in the Online Data Supplement). Those presenting a signal or significant association ($P<0.1$) in univariate models were tested in the multivariable Cox models (Statistical Methods in the Online Data Supplement) to identify independent parameters for survival end points. To assess the functional form of the association between all-cause mortality and continuous FMD, we implemented restricted cubic splines with 3 knots.[32] Subsequently, we aimed to analyze the prognostic value of FMD using a clinically relevant cutoff. For this purpose, the cutoff point for FMD (ie, 4.5%) was derived using 2 statistical approaches. First, we determined the optimal cut point of FMD predicting mortality under survival settings using the method proposed by Contal and O'Qingley.[33] We further confirmed this cut point by receiver operating characteristic analysis and selection of the maximum product of sensitivity and specificity according to the Liu method.[34] In addition, we sought to assess the incremental value of FMD over the best predictive model for reclassification improvement toward the survival end points by calculating the continuous Net Reclassification Index (NRI)[35] and the integrated discrimination improvement, as described in detail in the Online Data Supplement. Statistical analysis was performed by STATA package, version 11.1 (StataCorp, College Station, TX). Regarding our secondary hypothesis exploring the association of increased FMD in AL patients with markers of NO metabolism, *P* values were corrected by the Holm-Bonferroni method[36] to control the family-wise error rate. We deemed statistical significance at a=0.05.

RESULTS

Comparisons of FMD Between AL Patients and Matched Controls

Descriptive characteristics of AL and control groups are depicted in Table 1 and Online Tables I through III. Mean age in AL patients was 64.4±10.2 years and prevalence of female sex was 46.1%, which did not differ from controls. Prevalence of hyperlipidemia and hypertension in AL patients was lower than controls, whereas prevalence of diabetes mellitus, smoking, and coronary artery disease did not differ between groups. FMD was

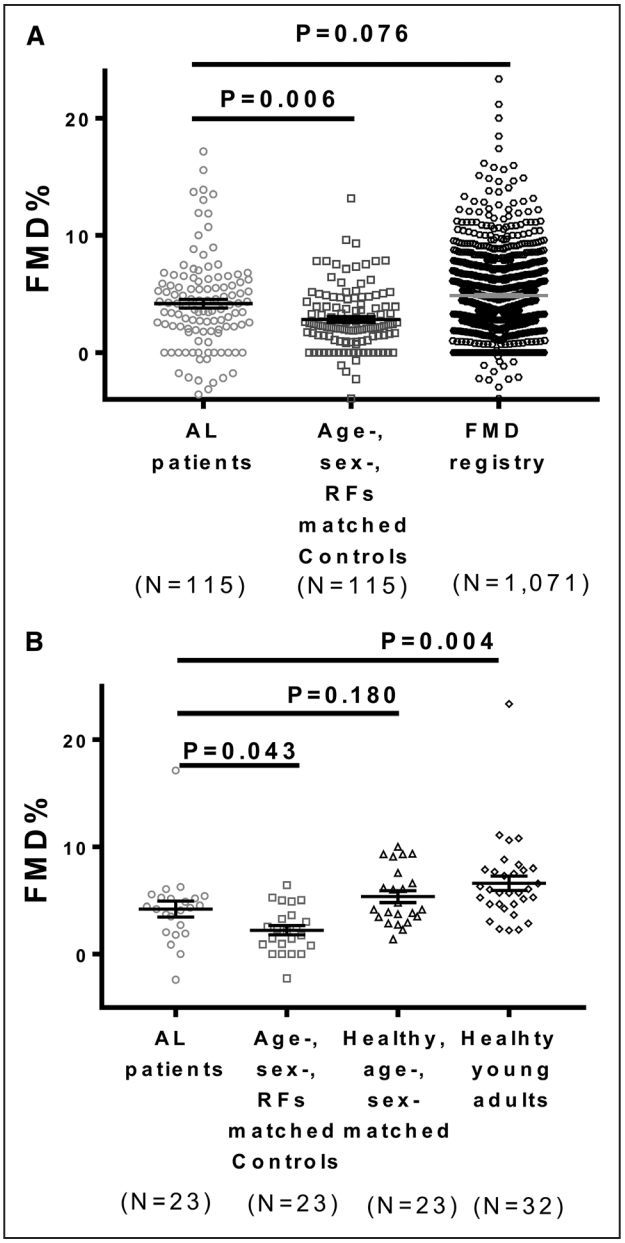

Figure 2. Flow-mediated dilatation (FMD) in light-chain amyloidosis (AL) patients and control populations. **A**, FMD was increased in 115 AL patients compared with age-, sex-, and cardiovascular risk factors–matched controls (n=115) but not to the total unmatched FMD registry (n=1071). **B**, In 23 triplets with different levels of matching, FMD in AL patients was increased in comparison to age-, sex-, and risk factor (RF)-matched controls, comparable to age- and sex-matched healthy adults with ideal cardiovascular risk profile and lower than young healthy controls with ideal cardiovascular risk profile. *P* values are derived from ANOVA with post hoc pairwise comparisons corrected by the Dunnett method. Bars represent median values and spikes SE, respectively. AL patients were matched 1:1 with subjects from the FMD registry using a hierarchical model by age, sex, and estimated glomerular filtration ratio stage and subsequently diabetes mellitus, coronary artery disease, smoking, and hyperlipidemia. Matching was not performed for arterial hypertension because low blood pressure is a manifestation of AL.

significantly higher in AL patients compared with non-AL–matched controls (n=115 per group; *P*=0.004; Table 1) and comparable to the FMD registry (Figure 2A). This difference was confirmed with allometrically scaled FMD (Table 1). Baseline and hyperemic flow stimulus, baseline brachial diameter, and shear stress were similar between AL patients and matched controls (Table 1). Importantly, FMD in AL group was higher in comparison to matched controls after adjustment for baseline brachial artery diameter and postocclusion hyperemic flow stimulus (*P*=0.005). After adjustment for parameters known to affect FMD[7,37] (ie, systolic and diastolic BP, hypertension, smoking, hyperlipidemia, and diabetes mellitus), this difference did not change (*P*=0.017). This difference in FMD remained significant after controlling for the presence of peripheral atherosclerotic plaque or coronary artery disease (*P*=0.008).

The triple 1:1-matched analysis (n=23 per group) revealed that FMD in AL patients (4.35% [2.04–5.26]) was significantly higher than their age-, sex-, and risk factor–matched counterparts (2.17% [0.82–3.64]; *P*=0.043), was comparable with their healthy age- and sex-matched controls (4.17% [3.46–7.6]), and lower than the ideally healthy group of young adults (5.86% [4.65–7.87]; *P*=0.004; Figure 2B).

Prognostic Importance of Vascular Markers in Patients With AL in the Derivation and Validation Cohort

After a median follow-up of 45 months, 48 patients died in the derivation cohort (41.7%). Early death is a common complication of AL[3]: 17 deaths (35%) occurred within the first 6 months and 27 deaths (56%) until the end of the first year of follow-up. In univariate analysis, FMD as a continuous variable was associated with higher risk of death (hazard ratio [HR], 1.08; 95% CI, 1.01–1.15; *P*=0.023), indicating that increasing FMD could be a marker of high-risk disease. In the smoothing plot of log HR versus the FMD (Online Figure I), we observed a curvilinear association between FMD and mortality that suggested the possible existence of a change point in the dose-response phenomenon. The estimated change point assessed by the Contal and O'Qingley method (log-rank test statistic, 11.1; *P*=0.007) and receiver operating characteristic analysis (area under the curve, 0.650; *P*=0.004), as described in the statistics section, was a cutoff value of FMD ≥4.5%. As shown in Figure 3A, FMD ≥4.5% was associated with increased risk of death and shorter survival (log-rank test, *P*=0.001). More specifically, the median overall survival of AL patients with FMD ≥4.5% was 21.3 months versus 71 months for patients with FMD <4.5%. A secondary analysis revealed that early mortality (within the first 6 months) was 44.4% for

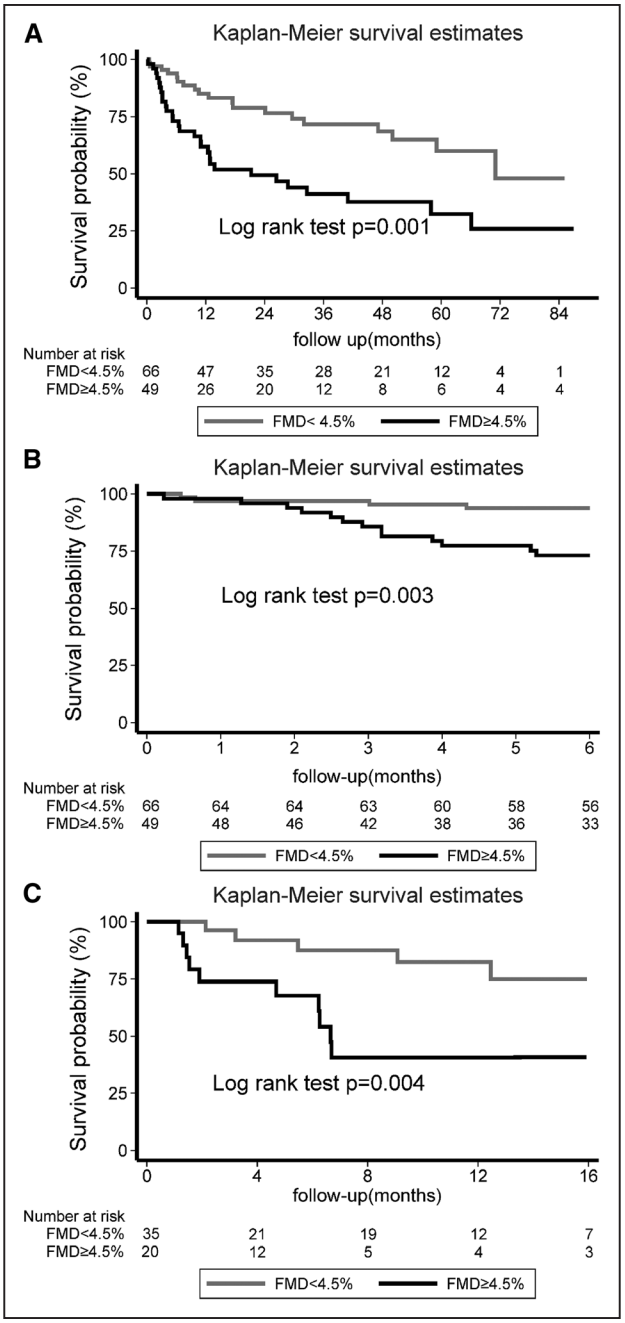

Figure 3. Kaplan-Meier curves for probability of survival in light-chain amyloidosis (AL) patients according to flow-mediated dilatation (FMD) values ≥4.5% or <4.5%.
In patients with increased FMD (≥4.5%) as compared with those with lower levels (<4.5%), Kaplan-Meier curves show inferior overall survival (**A**) until the end of follow-up (original AL cohort), (**B**) at 6-mo follow-up (original AL cohort), and (**C**) until the end of follow-up in the validation cohort. *P* was calculated by log-rank test.

subjects with FMD ≥4.5% versus 17.8% for AL patients with FMD <4.5% (log-rank test, *P*=0.003; Figure 3B).

High Mayo stage, low BP <100 mm Hg, and nerve involvement consisted the best prognostic model for mortality in the derivation cohort (Online Table IV). FMD ≥4.5% remained an independent prognostic factor associated with higher risk of death after adjusting for these factors (adjusted HR, 2.11; 95% CI, 1.17–3.82; *P*=0.013; Table 2). Further adjustment for AL-specific treatment did not attenuate this association (adjusted HR, 2.11; 95% CI, 1.17–3.83; *P*=0.013). Increased FMD was still independently associated with mortality (adjusted HR, 2.11; 95% CI, 1.13–3.95; *P*=0.019) after additionally controlling for preocclusion brachial artery diameter, peak hyperemic flow, and shear stress (Table 2). In contrast, hyperemic flow velocity or baseline brachial artery diameter were not related with mortality in the AL derivation cohort (*P*>0.1 for both). Age and sex are strong determinants of FMD,[18] whereas decreased FMD is associated with atherosclerosis.[19] After additional adjustment for age, sex, presence of subclinical and coronary atherosclerosis, FMD ≥4.5% remained an independent prognostic factor associated with higher risk of death (adjusted HR, 2.25; 95% CI, 1.20–4.20; *P*=0.011; Table 2). Increased FMD was also an independent predictor of mortality at 6 months after adjustment for the best prognostic model including Mayo stage and nerve involvement (adjusted HR, 4.36; 95% CI, 1.41–13.5; *P*=0.010; Online Table V). These results did not change after adjustment for NT-proBNP (N-terminal pro-B-type natriuretic peptide; HR, 2.42; 95% CI, 1.35–4.35; *P*=0.003), high-sensitive Troponin T (hsTnT; HR, 2.35; 95% CI, 1.26–4.39; *P*=0.007), or both (HR, 2.35; 95% CI, 1.25–4.40; *P*=0.007) instead of Mayo stage, even after additionally controlling for low systolic BP and nerve involvement (*P*<0.05 for all 3 models). In a sensitivity analysis, restrained to AL patients with heart involvement (n=77), increased FMD was still an independent predictor of mortality (HR, 1.92; 95% CI, 1.0–3.61; *P*=0.045). Finally, the association of increased FMD ≥4.5%, with all-cause mortality in AL, was confirmed in the validation AL cohort (n=55; HR, 4.2; 95% CI, 1.45–12.3; *P*=0.008 and *P*=0.004 by log-rank test; Figure 3C).

Reclassification Value of FMD as a Prognostic Factor in Patients With AL

FMD ≥4.5% correctly reclassified both high-risk AL patients who experienced the event (NRI among event subjects, 20.8%) and lower risk subjects who survived until the end of the follow-up (NRI among nonevent subjects, 40.3%) over the best prognostic model. Thus, a substantial fraction of AL patients would be reclassified into the correct risk category if FMD was additionally taken into consideration (overall NRI, 0.61; *P*=0.001; Table 3). Similarly, increased FMD conferred significant reclassification value for early mortality at 6 months as shown in Table 3 (overall NRI, 0.579; *P*=0.045) and on top of cardiac biomarkers (overall NRI, 0.633; *P*=0.002 and NRI, 0.561; *P*=0.003 over NT-proBNP and hsTnT, respectively). In addition, FMD ≥4.5% significantly increased integrated discrimination improvement over the best prognostic model for both early mortality and overall survival (Table 3).

Table 2. Multivariable Analysis of Factors Affecting Survival in 115 Patients With Light-Chain Amyloidosis

	Core Multivariable		Extended Multivariable Model 1*		Extended Multivariable Model 2†	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Disease-specific parameters						
Mayo stage						
Stage I						
Stage II–IIIA	3.56 (1.20–10.6)	0.022	3.75 (1.23–11.4)	0.020	3.42 (1.10–10.6)	0.034
Stage IIIB	9.36 (2.60–33.6)	<0.001	9.44 (2.46–36.2)	0.001	9.16 (2.36–35.6)	0.001
Nerve involvement	3.59 (1.90–6.77)	<0.001	4.16 (2.10–8.23)	<0.001	3.55 (1.79–7.06)	<0.001
SBP (≤100 mm Hg)	2.19 (1.14–4.20)	0.019	2.62 (1.24–5.53)	0.012	2.34 (1.16–4.77)	0.018
FMD-specific parameters						
FMD ≥4.5% or <4.5%	2.11 (1.17–3.82)	0.013	2.11 (1.13- 3.95)	0.019	2.25 (1.20–4.20)	0.011
Shear stress			0.919 (0.807–1.05	0.207		
Baseline diameter			0.880 (0.653–1.19)	0.207		
Hyperemic flow			0.995 (0.961–1.03)	0.765		
Atherosclerosis parameters						
Coronary artery disease					1.32 (0.500–3.50)	0.574
Presence of atherosclerotic plaque					1.10 (0.537–2.24)	0.799
Demographics						
Age					1.01 (0.977–1.04)	0.620
Sex					0.997 (0.476–2.09)	0.994

DBP indicates diastolic blood pressure; FMD, flow-mediated dilatation; HR, hazard ratio; and SBP, systolic blood pressure.

*In the extended multivariable model 1, the association of FMD with all-cause mortality was further adjusted for FMD-specific parameters including shear stress, baseline brachial diameter, and hyperemic flow.

†In the extended multivariable model 2, age, sex, presence of coronary artery disease, and presence of subclinical atherosclerosis as possible confounders affecting vascular reactivity were included.

Differences Between Low- and High-Risk AL Patients According to FMD

As shown in Online Table VI, patients with FMD ≥4.5% had more often heart involvement (79.6% versus 57.6%; *P*=0.013), increased baseline levels of hsTnT (*P*=0.007) and NT-proBNP (*P*=0.023), and thus had more often advanced Mayo stage disease (36.7% versus 18.2% for stage III; *P*=0.033) than patients with FMD <4.5%.

Sympathetic Stimulation by CPT

Patients with AL (n=19) showed increased and sustained vasodilation in response to sympathetic stimulation as assessed by CPT when compared with healthy controls (n=10; 4.39±2.52% versus 2.62±1.31% at T1, during CPT, and 4.43±3.23% versus 1.59±1.95% at T2, 3 minutes after CPT withdrawal, respectively; *P*<0.05 for both; Figure 4A and 4B). A significant group interaction was observed at T2, indicating that CPT-induced vasodilatation was prolonged in

Table 3. Reclassification Value of Flow-Mediated Dilatation Over the Best Predictive Model for Early and Overall All-Cause Mortality in 115 Light-Chain Amyloidosis Patients

	Continuous NRI					IDI	
	Subjects Correctly Reclassified, %	Subjects Incorrectly Reclassified, %	Net Reclassification Gain, %	Overall NRI (SE)	*P* Value	IDI (SE)	*P* Value
All-cause mortality at the end of the follow-up							
Events (n=48)	29 (60.4%)	19 (39.6%)	20.8	0.61 (0.189)* [0.231–0.992]	0.001	3.6 (1.8)	0.044
Nonevents (n=67)	47 (70.1%)	20 (29.8%)	40.3				
Early mortality at 6 mo							
Events (n=17)	13 (76.4%)	4 (23.5%)	52.9	0.579 (0.29) [0.095–1.06]	0.045	6.5 (3.0)	0.031
Nonevents (n=98)	51.5 (52.5%)	46.5 (47.5%)	5.0				

Best prognostic model for all-cause death at the end of the follow-up: Mayo stage (stage I vs II–IIIA vs IIIB), nerve involvement, and systolic blood pressure ≤100 mm Hg. Best prognostic model for early mortality: Mayo stage (stage I vs II–IIIA vs IIIB) and nerve involvement. IDI indicates integrated discrimination improvement; and NRI, net reclassification index.

*95% bias-corrected bootstrap CIs.

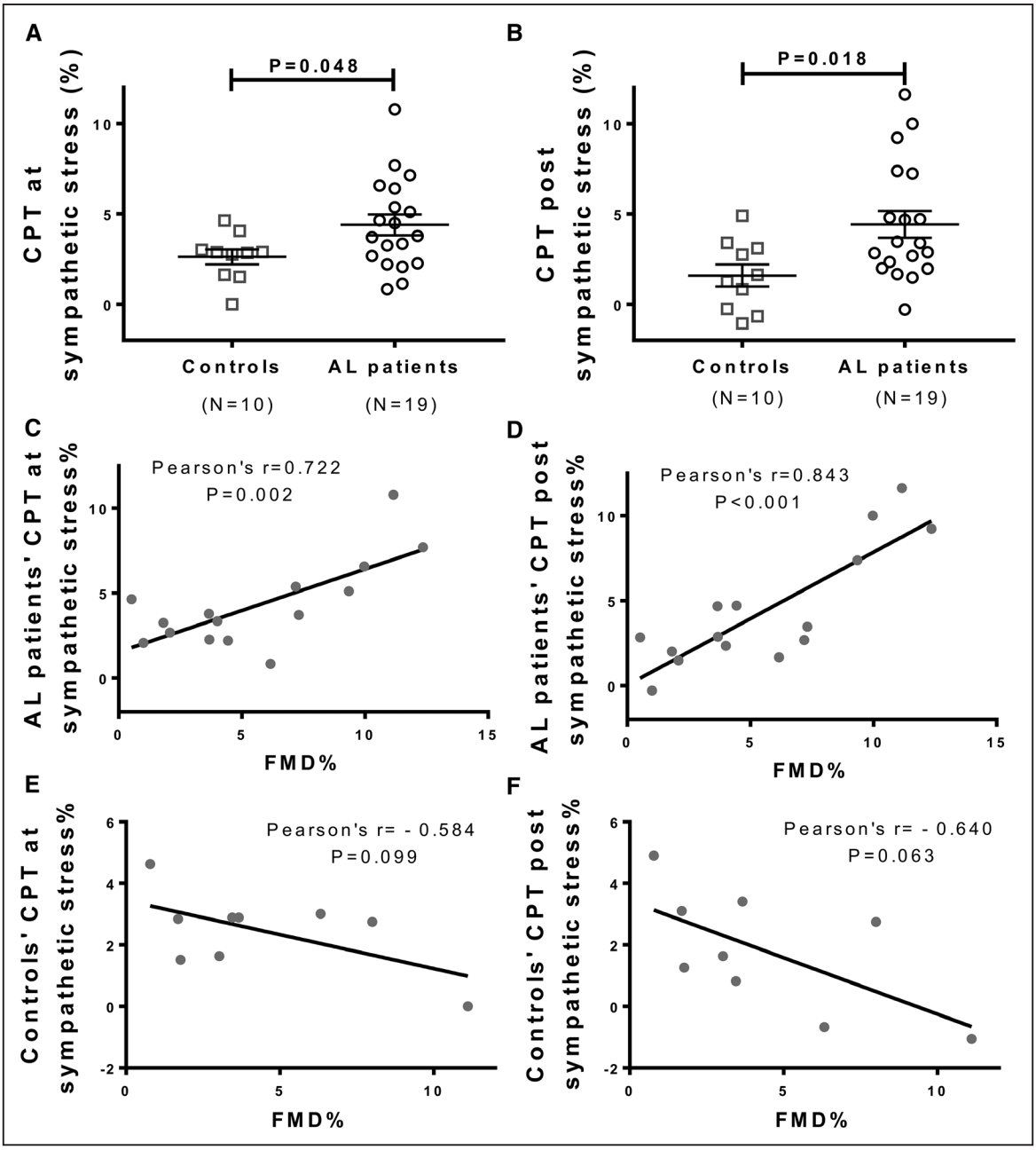

Figure 4. Flow-mediated dilatation (FMD) and autonomic function in light-chain amyloidosis (AL).
Cold pressure test (CPT) changes at sympathetic (**A**) and 3-min postsympathetic stress (**B**) are increased in AL patients (n=19) in comparison to controls (n=10). CPT changes at sympathetic and postsympathetic stress are related to baseline FMD in AL patients (**C** and **D**) but not in control subjects (**E** and **F**). *P* values in **A** and **B** are derived from independent samples *t* test. *P* values in **C**–**F** are derived from Pearson correlation test. Horizontal wide lines represent mean (**A** and **B**) values and error bars SE, respectively. Postsympathetic stress was assessed 3 min after removal of cold stimulus.

the AL group (*P* for interaction, 0.023). Importantly, in AL patients but not in controls, there was a significant correlation of FMD with maximum percentage vasodilation by CPT (Figure 4C through 4F). Patients with AL and controls did not differ in respect to baseline serum levels and changes in noradrenaline as shown in Online Table VII.

NO Pathway in AL

Characteristics of the AL patients with available blood samples to measure markers of NO pathway did not differ from the rest of the AL population (Online Table IX). Circulating levels of cGMP were increased ($P<0.001$) in the AL group, whereas nitrite and NTY levels did not differ compared with the control group (Figure 5A through 5C). Exhaled alveolar NO was increased in AL patients (n=26) as compared with healthy individuals (n=30; $P<0.001$; Figure 5D). When this population was ordered in 3 groups (ie, controls, AL patients with low FMD <4.5% and high FMD ≥4.5%), cGMP and exhaled alveolar NO gradually increased across ascending groups ($P<0.001$ for both; Figure 5G and 5H). These results

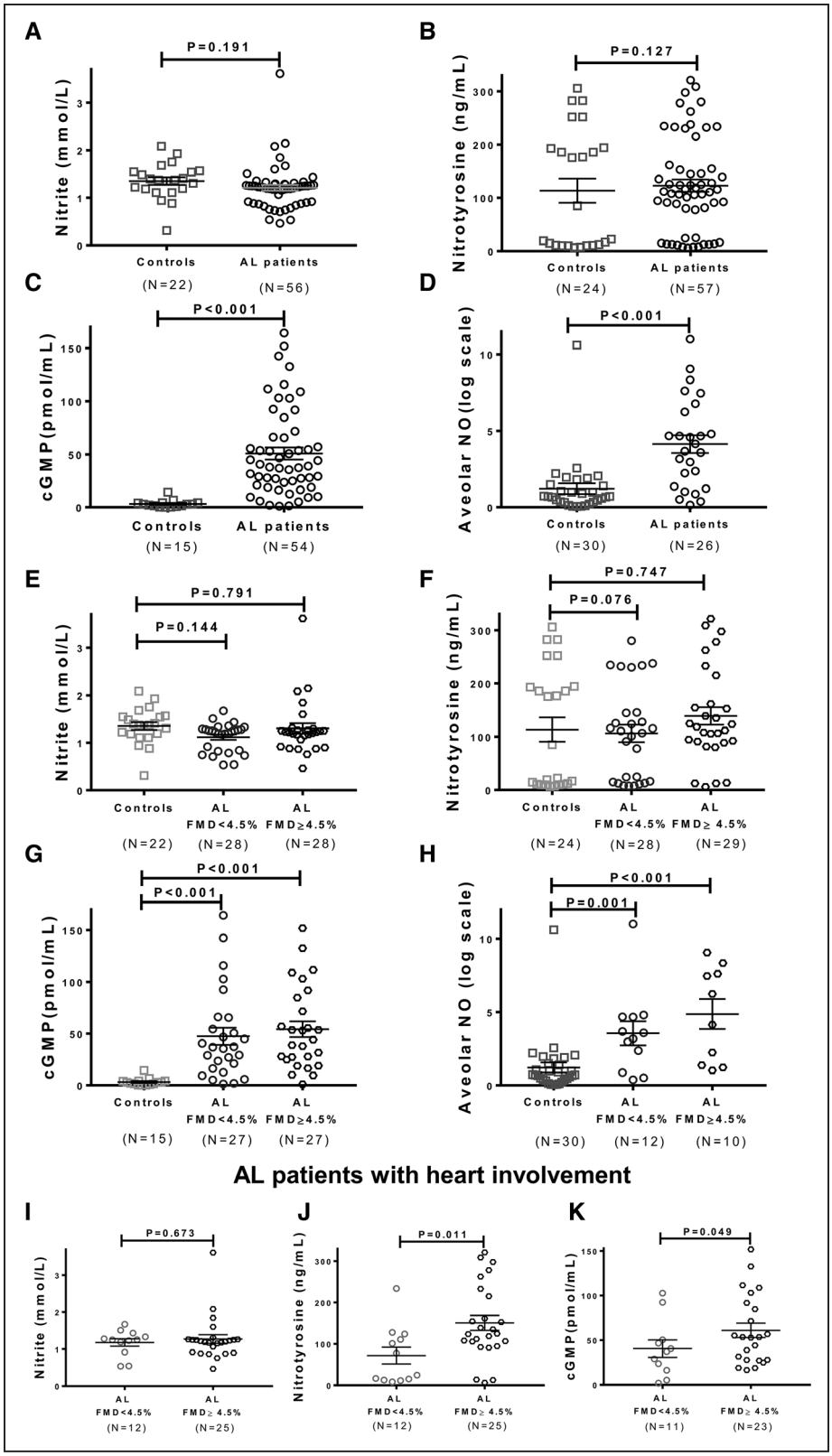

Figure 5. NO pathway in light-chain amyloidosis (AL).
Differences in plasma levels of (**A**) nitrites, (**B**) 3-nitrotyrosine (NTY), (**C**) cyclic GMP (cGMP), and (**D**) exhaled alveolar NO between controls and AL patients of the prospective (**A**–**C**) and the exhaled NO (**D**) cohorts. **E**–**H** are derived from the same research population as **A**–**D** but after discriminating AL patients according to low (<4.5%) and high (>4.5%) flow-mediated dilatation (FMD). **I**–**K**, Only AL patients with heart involvement are considered and compared according to FMD <4.5% or >4.5%. *P* values denote difference from controls as derived from independent samples *t* test (**A**–**D** and **I**–**K**) and ANOVA with post hoc pairwise comparisons corrected by the Dunnett method (**E**–**H**). Horizontal wide lines and error bars represent mean±SE, respectively.

did not materially change after adjusting for smoking and estimated glomerular filtration ratio stage, which significantly differed between controls (n=24) and AL patients (n=57; Online Table X).

In the high-risk group of patients with heart involvement, cGMP and NTY levels were significantly increased in patients with increased FMD ≥4.5% as compared with those with FMD <4.5% (Figure 5I through 5K). These findings were replicated after inverse rank normalization. In an exploratory analysis, FMD ≥4.5% remained significantly associated with increased all-cause mortality independently of cGMP and NTY (n=54; HR, 3.65; 95% CI, 1.58–8.4; *P*=0.002).

Changes of FMD After Treatment: a Prospective Analysis

In a subgroup of 15 patients with available pre- and posttreatment FMD measurements, persistently low posttreatment FMD (<4.5%) was associated with better response to treatment as compared with increased posttreatment FMD (*P*=0.021 by ordinal Kendal Tau test; Online Figure III). Persistently low FMD was also associated with lower mortality (*P*=0.026; Online Table XII).

DISCUSSION

To our knowledge, this is the first study that prospectively evaluates a dynamic marker of peripheral vasodilation in patients with systemic AL, a disease that is often accompanied by autonomic dysfunction, orthostatic hypotension, and low resting BP,[4,38] which are all associated with inferior survival.[3,39] Given that under conditions of decreased BP or low sympathetic activity, FMD is augmented,[11–14] we hypothesized that this dynamic marker of reactive vasodilation could confer clinically relevant prognostic value in patients with AL. Our findings are supporting this hypothesis, revealing 3 major implications: first, they indicate that arterial vasodilatory function is relatively augmented in a clinically important degree in AL; second, they provide a measurable marker as an indicator of increased reactive arterial dilatation in patients with AL; and third, suggest that FMD could serve as a novel biomarker for risk stratification of AL patients. Furthermore, in a secondary analysis aiming to explore the underlying mechanisms of our findings, we observed vascular autonomic dysfunction and multiple signals of augmented or preserved levels of systemic markers of NO pathway activity associated with increased FMD.

Systemic AL is a disease with high rates of early mortality due to cardiovascular complications.[3,40] The impact of the severity of cardiac amyloidosis in survival is so strong that disease staging is based on cardiac biomarkers. However, low BP (<100 mm Hg) was also found to predict mortality among patients at higher risk (ie, those at Mayo stage III).[3] Interestingly, in the current study, increased FMD was a predictor of mortality irrespective of the best prognostic model that included both advanced Mayo stage (stage IIIB) or NT-proBNP and troponin and low BP. The strongest association was observed with early mortality, with FMD ≥4.5%, increasing the odds for death by a factor of >4. Furthermore, this association remained significant even after adjusting for the presence of atherosclerosis, suggesting that increased reactive vasodilatation in AL patients may offset assumed benefits from the traditionally accepted healthy vascular profile reflected by increased FMD.[5–7] In addition, increased FMD substantially improved risk stratification by correctly reclassifying AL patients into higher or lower risk categories for early and late mortality. Importantly, our prognostic findings were confirmed in a temporal validation cohort with comparable clinical and vascular characteristics. As expected, patients from the validation cohort where followed for a shorter period. However, there was a clear signal of association between FMD ≥4.5% and mortality because of the known high incidence of early mortality in AL.[3]

Despite technical difficulties in FMD measurement, such as long learning curve, as well as lack of universally accepted reference values and standardized protocols,[7,37,41] these results indicate that FMD fulfills epidemiological criteria for further evaluation as a novel biomarker in AL and may be of clinical utility for a risk-guided treatment approach. Whether contemporary treatment for amyloidosis may affect FMD and, more importantly, whether such response to treatment would improve outcome in these patients is unknown. To that direction, our analysis of prospective data suggests that AL patients who achieve a hematologic response may retain low posttreatment FMD. Interestingly, despite the limited sample size, there was also a signal for improved survival associated with this FMD pattern. Several novel treatment regimens such as proteasome inhibitors and lenalidomide-based regimens may directly inhibit endothelial dilatation,[42,43] but whether they exert any effect on FMD in AL patients is unknown. Further research is warranted to explore the clinical value of FMD response to treatment of AL, as well as that of targeting pathways related with increased disease-specific FMD. In addition, these are the first epidemiological data indicating that increased reactive vasodilation in a selected population with hypotension is associated with mortality. Given that orthostatic hypotension has been shown to predict mortality in the general population,[44] our findings set the grounds for elucidating the role of FMD in identifying high-risk patients with conditions presenting low BP.

Subsequently, we explored possible mechanisms related to increased FMD in AL. Although, previous evidence has implicated AL with microvascular endothelial dysfunction,[45] FMD is not associated with microvascular endothelial function in humans.[46] Thus, our findings may

not be relevant to microvascular dysfunction. Furthermore, despite that our NO results cannot prove causality because of their cross-sectional nature and, therefore, cannot exclusively explain the prognostic utility of FMD in amyloidosis, they suggest that increased activity of NO as denoted by high cGMP plasma levels and exhaled NO in AL patients may contribute to increased reactive vasodilation. Notably, we found cGMP plasma levels markedly elevated by a factor of >10 as compared with controls, which was disproportionally high in relation to nitrite plasma levels in these patients. Although circulating cGMP may not substitute local levels and, therefore, may not directly reflect vascular smooth muscle cell reactivity to NO, these findings are suggestive of increased NO sensitivity of GC (guanylyl cyclase), the enzyme regulating downstream production of cGMP,[29] which might lead to enhanced downstream production of cGMP possibly contributing to increased FMD in AL patients.[29–31] Indeed, in AL patients with cardiac involvement comprising the majority of our AL population (67%), we found cGMP levels to be associated with increased FMD. Accordingly, increased or sustained NO availability may be detrimentally associated with hemodynamic vasodilatory indices in other diseases such as liver cirrhosis.[47] In support, overdilatation, expressed as markedly augmented FMD, has been previously reported in conditions related to orthostatic hypotension or lowering of sympathetic activity such as cirrhosis and vasovagal syncope.[9,10,12] Hypotension in AL[4,38] has been attributed to markedly reduced autonomic modulation in the heart and the peripheral vasculature mainly through sympathetic denervation.[4,48] Our findings regarding the underlying mechanisms are in agreement with studies where increased cGMP was found in conditions with cardiac dysautonomia.[49] Augmented cGMP can be linked with altered nNOS (neuronal NO synthase) activity, which could modify both NO bioavailability and NO-cGMP coupling at the level of nerve endings and peripheral autonomic ganglia in patients with AL[49]. Increased cGMP levels have been proposed to decrease neurotransmission in sympathetic neurons, whereas increased NO bioavailability in parasympathetic neurons has been shown to facilitate acetylcholine release.[49] It is well known that FMD is responsive to sympathetic stimulation or inactivation.[13,14,50] Accordingly, we found that FMD in AL patients was strongly associated with higher brachial artery dilatation in response to sympathetic stimulation compared with healthy controls. This reaction may be regarded as paradoxical, as it is contrasted to traditionally observed vasoconstriction in patients with atherosclerotic disease.[51]

Another point of interest is that NTY in AL was associated with increased FMD. In individuals without AL, nitrotyrosine is considered a marker of peroxynitrite load and nitrosative stress and is expected to be inversely associated with FMD.[52] Although there are no specific data in AL, in other β-sheet amyloidosis such as Aβ (amyloid-β) deposition and transthyretin amyloidosis, nitrosylation of tyrosine may be directly involved in disease pathogenesis.[53–55] Thus, our unexpected observation of increased NTY levels in high-risk AL patients with elevated FMD may reflect a parallel disease processes with nitrotyrosine being involved in amyloidosis progression. On the contrary, recent experimental evidence indicates that nitrotyrosine may induce unexpected vasodilatory response to β-adrenergic agonists through blocking of β2 adrenergic receptor internalization at nerve endings.[56] Interestingly, CPT may induce vasodilation through stimulation of postjunctional β2 adrenoceptors[51] and could, therefore, be enhanced by increased NTY local concentrations. Thus, the effect of local concentrations of NTY on paradoxical vasodilation by CPT observed in our AL population should be further explored.

Overall, considering that hyperemic change during performance of FMD was similar in both groups of high and low FMD and that CPT showed similar to FMD paradoxical vasodilatory response in AL, our results point to the direction of a generalized conductance vascular abnormality, suggesting that these patients are incapable of vasoconstricting in response to sympathetic activation. Thus, FMD may be considered a cumulative marker of increased risk of death in AL that may predominantly integrate autonomic maladaptation of vascular function with sustained vasodilation. Whether increased NO and associated nitrosative stress is actively involved in this process merits further investigation. To this end, a sensitivity analysis indicated that adjustment for cGMP and NTY levels did not affect the association of increased FMD with mortality. However, these survival results should be interpreted with caution because of the limited number of patients with available NO markers.

Certain limitations of our study should be acknowledged. First, it should be clarified that this study provides for the first time proof of concept on the clinical importance of assessing peripheral reactive vasodilation in AL and does not suggest a biomarker for immediate clinical application. FMD is known to present relatively wide variability,[7,37,41] both from a biological and technical point of view, which may hamper its validation as a biomarker in clinical practice. To address these methodological limitations, we used a standardized strict FMD protocol, which is reflected by a reproducibility performance comparable to current literature,[37] and statistically considered most parameters known to affect FMD to avoid residual confounding. Furthermore, confirmation of our survival results in a contemporary AL validation cohort indicates that FMD survival findings are reproducible in a single reference center setting. Thus, although FMD as a marker of endothelial dysfunction is not currently recommended for risk assessment in CVD because of its limited incremental value beyond existing biomarkers and risk scores, its use as a marker of reactive overdilation for wide clinical practice specifically in diseases related with autonomic

dysfunction and hypotension should be de novo considered. Such validation of FMD or of other markers of reactive vasodilatation needs independent confirmation in several other studies and definition of normal and reference values using a reproducible and universally consistent disease-specific methodology. Second, relatively low FMD values were found in the control group. This is not unexpected for 2 reasons: (1) we used the distal occlusion approach because of methodological advantages over the proximal occlusion technique,[7,57] which in general yields lower FMD values[58] and (2) control subjects were highly selected because of the matching process with AL cases and, therefore, presented at least similarly high prevalence of characteristics known to adversely affect FMD.[18–20] Indeed, our unmatched and age- and sex-only–matched healthy control groups presented comparable or higher FMD values with AL cases ranging closely to published literature.[37,58] Thus, low FMD of our strictly matched controls may reflect a high CVD risk factor burden of their AL counterparts (101 of 115 subjects had at least 2 CVD risk factors). AL patients in turn, despite their adverse cardiovascular profile, presented unexpectedly high FMD values, which were comparable to individuals with healthier CVD risk profile. These findings support the presence of inherent mechanisms in AL adversely augmenting FMD as discussed above. Finally, it should be noted that, apart for the CPT experiment, we applied a retrospective control patient method using a prospectively recruited FMD registry. However, control subjects were examined in our laboratory during the same recruitment period of the derivation and validation AL cohorts, using the same methods for documentation of population characteristics, as well as for FMD assessment. Finally, it should be acknowledged that with testing multiple potential mechanisms, there is the possibility of type I error attributable to multiple testing.

In conclusion, in patients with primary systemic AL, increased FMD was an independent predictor of all-cause mortality over validated prognostic factors and improved risk stratification. Furthermore, we found evidence of relatively increased reactive vascular dilatation, which was mainly associated with altered vascular autonomic function. Importantly, prospective data support an association between hematologic response and posttreatment FMD changes. Thus, FMD merits further investigation as a possible novel noninvasive biomarker to assess risk of mortality in AL patients aiming to guide risk-based treatment. In addition, these are the first prospective data providing a proof of concept that reactive overdilatation may identify high-risk patients in chronic hypotensive conditions.

ARTICLE INFORMATION

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