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# AXL receptor tyrosine kinase is increased in patients with heart failure☆,☆☆

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ABSTRACT

*Background:* AXL is a membrane receptor tyrosine kinase highly expressed in the heart and has a conspicuous role in cardiovascular physiology. The role of AXL in heart failure (HF) has not been previously addressed.
*Methods and results:* AXL protein was enhanced 6-fold in myocardial biopsies of end-stage HF patients undergoing heart transplantation compared to controls from heart donors ($P < 0.0001$). Next, we performed a transversal study of patients with chronic HF (n = 192) and a group of controls with no HF (n = 67). sAXL and BNP circulating levels were quantified and clinical and demographic data were collected.
sAXL levels in serum were higher in HF (86.3 ± 2.0 ng/mL) than in controls (67.8 ± 2.0 ng/mL; $P < 0.0001$). Also, sAXL correlated with several parameters associated with worse prognosis in HF. Linear regression analysis indicated that serum creatinine, systolic blood pressure and atrial fibrillation, but not BNP levels, were predictive of sAXL levels. Cox regression analysis indicated that high sAXL values at enrollment time were related to the major HF events (all-cause mortality, heart transplantation and HF hospitalizations) at one year follow-up ($P < 0.001$), adding predictive value to high BNP levels.
*Conclusions:* Myocardial expression and serum concentration of AXL is elevated in HF patients compared to controls. Furthermore, peripheral sAXL correlates with parameters associated with the progression of HF and with HF events at short term follow-up. All together these results suggest that sAXL could belong to a new molecular pathway involved in myocardial damage in HF, independent from BNP.


## 1. Introduction

Heart failure (HF), characterized by a ventricular systolic or diastolic dysfunction, constitutes the end-stage of many heart diseases. Population prevalence of HF averages 2 to 3% increasing to ≥10% at 70 years of age or older [1,2]. Epidemiological studies report a link between HF and low survival rates [3]. Several etiologies underlie the diagnosis of HF, including pathological remodeling of the myocardium after an infarct. Other important etiologies include cardiomyopathies, hypertension, valvular heart disease, and toxins. Therefore, we can consider HF as a multifactorial syndrome with abnormalities of cell signaling, that are reflected in the contractile function of the myocardium as well as in cardiac cell survival and death [4,5].

Receptor tyrosine kinases (RTKs) are membrane proteins recognizing extracellular signals leading to cellular responses such as proliferation, arrest or activation. AXL belongs to the Tyro3, Axl and MerTK (TAM) subfamily, of RTKs and is a protein of particular interest as a potential player in the pathophysiology of the failing heart. Although the TAM receptors were first cloned as orphan receptors, affinity purification techniques led to the identification of Growth Arrest-Specific 6 protein (GAS6) as the AXL ligand [6]. GAS6 has significant structure and sequence similarity with the vitamin K-dependent protein S, but lacks its anticoagulant activity [7]. Cells of the vasculature including

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endothelial cells, vascular smooth muscle cells and fibroblasts synthesize and express GAS6 and AXL [8,9]. According to the GeneAtlas data set, AXL is highly expressed in the heart, including cardiomyocytes, among other organs [10]. Membrane bound AXL can be shed by the effects of a Disintegrin and Metalloproteinase domain-containing protease (ADAM), leading to a soluble form that is detected in plasma as a stable complex with its ligand GAS6 [11,12].

The aim of this project was to analyze the AXL myocardial and serum levels in patients with HF and whether it could be useful in assessing the stage of the HF. Hence, we analyzed the relationship of sAXL with parameters that are affected in HF and estimated the value of sAXL in predicting events at one year follow-up.

## 2. Methods

### 2.1. Collection and analysis of human heart samples

Human heart samples were analyzed in 2 populations: control samples (n = 11) were non-diseased biopsies. These samples were from organ donors whose heart could not be used for transplantation because they did not match the requirements of the potential heart receptors. Left ventricle samples were collected at the time of organ donation.

Case samples (n = 15) were pathological human left ventricle biopsies from patients with severe HF obtained at the time of cardiac transplantation, as described previously [13]. Whenever it was possible, sample harvest of around 1 $cm^3$ myocardial biopsies was performed in a middle region between the apex and the base. The myocardial tissue was immediately immersed in liquid nitrogen. The Ethical Committee of the affiliated institution approved the protocol.

### 2.2. Western Blot analysis of sAXL protein

Myocardial proteins were extracted from left ventricle biopsies, if possible from the anterior wall. Samples were submerged in 1 mL of ice-cold protein lysis buffer containing: 50 mM Tris HCl pH 7.5 (T5941, Sigma), 150 mM NaCl (1.06404.5000, Merck), 1% Nonidet P-40 (Tergitol solution, NP40S, Sigma), 0.5% sodium deoxycholate (D6750, Sigma), 0.1% sodium dodecyl sulfate (L5750, Sigma), 1 mM phenylmethanesulfonyl fluoride (P7626, Sigma), 1 mM sodium orthovanadate (S6508, Sigma), 1 mM Pefabloc (11429868001, Roche) and complete Mini Protease Inhibitor Cocktail (11836153001, Roche). Samples were homogenized with an Omni TH homogenizer (Omni International Inc.). After 1 h of rotation at 4 °C, samples were centrifuged at 10,000 *g* at 4 °C for 30 min. The upper phase was collected and the total protein concentration was quantified with the Pierce BCA protein Assay method (23227, Thermo Scientific, Pierce) relative to a BSA standard curve. Thirty micrograms of total protein extract were loaded to NuPage® 4–12% Bis-Tris Gel (NP0322) and a western blot was performed with the Novex® gels methodology (Invitrogen). Proteins were transferred from the gel to a nitrocellulose membrane using a blot gel transfer (IB3010-01) and the iBlot® Dry Blotting System. After 1 h blockade of the membrane with phosphate buffered saline solution (PBS, Fisher Scientific), 0.1% Tween 20 (P1379, Sigma-Aldrich) and 5% of skimmed milk, it was incubated overnight at 4 °C with the AXL (c-20) primary antibody against the carboxy terminal protein of the full-length human protein diluted 1/1000 (sc-1096, Santa Cruz Biotechnology). Afterwards, the membrane was incubated during 1 h with an HRP-Rabbit anti-Goat secondary antibody diluted 1/5000 (31402 Thermo Scientific). Final detection of the AXL protein band was accomplished with the ECL kit Supersignal West Pico Chemioluminescent Substrate (34080, Thermo Scientific).

Quantification of the integrated density of the bands around 150 kDa was done with the ImageJ program (NIH, Maryland, USA). Each blot was loaded with 30 micrograms of a standard sample of human myocardial protein for calibration between blots. Results are given in relative units (RU) as the ratio between the integrated densities of each sample divided by the integrated density of the standard.

### 2.3. Patient enrolment and collection of clinical data

This was a transversal study of patients treated in a specialized outpatient HF clinic with chronic, stable HF with systolic dysfunction, characterized by an ejection fraction (EF) less or equal to 40% (mean evolution HF time of 7.5 ± 0.6 years). Participants were recruited in a consecutive manner based on presenting the inclusion criteria from May 2009 to November 2011 in the Hospital Clinic of Barcelona and from November 2010 to October 2011 in the Hospital Sant Pau of Barcelona. Patients belonged to New York Heart Association (NYHA) functional classes II, III or IV (mainly II and III). HF patients were diagnosed and assigned to a NYHA class by a group of cardiologists specialized in heart failure and heart transplantation according to the European Society of Cardiology criteria [2]. These patients were considered to have stable HF because they did not have a hospital admission with a diagnosis of HF during the previous month before enrolment.

Historical records from these patients were retrieved and an echocardiography taken within a year from the inclusion day was used for each patient. Clinical data such as ECG data, HF evolution time, functional capacity measured as 6-minute walking test (6MWT), cardiovascular risk factors (hypertension, dyslipidemia, diabetes mellitus and smoking), laboratory parameters and medication were entered for analysis. Ventricular dilation parameters, systolic function and the EF were assessed by echocardiography. One hundred ninety-two patients were included with a major reduced left ventricular function, and were designated as HF. Patients under 18 years of age were excluded as well as patients affected by other fibroproliferative diseases such as; kidney failure (creatinine levels >3 mg/dL), cirrhosis, bone metabolic disease, hyperthyroidism, pulmonary fibrosis, systemic sclerosis, macular degeneration and amyloidosis.

This study complies with the Declaration of Helsinki, the Ethical Committee of the affiliated institution approved the protocol and all subjects gave written, informed consent to participate in this study.

### 2.4. Patient short term follow-up

Follow-up data of 1 year ± 1 month were obtained from either a clinical or a telephone interview or from the clinical records. The events that were recorded were as follows: hospitalizations for either HF, acute coronary syndrome, cardiorespiratory arrest, ventricular tachycardia or fibrillation; device implantation of either a pacemaker, an implantable cardioverter–defibrillator (ICD), cardiac resynchronization therapy (CRT with or without ICD); percutaneous revascularization; cardiac surgery (for either heart transplantation, coronary revascularization or others); ictus and all-cause mortality (cardiac and non-cardiac deaths).

### 2.5. Controls characteristics

Controls were a group of subjects with no HF with a lower proportion of male subjects (46%) than the patients (85%, $P < 0.0001$). Controls mean age was 48.6 ± 2.0 years (n = 67) and was lower than the mean age of the patients 61.8 ± 0.8 years (n = 192, $P < 0.0001$). HF patients had higher proportions of all risk factors than controls: Hypertension $P < 0.0001$, Dyslipidemia $P < 0.0001$, Diabetes Mellitus $P < 0.01$, Current/former smoker $P < 0.0001$, and Previous AMI $P < 0.0001$.

### 2.6. Collection and analysis of serum and plasma samples

Blood samples were collected from an antecubital vein from all HF patients and controls. Serum and plasma samples from the HF patients were collected on the enrolment day.

To measure circulating levels of BNP, whole blood was collected in a chilled tube with the anticoagulant EDTA [14]. The sample was promptly put on ice after blood extraction and was centrifuged at RCF 1800 *g* for 10 min at 4 °C. The supernatant was collected, aliquoted and kept at −80 °C until analysis. Quantification of BNP plasma levels was performed and validated in the Biomedical Core Facility of the Hospital Clinic of Barcelona. Briefly, plasma BNP was quantified with a chemiluminometric immunoassay run on the ADVIA Centaur Immunochemistry analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). The intra-assay coefficient of variation was of 1.8–4.3% CV and the inter-assay coefficient of variation was of 2.3–4.7% CV.

Serum samples for sAXL analysis were kept at room temperature for at least 30 min after blood extraction, to let the clot to be formed and were later centrifuged at RCF 1800 g for 10 min at room temperature. The supernatant was collected, aliquoted and kept at −80 °C until analysis. A new detection method for sAXL quantification was devised in our laboratory using commercially available antibodies. Multiwell plates (96 wells, Rubilabor) were coated with the capture antibodies AF154 (R&D Systems) at a concentration of 2.0 μg/mL in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, pH 7.2, filtered through a 0.2 μm pore). The plates were sealed and left overnight at room temperature. The plates were washed three times with wash buffer (PBS with 0.05% Tween20) and then were blocked for 1 h at room temperature with PBS containing 1% BSA (Sigma). Serum samples were diluted 200 times in PBS containing 1% BSA. A standard curve was made by serial dilution of a purified sAXL protein produced by recombinant expression of the extracellular part of AXL (R&D Systems) and concentrations are given as ng/mL. A negative control without plasma sample was used and pooled-plasma samples were used in each plate as plate controls. After sample addition, the plates were washed three times with wash buffer (PBS with 0.05% Tween20). Then, 100 μL of the BAF154, diluted in PBS containing 1% BSA was added at a concentration of 50 ng/mL and incubated 2 h at room temperature. The aspiration/wash steps were repeated and 100 μL of the working dilution of Streptavidin-HRP (Sigma) were added to each well. The plate was covered and incubated for 20 min at room temperature. The aspiration/wash steps were repeated and a solution of tetramethyl-benzidine (slow kinetic form; Sigma) was added to the well. The reaction was stopped with sulfuric acid at 1 M concentration. The absorbance of wells was determined with an automatic plate reader at 450 nm. Intra-assay variability was determined by evaluating eight replicates of two standard samples. The mean %CVs was 6.45 and 9.21, respectively. Inter-assay mean %CV was determined in six different plates and gave the value of 8.32.

### 2.7. Data analysis

Logarithmic transformation with base e (ln) was applied to sAXL and BNP values to achieve a normal distribution. Ln(sAXL) and Ln(BNP) were back transformed and results are discussed in the text with the original sAXL and BNP values. The independent samples *T*-test was applied to analyze for ln(sAXL) differences between the control and the patients groups or between two groups of patients. Patients were stratified as patients with low sAXL levels (L-sAXL; sAXL values below the 3rd quartile 98.1 ng/mL) or high sAXL (H-sAXL; sAXL values equal or above the 3rd quartile 98.1 ng/mL). Differences in

continuous variables between both groups were assessed with a U Mann–Whitney test and differences in discontinuous variables with a Chi-Square test. Values are given as mean and standard error of the mean (SEM), unless otherwise stated. We analyzed whether the continuous variables that had different mean values in the two HF groups with L-sAXL <98.1 ng/mL or H-sAXL ≥98.1 ng/mL (Tables 1 and 3), correlated with ln(sAXL) serum values with the Pearson correlation factor. The variables that were considered for a putative correlation with ln(sAXL) serum values were: systolic blood pressure, diastolic blood pressure, 6-minute walk distance, serum creatinine levels, glomerular filtration rate, C reactive protein, uric acid, hemoglobin, hematocrit and hematies and lymphocyte count. We also assessed whether there was a correlation between the sAXL serum values and the established HF biomarker BNP. All variables correlated with ln(sAXL) serum values and were introduced in the linear regression model as independent variables and ln(sAXL) serum levels as the dependant variable. ENTER and STEPWISE methods were both used for introducing the independent variables.

The relationship between ln(sAXL) and the non-continuous variables that were different between the two HF groups (sAXL <98.1 ng/mL or sAXL ≥98.1 ng/mL, was evaluated by an unianova analysis. The variables valvular etiology and congestion signs did not present statistical differences in the unianova analysis and were not further studied. The variables dyslipidemia, NYHA classification and atrial fibrillation showed differences with the unianova analysis and were introduced in the linear regression model together with the continuous variables serum creatinine levels and systolic blood pressure. Receiver–Operator Characteristic Curve (ROC) analysis of sAXL and BNP for discriminating HF patients from controls was performed. The cut-off value for calculation of the specificity and sensitivity for sAXL was the nearest point to the upper-left corner and the point with highest Youden's index value. We considered the BNP cut-off point of 35 pg/mL, because is the upper limit of the reference interval used in our clinical setting.

HF evolution measured by time to any event or to the set of events all-cause mortality, heart transplantation and hospitalizations due to HF was analysed using statistical survival techniques. The 3rd quartile value of the sAXL distribution (98.1 ng/mL) or of the BNP distribution (362.7 pg/mL) were used as cut-off points for stratification in the Kaplan–Meier survival curves, and patients were divided in three groups with sAXL above the 3rd quartile, with BNP above the 3rd quartile or with both, sAXL and BNP, above the 3rd quartile. Patients lost at the follow-up (10 out of 192) were not included in the analysis. Univariate Cox regression model was evaluated with either all events or with the three HF events; all-cause mortality, heart transplantation or HF hospitalization or with only the two major events all-cause mortality, heart transplantation as outcomes. Hazard ratios were also calculated with the Cox proportional hazard analysis. A Cox regression analysis with a forward stepwise model was performed to verify whether the variable of sAXL values belonging to the 3rd quartile added prognostic value to the evaluation of patients with BNP levels belonging to the 3rd quartile.

All P values reported are 2 sided, and those that were less than 0.05 were considered to be statistically significant. Statistics were calculated with the SPSS version 18.0 (SPSS Inc. Chicago, Illinois).

## 3. Results

### 3.1. AXL protein is higher in myocardial samples from end-stage HF patients than in controls

Western blot analysis of myocardial biopsies from 26 individuals showed the presence of AXL as two high molecular weight bands of approximately 160, 140 kDa (Fig. 1A), and three other bands of lower molecular weight (120, 75 and 50 kDa), possibly corresponding to proteolytic fragments or splicing variants of the full length membrane receptor. The upper 160 and 140 kDa bands were quantified by densitometry and compared, showing that samples from 15 HF patients undergoing heart transplantation had a 6-fold increase in AXL immunoreactivity compared to 11 control donors with healthy hearts (1.2 ± 0.2 Relative Units (RU) versus 0.2 ± 0.05 RU; $P < 0.0001$, Fig. 1B).

### 3.2. sAXL is higher in HF patients than in controls and correlates with parameters associated with the progression of HF

sAXL levels in serum were higher in patients 86.3 ± 2.0 ng/mL than in controls 67.8 ± 2.0 ng/mL ($P < 0.0001$, Fig. 2A). Higher sAXL values were also encountered in patients with NYHA classes III–IV 100.2 ± 4.9 ng/mL (n = 54) compared to class II patients 80.9 ± 1.9 ng/ml (n = 138; $P = 0.0001$, Fig. 2B). Similar sAXL values were found in HF patients with different etiologies, with the exception of higher values in patients with valvular etiology, when compared against all other HF patients (Table 1 and Fig. 3).

Receiver–Operator Characteristic Curve (ROC) analysis of sAXL for discriminating HF patients from controls was performed. The area under the curve (AUC) was 0.715 and its 95% confidence interval estimate was from 0.649 to 0.782 ($P < 0.0001$). A cut-off point value of 71 ng/ml of sAXL gave a 70.3% of sensitivity and 64.2% of specificity.

**Table 1**
Baseline demographic, prior history and clinical characteristics of HF patients stratified by sAXL 3rd quartile level.

| | sAXL, ng/mL | | |
|---|---|---|---|
| Parameter | <98.1 | ≥98.1 | *P* |
| No. of patients | 144 | 48 | |
| Demographics | | | |
| Age, y, | 61 ± 1 | 64 ± 1 | ns |
| Male, n (%) | 123 (85) | 41 (85) | ns |
| Female, n (%) | 21 (15) | 7 (15) | ns |
| Risk Factors, n (%) | | | |
| Hypertension | 109 (77) | 34 (72) | ns |
| Dyslipidemia | 95 (68) | 20 (44) | <0.005 |
| Diabetes Mellitus | 50 (35) | 16 (33) | ns |
| Current/former smoker | 104 (73) | 28 (61) | ns |
| Previous AMI | 70 (50) | 20 (44) | ns |
| Clinical characteristics | | | |
| Body Mass Index, kg/m$^2$ | 28.1 ± 0.4 | 27.9 ± 0.8 | ns |
| NYHA classification, n (%) | | | |
| II | 112 (78) | 26 (54) | <0.005 |
| III–IV | 32 (22) | 22 (46) | <0.005 |
| Etiology, n (%) | | | |
| Idiopathic | 40 (28) | 13 (27) | ns |
| Ischemic | 75 (52) | 18 (38) | ns |
| Valvular | 12 (8) | 11 (23) | =0.01 |
| Hypertensive | 4 (3) | 1 (2) | ns |
| Other | 13 (9) | 5 (10) | ns |
| Heart rate, beats/min | 71.5 ± 1.2 | 72.2 ± 2.1 | ns |
| Systolic blood pressure, mmHg | 120.0 ± 1.9 | 108.9 ± 2.4 | < 0.005 |
| Diastolic blood pressure, mmHg | 73.3 ± 1.0 | 68.3 ± 1.5 | < 0.01 |
| Pulse pressure | 47.1 ± 1.6 | 40. 6 ± 2.2 | ns |
| Abdominal perimeter, cm | 102.8 ± 1.2 | 103.9 ± 3.1 | ns |
| 6-min walk distance, m | 418 ± 8.9 | 372 ± 17.4 | <0.05 |

AMI (acute myocardial infarction).

### 3.3. Patients' characteristics

Comparison of the demographic and clinical characteristics of HF patients classified as patients with serum sAXL levels lower than 98.1 ng/mL, the 3rd quartile value of sAXL (L-sAXL, n = 144) or patients with higher or equal serum sAXL levels than 98.1 ng/mL (H-sAXL, n = 48) is summarized in Tables 1 to 4. Lower systolic arterial pressure ($P < 0.005$) and lower diastolic arterial pressure ($P < 0.01$) and less functional capacity measured with the 6-minute walk test ($P < 0.05$) were found in the H-sAXL group when compared to the L-sAXL group (Table 1). Among common cardiovascular risk factors, lower proportion of dyslipidemia was found in the H-sAXL than in the L-sAXL group ($P < 0.005$, Table 1). When comparing the frequency of symptoms and signs between both groups, we found a higher proportion of patients with congestion signs, in the H-sAXL than in the L-sAXL group ($P < 0.01$, Table 2).

No differences in the echocardiographic characteristics were found between both groups of HF (Table 2). When comparing ECG findings, we encountered that the H-sAXL group had a higher percentage of patients with atrial fibrillation than the L-sAXL group ($P < 0.05$, Table 2). Patients in the H-sAXL group had higher values of BNP ($P = 0.001$), serum creatinine ($P < 0.001$), lower glomerular filtration rate ($P < 0.0001$), higher uric acid levels ($P < 0.05$) and higher C Reactive Protein ($P < 0.01$, Table 3). Analysis of blood parameters showed that the H-sAXL group had lower lymphocytes ($P < 0.05$) and erythrocyte count ($P = 0.001$), hemoglobin levels ($P < 0.05$) and hematocrit ($P < 0.05$, Table 3). No other differences in laboratory values were found between the two subsets of patients. Pharmacological treatment was very similar in both groups except for a lower intake of statins in the H-sAXL group ($P < 0.01$, Table 4) and for a higher proportion of

**Table 2**
Baseline HF symptoms and signs, ECG and echocardiographic parameters of HF patients stratified by sAXL levels.

| Parameter | sAXL, ng/mL <98.1 | ≥98.1 | P |
|---|---|---|---|
| No. of patients | 144 | 48 | |
| Symptoms and signs | | | |
| Paroxysmal nocturnal dyspnea, n (%) | 24 (18) | 6 (15) | ns |
| Reduction in exercise tolerance, n (%) | 56 (42) | 18 (50) | ns |
| Orthopnea, n (%) | 40 (30) | 11 (28) | ns |
| Syncope, n (%) | 19 (14) | 4 (9) | ns |
| Lower extremity edema, n (%) | 18 (13) | 6 (14) | ns |
| Congestion signs, n (%) | 4 (5) | 6 (27) | <0.01 |
| Jugular venous distension, n (%) | 7 (9) | 5 (24) | ns |
| Hepatojugular reflux, n (%) | 14 (18) | 4 (20) | ns |
| ECG parameters | | | |
| Sinusal rhythm, n (%) | 86 (61) | 24 (52) | ns |
| Atrial fibrillation, n (%) | 9 (6) | 8 (17) | <0.05 |
| QRS length, ms | 134.8 ± 3.2 | 134.5 ± 5.5 | ns |
| Interval PR, ms | 168.9 ± 4.0 | 168.6 ± 6.0 | ns |
| Necrosis Q waves, n (%) | 32 (34) | 7 (24) | ns |
| Intervent conduct disorders, n (%) | 77 (61) | 28 (70) | ns |
| Left bundle branch block, n (%) | 18 (21) | 8 (29) | ns |
| Pacemaker, n (%) | 77 (54) | 29 (60) | ns |
| Resynchronization therapy, n (%) | 19 (13) | 4 (8) | ns |
| Echocardiographic parameters | | | |
| LVESD, mm | 53.5 ± 1.0 | 53.6 ± 1.7 | ns |
| LVEDD, mm | 67.5 ± 0.8 | 66.9 ± 1.2 | ns |
| LVEF, % | 27.6 ± 0.6 | 26.5 ± 1.0 | ns |
| LAD, mm | 47.4 ± 0.7 | 50.5 ± 1.6 | ns |
| IVST, mm | 10.3 ± 0.1 | 10.8 ± 0.3 | ns |
| LVPWT, mm | 9.9 ± 0.1 | 10.1 ± 0.2 | ns |
| LVH, n (%) | 57 (42) | 24 (53) | ns |

Intervent conduct disorders (Interventricular conduction disorders), LVESD (left ventricle end-systolic diameter), LVEDD (left ventricle end-diastolic diameter), LVEF (left ventricle ejection fraction), LAD (Left atrial diameter), IVST (Interventricular septum thickness), LVPWT (Left ventricular posterior wall thickness), LVH (Left Ventricular Hypertrophy defined as IVST ≥ 11 mm).

patients taking diuretics, oral digoxin and antiarrhytmics in this group that almost reached significant differences.

### 3.4. Analysis of sAXL with other clinical variables

We next analyzed whether the continuous variables that had different mean values in the two HF groups with L-sAXL <98.1 ng/mL or H-sAXL ≥98.1 ng/mL (Tables 1 to 3), correlated with ln(sAXL) serum values with the Pearson correlation factor. We found that ln(sAXL) serum values directly correlated with ln(BNP) ($R = 0.270$, $P < 0.001$, $n = 167$) serum creatinine levels ($R = 0.36$, $P < 0.0001$, $n = 189$), C reactive protein ($R = 0.227$, $P < 0.01$, $n = 143$), uric acid ($R = 0.253$, $P < 0.01$, $n = 132$) and inversely with systolic blood pressure ($R = -0.323$, $P < 0.0001$, $n = 184$). diastolic blood pressure ($R = -0.238$, $P = 0.001$, $n = 183$), 6-minute walk distance ($R = -0.207$, $P < 0.01$, $n = 171$), glomerular filtration rate ($R = -0.296$, $P < 0.0001$, $n = 178$), hemoglobin ($R = -0.217$, $P < 0.01$, $n = 187$), hematocrit ($R = -0.207$, $P < 0.01$, $n = 189$), hematies count ($R = -0.243$, $P < 0.01$, $n = 183$) and lymphocytes count ($R = -0.211$, $P < 0.01$, $n = 188$).

Evaluation of the putative interaction between ln(sAXL) and the continuous variables that correlated with ln(sAXL) was done with a linear regression method with ln(sAXL) as a dependant variable. We found that serum creatinine levels ($P < 0.0001$) and systolic blood pressure ($P < 0.001$) were predictive of ln(sAXL) serum levels. And all other continuous variables, including BNP, were not predictive. When we introduced non-continuous variables in the linear regression model atrial fibrillation was predictive of ln(sAXL) ($P < 0.001$) together with serum creatinine levels and systolic blood pressure.

We also analyzed whether sAXL levels correlated with variables such as age, gender, and risk factors in controls. Higher sAXL values were quantified in female controls (73.2 ± 3.5 ng/mL, n = 36) than in male controls (64.5 ± 3.3 ng/mL, n = 31) but the difference was not statistically different. No differences of sAXL levels were found with controls age, and common cardiovascular risk factors (data not shown).

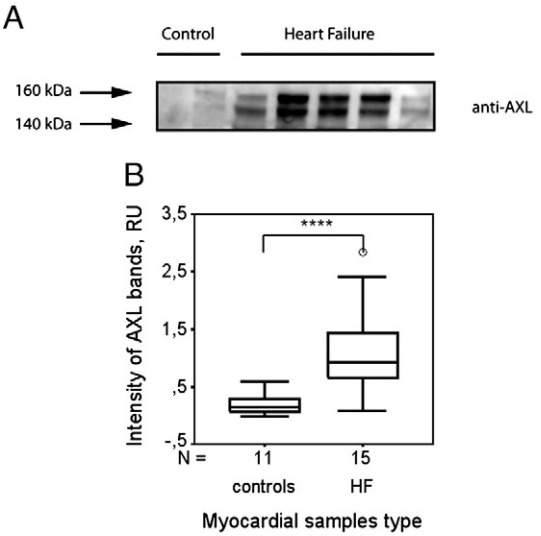


**Fig. 1.** Western Blot analysis of AXL expression in myocardial biopsies. A, representative western blot of myocardial biopsies from 2 healthy (control) and 5 pathological (heart failure) left ventricle samples. Thirty micrograms of protein were loaded in each lane. AXL (upper panel) appears as a doublet of 140–160 kDa bands. B, Quantification of the 140 and 160 kDa AXL bands by densitometry of 3 different blots with control (n = 11) and heart failure (n = 15) samples. Results were compared with a *T*-test (****$P < 0.0001$).

### 3.5. Analysis of sAXL in one year follow-up events

At 1 year ± 1 month after enrollment 52 patients out of 182 (29%) had had at least one event of any kind and the remaining 130 patients did not have any event (71%). When considering only major HF events (all-cause mortality, cardiac transplantation or HF hospitalizations), 33 patients had an event (18%) and 149 (82%) did not have any HF event. Kaplan–Meier survival curves and Cox regression analysis showed time course differences in patients that suffered from major HF events between patients with sAXL values lower or higher than the cut-off point of the 3rd quartile 98.1 ng/mL ($P < 0.001$, Fig. 4A). A 35% of patients with sAXL values higher than 98.1 ng/mL had suffered one of these events one year after enrollment, and their hazard ratio (HR) was 3.31. Such differences were also observed between patients with BNP values lower or higher than the cut-off point of the 3rd quartile 362.7 pg/mL ($P < 0.0001$, Fig. 4B). A total of 42% of patients with BNP values higher than 362.7 pg/mL presented a major HF event at one year follow-up, and the HR of these patients was 4.4. When we classified patients with a combination of both, sAXL and BNP, values above the 3rd quartile or not, we found higher differences in the Cox regression model ($P < 0.0001$, Fig. 4B) than when considering sAXL or BNP values alone. In this case, the percentage of patients in the high sAXL and BNP group that had undergone all-cause mortality, transplantation or HF hospitalizations rose to 62% and the HR to 6.77. Furthermore, a Cox regression analysis with a forward stepwise model showed that the addition of the variable of patients belonging to the sAXL 3rd quartile added prognostic value for major HF events to the variable of patients belonging to the BNP 3rd quartile ($p < 0.05$).

Remarkably, when considering only the major events all-cause mortality and heart transplantation, Kaplan–Meier curves and Cox regression analysis with patients with high sAXL also showed differences when compared to patients with low sAXL values ($P < 0.001$, HR = 4.40). Those differences were also found when analyzing patients according to their BNP levels ($P < 0.001$, HR = 5.14) and were higher when comparing patients with both high sAXL and BNP levels and the rest ($P < 0.0001$, HR = 8.54).

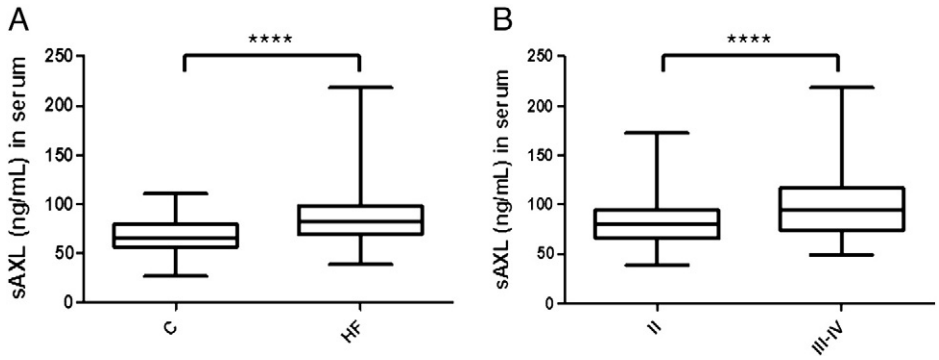


**Fig. 2.** Serum sAXL values from controls and HF patients. A, Boxplot of serum sAXL from controls (C) and heart failure (HF) patients. B, sAXL serum levels from HF patients belonging to NYHA functional class 2 (II) or 3–4 (III–IV). Ln-sAXL between groups was compared with a *T*-test (****$P < 0.0001$).

Kaplan–Meier curves and Cox regression analysis considering all types of events together also presented differences between patients with high and low sAXL ($P < 0.05$, HR = 1.85) but the differences were less than when considering only the major HF events or only all-cause mortality and heart transplantation. The survival curves with all events were also different for patients with BNP values above the 3rd quartile ($P < 0.001$, HR = 3.04) or with both BNP and sAXL values above the 3rd quartile ($P < 0.001$, HR = 4.13).

## 4. Discussion

In this report we demonstrate for the first time that AXL is expressed in the heart and that is increased in myocardial tissue from HF patients compared to healthy hearts and, in accordance with this over-expression, the serum concentration of sAXL is higher in HF patients than in controls. Furthermore, sAXL levels increase in aggravated HF, in patients with functional classes III–IV and with multiple clinical parameters that indicate a worse HF prognosis but no correlation with echocardiographic parameters was found. Also, linear regression analysis showed that BNP levels did not predict sAXL levels, suggesting that both proteins are elevated through independent mechanisms.

High sAXL values at the enrollment time were related to the HF events all-cause mortality, transplantation or HF hospitalizations at one year follow-up and it added predictive value to high BNP levels. All together these results, suggest that sAXL can be a novel player of the HF pathophysiology, acting in a BNP independent pathway.

The detection of AXL protein in myocardial biopsies confirms results in silico that showed a relatively high expression of *AXL* probes in heart and cardiomyocytes (Gene atlas, human probe 202686_s_at) [10]. Moreover, it suggests that at least part of the soluble receptor detected in serum could be originated in heart after specific proteolytic shedding, as has been shown in other cell types [12].

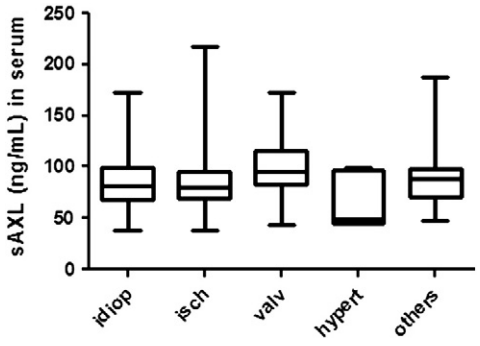


**Fig. 3.** Serum sAXL levels from HF patients with different HF etiologies. Boxplot of serum sAXL from HF patients divided in 5 different groups according to their etiology; idiopathic (idiop), ischemic (isch), valvular (valv), hypertensive (hypert) and others.

Currently, we do not know the role of AXL in HF and whether its expression is deleterious for the heart or if it is a compensatory mechanism. The GAS6/AXL interaction has demonstrated a prominent role in vascular physiology, including biological processes implicated in HF such as inflammation, tissue remodeling, vascular calcification and atherosclerosis [15,16]. Tjwa et al. [17] showed that GAS6 facilitates the interactions between endothelial cells, platelets, and leukocytes under inflammatory conditions. This mechanism is likely mediated by AXL, as AXL knockout mice present lower extravasated cells after vascular injury [18]. AXL also appears to have an important role in the vascular response to different injuries, including mechanical damage and hypertension-associated vascular remodeling [18–21]. A third process where AXL plays a prominent role is vascular calcification. AXL activation prevents the osteogenic differentiation of pericytes and vascular smooth muscle phosphate induced calcification [22,23]. In addition, Son et al. described the GAS6/AXL pathway as a central interface for the protective effects of statins on vascular smooth muscle calcification [24]. Finally, AXL has also been found to have a role in atherosclerosis [8,9]. Our results showed correlation of sAXL levels with the inflammatory parameter C-reactive protein (CRP) [25] in HF patients. On the other hand, we find a higher proportion of dyslipidemia in patients with lower values of sAXL, which would not sustain a role of AXL in the atherosclerosis process in this group of patients with HF.

**Table 3**
Baseline laboratory values of HF patients stratified by sAXL levels.

| | sAXL, ng/mL | | |
|---|---|---|---|
| Parameter | <98.1 | ≥98.1 | *P* |
| No. of patients | 144 | 48 | |
| BNP, pg/mL | 251.9 ± 37.1 | 456.6 ± 69.2 | =0.001 |
| Serum creatinine, mg/dL | 1.12 ± 0.03 | 1.36 ± 0.07 | <0.001 |
| Glomerular filtration rate, mL/min | 57.9 ± 0.7 | 52.2 ± 1.7 | <0.0001 |
| Sodium, mEq/L | 140.0 ± 0.3 | 139.3 ± 0.6 | ns |
| Potassium, mEq/L | 4.6 ± 0.04 | 4.5 ± 0.09 | ns |
| Aspartate aminotransferase, UI/L | 24.4 ± 0.7 | 31.9 ± 5.7 | ns |
| Alanine aminotransferase, U/L | 25.6 ± 1.3 | 33.6 ± 8.3 | ns |
| Bilirubin, mg/dL | 0.80 ± 0.04 | 0.88 ± 0.07 | ns |
| Uric acid mg/dL | 6.70 ± 0.17 | 7.84 ± 0.43 | <0.05 |
| Glucose, mg/dL | 114.9 ± 3.1 | 115.2 ± 4.3 | ns |
| Total Cholesterol, mg/dL | 169.0 ± 3.2 | 170.8 ± 5.8 | ns |
| HDL Cholesterol, mg/dL | 40.9 ± 0.8 | 40.1 ± 1.7 | ns |
| LDL Cholesterol, mg/dL | 103.1 ± 2.6 | 103.8 ± 5.3 | ns |
| Triglycerides, mg/dL | 127.3 ± 5.1 | 133.8 ± 12.0 | ns |
| C Reactive Protein mg/dL | 0.73 ± 0.16 | 1.34 ± 0.64 | <0.01 |
| Thyrotropin, mUI/L | 3.4 ± 0.7 | 2.3 ± 0.3 | ns |
| Thyroxine, ng/dL | 1.29 ± 0.02 | 1.35 ± 0.06 | ns |
| Hemoglobin, g/L | 138.3 ± 1.2 | 131.5 ± 2.8 | <0.05 |
| Hematocrit, L/L | 0.423 ± 0.003 | 0.407 ± 0.008 | <0.05 |
| Erythrocyte count, 10E12/L | 4.64 ± 0.04 | 4.35 ± 0.10 | =0.001 |
| Lymphocytes count, 10E9/L | 1.9 ± 0.05 | 1.6 ± 0.08 | <0.05 |
| Platelet count, 10E9/L | 222.3 ± 5.4 | 205.0 ± 8.2 | ns |

**Table 4**
Pharmacological treatment of HF patients stratified by sAXL levels.

| Pharmacological treatment | sAXL, ng/mL <98.1 | ≥98.1 | P |
|---|---|---|---|
| No. of patients | 144 | 48 | |
| ACEI, n (%) | 91 (66) | 34 (71) | ns |
| ARB, n (%) | 36 (27) | 7 (15) | ns |
| ACEI and/or ARB, n (%) | 127 (91) | 41 (85) | ns |
| Beta-blocker, n (%) | 132 (94) | 44 (92) | ns |
| Ca-antagonists, n (%) | 12 (9) | 3 (6) | ns |
| Antithrombotic and/or anticoagulant, n (%) | 110 (80) | 41 (85) | ns |
| Statins, n (%) | 95 (69) | 21 (44) | <0.01 |
| Antidiabetics, n (%) | 41 (30) | 11 (23) | ns |
| Diuretics, n (%) | 104 (75) | 42 (88) | 0.055 |
| Antialdosteronic agents, n (%) | 74 (54) | 28 (58) | ns |
| Digoxin, n (%) | 12 (9) | 9 (19) | 0.054 |
| Antiarrhythmics, n (%) | 26 (19) | 15 (31) | 0.054 |
| Nitrates, n (%) | 17 (12) | 8 (17) | ns |
| Hydralazine, n (%) | 5 (4) | 3 (6) | ns |
| Anemia treatment, n (%) | 6 (4) | 2 (4) | ns |

ACEI (Angiotensin Converting Enzyme Inhibitor), ARB (Angiotensin Receptor Blocker).

Experimentation with animal models is needed in order to ascertain whether there is a causative relation of AXL and any of these pathways in HF.

The lack of correlation of sAXL circulating levels and echocardiographic parameters, such as LVEDD, LVESD and LVEF, suggests that AXL shedding into blood follows different triggering signals than BNP. Natriuretic peptides are shed from the heart when there is an overload on any chamber of the heart, and hence there is a stretch situation [26,27]. It should be emphasized that even though lnBNP and lnsAXL values correlate; BNP does not appear to be a predictor of sAXL levels in a linear regression model.

Circulating sAXL in patients and controls correlates with several parameters that are altered in HF and that lead to a worse prognosis. In our results, higher sAXL levels are found in HF patients with more functional limitation determined either by the NYHA classification or by the formal 6MWT of exercise tolerance [28,29]. Also, HF patients with higher peripheral sAXL levels presented lower systolic and diastolic blood pressure. Low arterial pressure will compromise critical organ perfusion and is associated with a poorer prognosis [30]. Furthermore, we quantified increased serum sAXL levels together with increased kidney dysfunction determined by either higher serum creatinine levels or by lower glomerular filtration rates. Indeed, the GAS6/AXL pathway has been shown to be implicated in development of several renal pathologies [31,32]. Also, HF patients in the H-sAXL group had higher uric acid levels and sAXL was highly correlated with bilirubin and anemia in HF patients, three parameters that are associated with adverse outcomes in HF. [33–35]

Our ROC analysis of sAXL for discriminating HF patients from controls, shows that sAXL as a putative diagnostic marker has much less sensitivity and specificity than BNP. With our data, a BNP cut-off point of 35 pg/mL gave an 89.8% of sensitiviy and an 84.6% of specificity. In the acute setting, testing for the natriuretic biomarkers to rule out or to confirm the HF diagnosis is well established [28,36,37] and are indicated in Clinical Guidelines [2,29,38]. The natriuretic peptides are also well established HF prognostic markers. The higher rate of HF events we find in this group of HF patients with reduced ejection fraction and high BNP levels is in accordance with other results in the literature that have shown that the natriuretic peptides are powerful predictors of adverse outcomes in patients with HF. [39–44]

Other biomarkers such as cardiac necrosis markers, troponin I or T [45,46], or fibrosis markers such as soluble ST2 [47–49] and galectin-3 [50,51] have been shown to be predictive of hospitalization and death in patients with HF and have an additive prognostic value to natriuretic peptide levels. The carbohydrate antigen 125 (CA125) also has a predictive value of adverse outcomes in HF patients [52] and adds prognostic value to BNP [53]. Our results indicate that sAXL is also predictive of HF events at short-term follow-up and that the combination of an elevated sAXL with BNP was a better predictor of major HF events and of all-cause mortality and heart transplantation than either of the two markers alone.

The fact that AXL is 6-fold over expressed in pathological ventricular heart biopsies, suggests that the increased levels encountered in the peripheral blood samples from HF patients is shed from the heart.

## 5. Conclusions

Altogether, we have presented in this report quantification of AXL in heart biopsies by western blot and of sAXL in serum by a new enzyme-linked immunosorbent assay (ELISA). sAXL levels in HF correlate with BNP levels, the current marker of HF and with other clinical parameters related to aggravated HF. Remarkably, high sAXL levels predicted HF events at short term follow-up, and added predictive value to BNP. Based on these results, we conclude that sAXL appears to have a role in HF progression in a distinct molecular pathway than BNP.

## 6. Study limitations

The control group of the sAXL serum analysis was not matched with the HF group in age and cardiovascular risk factors. This is a serious

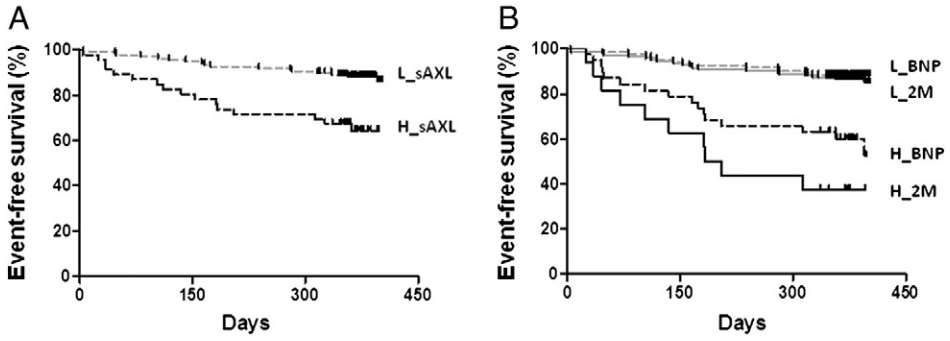


**Fig. 4.** Kaplan–Meier survival curve from HF patients divided according their sAXL and BNP levels and considering the major HF events (all-cause mortality, transplantation and HF hospitalizations). A, Kaplan–Meier event-free survival curve from patients that have sAXL values below (L_sAXL, dotted grey line) or above the 3rd quartile cut-off point (98.1 ng/mL, H_sAXL dotted black line). The differences in HF evolution were compared with the Cox Regression model ($P < 0.001$). B, Kaplan–Meier event-free survival curve from patients with BNP below (L_BNP, dotted grey line) or above the 3rd quartile cut-off point (362.7 pg/mL, H_BNP dotted black line, $P < 0.0001$). Kaplan–Meier event-free survival curve from patients that have the 2 markers, sAXL and BNP, levels above the 3rd quartiles cut-off points (H_2M black solid line) or that have one or both values below the 3rd quartiles cut-off points (L_2M grey solid line, $P < 0.0001$). $P < 0.05$ between H_BNP and H_2M.

limitation of this study, but analysis of sAXL levels in the control group indicated that was independent of the subject's age and sex. On the other hand, the difficulty of finding controls with several cardiovascular risk factors and no cardiovascular disease precluded the obtention of such samples.

This is a two center study from the same metropolitan area, with consecutive patients enrolled. And although it has some advantages, such as including a more homogeneous diagnosis, demographics and laboratory techniques, validation of these results in other centers or in multicentric studies will be needed to confirm the increase of sAXL in HF patients from other populations.

We were not able to obtain the cause of mortality from the records in most of the cases. Therefore, we have analyzed the variable all-cause mortality and could not do analysis with only cardiovascular causes of mortality.

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