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Original research article

Prognostic value of fibrosis-related markers in dilated cardiomyopathy: A link between osteopontin and cardiovascular events

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ABSTRACT

Introduction: Serum markers of fibrosis provide an insight into extracellular matrix (ECM) fibrosis in heart failure (HF) and dilated cardiomyopathy (DCM). However, their role as predictors of cardiovascular (CV) events in DCM is poorly understood.
Methods: This is an observational, prospective cohort study. 70 DCM patients (48 ± 12.1 years, ejection fraction – EF 24.4 ± 7.4) were recruited. Markers of collagen type I and III synthesis – procollagen type I and III carboxy- and amino-terminal peptides (PICP, PIIICP, PINP, PIIINP), fibrosis controlling factors – ostepontin (OPN), transforming growth factor (TGF1-β) and connective tissue growth factor (CTGF), and matrix metalloproteinases (MMP-2, MMP-9) and tissue inhibitor (TIMP-1), were measured in serum. All patients underwent endomyocardial biopsy. The end-point was combined with CV death and urgent HF hospitalization. Patients were divided into two groups: those who did (group 1, n = 45) and did not reach (group 2, n = 25) an end-point.
Results: Over a 12-month period of observation, 6 CV deaths and 19 HF hospitalizations occurred. Qualitative and quantitative measures of ECM fibrosis were similar in both groups. The levels of all of the markers of collagen synthesis, TGF1-β, MMP-9 and TIMP-1 were similar, however, OPN, CTGF and MMP-2 were significantly lower in group 1.
Conclusions: Invasively-determined fibrosis levels were not related with CV outcomes in DCM. Out of the 11 markers of fibrosis under study, only OPN was found to be related to CV outcomes. OPN is not only the pivotal protein controlling fibrosis, but may also serve as a biomarker associated with prognosis.

1. Introduction

Alterations in the extracellular matrix (ECM) result in cardiac fibrosis. Reactive and diffuse myocardial fibrosis is one of the main features of dilated cardiomyopathy (DCM) [1]. ECM fibrosis has numerous adverse effects on cardiac morphology and function, including an increase in collagen type I at the expense of collagen type III, which substantially increases left ventricular (LV) stiffness and alters LV filling, and produces abnormalities in collagen fiber alignment which then reduce myocardial force transmission, resulting in contractility impairment [2]. Another negative effect is the production of excess collagen tissue which may then result in either a conduction blockade or re-entrant arrhythmias [3]. Therefore, without doubt, ECM fibrosis contributes significantly to the transition from compensated and oligo-symptomatic DCM to overt heart failure (HF) [4]. Despite the well-known role of fibrosis in DCM, very few studies have studied the association between fibrosis and mortality. Moreover, in the majority of studies thus far, ECM fibrosis has been assessed non-invasively with cardiac magnetic resonance (CMR) [5,6,7] rather than via endomyocardial biopsy (EMB), the latter being the gold standard of myocardial structural assessment [8]. The most common localization of fibrosis in DCM is interstitial (pericellular). Post-mortem studies, along with paired LV and right ventricular (RV) biopsies, have shown that ECM fibrosis in DCM is diffuse. Thus, RV biopsy, which is the most commonly performed procedure, should provide data similar to that obtained from LV biopsy. The Masson's trichrome stain is particularly useful in the assessment of the main component of connective tissue e.g. collagen, which is the main component of interstitial fibrosis. In addition, myocardial fibrosis can be assessed indirectly by means of a wide array of imaging

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modalities. Although widely available, echocardiography and nuclear imaging have low specificity for ECM fibrosis detection, whereas positron emission tomography (PET) has high specificity but its availability is limited. Therefore, CMR is currently the method of choice for cardiac fibrosis assessment. CMR with delayed imaging following administration of gadolinium contrast allows the visualization of regional myocardial fibrosis via areas of late gadolinium enhancement (LGE) [5,6]. Moreover, assessment of diffuse fibrosis can be achieved with post-contrast enhanced T1 and T2 mapping. However, there are few studies with conflicting results on biopsy-determined fibrosis and outcomes in DCM and HF.

Increased collagen synthesis and degradation are indicated in the pathology of ECM fibrosis in DCM [9]. Measurement of collagen byproducts in blood is relatively straightforward and provides data which have led to insights into the dynamics of ECM fibrosis. These studies have shown that synthesis of collagen type I prevails over collagen type III during the ECM fibrosis process in DCM [10]. It is also known that transforming growth factor (TGF), acting via connective tissue growth factor (CTGF), and ostepontin (OPN) are both major cytokines implicated in the initiation and progression of the fibrosis process [11,12]. In addition, during ECM fibrosis, there has been found to be an overexpression of matrix metalloproteinases (MMPs) that escape the normal controls of tissue inhibitors (TIMPs) [13]. The various links between serum markers of fibrosis and invasively-determined ECM fibrosis have been extensively studied. However, to date, clear associations have only been found for two collagen synthesis by-products: C-terminal propeptide of procollagen type I (PICP) and the N-terminal propeptide of procollagen type III (PIIINP). The important role of various molecules, such as TGF, CTGF, cardiotrophin-1, OPN, MMPs and TIMPs in the fibrotic process have been described in animal models. Still, the significance of serum levels of these molecules and ECM fibrosis in humans is less clearly understood. Bearing in mind the crucial roles of these molecules in the pathology of fibrosis, it is of little wonder that they were thought to be associated with survival in patients with cardiac diseases. However, in the majority of studies so far, unselected HF populations have been investigated, and thus these findings may not necessarily apply to specific diseases, such as DCM.

In the present study, we examined the associations between biopsy-detected ECM fibrosis and a wide array of serum markers of fibrosis, including markers of collagen synthesis, fibrosis controlling factors, and the MMP/TIMP system with regards to cardiovascular (CV) outcomes in a contemporary DCM cohort.

2. Material and methods

2.1. Study population

This is a single-center, observational, prospective cohort study. Over a period of 14 months, 70 consecutive patients with DCM were recruited for this study. The diagnosis of DCM was confirmed according to the European Society of Cardiology (ESC) 2007 guidelines having excluded significant coronary artery disease, primary heart valve disease, congenital heart disease, and arterial hypertension [14]. All patients had significantly dilated LV (>117% of predicted LV end-diastolic diameter) and depressed systolic function with ejection fraction (EF) below 35%. For the purposes of inclusion, the patients had to have stable HF symptoms, in line with the New York Heart Association (NYHA) class I–III, for at least the preceding two weeks. Study participants were optimally treated according the current ESC guidelines and had their HF-medications appropriately up-titrated [15]. Furthermore, the presence of concomitant non-cardiac diseases, such as bone and joint diseases, chronic liver insufficiency, peripheral atherosclerosis, and neoplasms affecting collagen metabolism and the circulating levels of procollagens also served as exclusion criteria [16]. Each patient was observed for 12 months from the time of inclusion, with one interim visit after 3 months, and no patient was lost to follow-up. At the 12-month point of follow-up, CV death had occurred in 6 (8.6%) patients, and urgent HF hospitalization in 19 (27.1%) patients. Based on this data, patients were divided into two groups: those who did not have a combined end-point (group 1, n = 45), and those in whom a combined end-point occurred (group 2, n = 25). All patients were willing to participate, and signed informed consent forms which were approved, along with the study protocol, by the relevant institutional committees and the Ethical Committee.

Table 1
Baseline characteristics of the study population.

Parameter	group 1 (n = 45)	group 2 (n = 25)	p-value
Age [years]	46.6 ± 12.6	50.6 ± 10.9	0.19
Sex [male/female]	40 (89%)/5 (11%)	23 (92%)/2 (8%)	0.68
BMI [kg/m^2]	27.3 ± 5.2	27.1 ± 5.6	0.85
NYHA class	2.47 ± 0.76	2.76 ± 0.66	0.11
Duration [months]	**17.8 ± 28.2**	**36 ± 44.3**	**0.04**
LBBB [n, %]	16 (35.6%)	11 (44%)	0.49
LVESd/BSA [mm/m^2]	**28.2 ± 5.8**	**33.4 ± 8.2**	**0.003**
LVEDd/BSA [mm/m^2]	**33.8 ± 5.4**	**38.8 ± 8.4**	**0.004**
LVESvol/BSA [ml/m^2]	89.8 ± 41.7	107.5 ± 59.2	0.16
LVEDvol/BSA [ml/m^2]	118.1 ± 49.1	142.3 ± 73.8	0.13
EF [%]	25.1 ± 7.2	23.1 ± 7.6	0.29
E/E'(average sep + lat)	20.2 ± 12.5	20.8 ± 9.9	0.83
ECM fibrosis [n,%]	16 (35.6%)	8 (32%)	0.76
CVF [%]	6.7 ± 7	4.4 ± 3.6	0.4
CO [l/min]	**4.3 ± 1.3**	**3.4 ± 0.7**	**0.001**
PA mean [mmHg]	**19.7 ± 8.5**	**29.3 ± 12.1**	**0.001**
PCWP mean [mmHg]	**13 ± 7.5**	**19.7 ± 8**	**0.002**
PH [n, %]	**11 (24.4%)**	**16 (66.7%)**	**0.001**
VO_2peak [ml/kg/min]	**18.3 ± 6.1**	**14.5 ± 5.5**	**0.04**
Hb [g/dl]	14.7 ± 1.5	14 ± 1.7	0.11
creatinine [umol/l]	87.1 ± 20.2	110.4 ± 87.6	0.09
hs-troponin T [ng/ml]	0.02 ± 0.014	0.026 ± 0.023	0.21
hs-CRP [mg/dl]	10.7 ± 26.2	7.4 ± 18.4	0.59
NT-proBNP [pg/ml]	**2426.8 ± 4247**	**5069.7 ± 6852**	**0.05**
Beta-blocker [n, %]	44 (98%)	25 (100%)	0.45
ACE-I [n, %]	43 (95.6%)	23 (92%)	0.54
ARB [n, %]	2 (4.4%)	0	0.28
MRA [n, %]	42 (93.3%)	24 (96%)	0.64
Furosemide [n, %]	**23 (51.1%)**	**19 (76%)**	**0.05**
CRT/ICD [n, %]	14 (31.1%)	13 (52%)	0.08

Data are presented as mean ± SD or n (%).
BMI – body mass index, NYHA – New York Heart Association class, LBBB – left bundle branch block, LVESd/BSA – indexed to body surface area left ventricular end-systolic diameter, LVEDd/BSA – indexed LV end-diastolic diameter, LVESvol/BSA – indexed LV end-systolic volume, LVEDvol/BSA – indexed LV end-diastolic volume, EF – ejection fraction, E/E' (average sep + lat) – ratio of early mitral inflow E-wave and early myocardial E' velocity (E' – is an average of septal and lateral myocardial velocity), CVF – collagen volume fraction, CO – cardiac output; PA mean – mean pulmonary artery pressure, PH – pulmonary hypertension, VO_2peak – peak oxygen uptake, Hb – hemoglobin, CK-MB – myocardial fraction of creatine kinase, hs-troponin T – high sensitivity troponin T, hs-CRP – high sensitivity C-reactive protein, NT-proBNP – amino-terminal pro B-type natriuretic peptide, ACE-I – angiotensin converting enzyme inhibitor, ARB – angiotensin receptor type 1 blocker, MRA – mineralocorticoid receptor antagonist, CRT – cardiac resynchronization therapy, ICD – implantable cardioverter-defibrillator.
Bold values indicate that these particular parameters were statistically significant (p-value < 0.05).

2.2. Endomyocardial biopsy (EMB)

EMB procedures were performed by experienced operators via a femoral or jugular vein [17]. Long (104 cm), flexible, disposable biopsy forceps 7 French size with small jaws (Cordis®, Johnson & Johnson Co, Miami Lakes, FL, USA) were used for the procedure.

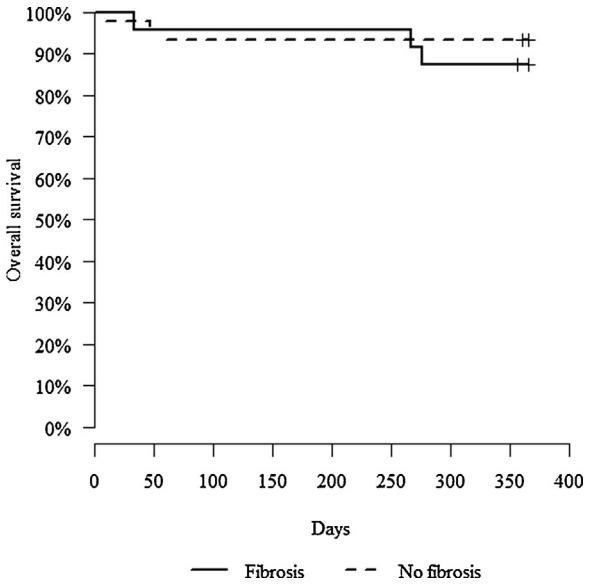

Fig. 1. Kaplan-Meier analysis of cumulative rates of survival in DCM patients with and without ECM fibrosis.

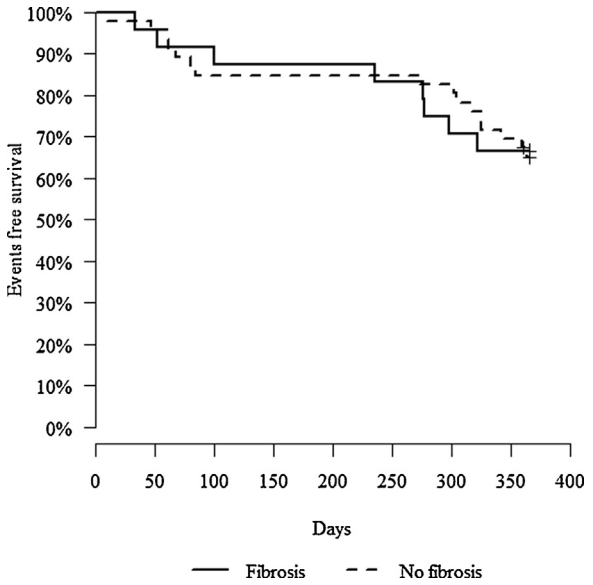

Fig. 2. 12-month event-free survival in groups stratified by fibrosis status. Kaplan-Meier analysis illustrates no difference in 12-month outcomes between the groups with and without ECM fibrosis.

Simultaneous fluoroscopic guidance and bioptom curvature enabled a precise biopsy of the right ventricular interventricular septum. Up to five myocardial samples were obtained, which were immediately stored in formalin for examination by light microscope. The presence of fibrosis was determined qualitatively by an experienced pathologist blinded to the clinical data. Specimens for fibrosis assessment were stained with Masson's trichrome, whereby fibrotic areas stain blue and normal muscle fibers stain red. We defined ECM fibrosis as the disproportionate accumulation of fibrillar collagen between intermuscular spaces previously devoid of collagen, which may also compress surrounding cardiomyocytes. Patients were diagnosed as either fibrosis-positive or negative.

2.3. Laboratory measurements

Venous blood samples were drawn on the day of the study after a 30-min supine rest, in a fasting state, in the morning. Standard biochemical measurements were performed immediately. Those included: creatinine (females: 44.0–80.0 umol/l and males: 62.0–106.0 umol/l), hs-CRP (<0.5 mg/dl), hs-troponin T (<0.014 ng/ml) and NT-proBNP (<125 pg/ml) all from Roche Diagnostics GmbH, Mannheim, Germany, and hemoglobin (Hb; 14.0–18.0 g/dl; Sysmex Corporation, Kobe, Japan). After centrifuge, supernatant was stored at −20 °C until assay. Concentrations of collagen synthesis markers and markers of collagen degradation were determined in plasma using commercially available ELISA tests as previously described [18]. Collagen type 1 (manufacturer's reference values – 46.7–178.9 pg/ml), Procollagen I N-Terminal Propeptide (PINP; 30.2–55.1 pg/ml), Procollagen III N-Terminal Propeptide (PIIINP; 2.69–63.56 ng/ml), Procollagen I C-Terminal Propeptide (PICP; 64–186 pg/ml = 0.064–0.186 ng/ml), Procollagen III C-Terminal Propeptide (PIIICP; 5.2–35.5 ng/ml), Connective Tissue Growth Factor (CTGF; 2.3–42.5 ng/ml) (all from Cloud Clone Corp. Houston, TX, USA); Osteopontin (OPN; 0.3–20 ng/ml; R&D Systems Inc., Minneapolis, US); RayBio MMP2 ELISA (∼400 ng/ml), RayBio MMP9 ELISA (2.0–139.4 ng/ml), and RayBio TIMP-1 ELISA (up to 400 ng/ml) (all from RayBiotech, Norcross, GA, USA) and TGF-β (4639–14757 pg/ml = 4.639–14.757 ng/ml) (Diaclone SAS, Besancon Cedex, France). All measurements were performed by technicians blinded to the sample status. Intra-assay and inter-assay coefficients of variation were <7%.

2.4. Statistical analysis

The data are presented either as mean ± SD, median (min–max), or count and percentages when appropriate. The normality of the distribution of variables was assessed with a Shapiro-Wilk test. Comparisons of clinical parameters in groups of patients with and without combined end-point were conducted with a t-Student test or with Mann-Whitney tests if a lack of normality was found. Nominal variables were compared with a chi^2 test. The two end-points for this investigation are as follows: (1) CV mortality and (2) combined end-point of CV mortality and urgent HF hospitalization. Survival data were analyzed using the Kaplan-Meier method, and compared with the log-rank test. To examine the associations of serum markers of fibrosis with end-points of interest (unadjusted analyses and analyses adjusted for age, duration of disease, CVF, EF and NT-proBNP) Cox proportional hazard were performed. Calculations for the optimal cut-off values of serum OPN and for other markers of fibrosis [in order to determine the cut-off values for adverse outcomes] were carried out using a receiver operating characteristic (ROC) curve. Furthermore, patients were compared according to an OPN optimal cut-off value, derived from ROC analysis, with the use of a log-rank test. All results were considered statistically significant when p was <0.05. All the analyses were conducted in R software, version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Outcome data and baseline characteristics

Table 1 shows the comparison of baseline parameters between the two groups. Patients in whom the combined end-point occurred (group 2) had a significantly longer duration of disease and larger LV diameters; however, LV volumes and EF were similar between the two groups. Furthermore, patients from group 2 had lower cardiac output, higher mean pulmonary and capillary wedge

pressures, and thus, were more often diagnosed with secondary pulmonary hypertension. Patients from group 1, (i.e. patients with event-free survival) had better exercise tolerance, lower NT-proBNP, and were less likely to be on Furosemide. However, patients from both groups did not differ in terms of age, sex, NYHA class, the frequency of LBBB, the presence of ECM fibrosis, hemoglobin levels, or kidney function. Lastly, both groups were on similar and optimal HF therapy.

3.2. ECM fibrosis and cardiovascular outcomes in DCM

Biopsy-detected ECM fibrosis was diagnosed in 24 (34.3%) patients. Figs. 1 and 2 show the Kaplan-Meier curves for CV mortality and combined end-point for patients stratified according to their ECM fibrosis status, respectively. At the 12-month point of observation, there was an equal percentage of deaths in patients with and without ECM fibrosis (HR 0.52; 95% CI 0.1–2.6; p = 0.43). Furthermore, patients with and without fibrosis did not differ in terms of the occurrence of combined end-point (HR 1.01; 95% CI 0.43–2.4; p = 0.98).

3.3. Comparison of serum markers of fibrosis between patients with and without combined end point

Comparison of serum markers of fibrosis values between patients with and without combined end point is presented in Table 2. All markers of collagen synthesis were similar between two groups. Among fibrosis controlling factors, OPN and CTGF levels were significantly lower in patients without an event (group 1), whereas TGF values were similar in both groups. Finally, only MMP-2 levels were significantly lower in group 2, but MMP-9 and TIMP-1 values did not differ between groups.

3.4. Serum markers of fibrosis and CV outcomes in DCM

Cox proportional hazard analysis showed that, out of all of the markers of fibrosis under study, only OPN was a predictor of CV death in an unadjusted (HR 1.31; 95% CI 1.11–1.51; $p < 0.002$) and an adjusted (HR 1.54; 95% CI 1.09–2.17; $p < 0.01$) model (Table 3). Moreover, OPN was also the only predictor of combined end point in unadjusted (HR 1.21; 95% CI 1.08–1.36; $p < 0.001$) and adjusted (HR 1.24; 95% CI 1.01–1.51; $p < 0.05$) models (Table 4).

Table 2
Comparison of the serum markers of fibrosis between patients with (group 2) and without (group 1) a combined end- point.

Parameter	group 1 (n = 45)	group 2 (n = 25)	p-value
PICP [ng/ml]	0.18 (0.06–0.38)	0.14 (0.07–0.46)	0.83
PINP [pg/ml]	136.5 (31–1050)	107.1 (25.2–625)	0.14
PIIICP [pg/ml]	225.1 (70.3–1165)	197.2 (83.5–937)	0.52
PIIINP [ng/ml]	4.24 (2.18–6.08)	4.5 (2.5–5.8)	0.25
Col-1 [pg/ml]	59.7 (23.1–206.5)	56.4 (31.2–233.4)	0.95
OPN [ng/ml]	**2.9 (0.9–7.9)**	**3.7 (0.7–19.4)**	**0.05**
TGF-β1 [ng/ml]	2.39 (1.06–6.97)	2.07 (0.65–6.31)	0.06
CTGF [ng/ml]	**3.37 (0.65–23.87)**	**5.34 (0.48–11.7)**	**0.05**
MMP-2 [ng/ml]	**5.84 (2.7–12.9)**	**6.67 (2.97–14.8)**	**0.05**
MMP-9 [ng/ml]	2.22 (0.28–6.96)	1.75 (0.29–9.25)	0.16
TIMP-1 [ng/ml]	14.2 (1.8–33.9)	20.1 (2.4–36.2)	0.07

Data are presented as median (range).
PICP – procollagen I C-terminal propeptide; PINP – procollagen I N-terminal propeptide; PIIICP – procollagen III C-terminal propeptide; PIIINP – procollagen III N-terminal propeptide; Col-1–collagen type I; OPN – osteopontin; TGF-β – transforming growth factor beta; CTGF – connective tissue growth factor; MMP-2–matrix metalloproteinase type 2; MMP-9–matrix metalloproteinase type 9; TIMI-1–tissue inhibitor type 1 of matrix metalloproteinase.
Bold values indicate that these particular parameters were statistically significant (p-value < 0.05).

Table 3
Cox proportional hazard regression (HR) analysis, using serum markers of fibrosis, for incidence of CV mortality.

	Model 1				Model 2			
	HR	95% CI		p-value	HR	95% CI		p-value
PICP	2.7	<0.001	>1000	0.83	0.34	<0.001	>1000	0.85
PINP	0.99	0.98	1.01	0.64	0.99	0.99	1.07	0.61
PIIICP	0.99	0.99	1.01	0.79	0.99	0.99	1.01	0.73
PIIINP	1.01	0.99	1.01	0.94	1.01	0.99	1.01	0.83
TGF-β1	1.01	0.99	1.001	0.38	1.01	0.99	1.01	0.23
CTGF	0.98	0.78	1.26	0.89	0.97	0.74	1.26	0.81
COL-1	1.01	0.99	1.01	0.41	1.02	0.99	1.01	0.44
OPN	**1.31**	**1.11**	**1.55**	**0.002**	**1.54**	**1.1**	**2.17**	**0.01**
MMP-2	1.03	0.71	1.49	0.86	1.03	0.71	1.48	0.89
MMP-9	0.99	0.99	1.01	0.33	0.99	0.99	1.001	0.33
TIMP-1	1.01	0.99	1.01	0.67	1.01	0.99	1.002	0.65

PICP – procollagen I C-terminal propeptide; PINP – procollagen I N-terminal propeptide; PIIICP – procollagen III C-terminal propeptide; PIIINP – procollagen III N-terminal propeptide; Col-1–collagen type I; OPN – osteopontin; TGF-β – transforming growth factor beta; CTGF – connective tissue growth factor; MMP-2 – matrix metalloproteinase type 2; MMP-9 – matrix metalloproteinase type 9; TIMI-1 – tissue inhibitor type 1 of matrix metalloproteinase.
Bold values indicate that these particular parameters were statistically significant (p-value < 0.05).

3.5. Role of osteopontin in predicting CV outcomes in DCM

ROC analyses were conducted to identify the optimal OPN plasma level for the prediction of CV mortality and combined end point. The best cut-off value for OPN level for the prediction of CV death was 4.98 ng/ml, with a sensitivity of 60% and specificity of 90.3% (area under curve – AUC 0.768) (Fig. 3). Death rates were calculated by a Kaplan-Meier analysis according to the OPN cut-off value determined by the ROC curve (Fig. 4). There was a trend that patients with OPN ≥ 4.98 ng/ml had lower survival (HR 5.36; 95% CI 0.89–32.1; p = 0.06) than those with OPN <4.98 ng/ml but no clear statistical significance was observed. Furthermore, based on ROC analysis, the optimal OPN cut-off value for the prediction of combined end point was 3.49 ng/ml, with a sensitivity of 52.4% and specificity of 62.5% (AUC of 0.623) (Fig. 5). However, patients split according to the OPN cut-off value of 3.49 ng/ml did not differ in terms of the frequency of combined end-point (HR 1.5; 95% CI 0.64–3.5; p = 0.35) (Fig. 6).

Table 4
Cox proportional hazard regression (HR) analyses, using serum markers of fibrosis, for incidence of combined end-point.

	Model 1				Model 2			
	HR	95% CI		p-value	HR	95% CI		p-value
PICP	4.08	0.04	413.7	0.55	0.34	0.01	120.7	0.72
PINP	0.99	0.99	1.02	0.63	0.99	0.95	1.02	0.47
PIIICP	0.99	0.95	1.05	0.71	0.99	0.97	1.02	0.61
PIIINP	1.02	0.99	1.07	0.28	1.05	0.99	1.07	0.32
TGF-β1	0.99	0.98	1.02	0.14	0.99	0.99	1.04	0.81
CTGF	1.02	0.93	1.13	0.62	0.99	0.88	1.13	0.95
COL-1	0.99	0.96	1.02	0.81	1.05	0.97	1.06	0.95
OPN	**1.21**	**1.08**	**1.36**	**0.001**	**1.24**	**1.01**	**1.51**	**0.03**
MMP-2	1.16	0.98	1.37	0.08	1.09	0.97	1.32	0.37
MMP-9	0.99	0.99	1.05	0.29	0.99	0.93	1.01	0.23
TIMP-1	1.03	0.99	1.08	0.14	1.02	0.98	1.07	0.32

PICP – procollagen I C-terminal propeptide; PINP – procollagen I N-terminal propeptide; PIIICP – procollagen III C-terminal propeptide; PIIINP – procollagen III N-terminal propeptide; Col-1–collagen type I; OPN – osteopontin; TGF-β – transforming growth factor beta; CTGF – connective tissue growth factor; MMP-2 – matrix metalloproteinase type 2; MMP-9 – matrix metalloproteinase type 9; TIMI-1 – tissue inhibitor type 1 of matrix metalloproteinase.
Bold values indicate that these particular parameters were statistically significant (p-value < 0.05).

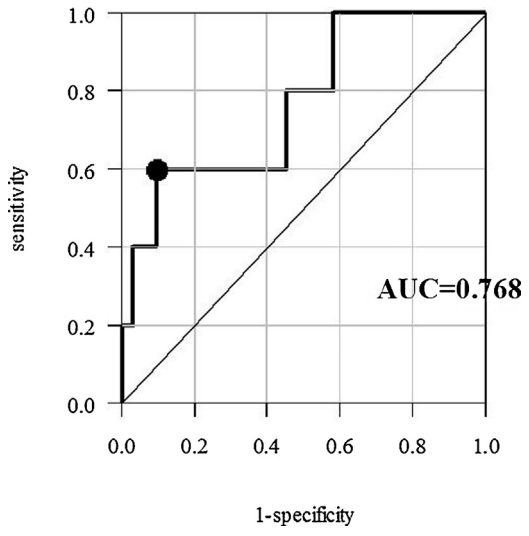

Fig. 3. ROC curve for prediction of CV mortality. The AUC of osteopontin is 0.768.

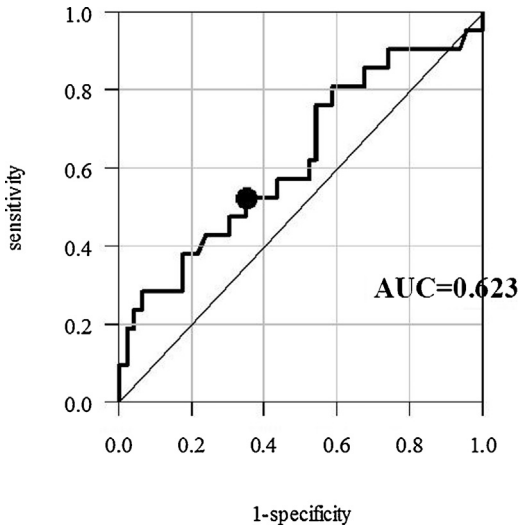

Fig. 5. ROC curves for prediction of event-free survival. The AUC of osteopontin is 0.623.

4. Discussion

The main findings of the study can be summarized as follows: biopsy-detected ECM fibrosis was found to be unrelated to CV mortality, and combined end-point, in a contemporary DCM cohort. Secondly, out of 11 serum markers of fibrosis, only OPN was associated with CV mortality and morbidity. However, when patients were stratified by the OPN cut-off value, derived from ROC analyses, they did not significantly differ in terms of CV mortality and combined end-point.

4.1. Invasively determined ECM fibrosis and cardiovascular outcomes in DCM

Surprisingly, there is a scarcity of data regarding invasively-determined ECM fibrosis and CV mortality. One study, outside the scope of HF research, which looked at valvular diseases, showed that ECM fibrosis (assessed either by way of histologic analysis of samples obtained during open-heart surgery or with contrast-enhanced magnetic resonance) predicted all-cause mortality following aortic valve replacement [19]. Within the HF field, there is currently a single study showing an association between biopsy-detected fibrosis and all-cause death, published by Aoki et al. in 2011 [20]. Of note, fibrosis was associated with mortality only in patients with HF and reduced EF, which constituted 53% of the study population, whereas in patients with preserved EF no such relationship was observed. Furthermore, the study population was not optimally treated according to any current practice since only 68% of patients were on ACE-I, 73% were on beta-blockers, and only 34% were on Spironolactone, which significantly reduces the applicability of the results to contemporary cohorts. On the other hand, Vigliano et al. did not find any relationship between invasively-determined fibrosis and prognosis in DCM [21].

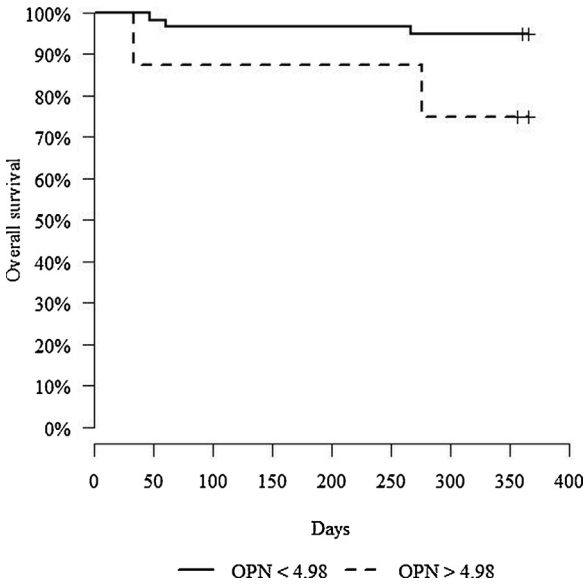

Fig. 4. Kaplan-Meier 12-month survival curve for OPN cut-off value of 4.98 ng/ml. No difference in the CV mortality was found between the groups stratified by OPN value derived from the ROC analysis.

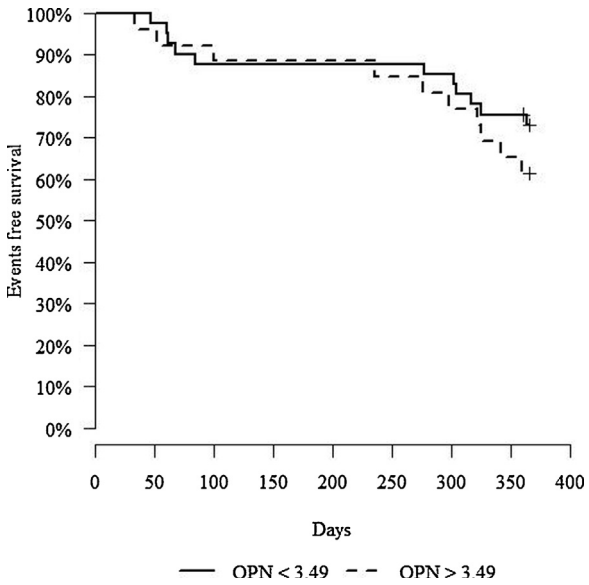

Fig. 6. 12-month event-free survival according to osteopontin (OPN) cut-off value of 3.49 ng/ml. No difference in the 12-month outcome was found between the groups stratified by OPN value derived from the ROC analysis.

Similarly, Nunes et al. reported a lack of association between biopsy-detected fibrosis and prognosis in DCM, and also in Chagasic cardiomyopathy [22]. Even in the landmark study on the role of MRA treatment in HF, Izawa et al. only reported amelioration of fibrosis in a Spironolactone-treated group; however, no data were provided on the relationship between fibrosis and outcomes [23]. In our study, we included a homogenous group of consecutive DCM patients with significantly remodeled and functionally impaired LV, and found biopsy-detected ECM fibrosis in approximately one-third of these patients. Thus, the distribution of fibrosis in our cohort is in accordance with that typically observed in other studies. Based on survival analyses, we did not observe any associations between fibrosis and mortality or HF-related hospitalizations. There are several potential explanations as to why we and others have not found a link between invasively-determined fibrosis and CV outcomes. It may be that the observation period of 12 months is too short to find any outcome differences; however, Kaplan-Meier curves, especially for combined CV outcomes, are quite well superimposed. Other factors that should be taken into account are the limitations of EMB biopsy in the determination of ECM fibrosis, such as sampling error or the sampling of only superficial endo-myocardial layers, when in fact it may happen that fibrosis is located much deeper within the myocardium. Therefore, we cannot rule out that by relying on biopsy instead of magnetic resonance imaging, we may have missed a substantial number of patients with mid-myocardial or epicardial fibrosis. The rapid development of magnetic resonance imaging which allows accurate measurements of fibrosis in the whole myocardium will likely supplant EMB in the future. Finally, the famous quote, formulated by Rose and Beck in 1985, that DCM is "a serious condition with remarkably few morphological features of myocardial damage" is still valid and applicable to practice after more than 30 years [24].

4.2. Serum markers of fibrosis and cardiovascular outcomes in DCM

4.2.1. Markers of collagen synthesis

Markers of collagen synthesis are easily available molecules that may provide a basis for insights into the dynamics of the fibrosis process. Ruiz-Ruiz et al. observed that PICP had additive prognostic value in patients with decompensated HF [25]. On the other hand, PICP and PIIINP were not associated with mortality and urgent hospitalizations in HF patients with preserved HF [26]. In the landmark CARE-HF trial, increased PIIINP and low MMP-1 were found to be associated with adverse CV outcomes but were not predictors of patient response to CRT [27]. In line with this, plasma PIIINP independently predicted event-free survival in chronic systolic HF patients [28]. Uniquely, in our study we simultaneously examined four collagen type I and III byproducts (PICP, PINP, PIIICP and PIIINP) plus mature collagen type I, and found no differences in their levels of blood expression between DCM patients with and without an event. Consequently, none of the studied markers of collagen synthesis was associated with CV outcomes. In contrast to previous studies, we examined a very homogenous and relatively young group of DCM patients, whereas, in previous studies a mixture of DCM and ischemic or hypertensive HF populations as well as much older patients were recruited. On the other hand, it should be acknowledged that it would be over-simplistic to claim that all serum-measured collagen byproducts stem directly from the heart since collagen metabolism takes places in numerous tissues, both in healthy patients and those with disease. Although we attempted to correct for this by not including patients with known diseases that are characterized by increased collagen metabolism turnover (Methods section), we were not able to eliminate physiological metabolism as a source of these markers, which may be altered in a chronic under-perfusion state of HF. Nevertheless, our findings seem to be robust, and clearly showed no associations between collagen metabolism and CV outcomes in DCM.

4.3. Fibrosis controlling factors and MMP/TIMP system

In the DCM field, the majority of data centers on OPN as a marker; nevertheless, the prognostic value of OPN is still not entirely clear, and the relationship of other serum markers of fibrosis to CV outcomes is even less well understood. Almost a decade ago, Rosenberg et al. reported that OPN was an independent predictor of death in a large cohort of patients with HF due to ischemic and dilated cardiomyopathy [29]. Although DCM patients comprised the majority of the study population, survival analyses were performed for the whole cohort, and it is not known whether OPN would still be an independent predictor in the DCM-only subgroup. In a more recent study, Behnes et al. showed that OPN was associated with all-cause mortality in a largely heterogeneous group of subjects presenting to the emergency department, suspected of having acute HF [30]. The potential importance of OPN as a prognostic marker was also observed in patients with acute ST elevation myocardial infarction treated with primary angioplasty [31]. Furthermore, baseline OPN predicted the development of right ventricular failure following LV assist device implantation in patients with advanced HF [32]. Although TGF and CTGF are crucial cytokines involved in the pathogenesis of fibrosis, there is a dearth of data on their role as prognostic markers. Osmancik et al. observed that in responders to CRT, blood concentrations of TGF decreased significantly during follow-up, whereas in non-responders TGF significantly increased [33]. In addition, the concentration of TGF-β1 was found to be a significant predictor of death during follow-up. Similarly, the prognostic value of the levels of crucial enzymes MMPs/TIMPs during ECM remodeling and fibrosis has been as yet poorly explored. Jungbauer et al. found that TIMP-1 along with NT-proBNP and troponin independently added some prognostic information in unselected chronic HF [34]. We observed that OPN clearly outperformed the other markers of ECM metabolism under study as a predictor of CV outcomes, particularly CV mortality. It appears that, despite their integral role in ECM fibrosis, other previously studied markers are not of use for the purposes of predicting CV outcomes.

4.4. Study limitations

We would like to acknowledge potential limitations to the present study. Firstly, a period of 12 months is relatively short; however, over that time period, 6 (8.6%) CV deaths occurred which indicates that DCM is a serious cardiac disease with high mortality and morbidity. As has been already underlined, EMB has several shortcomings, including sample error, the patchy distribution of fibrosis, or the fact that samples were harvested from the right ventricular (RV) septum. On the other hand, there are studies, including autopsies, showing that RV biopsies are equivalent to LV biopsies.

5. Conclusions

Invasively-determined ECM fibrosis levels were not related with CV outcomes in DCM. Out of the 11 markers of fibrosis under study, only OPN was found to be related to CV outcomes, whereas levels of markers of collagen synthesis, TGF, CTGF and MMPs/TIMPs did not have any predictive power. Osteopontin is not only the pivotal protein controlling ECM fibrosis, but is also an important biomarker associated with prognosis.

Conflict of interest

None.

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