Cardiovascular Pathology 67 (2023) 107573

Contents lists available at ScienceDirect

Cardiovascular Pathology

journal homepage: www.elsevier.com/locate/carpath

Primary myocardial fibrosis — a distinct entity characterized by heterogeneous histology

Lasse Pakanen[a,b,*], Henrik Appel[c], Anne Ahtikoski[d], Pernille Heimdal Holm[e], Mervi Kreus[c,f], Kristine Boisen Olsen[e], Jytte Banner[e], Bo Gregers Winkel[g], Heikki Huikuri[c], Riitta Kaarteenaho[c,f], Juhani Junttila[c,h]

[a] Forensic Medicine Unit, Finnish Institute for Health and Welfare, Oulu, Finland
[b] Department of Forensic Medicine, Research Unit of Biomedicine and Internal Medicine, Medical Research Center (MRC) Oulu, University of Oulu, Oulu, Finland
[c] Research Unit of Biomedicine and Internal Medicine, Medical Research Center (MRC) Oulu, University of Oulu and Oulu University Hospital, Oulu, Finland
[d] Department of Pathology, The Welfare District of Southwest Finland, Turku University Hospital and University of Turku, Turku, Finland
[e] Section of Forensic Pathology, Department of Forensic Medicine, University of Copenhagen, Copenhagen, Denmark
[f] Center of Internal Medicine and Respiratory Medicine, Medical Research Center (MRC) Oulu, University of Oulu and Oulu University Hospital, Oulu, Finland
[g] Department of Cardiology, Rigshospitalet, Copenhagen, Denmark
[h] Biocenter Oulu, University of Oulu, Oulu, Finland

ARTICLE INFO

Article history:
Received 7 July 2023
Revised 28 August 2023
Accepted 28 August 2023

Keywords:
Ischemic heart disease
Medicolegal autopsy
Myocardial fibrosis
Primary myocardial fibrosis
Sudden cardiac death

ABSTRACT

Primary myocardial fibrosis (PMF), defined as myocardial fibrosis in the absence of identifiable causes, may represent a common alternative phenotype in various cardiomyopathies and contribute to sudden cardiac death (SCD). No previous definitions of histopathological characteristics exist for PMF. We aimed to evaluate whether common features of fibrosis could be identified. PMF cases (n = 28) were selected from the FinGesture cohort consisting of 5,869 SCD victims that underwent a medicolegal autopsy. Twelve trauma controls and 10 ischemic heart disease cases were selected as reference groups. Further 3 PMF cases and 5 ischemic heart disease cases from autopsies performed in the University of Copenhagen, Denmark, were selected for a validation substudy. Relative area of fibrosis, amount of diffuse and perivascular fibrosis, and location of fibrosis were assessed from left ventricle myocardial samples stained with Masson trichrome. Further evaluations were performed with alpha-smooth muscle actin (α-SMA), vimentin, and CD68 stainings. Mean relative area of fibrosis was 5.8 ± 10.7%, 1.0 ± 0.7%, and 7.0 ± 7.4% in PMF, trauma controls, and ischemic cases, respectively. Fibrosis in the PMF group was mostly located in other sites than the endocardium. Most cases with fibrosis had vimentin-positive but α-SMA-negative stromal cells within fibrotic areas. Histopathologically, PMF represents a heterogeneous entity with variable fibrotic lesions affecting the whole myocardium and a suggested significant role of fibroblasts. These findings may bring validation to PMF being a common manifestation of cardiomyopathies. Evidently, PMF stands out as a particular entity demanding special attention as a cause of SCD.

1. Introduction

Myocardial fibrosis is a common manifestation of a wide spectrum of cardiac diseases. Fibrosis is usually divided into macroscopic or microscopic scarring, as well as interstitial and perivascular fibrosis [1]. Scarring, or replacement fibrosis, is commonly associated with coronary artery disease with related ischemic injuries. The latter types are considered reactive and are seen not only in ischemic disease but also in multitudinous nonischemic conditions [2]. Fibrosis disrupts the normal cellular electrical activity and rigidifies the myocardium, which together may contribute to heart failure and provoke sudden cardiac death (SCD) [3].

Although the reparative fibrosis is a critical event in myocardial infarction protecting the ventricle wall from rupture, fibrotic remodeling of the myocardium in nonischemic heart diseases can

Abbreviations: α-SMA, alpha-smooth muscle actin; ARVC, arrhythmogenic right ventricular cardiomyopathy; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; PMF, primary myocardial fibrosis; PMI, postmortem interval; SCD, sudden cardiac death; VUS, variant of uncertain significance.

* Corresponding author. Lasse Pakanen, Finnish Institute for Health and Welfare, P.O. Box 310, Oulu FI-90101, Finland. Tel. +358 29 524 7906.
E-mail address: lasse.pakanen@thl.fi (L. Pakanen).

https://doi.org/10.1016/j.carpath.2023.107573

also reflect a reparative process or be the main cellular process causing dysfunction [4]. The excess extracellular matrix in fibrotic myocardium is mainly produced from activated myofibroblasts derived from resident myocardial fibroblasts [5]. Transdifferentiating a fibroblast to myofibroblast is known to take place during cardiac stress that can be caused by mechanical overload or fibroblast stimulation during ischemia or inflammation [4,6]. Myofibroblasts can be immunohistochemically identified and separated from fibroblasts by their expression of alpha-smooth muscle actin (α-SMA) [6]. Even though α-SMA is not a specific marker for myofibroblasts of heart or other organs, as demonstrated in single-cell sequencing [7], it is the most commonly used marker for myofibroblast differentiation in histopathological studies. Expression of α-SMA is additionally seen in vascular smooth muscle and the pericytes of arterioles and venules [7,8].

Primary myocardial fibrosis (PMF) has been defined as myocardial fibrosis in the absence of identifiable causes [9]. Hookana et al. [10] reported the prevalence of PMF among autopsied nonischemic SCD cases to be 13.6%, with PMF being the most frequent cause of SCD in victims under 40 years of age. Other studies have reported nonspecific fibrosis as an autopsy finding in 12–32% of deaths considered as SCD [11–15]. Based on postmortem genetic analysis, variants associated with arrhythmogenic right ventricular cardiomyopathy (ARVC), dilated cardiomyopathy (DCM), and hypertrophic cardiomyopathy (HCM) were previously found among PMF cases as part of the FinGesture (Finnish Genetic Study for Arrhythmic Events) cohort, and it is speculated that PMF may represent a common alternative phenotype in these diseases [9].

Autopsy findings in genetic disorders affecting the heart may vary from structurally normal heart to extensive fibrosis and hypertrophy [16]. ARVC, DCM, and HCM each have their own histopathological features of fibrosis, including location and type [17]. Yet, acquired conditions, such as ischemic heart disease, hypertensive heart disease, and aortic stenosis are more common causes of myocardial fibrosis and can lead to extensive fibrotic lesions [18].

A systematic histopathological evaluation of myocardial fibrosis has not been carried out in PMF cases before. A rigorous histological examination could help recognize common patterns in PMF and bring light upon the nature of this unclear entity. Hence, the aim of this study was to assess whether common features exist as to the location or other characteristics of fibrosis among these cases and to compare the findings with non-fibrotic hearts as well as with hearts with known ischemic disease.

2. Materials and methods

The study is a retrospective autopsy-based collaboration study between University of Oulu, Finland and University of Copenhagen, Denmark.

FinGesture study protocol was approved by the Ethics Committee of Northern Ostrobothnia Hospital District and complies with the Declaration of Helsinki. Permissions to use medicolegal autopsy material and gather data from the documents of medicolegal cause-of-death investigation were obtained from the Finnish Institute for Health and Welfare (Document record number: THL/697/5.05.00/2017), and from the Regional State Administrative Agency of Northern Finland (PSAVI/2904/05.07.00/2018). Furthermore, approval to process data from University of Copenhagen was obtained (514-0774/22-3000).

2.1. Study and control groups

A study group (PMF, n = 28) consisted of cases selected from the FinGesture cohort, which includes 5,869 SCD victims that have undergone a medicolegal autopsy in the Finnish Institute for Health and Welfare, Oulu, Finland, or at the Department of Forensic Medicine, University of Oulu, Oulu, Finland during 1998–2017. The FinGesture study has been described in detail earlier [10,19]. For this study, we selected all cases in which the main cause of death was an undetermined cardiomyopathy with varying degrees of interstitial, diffuse or patchy myocardial fibrosis without hypertrophy, other structural abnormalities, or determinable etiology, and with age at death no more than 45 years, as well as the postmortem interval (PMI; time between death and autopsy) no more than 5 days. The causes of death were determined by experienced forensic pathologists based on complete medicolegal autopsy including macroscopic and histological examination, as well as necessary ancillary investigations such as toxicology.

As reference groups, we randomly selected a group of cases with a traumatic cause of death and with no known cardiac disease (trauma controls, n = 12), as well as a group with ischemic heart disease as the main cause of death (n = 10). All cases had a PMI no more than 5 days. The trauma controls were age-matched with the PMF cases. However, no such matching was done with ischemic cases, because ischemic heart disease is extremely rare as a cause of death in the young. All cases had undergone a medicolegal autopsy during the study period.

2.2. Subset analysis for further validation and classification

To further assure the quality of measurements of the fibrotic involvement in the myocardium and to assess whether cases can be classified based on the amount of fibrosis, we performed a subset analysis. For this, we selected 3 PMF cases and 5 cases with ischemic heart disease from autopsies performed at the Department of Forensic Medicine, University of Copenhagen, Copenhagen, Denmark during 2007–2009. The Danish cases were pooled with a random set of 15 Finnish cases (5 cases from each group).

2.3. Stainings

Myocardial tissue samples were collected as part of the routine forensic autopsy protocol. Each autopsy case had 1–4 samples (median 2 samples per case in each group) taken from different locations of the left ventricle per autopsy case. Myocardial tissue samples were immediately formalin-fixed and embedded in paraffin. Hematoxylin-eosin-stained slides were re-evaluated by an experienced cardiac pathologist (A.A.). Totally 102 samples were selected for preliminary histological analysis. The most representative samples revealing total cross-section of the left ventricle were selected for further analyses. Histochemical staining of Masson trichrome was used for the evaluation of fibrosis in all representative samples.

Immunohistochemical staining was performed in serial, consecutive 3.5 μm thick tissue sections. CD34 staining (1:1000 monoclonal mouse anti-CD34, Novocastra, Leica Biosystems, Cat# NCL-L-END) was used for identifying endothelial cells lining the endocardium. To identify the phenotype of stromal cells within fibrosis, myofibroblasts and fibroblasts were detected using α-SMA clone 1A4 (1:1000, Dako, Cat# M0851) and anti-vimentin clone V9 (1:2000, Dako, Cat# M0725) stainings. Anti-CD68, clone PG-M1 (1:100 Dako, Cat# M0876) staining was further used for identifying macrophages in lesions with α-SMA positivity. Envision kit (Dako K5007) was used for the detection of antibodies. All stainings were carried out using manufacturers' protocol. The immunohistochemical stainings were successfully conducted in 25 PMF, 9 ischemic, and 7 trauma control cases. Phosphate-buffered saline and mouse isotype controls (Invitrogen, Carlsbad, CA, USA) were used as negative controls.

Whole slide images were acquired with a Leica-Aperio AT2 (Leica Biosystems, Nussloch, Germany) at 40x magnification in Biobank Borealis of Northern Finland, Oulu University Hospital.

2.4. Analyses of fibrosis in tissue samples

Total area without epicardial adipose tissue, as well as the number and area of fibrotic lesions were measured from digitized Masson trichrome stainings with an open-source software ImageJ [20] using freehand area selection tool. A relative area of fibrosis was determined by calculating the percentual proportion of total fibrotic area to the total area of tissue section. Measurements were conducted blinded by 2 independent researchers (H.A. and L.P.). The results of H.A. were used for statistical analyses, and the results of L.P. to measure inter-rater reliability. In the subset analysis, digitized slides stained with Masson trichrome were cross-evaluated blinded by L.P. and P.H.H.

Digitized slides stained with Masson trichrome were also examined with virtual microscopy software Aperio ImageScope (Leica Biosystems, Buffalo Grove, IL, USA). The amount of diffuse fibrosis and perivascular fibrosis were assessed semiquantitatively on scale: 0–3 (0 = none, 1 = slight, 2 = moderate, and 3 = abundant). A mean was calculated of these 2 parameters, and the resulting score was classified as follows: 0–1.5: none or slight; 2–3: moderate or abundant. Location of fibrosis was assessed in 4 categories: epicardium, midwall, endocardium, and transmural.

2.5. Analysis of fibroblasts and myofibroblasts

The number of vimentin-positive stromal cells, for example, fibroblasts, was assessed semiquantitatively with ImageScope in 3 categories, namely as decreased, no difference, and increased, in relation to non-fibrotic areas. Alpha-SMA-positive stromal cells, for example, apparent myofibroblasts, were assessed as present or absent. Care was taken not to interpret the vimentin-positive stromal cells associated to neovascularization or inflammatory cells as fibroblasts and vascular smooth muscle cells or pericytes as myofibroblasts. The evaluation was conducted blinded by a cardiac pathologist (A.A.), and the results were re-evaluated by an independent observer (H.A.).

2.6. Statistical analyses

Statistical analyses were done with IBM SPSS Statistics for Windows, version 27.0 (IBM Corp., Armonk, NY, USA). Inter-rater reliabilities were analyzed with Pearson's correlation coefficient and with Cohen's kappa coefficient regarding continuous and categorical variables, respectively. Statistical differences were estimated with Student's t-test, or analysis of variance for continuous variables, and Chi-squared test or Fisher's exact for categorical variables. Effect sizes were measured with Cohen's d and Cramer's V for continuous and categorical variables, respectively.

3. Results

3.1. Demographics of the study subjects

Demographics of the study subjects are presented in Table 1. The ischemic group had a higher mean age compared with the other groups, but no significant differences arose regarding sex distribution, PMI, or BMI. Heart weight was significantly greater in ischemic cases than in PMF cases ($p = .001$), or trauma controls ($p < .001$). Demographic data of cases selected for the subset analysis are presented in Table 2.

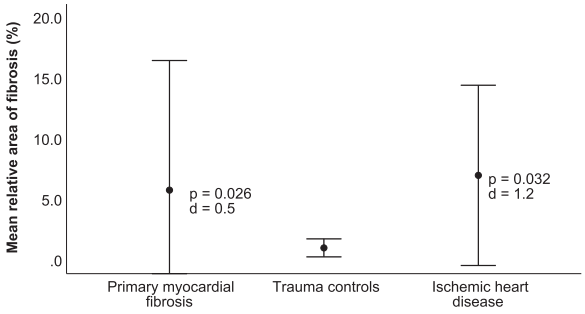

Fig. 1. Mean relative area of fibrosis in primary myocardial fibrosis, trauma controls, and ischemic heart disease. Error bars represent standard deviation. Statistical significances are reported compared with trauma controls.

3.2. Extent of myocardial fibrosis

The mean relative area of fibrosis was higher in PMF group than in trauma controls (5.8 ± 10.7% vs. 1.0 ± 0.7%; $p = .026$, d = 0.5; Fig. 1). Three PMF cases had the highest relative area of fibrosis (29.2%, 38.26%, and 42.5%) even though the mean relative area of fibrosis was highest in the ischemic group (7.0 ± 7.4%). A significant difference arose between the ischemic group and trauma controls ($p = .032$, d = 1.2), but not between the ischemic and PMF groups ($p = .742$, d = 0.1).

Diffuse and perivascular fibrosis (Fig. 2) was more often moderate or abundant in PMF and ischemic cases than in trauma controls, though the differences did not reach statistical significance (Table 3).

3.3. Location of fibrosis

Fibrosis was mostly located in other sites than the endocardium (Fig. 3) in PMF cases when only the cases with relative area of fibrosis ≥2% and moderate or abundant diffuse and perivascular fibrosis were included (Table 3). However, the differences did not reach statistical significance due to the small group sizes (PMF vs. trauma controls: $p = .375$, V = 0.5; PMF vs. ischemic group: $p = .242$, V = 0.4).

3.4. Fibroblasts and myofibroblasts in fibrotic areas

An increase in the number of vimentin-positive but α-SMA-negative stromal cells, apparent fibroblasts in or around fibrotic area was observed in 6 of the 8 ischemic cases (75%) (Fig. 4) and 14 of the 25 PMF cases (56%) (Fig. 5). One trauma control case (12.5%) had slight increase in the perivascular fibroblast population although fibrosis per se was not observed (Fig. 6). Alpha-SMA-positive stromal cells, interpreted as myofibroblasts, were found in 3 ischemic cases (37.5%) (Fig. 4) and one PMF case (4%) (Fig. 5) but none of the trauma controls (Fig. 6). All lesions with myofibroblasts were associated with various infiltrations of CD68-positive macrophages. One ischemic case had multiple lesions with varying ages of ischemia and fibrosis in the same histological sample. Representative images of these lesions are presented in the Supplemental Figure. All the lesions seemed to have increased numbers of vimentin-positive fibroblasts, but only the scar-type loose fibrosis devoid of necrosis was associated with focal α-SMA-positive myofibroblasts. Fibrotic areas totally devoid of fibroblasts and myofibroblasts were seen in 2 ischemic and 1 PMF case, all associated with sclerotic endocardial fibrosis.

Table 1
Demographics of the subjects with primary myocardial fibrosis, trauma controls, and ischemic heart disease

	Group			
	Primary myocardial fibrosis	Trauma controls	Ischemic heart disease	All
n (%)	28 (56.0)	12 (24.0)	10 (20.0)	50 (100.0)
Sex, n (%)				
Men	19 (67.9)	9 (75.0)	6 (60.0)	34 (68.0)
Women	9 (32.1)	3 (25.0)	4 (40.0)	16 (32.0)
Age, years, mean ± SD	36 ± 8	29 ± 9	70 ± 11	41 ± 17
BMI, kg/m^2, mean ± SD	23.7 ± 4.8	24.5 ± 4.9	26.0 ± 5.0	24.3 ± 4.8
Heart weight, g, mean ± SD	352 ± 71	311 ± 49	466 ± 130	365 ± 96
PMI, days, mean ± SD	3 ± 1	3 ± 1	4 ± 1	3 ± 1

BMI, body mass index; PMI, postmortem interval; SD, standard deviation.

Table 2
Demographics of the subjects in subset analysis with Finnish and Danish cases

	Primary myocardial fibrosis		Trauma controls		Ischemic heart disease	
	Finnish	Danish	Finnish	Danish	Finnish	Danish
n	5	3	5	0	5	5
Sex, n (%)						
Men	3 (60.0)	3 (100.0)	3 (60.0)		4 (80.0)	5 (100.0)
Women	2 (40.0)	0 (0.0)	2 (40.0)		1 (20.0)	0 (0.0)
Age, years, mean ± SD	36 ± 8	37 ± 7	29 ± 9		70 ± 11	42 ± 4
BMI, kg/m2, mean ± SD	22.7 ± 4.3	24.6 ± 2.7	22.9 ± 3.8		26.1 ± 4.1	25.0 ± 4.2
Heart weight, g, mean ± SD	373 ± 82	350 ± 66	318 ± 60		482 ± 140	470 ± 96
PMI, days, mean ± SD	3 ± 2	2 ± 1	3 ± 1		4 ± 1	3 ± 1

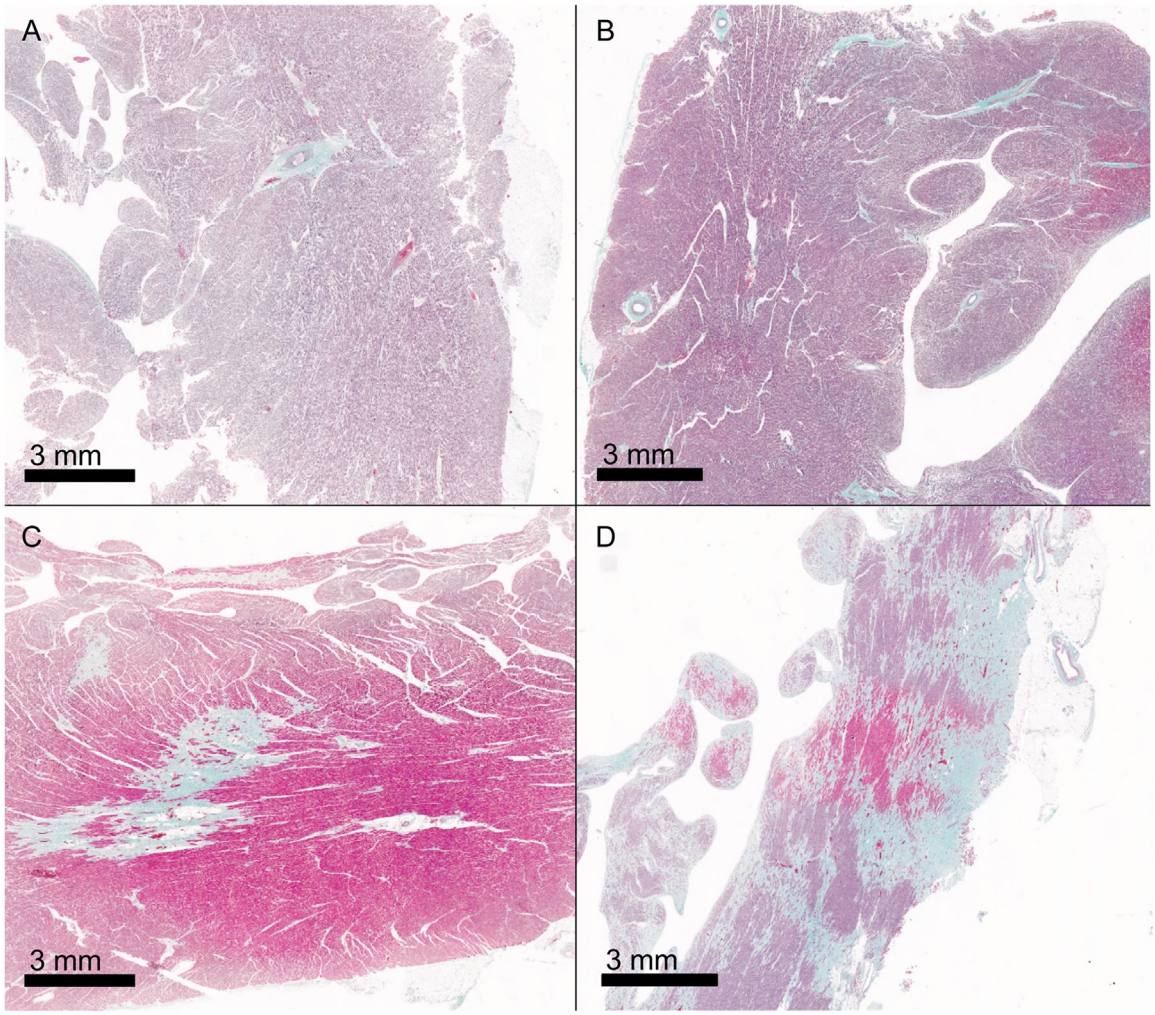

Fig 2. Examples of diffuse myocardial fibrosis assessed semiquantitatively on scale: 0–3, where 0 = none (A), 1 = slight (B), 2 = moderate (C), and 3 = abundant (D). The tissue samples were taken from the left ventricle and stained with Masson trichrome.

Table 3
Results of the histological findings of primary myocardial fibrosis, trauma controls, and ischemic heart disease

Variable	Group		
	Primary myocardial fibrosis	Trauma controls	Ischemic heart disease
Mean sample area without epicardial adipose tissue, mm^2 ±SD	280.2 ± 95.5	257.6 ± 71.3	282.8 ± 43.2
Number of fibrotic areas, mean ± SD	4 ± 3	5 ± 3	7 ± 4
Mean size of fibrotic areas, mm^2 ± SD	3.9 ± 7.9	0.5 ± 0.3	4.2 ± 5.6
Diffuse and perivascular fibrosis	n (%)	n (%)	n (%)
None or slight	13 (46.4)	8 (66.7)	4 (40.0)
Moderate or abundant	15 (53.6)	4 (33.3)	6 (60.0)
Location of fibrosis[1]	n (%)	n (%)	n (%)
Endocardium exclusively	2 (28.6)	1 (100.0)	3 (75.0)
Other sites	5 (71.4)	0 (0.0)	1 (25.0)

SD, standard deviation.
[1] Cases with moderate or abundant diffuse and perivascular fibrosis, and relative area of fibrosis ≥2%.

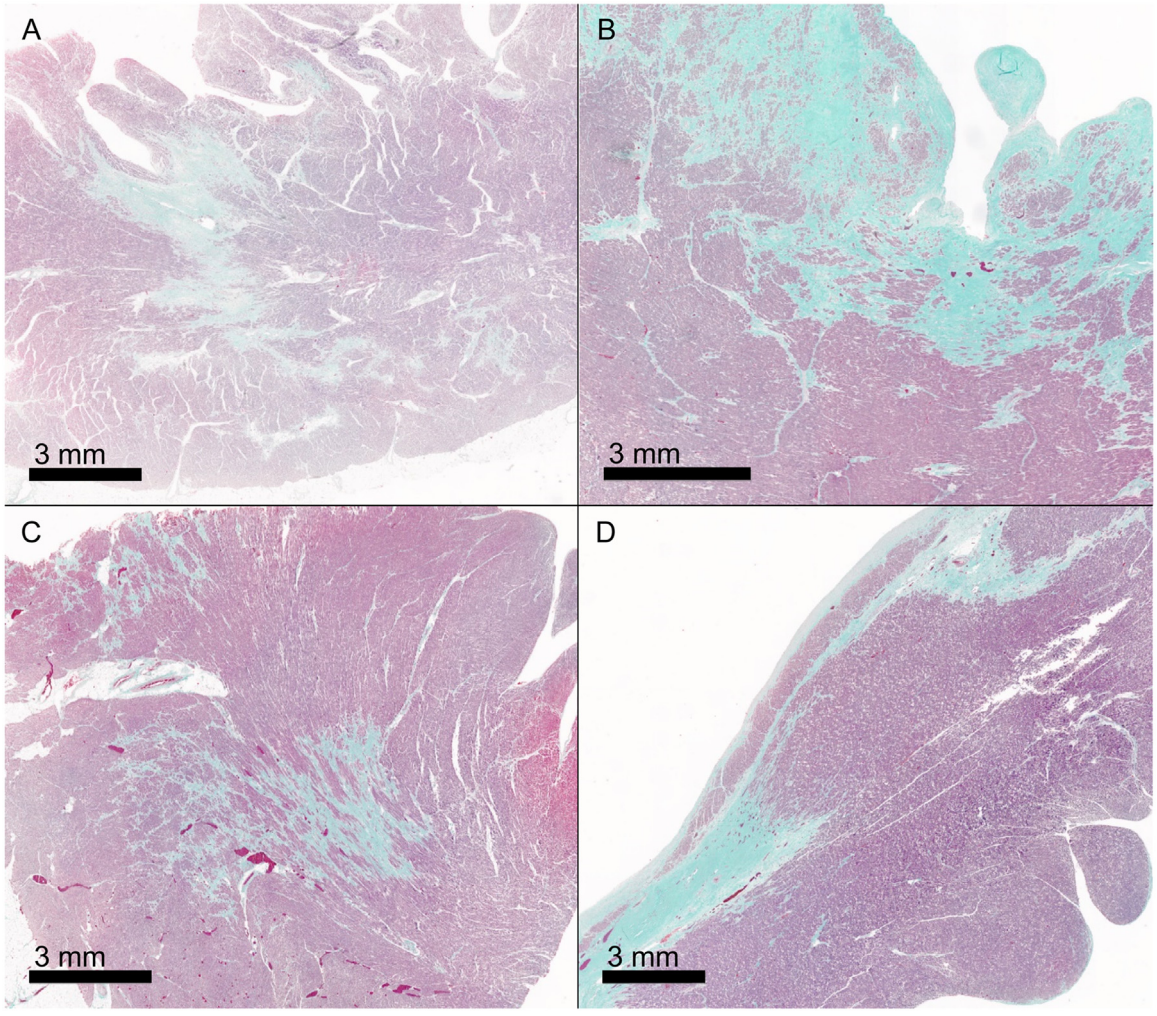

Fig. 3. Examples of the location of myocardial fibrosis assessed in 4 categories: transmural (A), endocardium (B), midwall (C) and epicardium (D). The tissue samples were taken from the left ventricle and stained with Masson trichrome.

3.5. Genetic variants of PMF cases

Four PMF cases had a previously identified mutation: 2 cases with variants associated with HCM (genes *MYH7, TPM1*), one case with 2 variants associated with ARVC (*DSP*) and DCM (*LMNA*), and one case with a variant of uncertain significance (VUS) (*DTNA*). Histological characteristics of these cases are presented in Table 4.

3.6. Underlying factors affecting fibrosis

Mean BMI was marginally higher in cases with no or slight diffuse and perivascular fibrosis (25.5 ± 4.1 kg/m^2 vs. 23.2 ± 5.3 kg/m2), though the difference did not reach statistical significance ($p = .098$, d = 0.5). The amount of diffuse and perivascular fibrosis did not differ according to heart weight, and no correlations arose between relative area of fibrosis and BMI, or heart weight.

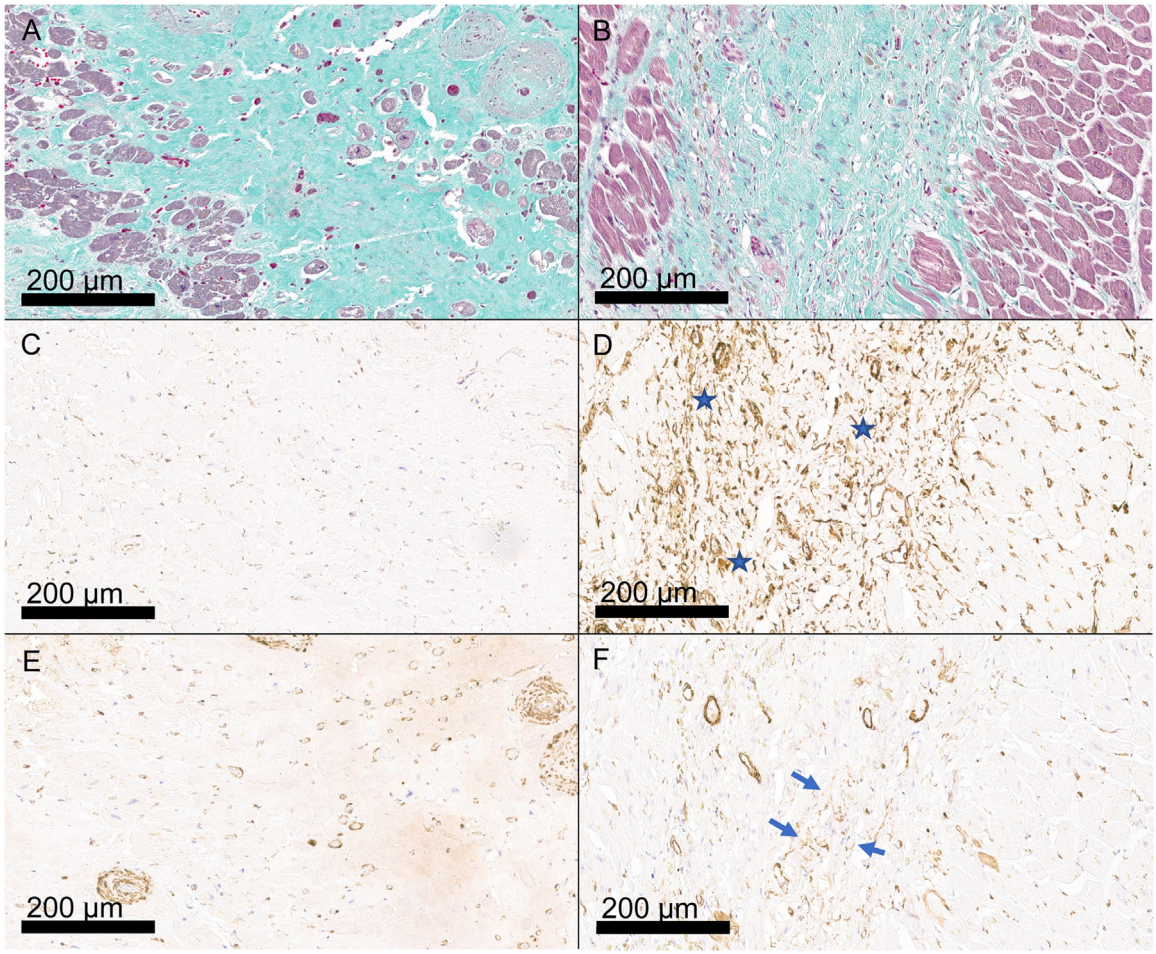

Fig. 4. Two cases from the ischemic group. A, C, E: sclerosing fibrosis without fibroblast proliferation or myofibroblasts. B, D, F: fibrosis with fibroblast proliferation (stars) and myofibroblasts (arrows). A–B Masson trichrome, C–D vimentin, E–F alpha-smooth muscle actin.

Table 4
Histological characteristics of primary myocardial fibrosis cases with identified mutations.

Case	1	2	3	4
Affected gene(s)	*MYH7*	*TPM1*	*DSP, LMNA*	*DTNA*
Total relative area of fibrosis, %	0.46	2.57	29.22	0.21
Diffuse and perivascular fibrosis	moderate/abundant	moderate/abundant	moderate/abundant	none/slight
Average size of fibrotic area, mm^2	0.40	1.10	38.26	0.20
Location of fibrosis	-	transmural	endocardium	-

3.7. Validation and classification

In the subset analysis, the cases were classified according to the relative area of fibrosis, and cutoff values were determined based on the fibrosis levels in the larger set: low <1 %, moderate 1–5%, and abundant >5%. Half the PMF cases were classified as moderate and the other half as low, whereas most of the ischemic cases were moderate or abundant (Fig. 7).

3.8. Evaluation of interobserver variability

No interobserver differences (H.A., L.P.) emerged since intraclass correlation coefficients were 0.992 and 0.996 when measuring the total area of sample and the area of fibrosis, respectively. Kappa values were 0.561, 0.550, and 0.538 for measurements of diffuse fibrosis, perivascular fibrosis, and location of fibrosis, respectively. In the subset analysis, intraclass correlation coefficients in measurements between L.P. and P.H.H. were 0.941 (total sample area) and 0.972 (fibrosis area).

4. Discussion

To our knowledge, this is the first study to describe histopathological characteristics of fibrosis in PMF. We suggest that certain patterns of fibrosis distribution could be recognized in relation to trauma controls and cases with ischemic heart disease.

The relative area of fibrosis was significantly higher in PMF cases than in trauma controls with the effect size reaching medium level. Clinically speaking, the fibrotic area in PMF may not seem very prominent with a mean of 5.8% of the sample area. However, the total amount of fibrosis does not necessarily correspond to the clinical outcome, as different fibrosis patterns have differing effects on conduction abnormalities [21]. Thus, even relatively small fibrotic lesions may act as substrate for lethal arrhythmias, which is probably the mechanism of death in SCDs caused by PMF. Few data have been previously published on the amount of fibrotic tissue in normal myocardium. Miles et al. [22] measured the relative content of collagen with an automated approach and found percentual contents of 9.5%, 8.6%, and 15.2% for the left ventricle, septum, and

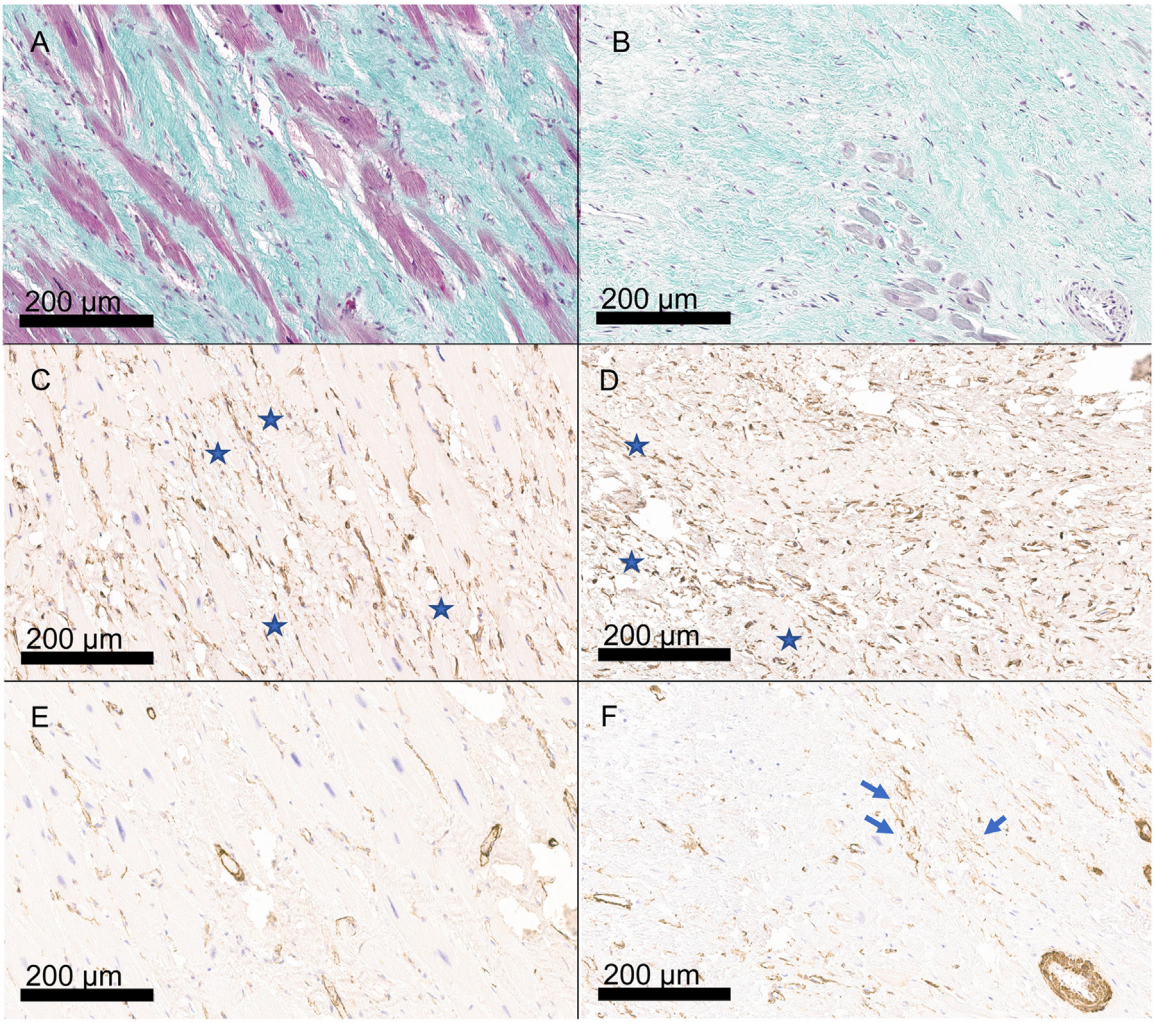

Fig. 5. Two cases from the primary myocardial fibrosis group. A, C, E: fibrosis with mild increase in fibroblasts (stars) but no myofibroblasts. B, D, F: fibrosis with increased number of fibroblasts (stars) and focal myofibroblasts (arrows). A–B Masson trichrome, C–D vimentin, E–F α smooth muscle actin.

right ventricle, respectively. These results cannot, however, be directly compared with the present findings of relative fibrosis. In the subset analysis here, a cutoff value of 1% was used, which correctly identified all trauma control subjects, though this approach did not count for potential diffuse fibrotic lesions.

The PMF cases exhibited quite large variation in the measured areas of fibrosis, which was also evident in the subset analysis. Also, semiquantitative estimation of diffuse and perivascular fibrosis showed variability when only a little more than half the cases expressed moderate or abundant fibrosis. The varying distributions point to a certain heterogeneity in the level of fibrosis, which may reflect differences in disease stages. Also, differences in disease progress may be related to the underlying disorder if PMF indeed represents a common phenotype for specific cardiomyopathies, as speculated previously [9].

A notable feature was the location of fibrosis in the samples with distinct fibrosis. In most PMF cases, fibrosis was found in other sites than the endocardium exclusively, suggesting that the disease affects the whole myocardium. Specific cardiomyopathies are known to have some differences in their distribution of fibrosis. In HCM, most prominent fibrosis is seen in the midventricular layer, though also epicardial, endocardial, and trabecular sites are affected [23]. DCM, in contrast, typically exhibits endocardial thickening and fibrosis [24]. In ARVC, fibrosis gradually progresses from epicardium to the whole myocardium [25]. In contrast to specific cardiomyopathies and PMF, ischemic fibrosis can be quite focal. As in the present study, the endocardium is often affected, but also epicardial and transmural locations are seen depending on the extent of the ischemic lesion [17].

Both reactive- and replacement-type fibrosis were present in PMF cases, and extensive scarring was seen in 3 cases. Replacement fibrosis is thought to result from cardiomyocyte death, commonly seen after myocardial infarction [1], but many cardiomyopathies also exhibit replacement fibrosis as a histopathological feature [17], especially at the end-stage [23]. Furthermore, replacement-type fibrosis was always accompanied by interstitial fibrosis in ischemic heart disease, and fibrosis was seen primarily in perivascular locations in the study of Istrătoaie et al. [26] This was also the case in our study, where most ischemic cases had moderate to abundant diffuse and perivascular fibrosis, as well as an extensive relative area of fibrosis. Interestingly, the average size of the measured fibrotic lesions was similar in both PMF and ischemic cases, but somewhat greater variation was seen in the PMF samples.

As expected, myocardial fibrosis in ischemic injury was related to an increase in the number of fibroblasts. Only 3 cases with sclerotic fibrosis located to endocardium were devoid of fibroblasts: 2 of them associated with ischemic injury and 1 with PMF. One trauma control had mild increase in the number of fibroblasts in a slightly expanded perivascular area. This could be due to

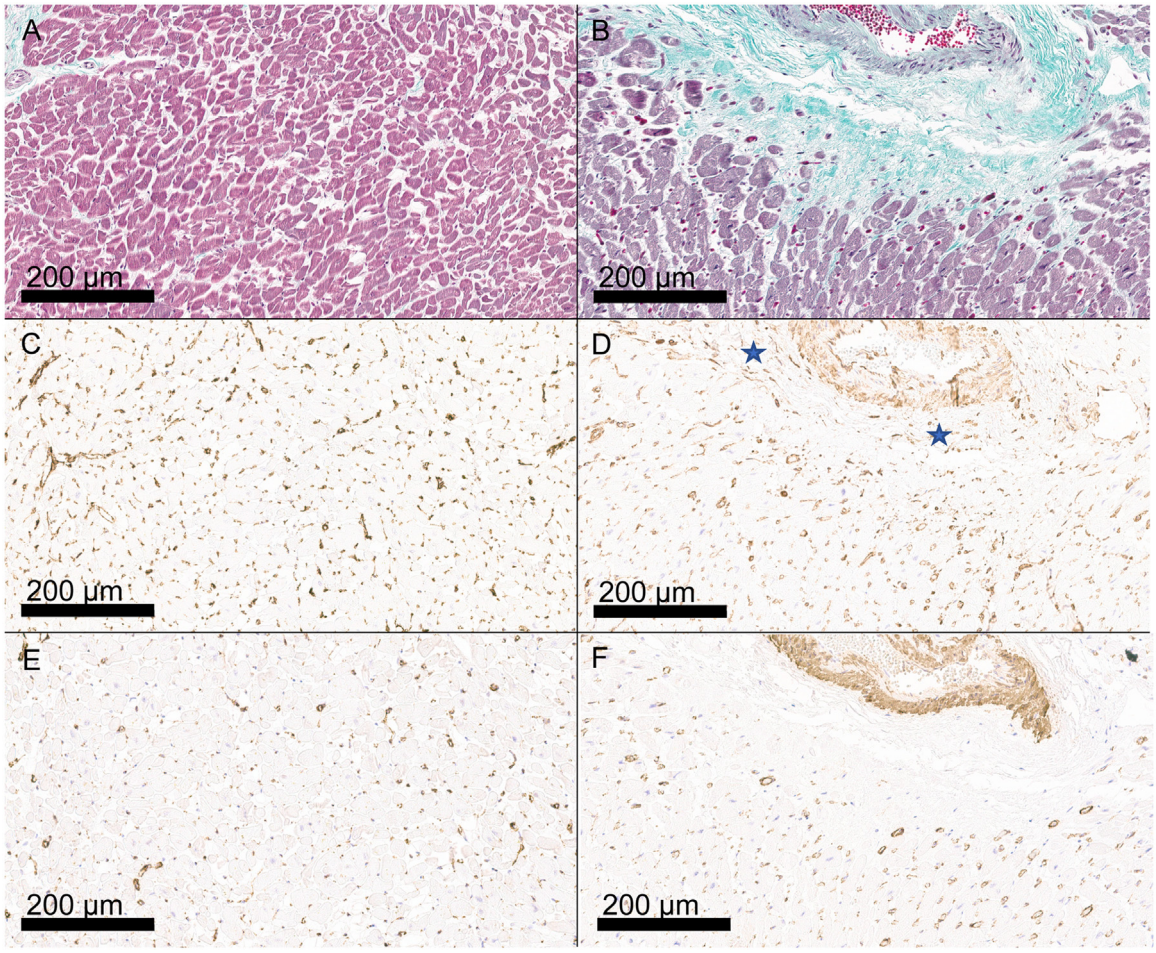

Fig. 6. Two cases from the trauma control group. A, C, E: no fibrosis. B, D, F: slight perivascular fibrosis with fibroblasts (stars) but no myofibroblasts. A–B Masson trichrome, C–D vimentin, E–F alpha-smooth muscle actin.

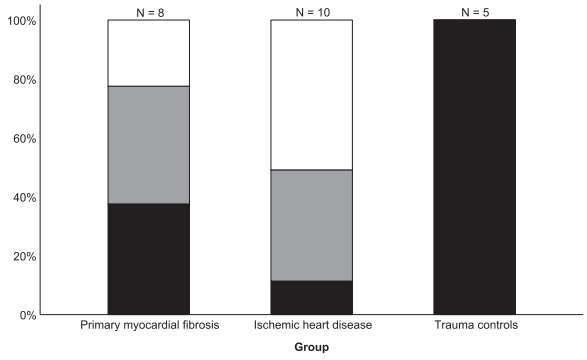

Fig. 7. Distribution of cases according to relative area of fibrosis in subset analysis. Bar colors indicate fibrosis levels: low, <1% (black); moderate, 1–5% (gray); abundant, >5% (white).

an undiagnosed hypertension, which perivascular fibrosis is often related to. [4] Alpha-SMA-positive myofibroblasts were only seen in scar-type fibrosis with at least mild infiltrates of macrophages reflecting a proliferative phase in the fibrotic event. Myofibroblasts were mainly present in the lesions of the ischemic group, except for one PMF case with extended transmural fibrosis and focal proliferation of myofibroblasts. Myofibroblasts were not found in the interstitial or perivascular fibrosis of either PMF or ischemic cases. This finding is consistent with the study of Suurmeijer et al. [27] where they found that while post-infarct scars were occupied by myofibroblasts, these cells were absent in interstitial fibrosis. Similar results were reported by Istrătoaie et al. [28] who studied different types of chronic heart diseases. The role of fibroblasts and activated myofibroblasts in ischemic injury has been extensively studied and reported in several reviews (e.g., Frangogiannis et al. [29]), but their role in non-ischemic myocardial fibrosis has received considerably less attention. While fibroblasts and myofibroblasts have not been histologically characterized earlier in PMF, John et al. [30] reported a heterogeneous increase of collagen content, and Hookana et al. [31] increased type I collagen synthesis in SCD victims with idiopathic myocardial fibrosis reflecting activation of fibrogenic cells. The present study further suggests an active role of fibroblasts in the fibrotic event of PMF and only a minor role or, alternatively, a short period of existence of myofibroblasts when compared with ischemic injury. Myofibroblasts with α-SMA-positivity have been observed to appear within a few days after infarction, but they lose their α-SMA-expression in a few weeks in mouse heart even though the cells persist throughout the whole lifespan of the scar [32]. Similar results of decreased α-SMA-positivity in 21–28 days after reperfusion have been reported in canine heart [29]. Willems et al., [33] however, presented results that α-SMA-positive stromal cells ultrastructurally resembling myofibroblasts were found in myocardial scars of up to several years old in humans. The presence of myofibroblasts in both ischemic and PMF cases was associated with at least a slight infiltrate of macrophages consistent with the knowledge that immune cells contribute to the activation of fibroblasts and differentiation

to myofibroblasts [34]. Necrosis or considerable infiltrates of inflammatory cells were not found in any of the PMF cases suggesting that the fibrotic event of PMF is different than the well-characterized infarct-type remodeling of the myocardium. This is a novel observation that requires further studies to clarify the specific mechanism behind PMF.

SCDs due to PMF affect foremost young people [10]. Ischemic heart disease, hypertensive heart disease, and valvular diseases are frequent in the older population, and fibrotic changes detected in autopsy are usually attributed to these conditions. In younger people, genetic conditions are more prevalent behind SCDs with estimations reaching as high as half the cases [35]. The genetic basis of many congenital pathologies, such as certain cardiomyopathies and channelopathies, have been identified [36]. Many pathogenic and likely pathogenic mutations, and VUSs were also identified in the FinGesture PMF population [9] of which the PMF cases in the present study were derived. Intriguingly, the case with 2 known pathogenic mutations was one of which had extensive fibrotic lesions. It may be likely that the fibrotic lesions in these cases are due to the detected mutations, but a definite diagnosis cannot be made, because of the absence of morphological changes commonly seen in these cardiomyopathies. However, our findings highlight the possibility of (yet unknown) genetic disorders behind PMF.

Myocardial fibrosis is known to accompany myocardial hypertrophy [18]. Although the mean heart weight in the ischemic group was clearly hypertrophic and significantly greater than in PMF group, heart weight did not in itself explain the occurrence of fibrosis. Another well-known risk factor for fibrosis is obesity through a variety of mechanisms [37]. Those with little diffuse and perivascular fibrosis had a slightly higher mean BMI, though the difference was not significant. No such association was either found between myocardial collagen content and BMI in noncardiac causes of death in the study of Miles et al. [22] in which the mean BMI of studied subjects was slightly higher than here, 28.7 kg/m^2.

4.1. Strengths and limitations

The main strength of this study is that the FinGesture cohort represents a large and unique study population, which provides excellent opportunities to study even uncommon disease entities. Even so, the rarity of SCD due to PMF restricted the number of cases available for study, thus limiting the usefulness of statistical methods. Lack of exact data on all sampling sites poses some challenges to interpretation of data. Sampling was focused on abnormal-looking areas, and the fibrosis in PMF cases is often not macroscopically visible, which may emphasize replacement-type scarring in the study population, specifically in the ischemic group. Furthermore, the distribution of cases on a long time span may be a limiting aspect, as autopsy methods and interpretation of findings have developed in recent years [16]. However, the medicolegal autopsies performed in the study population were done rigorously by experienced forensic pathologists using contemporary guidelines including full macroscopic and microscopic examinations as well as requisite supplementary examinations such as toxicology. Moreover, the selected short PMI of the cases allowed re-evaluation with complementary stainings of the study samples to compensate for the lack of possible inconsistencies in the original histological examinations.

Quantifying myocardial fibrosis reliably is challenging, and different approaches limit the possibilities of comparing findings between studies [38]. Automated processes are available for detecting fibrosis [39], but they carry their own drawbacks related to contrast issues, for instance. This is especially true when estimating diffuse fibrosis, which is why a manual approach was applied here. Despite the limitations of this method, the results were repeatable as was demonstrated by excellent inter-rater reliability.

4.2. Conclusions

In histopathological terms, PMF represents a heterogeneous entity with variable fibrotic lesions affecting the whole myocardium and suggesting a significant role of fibroblasts behind fibrosis. These observations may bring validation to the theory that PMF is a common manifestation of various cardiomyopathies. In any case, it is evident that PMF stands out as a particular entity demanding special attention as a cause of SCD. In addition to genetic studies, the histological morphology of PMF should be further analyzed to elucidate possible common features of this disease complex.

Declaration of Competing Interest

Riitta Kaarteenaho and Juhani Junttila have received a state subsidy of the Oulu University Hospital. Other authors declare no conflicts of interest. This work did not receive any specific funding outside the authors' organizations.

Acknowledgments

The authors are grateful to Ms Riitta Vuento for processing samples.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.carpath.2023.107573.

References

[1] Anderson KR, Sutton MG, Lie JT. Histopathological types of cardiac fibrosis in myocardial disease. J Pathol 1979;128:79–85.
[2] Jellis C, Martin J, Narula J, Marwick TH. Assessment of nonischemic myocardial fibrosis. J Am Coll Cardiol 2010;56:89–97.
[3] López B, Ravassa S, Moreno MU, José GS, Beaumont J, González A, et al. Diffuse myocardial fibrosis: mechanisms, diagnosis and therapeutic approaches. Nat Rev Cardiol 2021;18:479–98.
[4] Frangogiannis NG. Cardiac fibrosis. Cardiovasc Res 2021;117:1450–88.
[5] Humeres C, Frangogiannis NG. Fibroblasts in the infarcted, remodeling, and failing heart. JACC Basic Transl Sci 2019;4:449–67.
[6] Tarbit E, Singh I, Peart JN, Rose'Meyer RB. Biomarkers for the identification of cardiac fibroblast and myofibroblast cells. Heart Fail Rev 2019;24:1–15.
[7] Tallquist MD. Cardiac fibroblast diversity. Annu Rev Physiol 2020;82:63–78.
[8] Alex L, Frangogiannis NG. Pericytes in the infarcted heart. Vasc Biol 2019;1:H23–31.
[9] Junttila MJ, Holmström L, Pylkäs K, Mantere T, Kaikkonen K, Porvari K, et al. Primary myocardial fibrosis as an alternative phenotype pathway of inherited cardiac structural disorders. Circulation 2018;137:2716–26.
[10] Hookana E, Junttila MJ, Puurunen V-P, Tikkanen JT, Kaikkonen KS, Kortelainen ML, et al. Causes of nonischemic sudden cardiac death in the current era. Heart Rhythm 2011;8:1570–5.
[11] Yazdanfard PD, Christensen AH, Tfelt-Hansen J, Bundgaard H, Winkel BG. Non–diagnostic autopsy findings in sudden unexplained death victims. BMC Cardiovasc Disord 2020;20:58.
[12] Raju H, Parsons S, Thompson TN, Morgan N, Zentner D, Trainer AH, et al. Insights into sudden cardiac death: exploring the potential relevance of non-diagnostic autopsy findings. Eur Heart J 2019;40:831–8.
[13] Chugh SS, Kelly KL, Titus JL. Sudden cardiac death with apparently normal heart. Circulation 2000;102:649–54.
[14] Finocchiaro G, Papadakis M, Robertus J-L, Dhutia H, Steriotis AK, Tome M, et al. Etiology of sudden death in sports: insights from a United Kingdom Regional Registry. J Am Coll Cardiol 2016;67:2108–15.
[15] Lahrouchi N, Raju H, Lodder EM, Papatheodorou S, Miles C, Ware JS, et al. The yield of postmortem genetic testing in sudden death cases with structural findings at autopsy. Eur J Hum Genet 2020;28:17–22.
[16] Basso C, Aguilera B, Banner J, Cohle S, d'Amati G, de Gouveia RH, et al. Guidelines for autopsy investigation of sudden cardiac death: 2017 update from the Association for European Cardiovascular Pathology. Virchows Arch 2017;471:691–705.
[17] Eijgenraam TR, Silljé HHW, de Boer RA. Current understanding of fibrosis in genetic cardiomyopathies. Trends Cardiovasc Med 2020;30:353–61.
[18] Espeland T, Lunde IG, Amundsen BH, Gullestad L, Aakhus S. Myocardial fibrosis. Tidsskr Nor Laegeforen 2018;138(16).
[19] Kaikkonen KS, Kortelainen M-L, Linna E, Huikuri HV. Family history and the risk of sudden cardiac death as a manifestation of an acute coronary event. Circulation 2006;114:1462–7.

[20] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671–5.
[21] Verheule S, Schotten U. Electrophysiological consequences of cardiac fibrosis. Rev Cells 2021;10:3220.
[22] Miles C, Westaby J, Ster IC, Asimaki A, Boardman P, Joshi A, et al. Morphometric characterization of collagen and fat in normal ventricular myocardium. Cardiovasc Pathol 2020;48:107224.
[23] Galati G, Leone O, Pasquale F, Olivotto I, Biagini E, Grigioni F, et al. Histological and histometric characterization of myocardial fibrosis in end-stage hypertrophic cardiomyopathy. A clinical-pathological study of 30 explanted hearts. Circ Heart Fail 2016;9:e003090.
[24] Jain P, Arava S, Seth S, Lalwani S, Ray R. Histological and morphometric analysis of dilated cardiomyopathy with special reference to collagen IV expression. Indian J Pathol Microbiol 2017;60:481–6.
[25] Pilichou K, Thiene G, Bauce B, Rigato I, Lazzarini E, Migliore F, et al. Arrhythmogenic cardiomyopathy. Orphanet J Rare Dis 2016;11:33.
[26] Istrătoaie O, Ofiţeru A-M, Nicola G-C, Radu RI, Florescu C, Mogoantă L, et al. Myocardial interstitial fibrosis - histological and immunohistochemical aspects. Rom J Morphol Embryol 2015;56:1473–80.
[27] Suurmeijer AJH, Clément S, Francesconi A, Bocchi L, Angelini A, Van Veldhuisen DJ, et al. α-Actin isoform distribution in normal and failing human heart: a morphological, morphometric, and biochemical study. J Pathol 2003;199:387–97.
[28] Istrătoaie O, Pirici I, Ofiţeru A-M, Grosu F, Brînzea A, Olar L, et al. Evaluation of cardiac microvasculature in patients with diffuse myocardial fibrosis. Rom J Morphol Embryol 2016;57:1351–6.
[29] Frangogiannis NG, Michael LH, Entman ML. Myofibroblasts in reperfused myocardial infarcts express the embryonic form of smooth muscle myosin heavy chain (SMemb). Cardiovasc Res 2000;48:89–100.
[30] John BT, Tamarappoo BK, Titus JL, Edwards WD, Shen WK, Chugh SS. Global remodeling of the ventricular interstitium in idiopathic myocardial fibrosis and sudden cardiac death. Heart Rhythm 2004;1:141–9.
[31] Hookana E, Junttila MJ, Kaikkonen KS, Porvari K, Kaija H, Risteli J, et al. Increased type I collagen synthesis in victims of sudden cardiac death due to idiopathic myocardial fibrosis. Ann Med 2014;46:318–23.
[32] Fu X, Khalil H, Kanisicak O, Boyer JG, Vagnozzi RJ, Maliken BD, et al. Specialized fibroblast differentiated states underlie scar formation in the infarcted mouse heart. J Clin Invest 2018;128:2127–43.
[33] Willems IEMG, Havenith MG, De Mey JGR, Daemen MJ. The α-smooth muscle actin-positive cells in healing human myocardial scars. Am J Pathol 1994;145:868–75.
[34] Lafuse WP, Wozniak DJ, Rajaram MVS. Role of cardiac macrophages on cardiac inflammation, fibrosis and tissue repair. Cells 2020;10:51.
[35] Thiene G. Sudden cardiac death in the young: a genetic destiny? Clin Med 2018;18(Suppl 2):s17–23.
[36] Bezzina CR, Lahrouchi N, Priori SG. Genetics of sudden cardiac death. Circ Res 2015;116:1919–36.
[37] Mahajan R, Lau DH, Sanders P. Impact of obesity on cardiac metabolism, fibrosis, and function. Trends Cardiovasc Med 2015;25:119–26.
[38] Schipke J, Brandenberger C, Rajces A, Manninger M, Alogna A, Post H, et al. Assessment of cardiac fibrosis: a morphometric method comparison for collagen quantification. J Appl Physiol 2017;122:1019–30.
[39] Gratz D, Winkle AJ, Dalic A, Unudurthi SD, Hund TJ. Computational tools for automated histological image analysis and quantification in cardiac tissue. MethodsX 2019;7:22–34.