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ORIGINAL PAPER

Histopathological myocardial changes in patients with severe aortic stenosis referred for surgical valve replacement: a cardiac magnetic resonance correlation study

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Aims Myocardial fibrosis (MF) takes part in left ventricular (LV) remodelling in patients with aortic stenosis (AS), driving the transition from hypertrophy to heart failure. The structural changes that occur in this transition are not fully enlightened. The aim of this study was to describe histopathological changes at endomyocardial biopsy (EMB) in patients with severe AS referred to surgical aortic valve replacement (AVR) and to correlate them with LV tissue characterization from pre-operative cardiac magnetic resonance (CMR).

Methods and results One-hundred fifty-eight patients [73 (68–77) years, 50% women] were referred for surgical AVR because of severe symptomatic AS, with pre-operative CMR ($n = 143$) with late gadolinium enhancement (LGE), T1, T2 mapping, and extracellular volume fraction (ECV) quantification. Intra-operative septal EMB was obtained in 129 patients. MF was assessed through *Masson's Trichrome* histochemistry. Immunohistochemistry was performed for both inflammatory cells and extracellular matrix (ECM) characterization (Type I Collagen, Fibronectin, Tenascin C). Non-ischaemic LGE was present in 106 patients (67.1%) [median fraction: 5.0% (2.0–9.7)]. Native T1 was above normal [1053 ms (1024–1071)] and T2 within the normal range [39.3 ms (37.3–42.0)]. Median MF was 11.9% (6.54–19.97), with predominant type I collagen perivascular distribution (95.3%). Sub-endocardial cardiomyocyte ischaemic-like changes were identified in 45% of EMB. There was no inflammation, despite ECM remodelling expression. MF quantification at EMB was correlated with LGE mass ($P = 0.008$) but not with global ECV ($P = 0.125$).

Conclusion Patients with severe symptomatic AS referred for surgical AVR have unspecific histological myocardial changes, including signs of cardiomyocyte ischaemic insult. ECM remodelling is ongoing, with MF heterogeneity. These features may be recognized by comprehensive CMR protocols. However, no single CMR parameter captures the burden of MF and histological myocardial changes in this setting.

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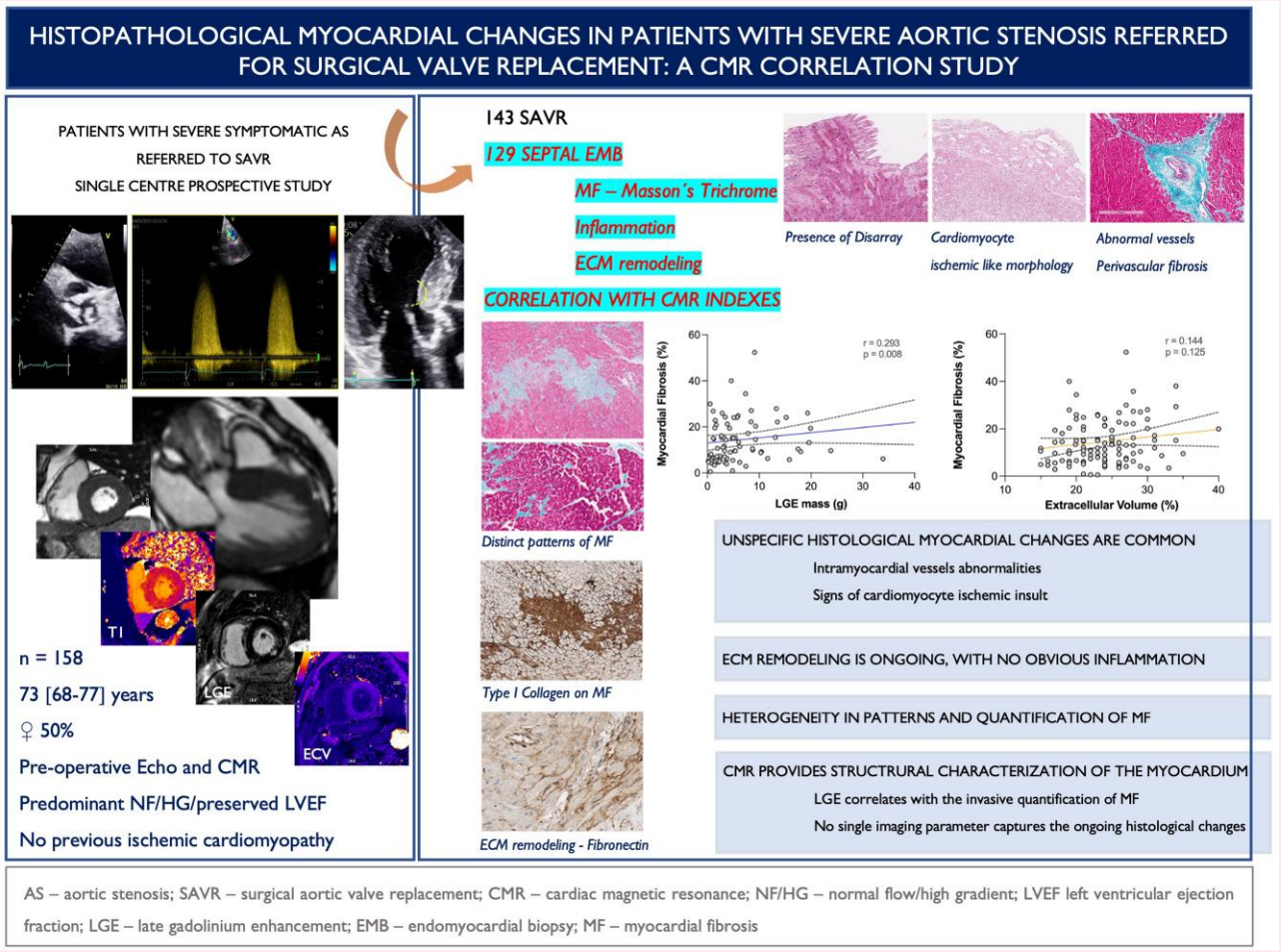
Graphical Abstract



Keywords aortic stenosis • cardiac magnetic resonance • myocardial remodelling • myocardial fibrosis • endomyocardial biopsy • myocardial histopathology

Introduction

Calcific aortic stenosis (AS) is the most common valvular heart disease, progressing with a chronic sustained increase in the haemodynamic load to the left ventricle (LV). Myocardial remodelling in this setting starts from less than severe stages of the disease,[1] happening with variable degrees of myocardial fibrosis (MF). This is an independent marker of LV decompensation, heart failure development, and adverse outcomes in patients with severe AS.[2]

Endomyocardial biopsy (EMB) is the gold standard method to evaluate cardiomyocyte adaptation and extracellular matrix (ECM) composition. Collagen volume fraction (CVF) is correlated with myocardial collagen content, being a recognized quantitative measurement of MF.[3] Patients with severe AS undergoing aortic valve replacement (AVR) may have a combined pattern of both replacement and diffuse fibrosis, this last being deemed reversible after pressure overload relief.[4] However, the sequential relation between reactive and replacement fibrosis lacks confirmation, as distinct pathophysiology pathways may ensue. ECM remodelling and the potential role of inflammation for the progression of reactive fibrosis are also not fully elucidated. Lastly, clinical correlation data centred on histopathology findings from EMB in patients with severe AS are inconsistent.[5]

Currently, cardiac magnetic resonance (CMR) is the best imaging modality that offers a direct, whole-heart assessment of MF, circumventing the limitations of EMB in terms of invasiveness, sampling, cost, and technical demands.[6] Myocardial tissue composition, cellular and extracellular compartments' expansion, and both diffuse and replacement fibrosis may all be extracted from multi-parametric comprehensive protocols.[7] ECV, as derived from combined pre- and post-contrast T1 mapping, seems to be particularly sensitive to extracellular space expansion and diffuse fibrotic burden.[8] Nevertheless, correlation data from distinct clinical cohorts are far from uniform, and some of these studies did not find associations between the ECV and CVF in EMB samples.[3]

Our study's main hypothesis is that in patients with severe AS, there are structural and functional myocardial changes that may be identified and characterized by CMR. We tried to evaluate if the combination of the conventional study of myocardial hypertrophy and fibrosis at EMB in this setting, with the specific assessment of the presence of inflammation and ECM remodelling at immunohistochemistry, may provide additional information towards the mechanisms behind ECM expansion. Our aim was therefore to assess the histopathology changes at EMB in a group of patients with severe AS referred to surgical AVR and to

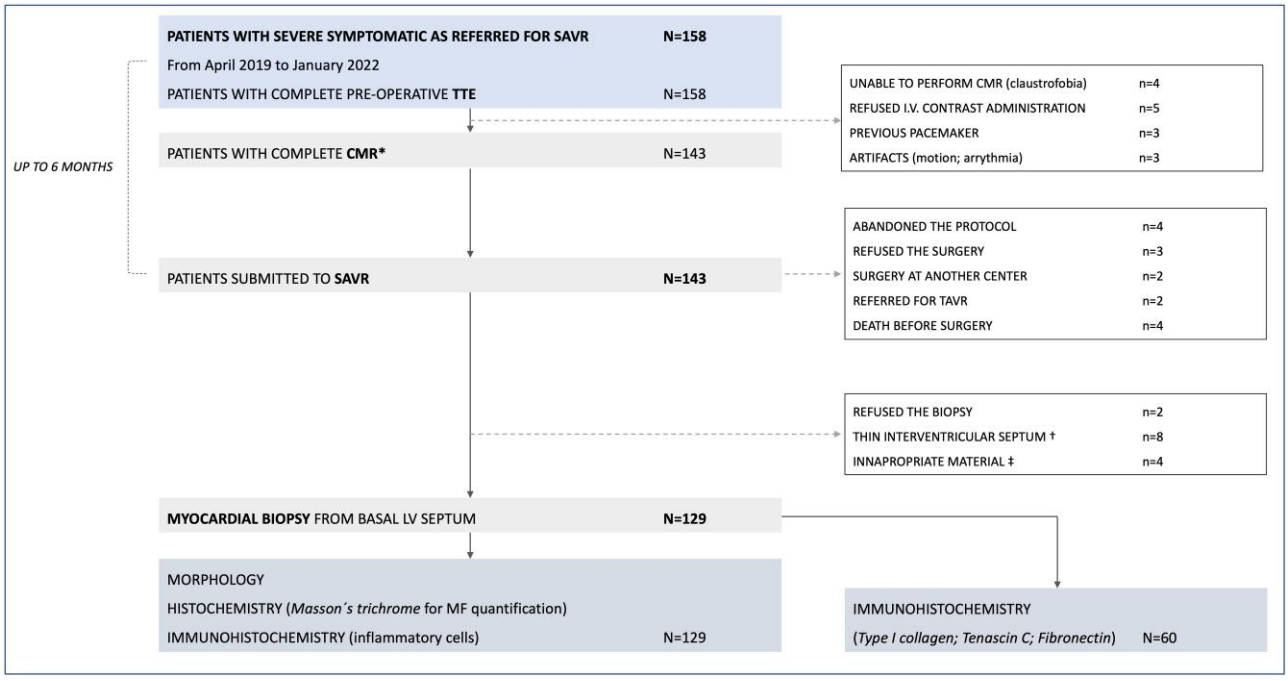


Figure 1 Study flowchart. * Complete CMR when appropriate for both T1 and T2 mapping estimation and extracellular volume calculation, beyond the assessment and quantification of LGE; † As considered by the surgical team; ‡ To scarce biopsy material to perform the histomorphology and immunohistochemistry study.

find correlations with non-invasive markers of LV tissue characterization, obtained from a pre-operative comprehensive CMR imaging protocol.

Methods

Study population and study design

We prospectively evaluated 158 patients undergoing surgical AVR at our tertiary centre between April 2019 and January 2022 for isolated severe symptomatic AS, defined according to European guideline on valvular heart disease.[9] Patients with previous history of myocardial infarction and ischaemic cardiomyopathy were excluded. Additional exclusion criteria are detailed at Supplementary data online, *Material/Methods*. This prospective study is part of a correlation research protocol in patients with severe symptomatic AS, dedicated to LV adaptation and ECM remodelling, as assessed by multi-modality imaging and histopathology from EMB. The study design and procedural schedule are depicted in *Figure 1*. This was previously specified and approved by the ethical committee of Nova Medical School University (number 61/2018/CEFCM) in line with the principles of the Helsinki Declaration. All participants provided written informed consent.

Clinical data and evaluation for aortic valve stenosis

Clinical parameters, 12-lead electrocardiogram, and transthoracic echocardiography (TTE) were collected at the study inclusion before AVR. CMR was carried out within 2 weeks after patient inclusion alongside blood sample for haematocrit, creatinine, high-sensitivity cardiac troponin I, and N-terminal pro–b-type natriuretic peptide. Both TTE and CMR studies were performed within 6 months prior to AVR. All patients underwent a comprehensive TTE by experienced cardiologists before AVR, using commercially available ultrasound systems (Vivid E9 and E95; GE Healthcare, Chicago, IL, USA) with a 4D probe (GE 4V-D and 4Vc-D Matrix probes for Vivid E9 and E95, respectively) in accordance with current guidelines.[10,11] Detailed echocardiographic measurements and derived indexes are described at Supplementary data online, *Material/Methods*.

If clinically justified for coronary artery disease exclusion, patients performed a coronary angiography before intervention and coronary revascularization was added to AVR when indicated.

Cardiac magnetic resonance

A pre-operative CMR study was performed at 1.5T equipment (Magnetom Avanto; Siemens Medical Solutions, Erlangen, Germany) using a clinical scan protocol, as previously published.[12] Details concerning post-contrast late gadolinium enhancement (LGE) imaging, native and post-contrast T1 mapping, ECV quantification, T2*, and T2 mapping are specified in Supplementary data online, *Methods*. As per institutional protocol, our native T1 values, as derived from healthy controls, were considered normal for the interval between 972 and 1029 ms.

EMB and histological analysis

EMB samples were obtained either from intraoperative septal biopsy as per protocol design (harvested with a scalpel from the basal interventricular septum, preferably with endocardium inclusion) or from complementary septal myectomy, performed by the surgical team at the time of surgical AVR because of asymmetric septal hypertrophy or at surgeon's discretion. To assess cardiomyocyte adaptation, sub-endocardial changes, fibrosis, and potential presence of inflammation, a combined histochemistry and immunocytochemistry protocol with distinct markers was established. In a randomly selected group of 60 EMB, specific ECM remodelling markers were studied. Supplementary data online, *Table S1* specifies each technique and specific evaluated marker, with potential derived information. For the

sake of representativity, additional distal interventricular septum biopsies were randomly requested to the surgical team in 15 patients.

Technical data involving pre-analytical steps for EMB processing, histomorphology, and immunohistochemistry studies are detailed in Supplementary data online, *Methods*. We used an automatic algorithm for MF quantification, previously described and assessed by our group.[13] Percentage of fibrosis was calculated as [area of fibrosis/(area of cardiomyocytes + area of fibrosis) × 100], with the exclusion of dense endocardial fibrosis (see Supplementary data online, *Figure S1*).

Statistical analysis

Categorical variables are presented as count (percentage), and the difference between groups was analysed by chi-square and Fisher's exact tests. Continuous variables with normal and non-normal distribution were described by the mean ± SD or median ± interquartile range and compared using Student's *t*-test or Mann–Whitney *U* test, respectively. Analysis of variance and Kruskal–Wallis test were performed for comparison amongst multiple groups.

Relationships amongst imaging and EMB-derived variables were assessed for patients with complete data (129 patients), using either Pearson's or Spearman's correlation coefficient, as appropriate.

The inter-observer variability of the quantification of LGE and MF, using the automatic algorithm, was both assessed by calculating the intra-class correlation coefficient analysis on a random sample of 30 pre-operative CMR studies and 30 EMB by two independent operators, respectively. Both analyses showed very good reproducibility, with an intra-class correlation coefficient of 0.98 [95% confidence interval (CI) 0.95–0.99, $P < 0.001$] for LGE and 0.93 (95% CI 0.90–0.98, $P < 0.001$) for MF.[13]

A two-sided *P*-value of <0.05 was considered statistically significant. The statistical analysis was performed with IBM SPSS Statistics 26.0 (IBM Corp, Armonk, NY, USA).

RESULTS

Clinical- and surgery-related data

From a total of 158 patients included in the protocol, median age of 73 (68–77) years, 79 (50%) of them being women, 143 were submitted to elective surgical AVR (*Figure 1*) for severe symptomatic AS (see Supplementary data online, *Table S2*). They were all ambulatory patients, mainly with significant functional impairment in daily activities (94.9% of them in NYHA ≥II functional class). The great majority of patients (93%) had a bioprosthetic implantation, and concomitant surgical revascularization was performed in 24 (15.2%) patients.

Pre-operative echo and CMR data

The classical phenotype of high gradient, normal flow, preserved LV ejection fraction (EF) was present in 136 (86%) patients (*Table 1*). Thirteen patients had reduced LV EF, five with a low-flow state, despite high gradients. Nine patients had low gradient AS (all of them with a mean gradient between 30 and 40 mmHg), two of them with reduced EF, and the remaining with paradoxical AS (see Supplementary data online, *Figure S2*). Moderate mitral valve regurgitation was present in 31 (19.6%) patients. This was considered functional mitral regurgitation except in one of the cases with a leaflet prolapse. The mean maximum reported basal septum thickness was 16 ± 2.6 mm. Both global and basal anteroseptal native T1 values were considered above normal for our institutional cut-off. Native T2 myocardial values did not favour the presence of myocardial inflammation. Neither ECV estimation nor T2* values supported myocardial amyloid or iron deposition, respectively.

Non-ischaemic LGE was identified in 106 (67.1%) patients, representing 5% (2.0–9.7%) of global LV mass, though exclusive insertional LGE occurred in 26 (16.5%) patients. Amongst those with LGE, this was mostly frequent in the basal anterior interventricular septum (39%) and mid inferior interventricular septum (38%), as showed in a previous report on this same cohort.[14] In two patients, a small subendocardial ischaemic scar was identified outside the interventricular septum despite the absence of previous history of myocardial infarction.

Table 1 Aortic valve stenosis and left ventricular characterization at pre-operative echo and CMR study

Total study population: *n* = 158	
Aortic valve characterization—TTE[a]	
Mean aortic gradient, mmHg	60.9 ± 17.4
Aortic valve area, cm^2	0.72 ± 0.17
LV adaptation—TTE and CMR (patients with complete pre-operative CMR: *n* = 143)	
Stroke volume index, mL/m^2	47.2 ± 10.7
Relative wall thickness	0.53 ± 0.12
LV ejection fraction, %	58.2 ± 9.2
Maximum septal thickness (echo), mm	16.0 ± 2.6
Global longitudinal strain at echo, %	14.9 ± 3.6
LV mass by CMR, g	145.2 ± 51.7
LV indexed mass by CMR, g/m^2	79.3 ± 25.9
LV telediastolic indexed volume, mL/m^2	65.9 ± 33.0
Geometric remodelling, g/mL	0.95 (0.82–1.06)
LV ejection fraction by CMR, %	59.4 ± 10.3
Tissue characterization (patients with complete pre-operative CMR: *n* = 143)	
Global native T1, ms	1053 (1024–1071)
Septal native T1, ms	1074 (1046–1103)
Global native T2, ms	39.3 (37.3–42.0)
Septal native T2, ms	41.4 (38.7–43.6)
T2*, ms	30.6 (26.1–35.5)
Global ECV, %	23.0 (20.0–27.0)
Septal ECV, %	23.0 (19.0–26.0)
LGE, *n*	106 (67.1%)
Junctional LGE, *n*	26 (16.5%)
LGE mass, %	5.0 (2.0–9.7)

Values are median (interquartile range), mean ± standard deviation.
CMR, cardiac magnetic resonance; ECV, extracellular volume, geometric remodelling: LV mass/LV end-diastolic volume; LGE, late gadolinium enhancement; LV, left ventricle; TTE, transthoracic echocardiography.
[a]Trace or mild aortic valve regurgitation was present in 101 patients (63.9%).

Histomorphology and immunohistochemistry at EMB

A mean period of 3.4 ± 2.1 months elapsed between imaging studies and surgical AVR. EMB was performed in 129 patients (*Figure 1*), with no specific associated complication, either as per protocol procedure or as a myectomy surgical sample (*Table 2*). Myocyte hypertrophy was an invariable finding as individual cell measurements were all above 20 μm (*Figure 2A*). There was elastic fibres deposition (*Figure 2B*), representing approximately one-third of the endocardial thickness, in the whole group of patients with endocardium inclusion. Abnormal

Table 2 Findings at EMB concerning histomorphology and immunohistochemistry

Morphology	***n* = 129**
Sample thickness, mm	2.73 ± 1.28
Sample area, mm^2	11.95 (7.15–29.90)
Endocardium inclusion	114 (88.4%)
Cardiomyocyte diameter, µm[a]	36.38 ± 7.12
Endocardium thickness[b], µm	134.0 (76.5–205.7)
Elastic deposition[c]	114 (88.4%)
Elastic/endocardium ratio	0.38 ± 0.18
Disarray[d]	20 (15.5%)
Vascular abnormalities[e]	79 (61.2%)
Perivascular fibrosis	123 (95.3%)
Replacement fibrosis[f]	41 (31.8%)
Quantification of fibrosis, %	11.90 (6.54–19.97)
Cardiomyocyte ischaemic like changes	58 (45%)
Immunohistochemistry[g]	***n* = 129**
Alpha-actin	89 (69%)
CD45+	107 (82.9%)
CD45+/field[h]	3.0 (1.1–6.0)
CD3+	102 (79.1%)
CD20+	7 (5.4%)
CD68+	68 (47.3%)
Immunohistochemistry for collagen type and ECM remodelling, *n* = 60	
Perivascular Type I collagen	60 (100%)
Tenascin C	15 (25.0%)
Fibronectin	49 (81.7%)

Values are median (interquartile range), mean ± standard deviation.
[a]Transversal measurement performed at the centre cell plane (cross-section encompassing the nucleus at haematoxylin–eosin × 40).
[b]As the mean of 5–10 measurements.
[c]Identified at elastic van Gieson's stain.
[d]Considered when in the presence of cellular interlacing, whirling, or herringbone architecture arrangement.
[e]Considered for abnormal vessel walls (intimal and medial smooth muscle cell hyperplasia; luminal distortion and narrowing).
[f]Defined for large coalescent areas of fibrosis.
[g]Caspase-3 was un-interpretable; CD90 had inconsistent ground noise positivity and was also considered un-interpretable.
[h]Number of positive cells per ×20 amplification field of view.

cardiomyocyte architecture, i.e. disarray, was identified in 20 patients (*Figure 2C*), and our samples were notable for the presence of vascular abnormalities (61.2% of the cases) (*Figure 2D*) (see Supplementary data online, *Figure S3*). Our quantification covered both interstitial and replacement type of fibrosis, totalizing a median of 11.90% (6.54–19.97%). This included microscars, large coalescent areas of substitution fibrosis, as well as highly prevalent perivascular involvement (*Figure 2F–H*). None of the samples had histological evidence of myocardial infarction.

In 45% of the cases, we found the presence of sub-endocardial groups of cardiomyocytes with abnormal morphology, namely with cytoplasmatic enlargement, vacuolization and myofibre derangement (*Figure 3*), as described in stunned and hibernating myocardium.[15]

In about two-thirds of the cases, there was a positive stain for alpha-smooth muscle actin (α-SMA) at the sub-endocardium, mostly distributed as isolated 'islands' (see Supplementary data online, *Figure S4*), with variable grades of positivity. The expression of this contractile protein stands for the presence of myofibroblasts. We also searched for resident fibroblasts using anti-CD90 antibodies, but this was considered non-assessable due to ground noise positivity and simultaneous cardiomyocyte marking. There was no inflammatory myocardial infiltration, with random and scant distribution of leucocytes, with T-cell type (CD3+) predominance, and occasional presence of monocyte lineage cells (see Supplementary data online, *Figure S5*). Specific evaluation was made to investigate the presence of cardiomyocyte apoptosis through immunohistochemistry for caspase-3. However, this had diffused and weak intra-cytoplasmic positivity despite appropriate positive control, which hindered interpretation. In the group of 60 randomly selected patients, we found positivity for Type I collagen at coalescent scars previously identified at *Masson's Trichrome*, invariably following the perivascular distribution of fibrosis (*Figure 4*). It became demonstrated that Tenascin C was occasionally expressed outside the cardiomyocytes, at the perivascular ECM. Fibronectin was widely distributed, with predominant expression at the membrane of cardiomyocytes, in areas of perivascular fibrosis (see Supplementary data online, *Figure S6*).

Additional distal myocardial biopsies were collected in eleven cases, reported by the surgical team as smaller than proximal samples, mostly from the mid to distal interventricular septum. There were no specific differences in relation to the corresponding proximal EMB in terms of both morphology, histo-, and immunohistochemistry (see Supplementary data online, *Table S3*).

Relationships between pre-operative imaging characterization, invasive quantification of fibrosis, and histopathology findings

To assess the relationship between LV remodelling and non-invasive markers of both replacement and interstitial fibrosis/ECM expansion, we correlated several CMR-derived indexes (see Supplementary data online, *Table S4*; *Figure 5*). We found a significant, despite the modest correlation between LV mass and LGE ($r = 0.36$; $P < 0.001$).

The relationship between the invasive quantification of MF and CMR tissue characterization parameters was evaluated as to try to find non-invasive imaging markers of MF. Invasive MF was correlated to LGE mass ($r = 0.29$; $P = 0.008$), but not with global native T1 and ECV. However, a correlation between MF and ECV was detected for the basal septum assessment ($r = 0.24$, $P = 0.011$). *Figure 6* includes the co-registered imaging and histopathology data from one of the patients.

Neither the prevalence of significant coronary artery disease nor the prevalence of dysplastic vessels at EMB differed in patients with sub-endocardial ischaemic-like changes (52.2% vs. 47.8%, $P = 0.493$; 65.5% vs. 57.7%, $P = 0.469$, for the presence of significant coronary artery disease and vascular dysplasia in patients with and without sub-endocardial changes, respectively).

We additionally tried to assess if routine blood biomarkers of both cardiac cell injury and loading have relationships with CMR-derived indexes and MF. Troponin level was significantly correlated with LGE mass ($r = 0.272$; $P = 0.006$), but none of the biomarkers were correlated with the invasive quantification of MF.

Discussion

From this observational and correlation study with EMB in the setting of severe AS, we showed that (i) there are unspecific histological changes

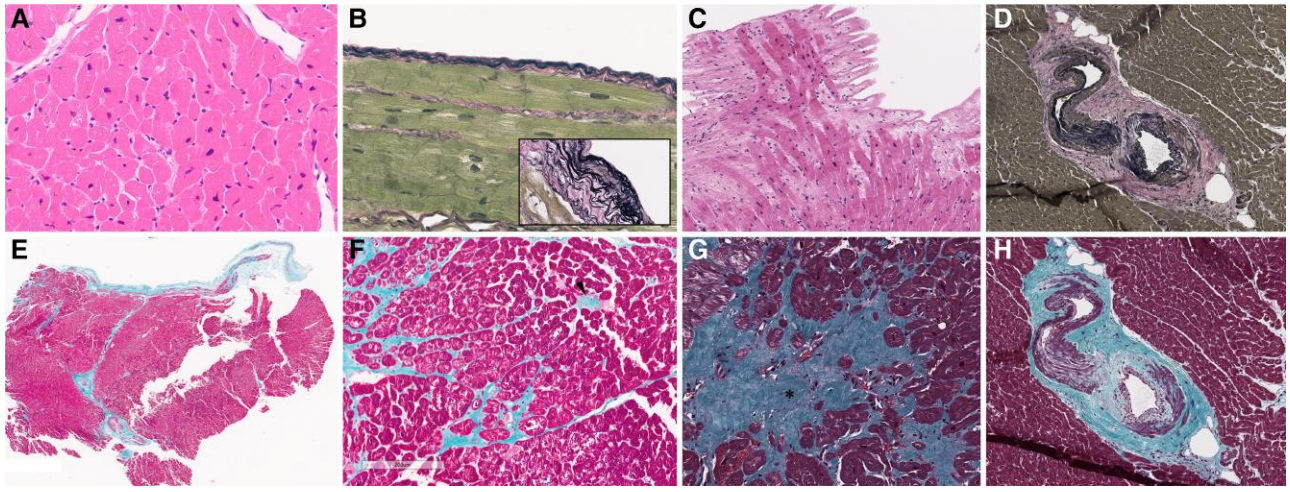


Figure 2 Histomorphology findings. (*A*) Cardiomyocyte hypertrophy in cross-section (haematoxylin–eosin × 100); this was an invariable finding at EMB; (*B*) mild sub-endocardial elastosis at elastic van Gieson's stain (×100). Detail of elastic fibres (box) from another patient with prominent endocardial thickening (×200); (*C*) herringbone architecture pattern defining myocardial disarray (haematoxylin–eosin × 100), which was present in 15.5% of the samples; (*D*) intra-myocardial dysplastic arteries put in evidence at elastic van Gieson's stain (×40), with media thickening, elastic extra-deposition, and luminal distortion; (*E*) overview of a biopsy specimen with endocardial inclusion (Masson's Trichrome × 10); (*F*) coalescent areas of interstitial fibrosis described as microscars (arrowhead) (Masson's Trichrome × 40); (*G*) large area of replacement fibrosis (*) (Masson's Trichrome × 100); (*H*) extensive perivascular fibrosis (Masson's Trichrome × 40, same area as *D*).

in the myocardium, and these include sub-endocardial ischaemic-like changes, in patients with no previous history of ischaemic cardiomyopathy; (ii) intra-myocardial vascular abnormalities are prevalent and there is evidence of ECM remodelling, with no obvious inflammation; (iii) a comprehensive pre-operative CMR protocol involving both morpho-functional LV characterization, LGE quantification, mapping derived indexes, and their combined assessment may yield a summed indication of some of these structural changes. These data may provide insights towards the pathophysiology behind LV hypertrophy and the topography of MF. Indeed, structural correlates demonstrated that both cardiomyocyte and LV hypertrophy are just parts of a complex process of adaptation to chronic pressure overload. The novelty of this study was to demonstrate the heterogeneous nature of myocardial structural remodelling in patients with severe AS and the same clinical indication for intervention. Perfusion impairment was deduced in some of the patients from the invasive structural assessment. In line with previous studies, we confirmed that no single CMR parameter captures the burden of MF and histological myocardial changes in this setting.

Histopathology findings at EMB

To our knowledge, ours is one of the few studies with a large number of EMB performed during surgical AVR in a mostly homogeneous cohort of patients with classical severe symptomatic AS, with pre-operative CMR evaluation for correlation purposes. In line with ancillary data from Krayenbuehl *et al.*,[16] we confirmed the presence of classical signs of cardiomyocyte adaptation and ECM changes involving MF in this setting. The presence of myocardial disarray stands for the unspecific findings of EMB across the spectrum of myocardial diseases. As previously demonstrated by our group,[17] this is not specific for hypertrophic cardiomyopathy and might have occurred in our cohort because of localized interventricular septum hypertrophy in the context of a pressure overload condition. We were also able to confirm the findings from the landmark correlation study of Treibel *et al.*[3] We found the same proportion of MF and we described endocardial thickening with dense fibrosis, pericellular fibrosis, and coalescent areas, with a high proportion of perivascular involvement. Even though, we did not assess the specific endo-mid MF gradient. We excluded the dense endocardial fibrosis from the quantification of MF, since in some biopsy specimens, there was no endocardium included. This was performed to also include biopsies without endocardium in the analysis.

One of our most interesting findings deals with the presence of morphological changes amongst groups of sub-endocardial cardiomyocytes in 45% of the cases, as described in the setting of myocardial stunning or hibernation.[18] We interpreted these findings as chronic myocardial hibernation with reduced blood flow. This is probably unrelated to the presence of significant coronary artery disease. None of the patients had evidence of an ischaemic scar at histomorphology. The prevalence of these changes at the sub-endocardium was much higher than the prevalence of significant coronary artery disease, and these were not more prevalent in patients submitted to surgical revascularization. We did not specifically assessed perfusion in our cohort, but a sub-normal myocardial flow gradient might explain these changes, as reported.[19] The invariable presence of cardiomyocyte hypertrophy and high prevalence of dysplastic changes in intra-myocardial vessels stand for a potential disproportion between tissue perfusion and unmet increased metabolic needs, namely at sub-endocardial location. Prominent perivascular fibrosis is also probably related with a perfusion imbalance. We confirmed the presence of predominant Type I Collagen, which happens to be qualitatively distinct, as stiff, and heavily cross-linked,[20] at immunohistochemistry. This follows the sub-endocardial vessel web and may interfere with vascular compliance. Ultimately, cardiomyocyte changes in morphology probably reflect altered cell metabolism, functional impairment, and yet-to-be-proved cell dedifferentiation.[15] From morphological swelling (oncosis), it might be supposed that programmed cell death is simultaneously occurring. Unfortunately, we were unable to confirm this from caspase-3 immunohistochemistry.

The absence of an obvious inflammatory infiltrate was demonstrated in our cohort. Nevertheless, active ECM remodelling is ongoing, as we

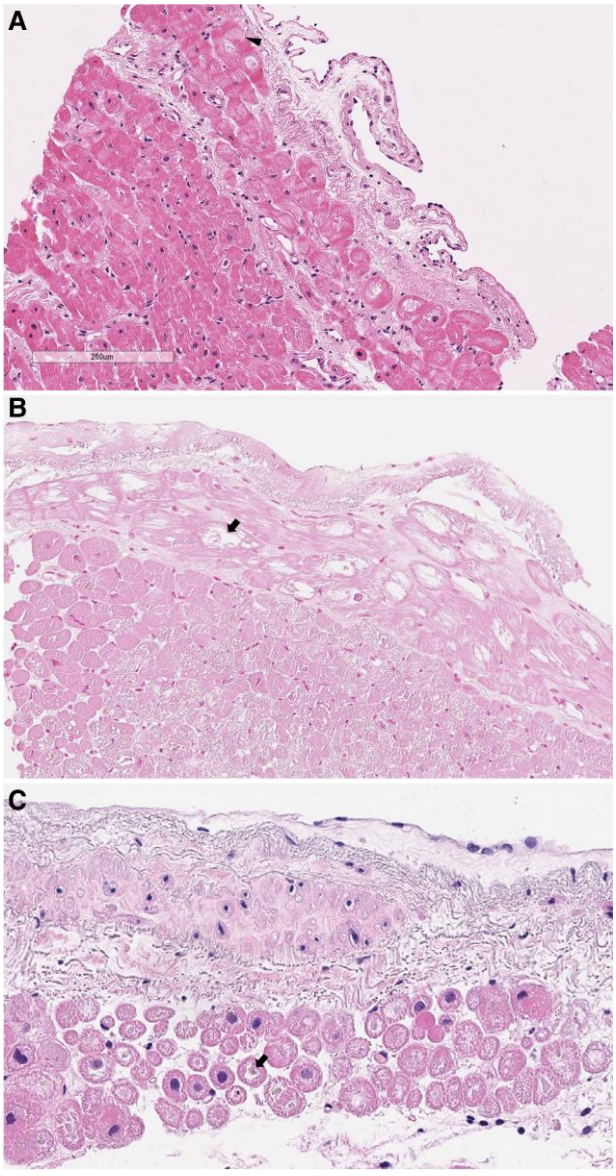


Figure 3 (*A–C*) Examples of abnormal sub-endocardial cardiomyocyte morphology, present in 45% of the samples with endocardial inclusion. At haematoxylin–eosin × 100 it is possible to identify cytoplasmatic enlargement, vacuolization (arrow) and myofibre detachment (arrowhead).

found in a smaller sub-group of patients, randomly selected to be evaluated for ECM remodelling markers. Tenascin C expression and the extensive positivity for tissue fibronectin corroborate the qualitative change in ECM components. Immune cells also participate in this change, as identified from the presence of intra-myocardial monocyte lineage cells (CD68+), at least in almost half of the cases. We also identified α-SMA in a high proportion of cases, which stands for the previous occurrence of a phenotype switch of cardiac fibroblast to their myofibroblast counterpart. Additionally, we tried to assess the presence of resident fibroblasts, as myofibroblast conversion may not be required for activation of a pro-fibrotic environment.[21] However, anti-CD90 evaluation was un-interpretable. Together with variable α-SMA expression, this confirms the challenging identification of potential effector cells responsible for ECM remodelling, either to their limited protein expression or unspecific available cell markers for cardiac fibroblast populations.[22] In clinical terms, the variable expression of ECM markers means different stages of myocardial remodelling in patients with severe AS and the same clinical indication for intervention. This could determine distinct post-surgical reverse remodelling and prognosis. The identification of specific markers of fibroblasts and MF promoters may ultimately lead to anti-fibrotic therapeutic targeting.

Non-invasive correlation with histopathology

For long time LV remodelling in terms of mass, volumes and function have been categorized for prognostic purposes and stratification in patients with AS. However, these are not as strong as CMR-derived tissue characterization parameters, namely replacement fibrosis (LGE)[2] and ECM surrogate markers of diffuse fibrosis (ECV).[23] Indeed, we found positive association between LGE and LV mass, further indicating that replacement fibrosis is more intimately related to more advanced

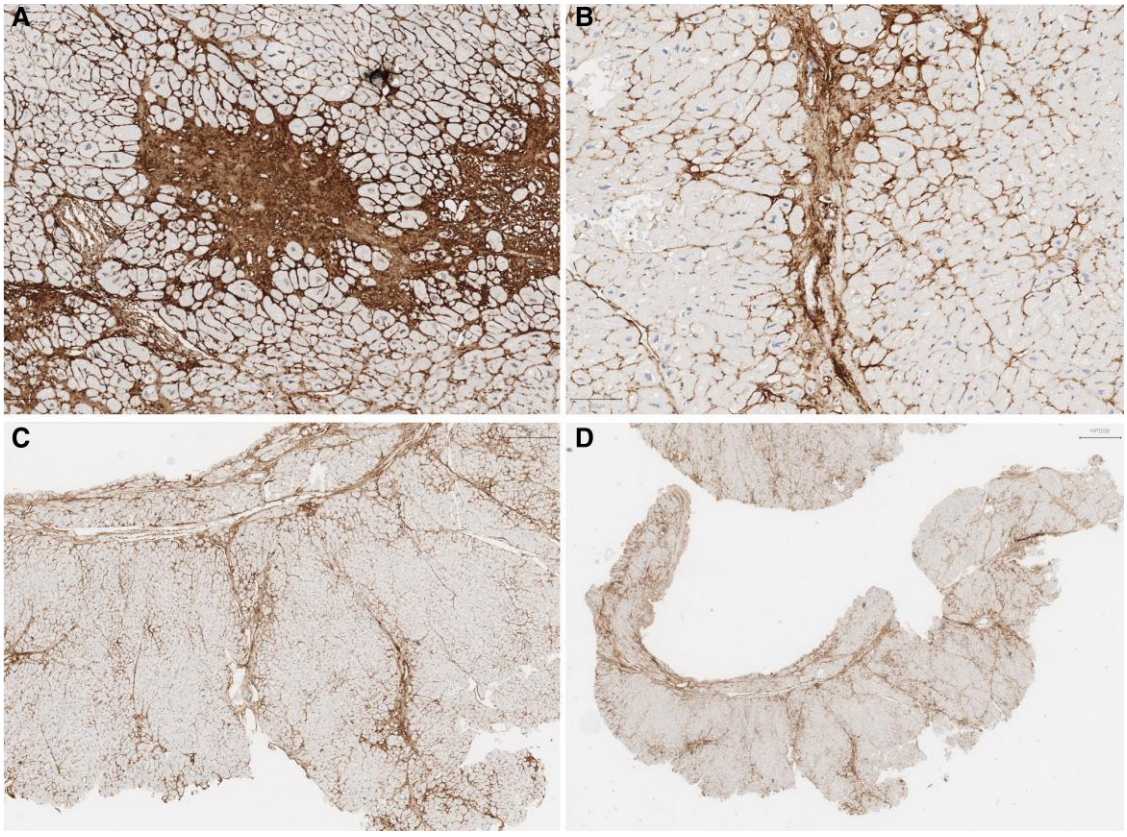


Figure 4 (*A–D*) Type I collagen immunohistochemistry. In *A* (×100), there is positivity at an area of coalescent fibrosis, previously identified at Masson's Trichrome stain. From *B* to *D* (same case at ×40, ×20 and ×10), there is centripetal distribution of positive staining, towards the intramyocardial vessel web.

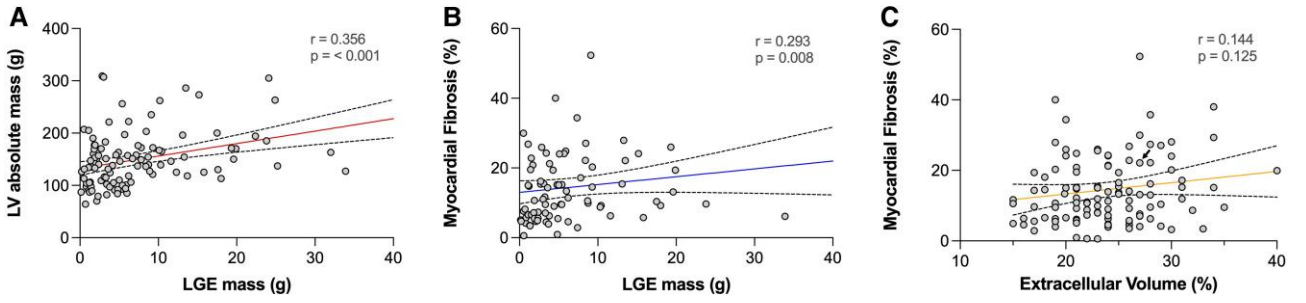


Figure 5 Correlations amongst LV mass and LGE as assessed by CMR (*A*) and between invasive MF quantification and CMR-derived quantifications of LGE (*B*) and ECV (*C*). The arrow in *C* refers to the patient in *Figure 6*.

stages of LV geometric remodelling, as previously demonstrated.[8,24] These significant correlations are probably related to our findings in what concerns troponin levels. Despite the absence of a single blood biomarker that reflects specific single tissue remodelling and fibrosis, troponin may reflect cardiomyocyte ongoing injury and necrosis, which stands the basis for replacement fibrosis and LGE.

We found that MF correlates with LGE mass, as expected from two distinct parameters that are evaluating the same pathophysiological process. This is in line with previous studies,[3,25] and here, we considered two main implications: (i) there is a correlation between replacement and diffuse type of MF; and (ii) LGE assessment may be interpreted as a whole heart biopsy, reflecting fibrosis at EMB. Even so, it should be noted that the invasive assessment is mostly reflecting the sub-endocardial layer and may be limited by sampling bias. Some of the structural findings, including MF quantification, may have been influenced by basal septum location (increased and asymmetric wall thickening; higher sub-endocardial pressure determined by the higher ratio of LV cavity at the basal segments). For the sake of EMB representativity,

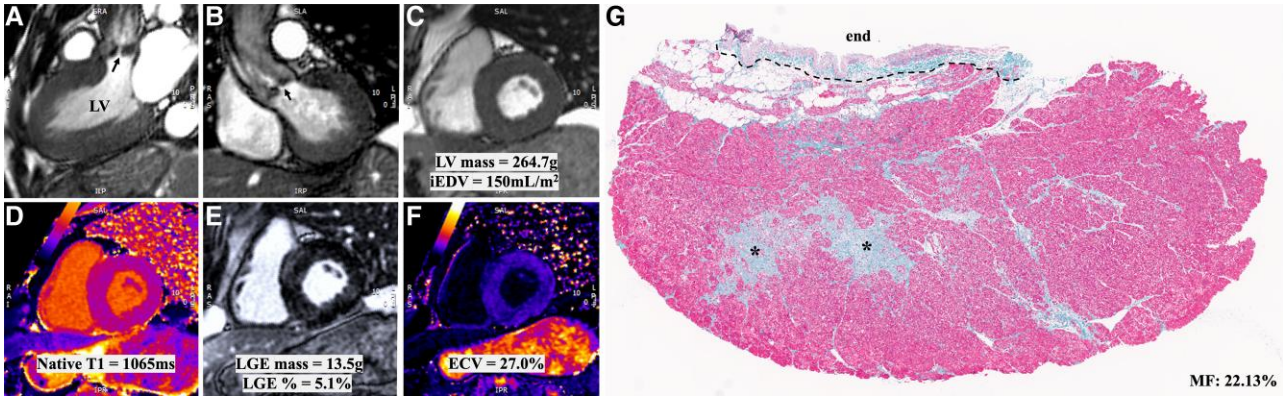


Figure 6 Co-registered pre-operative CMR data and invasive quantification of MF from one of the patients of the cohort (marked with an arrow in *Figure 5C*). * in *G* represents areas of large coalescent substitution fibrosis.

we tried to overcome some of these sampling issues with a more distal LV biopsy in some of the cases, and our structural findings were nearly the same.

Except for septal ECV, MF quantification did not correlate with global ECV, and we could not confirm previous validation data of this measurement for the assessment of MF.[26] As previously described, a significant overlap in ECV fraction values between patients with distinct AS severity and healthy volunteers may exist.[27] Differences from our cohort regarding the severity of AS, symptomatic status, concomitant ischaemic cardiomyopathy, LV dysfunction, and magnitude of invasive MF (some cohorts reporting more than 30%) may also explain our findings. The vascular compartment and perfusion status of our cohort, which was not specifically evaluated, may also have interfered with T1 values and MF correlation, as previously proposed by Mahmod *et al.*[28] Indeed, our previous discussion towards abnormal intramyocardial vessels at EMB may be part of this interplay.

Contrary to one previous publication,[29] T2 mapping had no added value for multi-parametric CMR characterization. This was supported by the absent inflammatory infiltrate at histology and completely normal T2 values.

According to previous investigations,[30] we demonstrated that the combination of multiple CMR-derived indexes may yield myocardial tissue characterization, but no single parameter described with certainty what was happening at the invasive histological level. As proposed by Treibel *et al.*,[3] the combination of CMR indexes may improve the identification of adverse LV remodelling and estimate the burden of histological MF. However, and for ECV in particular, we were unable to prove its direct correlation with MF. Both diffuse and replacement fibrosis might contribute to ECV expansion, but this happens as part of a complex ECM remodelling process.

From the structural changes, we can say that cardiomyocyte adaptation and hypertrophy elapse with interaction with ECM components and immune cell mediation, with no inflammation. In this setting, it might be speculated that perfusion impairment drives the activation of fibrosis effector cells, i.e. myofibroblasts. Abnormal intra-myocardial vessels, pronounced perivascular collagen deposition with qualitative change, and features of an ischaemic insult of the cardiomyocytes at sub-endocardial location might reflect the underlying pathophysiology.

In practical terms, the assessment of LV geometric remodelling and tissue characterization, with both LGE and combined indexes for the study of the extracellular compartment at CMR, might be useful to identify patients with more advanced LV remodelling. This would better select patients benefiting from earlier valve intervention, as is being evaluated in ongoing clinical trials. As we found signs of sub-endocardial ischaemic-like changes in the absence of ischaemic cardiomyopathy, future developments on CMR perfusion protocols could be of interest. This would not probably outperform current patient stratification[31] but should enlighten the pathophysiology underlining ECM remodelling and MF.

Limitations

As a single observation in time, this study has limitations. It represents a snapshot assessment of what has been happening in the myocardium from the beginning of aortic valve disease. As a group of patients referred to our institution for surgical AVR, we did not evaluate or had access to data concerning the length of time elapsed since the beginning of symptoms or clinical indication for surgery, which might have been important to interpret heterogeneity on both ultra-structural findings and MF quantification.

The native T1 values of the cohort were compared with our institutional normal values of myocardial relaxation, as derived from healthy controls, but not from an age/co-morbidity-matched population of the cohort without AS. In this way, we were not able to conclude if the elevated T1 values were strictly related to aortic valve disease. This could have interfered with the correlation between CMR-derived data and invasive measurements. However, we believe that our results mainly reflect those from the typical clinical profile of patients with severe AS who are being referred for surgical AVR.

We did not specifically assess myocardial perfusion on pre-operative CMR. This could have been useful not only to correlate with histological changes such as vessel abnormalities and signs of myocardial ischaemia but also to interpret these last findings according to the presence of coronary artery disease and its possible independent meaning. Targeted ultra-structural evaluation of cardiomyocyte changes at the sub-endocardium could confirm the presence of an ischaemic insult through the identification of intra-cytoplasmic glycogen and organelle changes.[18]

Myocardial infiltration, oedema, and inflammation remain important potentially confounding sources of increased ECV, as imaging markers do not measure fibrous tissue directly, but the total interstitial space instead. We did not specifically assess volume status/congestion, and this could have been important to interpret both ECV correlations and T2 mapping.[32]

Lastly, technical issues might have interfered with our results in terms of comparison with previous correlation studies. 1.5T CMR is less sensitive to T1 native-derived indexes (less discriminatory power), and this could explain some of our results in the assessment of the extracellular

compartment, with possible overlap measurements when compared with studies with 3T CMR. Quantification of MF through Picrosirius red staining, a common histochemistry technique for MF, is usually overrated when compared to *Masson's Trichrome*, as this may stain other ECM components such as fibronectin. This could have also influenced our correlation results as fibronectin was extensively expressed.

As caspase-3 was not interpretable, additional markers of apoptosis (terminal deoxynucleotidyl transferase-mediated nick-end labelling), oncosis (complement 9), and autophagic cell death (ubiquitin)[33] may eventually enlighten future research. A typically perivascular marker such as fibroblast-specific protein-1 could be an alternative to CD90 in trying to identify myocardial-activated fibroblasts.[22] Targeted nuclear imaging with specific ligands for fibroblast activation protein could pursue this aim non-invasively.

Conclusions

Patients with severe AS referred for surgical treatment because of similar clinical indications and no previous history of ischaemic cardiomyopathy have diverse histopathology myocardial changes. These include intra-myocardial vessel abnormalities and signs of cardiomyocyte ischaemic insult. ECM remodelling with no obvious inflammation is ongoing, with qualitative and quantitative heterogeneity of MF. Some of these features might be characterized by comprehensive CMR protocols, but neither single nor combined imaging parameters fully describe the histological structural changes taking place.

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Supplementary data

Supplementary data are available at *European Heart Journal - Cardiovascular Imaging* online.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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