Distinct fibrosis pattern in desmosomal and phospholamban mutation carriers in hereditary cardiomyopathies

Short title: Fibrosis patterns in arrhythmogenic cardiomyopathy

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Abstract

Background: Desmosomal and phospholamban (PLN) mutations are associated with arrhythmogenic cardiomyopathy. Ultimately, most cardiomyopathy hearts develop significant cardiac fibrosis.

Objective: To compare the fibrosis patterns of desmosomal and p. Arg14del PLN associated cardiomyopathies with the pattern in hearts with other hereditary cardiomyopathies.

Methods: A mid-ventricular transversal slice was obtained from hearts of 30 patients with a cardiomyopathy with a known underlying mutation, and from 8 controls. Fibrosis and fatty changes were quantitatively analyzed using digital microscopy.

Results: Hearts from patients with desmosomal mutations (n=6) showed fibrosis and fibrofatty replacement in the left ventricle (LV) outer myocardium, mainly in the posterolateral wall, and in the right ventricle. A similar phenotype, but with significantly more severe fibrotic changes in the LV, was found in the PLN mutation group (n=8). Cardiomyopathies associated with lamin A/C (n=5), sarcomeric (n=8) and desmin (n=3) mutations all showed a different pattern than the desmosomal and PLN mutation carriers. The posterolateral LV wall appeared to be the most discriminative area with fibrosis and fatty changes predominantly at the outer compact myocardium in 13/14 (93%) hearts with desmosomal and PLN mutations, in 0/13 (0%) hearts with lamin A/C and sarcomeric mutations and in 1/3 (33%) desminopathy hearts ($p<0.001$).

Conclusion: Desmosomal- and PLN-associated cardiomyopathies have a distinct fibrosis pattern as compared to other hereditary cardiomyopathies. The posterolateral LV wall appeared to be the most discriminative region between mutation groups. These results may provide a roadmap for cardiac imaging interpretation and may help in further unraveling of disease mechanisms.

Keywords: heart, cardiomyopathy, fibrosis, genetics, mutation, histology

Introduction

Hereditary cardiomyopathies are disorders leading to declined functionality of the heart muscle and have been associated with different pathogenic genetic mutations.[1] Currently patients with cardiomyopathy are categorized based on their clinical presentation and phenotype. Cardiomyopathies are grouped in broad descriptive categories, and are classified as arrhythmogenic (ACM), dilated (DCM), hypertrophic (HCM), restrictive and left ventricular non-compaction cardiomyopathies.

In recent years knowledge about the underlying pathogenic mutations has increased dramatically. Knowledge about mutated genes in an individual patient can help to determine prognosis and to optimize treatment.[2, 3] In addition, identification of pathogenic mutations in probands enables appropriate cascade screening in their frequently still asymptomatic family members. Cascade screening contributes to assessment of disease predisposition and may be important for risk stratification. Therefore it has been recognized that more comprehensive nosology is needed for advanced genotype-phenotype correlation.[2, 4, 5] We hypothesized that more knowledge of the pattern of pathological changes on the tissue level observed in different groups of mutations, will contribute to further improvement of this correlation.

Histologically observed cardiac fibrosis is a hallmark feature seen in most cardiomyopathies. A multitude of mechanisms accompanied with stress and injury can cause changes in the extracellular matrix leading to increased production of collagen by interstitial fibroblasts. Fibrosis is a major cause of cardiac stiffness, diastolic and systolic impairment, arrhythmias, and can eventually lead to heart failure.[6] Knowledge about the pattern of fibrosis in relation to the pathogenic mutation will improve further detailed classification of cardiomyopathies and may eventually lead to better understanding of disease mechanisms.

Desmosomal and phospholamban (PLN) mutations are notorious for their association with life-threatening ventricular arrhythmias in ACM. We recently demonstrated that p. Arg14del in PLN has a specific pattern of fibrosis in the heart that is independent of clinical presentation.[7, 8] This p. Arg14del

mutation is a founder mutation in the Netherlands and is the most frequently observed single individual pathogenic mutation in ACM in this country.[2, 9, 10] The aim of this study was to compare the distribution pattern of desmosomal- and p. Arg14 del PLN- associated cardiomyopathies with the pattern in cardiomyopathies associated with other pathogenic mutations.

Materials and Methods

The study met the criteria of the code of proper use of human tissue that is used in the Netherlands. The study was approved by the scientific advisory board of the biobank of the University Medical Center Utrecht, Utrecht, the Netherlands (protocol no. 12/387).

Thirty hearts with cardiomyopathy and a known cardiac pathogenic mutation were obtained at transplantation (n=24) or autopsy (n=6). At the time of transplantation or death all patients had severe heart failure with reduced LV ejection fraction. Initially, at the time of clinical presentation, the cardiomyopathy of these patients had been classified as DCM, ACM or HCM by their cardiologist. For the initial diagnosis ACM the Revised Task Force criteria were used.[11] Four non-cardiomyopathy hearts served as control group: three donor hearts rejected for transplantation and one heart obtained at autopsy of a patient with a non-cardiac cause of death. Another four explanted hearts from patients with ischemic heart disease served as a second positive control group with fibrosis.

We used a systematic methodology for high resolution digital cardiac fibrosis quantification as previously described (supplementary methods and supplementary figure 1).[7] In short fibrosis and fatty changes were quantified in microscopic slides from a total midventricular heart slice. For comparison between different mutation groups the heart was divided into 6 regions for the left ventricle (posterolateral, posterior, septal, anterior, anterolateral, and lateral part) and 2 regions for the right ventricle (posterior and anterior).

In addition to the quantification, the measured fibrosis of the LV (including septum) of every heart was divided into 4 categories: (sub)endocardial fibrosis [fibrosis of the (sub)endocardium, mural

thrombi were not measured], interstitial / perivascular fibrosis [fibrosis with diffuse distribution in between cardiomyocytes / increased perivascular fibrosis], replacement fibrosis [replacement of areas of myocardium by connective tissue] and fibrofatty replacement [replacement of myocardium by fibrosis and adipocytes]. The relative contribution of each category to the total amount of fibrosis was estimated (observers SS and AV).

Statistical analysis

Statistics were performed using IBM SPSS Statistics (IBM Corporation, Armonk, New York, United States). Categorical data were compared using a Fisher's exact test. The Mann-Whitney test was used to compare continues variables between 2 groups. The Kruskal-Wallis test was used to compare continues variables between multiple groups. A p-value of <0.05 was considered significant.

Results

Patients were grouped in functional groups of gene mutations: desmin filament network, PLN (calcium handling regulator), desmosome, nuclear envelope and sarcomeric (table 1). The fibrosis pattern of the hearts of 6/8 p. Arg14 del PLN patients has been described before.[7]

Patient characteristics

Patient characteristics are summarized in table 2. The mean age was 36 ± 15 years; 18 patients (60%) were male. Before heart transplantation or death all patients had a severely reduced LV ejection fraction and suffered from severe heart failure. The initial clinical diagnosis that was noted in the medical file by the treating cardiologist (ACM, DCM or HCM) at the time of the cardiomyopathy diagnosis varied among groups (table 2). Half of all patients (47%) had previous implantation of a left ventricle assist device (LVAD) before the heart was obtained at transplantation or autopsy. On routine coronary angiography for pre-heart transplant / LVAD implantation evaluation, two patients

had coronary artery disease for which single-vessel percutaneous coronary intervention had been performed (1 with a PLN mutation and 1 with a lamin A/C mutation).

The total amount of fibrosis

The total amount of fibrosis at the midventricular level per condition in both ventricles is shown in figure 1. The four controls without heart disease revealed almost no fibrosis (median [IQR] 3% [2-4]). On average the hearts with a desminopathy (25% [24-26]) and the hearts with a p. Arg14del PLN mutation (25% [22-36]) had the most fibrosis, followed by the sarcomeric gene mutations (20% [16-28]), desmosomal mutations (17% [14-26]) and the lamin A/C mutations (14% [8-30]; p=0.67 between mutation groups; p<0.001 for control versus cardiomyopathy).

Distribution of fibrosis and adipose tissue

We observed distinct distribution patterns of fibrosis and adipose tissue that are related to mutation groups (table 3). The desmosomal and p. Arg14del PLN groups revealed a different pattern than the other mutations groups with fibrofatty replacement in the RV and epicardial fibrosis and fibrofatty replacement in the LV, most pronounced in the posterolateral wall. The p. Arg14del PLN hearts showed significantly more fibrosis in the LV free wall than hearts in the desmosomal group (p=0.02), whereas a trend of more fatty changes in the RV of the desmosomal group than in the p. Arg14del PLN group was observed (p=0.08; table 3).

Examples of Masson's trichrome stains of the myocardium of the LV per condition are shown in figure 2. The stains in figure 2 show that the fibrosis was not evenly distributed throughout the LV wall. Masson's trichrome stains of all patients, including the exact genetic mutation, and normal controls are shown in supplementary file 2. Schematic overviews of the distribution of fibrosis and adipose tissue for each layer in the left ventricular free wall (trabecular layer, inner compact myocardium, outer compact myocardium) per condition are shown in figure 3. The average percentage of fibrosis per layer in the different mutation groups is shown in supplementary figure 3.

Schematic overviews and Masson's trichrome stains of the controls with ischemic heart disease are shown in supplementary figure 4. In supplementary figure 5 the schematic overviews of the distribution of fibrosis in the subgroups of the heterogenous sarcomeric group are presented.

Types of fibrosis

The different types of fibrosis per mutation group in the LV are shown in figure 4. Interstitial fibrosis contributed significantly to total fibrosis in all mutation groups. Fibrofatty replacement contributed significantly to total fibrosis in the desmosomal, p. Arg14del PLN and desminopathy groups. Replacement fibrosis was most abundantly present in some hearts of the sarcomeric group, especially in the hearts with a MYBPC3 mutation that first presented with a hypertrophic cardiomyopathy. In all studied hearts some (sub)endocardial fibrosis was observed.

Posterolateral wall of the left ventricle

The posterolateral wall of the LV was the most distinctive area when trabeculated (endocardial), inner compact and outer compact (epicardial) myocardium were compared. Therefore subgroup analysis was performed in this region where these three layers of the wall were compared and divided into 3 groups: fibrosis and fatty changes epicardial > endocardial, epicardial < endocardial (more than 5% difference) and epicardial = endocardial (difference ≤5%). Strikingly, 5/6 (83%) hearts of the desmosomal and all hearts in the PLN (8/8) group showed more fibrosis/fatty changes in the epicardial than in the endocardial area. In one heart of the desmosomal group 1 slide was missing in the posterolateral area, in this heart the areas in the 2 adjacent slides were taken to make this calculation. In all hearts of the Lamin A/C group (5/5) the opposite pattern was observed with more fibrosis in the endocardial area than in the epicardial area. 7/8 (88%) of the hearts with a sarcomeric mutation also had more endocardial than epicardial fibrosis. In the desminopathy group 1/3 (33%) hearts showed more epicardial than endocardial alterations, whereas the other 2 hearts did not reveal differences between both wall layers. The latter can be explained by the fact that fibrosis in

desminopathy hearts is more pronounced in the anterior and posterior wall than in the posterolateral wall. Differences among mutation groups were significant ($p<0.001$), results are shown in supplementary figure 6.

Discussion

By applying our previously developed method for high resolution systematic digital histological quantification of cardiac fibrosis and adipose tissue, we aimed to unravel the distribution pattern of cardiac fibrosis in desmosomal and p. Arg14del PLN cardiomyopathy hearts and compare this pattern with fibrosis in other hereditary cardiomyopathies. The study has two important results: first, we found that cardiomyopathies associated with desmosomal or the p. Arg14del PLN mutations, have a distinct fibrosis pattern. Especially the posterolateral wall of the LV appeared to be highly discriminating. Second, p. Arg14del PLN cardiomyopathy hearts revealed significantly more fibrosis in the LV free wall than hearts with desmosomal mutations. Both desmosomal and p. Arg14del PLN mutations are notorious for their association with life-threatening ventricular arrhythmias.

Desmosomal mutations

Desmosomes are located in the intercalated discs that connect cardiomyocytes. Desmosomal mutations have been associated with ACM that was first described as fibrofatty replacement of the right ventricle (RV) in the classical triangle of dysplasia consisting of the right ventricular inflow tract, outflow tract and apex. Later it was recognized that ACM is not limited to the RV and that the LV is frequently involved not only in end stage disease, but also in early stages.[12, 13] In addition, desmosomal ACM may show predominant LV involvement, particularly in desmoplakin mutation carriers.[14] In the present study patients with desmosomal mutations revealed RV fibrofatty changes and fibrosis with fatty changes in the outer part of the LV wall, predominantly in the posterolateral part. Our observations in the desmosomal group are in line with earlier observations in autopsy studies from ACM patients with unknown mutations.[15] Also in transgenic mouse models this pattern

of fibrosis has been described.[16] In addition, our results in the LV confirm late gadolinium enhancement in cardiovascular magnetic resonance studies that typically involves the subepicardial and midwall layers of the inferolateral region of the LV in ACM.[12-14]

Phospholamban mutations

We recently demonstrated that hearts from patients with a p. Arg14del PLN mutation also have a pattern of RV fibrofatty replacement and LV fibrosis with fatty changes mostly in the posterolateral wall, independently of clinical presentation.[7, 8] To the best of our knowledge the present study is the first histological study showing that hearts with the p. Arg14del PLN mutation have significantly more fibrosis in the LV and a trend towards less adipose tissue in the RV as compared to hearts with desmosomal mutations. Our results confirm recent observations in a cohort of 153 Dutch ACM patients and in a combined USA and Dutch cohort of 577 patients. In these cohorts also more LV involvement in the PLN mutation patients was found compared to desmosomal mutations using electrocardiographic and imaging criteria (echocardiography, cardiac magnetic imaging, RV/LV cine-angiography).[2, 17]

Pathophysiological mechanism

The segregation we find in fibrosis patterns suggests that different groups of mutations make the cardiomyocyte vulnerable for different stressors with potential damaging mechanisms that are not evenly distributed over the various regions of the myocardium. We speculate that the pattern of predominantly RV and LV (posterolateral) epicardial fibrosis / fibrofatty replacement is induced by increased sensitivity to wall stress on the heart (figure 5). It has been demonstrated that exercise induces 125% increase in end-systolic wall stress in the RV compared with only 14% in the LV.[18] This suggests that the RV is more vulnerable for wall stress. In addition it has been demonstrated in 2-[fluorine 18]fluoro-2-deoxy-d-glucose (FDG) positron emission tomography (PET)/computed tomography (CT) studies that under fasting conditions FDG uptake is 25% higher in the posterolateral

wall as compared to the septal region.[19] It was suggested that these regional differences in FDG uptake might be due to increased myocardial wall stress with increased metabolic demand, indeed suggesting that also the posterolateral wall is exposed to relatively high wall stress.[20]

Among mutations with this pattern of RV and LV (posterolateral) epicardial fibrosis / fibrofatty replacement are desmosomal mutations that lead to instability of the intercalated disk which will make the cadiomyocytes more vulnerable for wall stress. We also observed this pattern in PLN cardiomyopathy. There are two possible pathophysiological explanations why the p. Arg14del PLN mutation would lead to increased sensitivity to wall stress. First, it has recently been described that in hearts with this mutation aggregates of remnant PLN protein are found in cardiomyocytes.[8] These aggregates replace parts of the contractile elements in the sarcolemma making the cell instable and vulnerable for mechanical stress. Second, it has been suggested that disturbed calcium handling in p. Arg14del PLN cardiomyopathy, with activation of calmodulin dependent kinase II and calcineurin A, may lead to maladaptive remodeling of the macromolecular protein complex that forms the intercalated disk.[21] This may lead to changes comparable to those in desmosomal mutations. In desminopathies we also observed predominantly epicardial fibrosis, however not concentrated in the posterolateral wall. Desmin is an important part of the intermediate filaments that give structure to cardiomyocytes. Therefore dysfunction of desmin may also make the cardiomyocytes more vulnerable for mechanical forces.

In cardiomyopathies with fibrosis predominantly in the trabecular and the inner compact myocardial layer we postulate that there might be increased energy need of cardiomyocytes (figure 5). These layers are far away from the epicardial coronary arteries making them more vulnerable for shortness of energy. Mutations in the proteins of the sarcomere or titin lead to inefficient function of the sarcomere leading to increased energy need for contraction.[1] Interestingly, in an elegant pathology study in hearts with DCM of undetermined genotype the same pattern was described with the most interstitial fibrosis in the subendocardial regions.[22] This suggests that this pattern might be a common phenotype of (idiopathic) DCM due to various causes. Also in HCM there is a mismatch of

energy demand and supply, that is enhanced by alterations in intramyocardial branches of the coronary arteries.[1]

Clinical implications

Our results may have clinical implications. Techniques of late gadolinium enhancement in cardiovascular magnetic resonance for myocardial fibrosis detection have improved dramatically in recent years and could be integrated in the monitoring and therapeutic management of many patients. In addition novel magnetic resonance techniques with or without using contrast agents look promising for cardiac fibrosis detection.[23] We observed that in the LV posterolateral wall the pattern of fibrosis and fatty changes is highly distinctive for groups of mutations. Therefore, the present study may provide a roadmap for further *in vivo* cardiac fibrosis assessment in patients. From the present series of patients we did not have magnetic resonance images available, but correlation between radiology and histology would be an interesting topic for future studies.

Limitations

Our study is limited by the fact that groups of hearts with mutated genes are small. However, our study is a start of systematic analysis of hearts with known mutations. We think that in coming years cardiovascular pathologists around the world can work in collaboration with cardiologists and clinical geneticists to create databases and biobanks of cardiomyopathy patients that include clinical variables, pathogenic mutations and detailed examination of cardiac tissue. For this it is important that pathologists work according to standardized protocols when examining hearts and initiatives to create such a protocol e.g. under the auspices of the Society for Cardiovascular Pathology and the Association of European Cardiovascular Pathology are urgently needed.

Another limitation is the fact that we only studied end stage disease. Future studies using hearts of sudden death cases can reveal whether these patterns are also observed in earlier stages of the disease. In addition, we studied the hearts at the midventricular level. Since changes of the

myocardium in cardiomyopathy hearts can be focal or segmental, we may have missed some structural changes.

In conclusion, the pattern of fibrosis and fatty changes in cardiomyopathy hearts with desmosomal and PLN mutations is distinctive from other genetic cardiomyopathies. The posterolateral wall of the LV appeared to be the most discriminating area for the identification of the different groups of mutations. PLN hearts revealed significantly more fibrosis than hearts with desmosomal mutations in the left ventricular free wall.

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Disclosure/Conflict of Interest

None.

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Figures

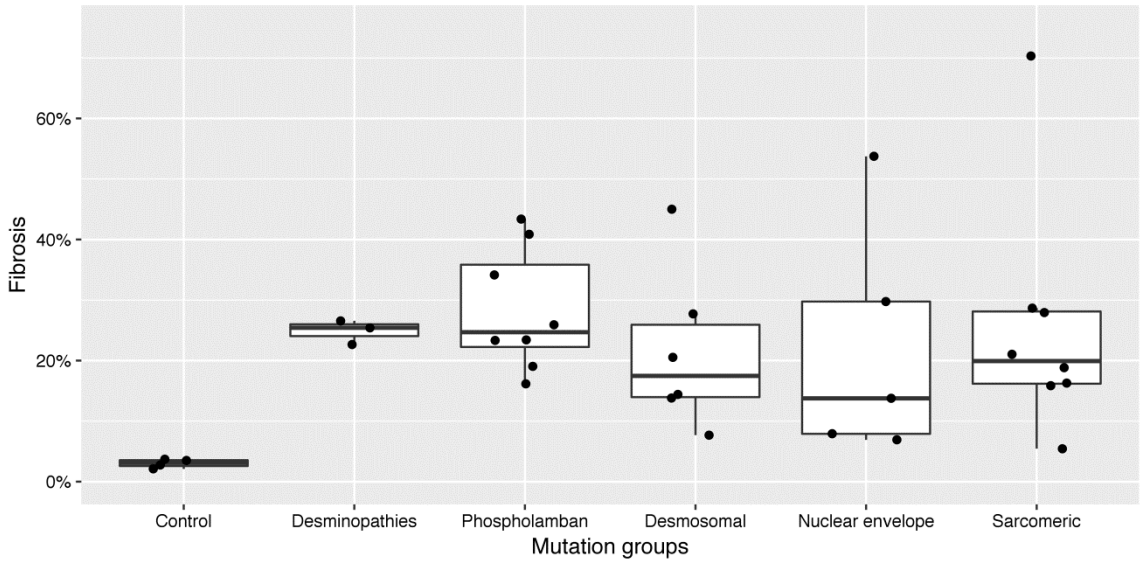

Figure 1. Boxplots of mean percentage of fibrosis in both ventricles in the different mutation groups. Dots show individual data values. P=0.67 between mutation groups. P<0.001 for control versus cardiomyopathy.

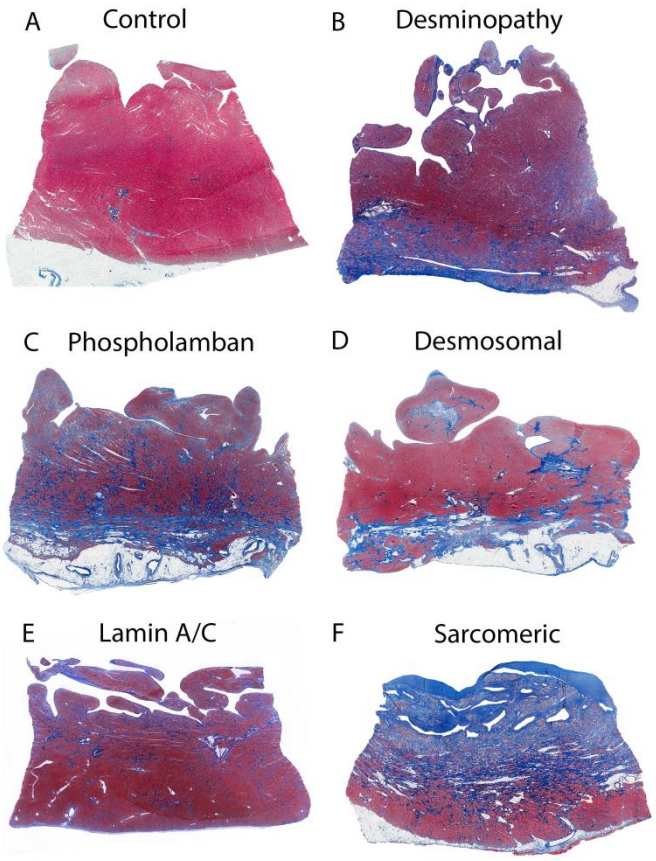

Figure 2. Histological findings in control heart and hearts with a pathogenic mutation. Control heart with almost no fibrosis (A); desminopathy heart (B), phospholamban (C) and desmosomal (D) mutations with fibrosis in the outer layer of the myocardium; hearts with a mutation in the genes encoding lamin A/C (E) and sarcomeric (F) proteins with trabecular fibrosis and fibrosis in the inner layer of the compact myocardium. The slides were stained with Masson's trichrome that stains fibrosis in blue, cardiomyocytes in red and adipocytes in white.

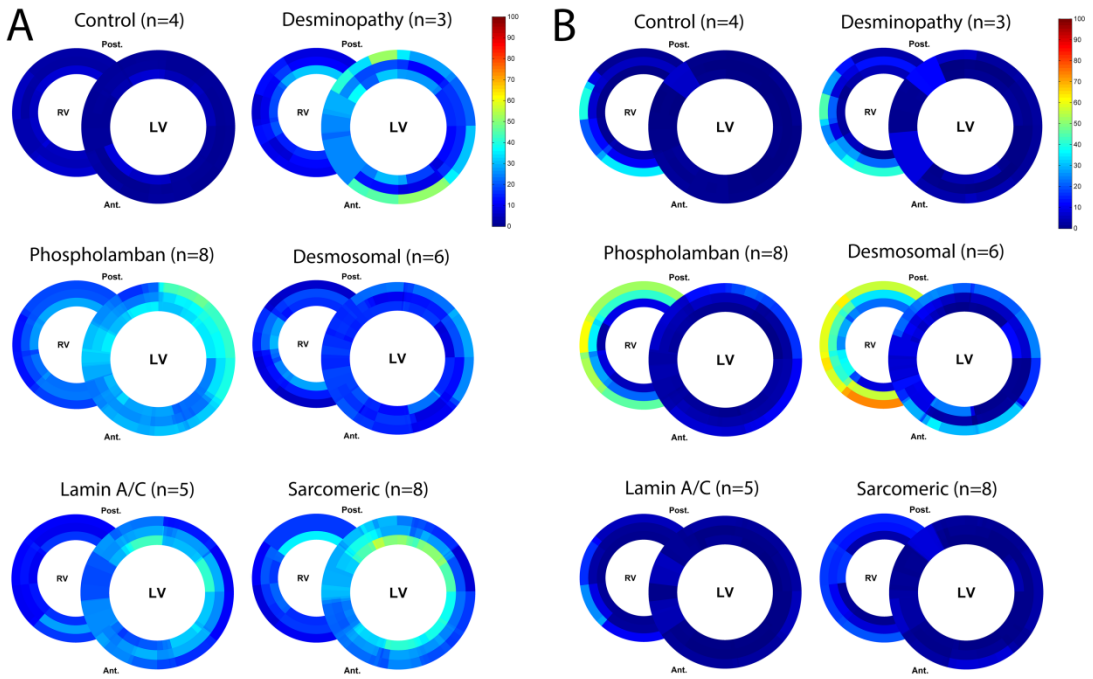

Figure 3. Schematic overview of average fibrosis (A) and adipose tissue (B) in the myocardium of heart slices of control hearts and the different groups of mutations. The results of the digital quantification in mean percentage of fibrosis and adipose tissue per group are shown using a colorscale. LV, left ventricle; RV, right ventricle; Ant., anterior; Post., posterior.

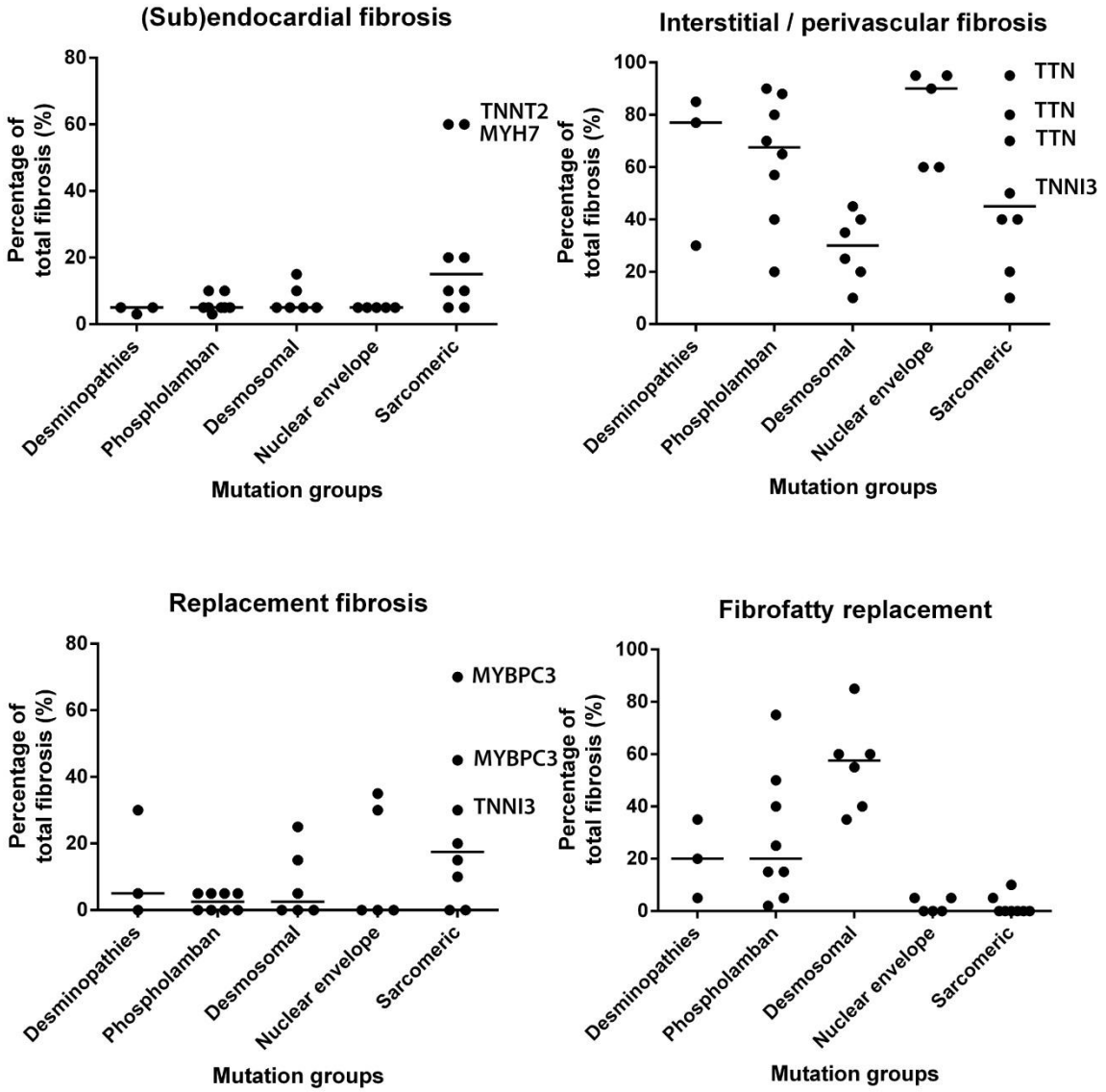

Figure 4. Relative contribution of different types of fibrosis to total fibrosis. The graphs show the contribution of each subtype of fibrosis to the total fibrosis in the left ventricle including septum. In the heterogeneous sarcomeric group the genes with the pathogenic mutation are shown for the highest values per graph.

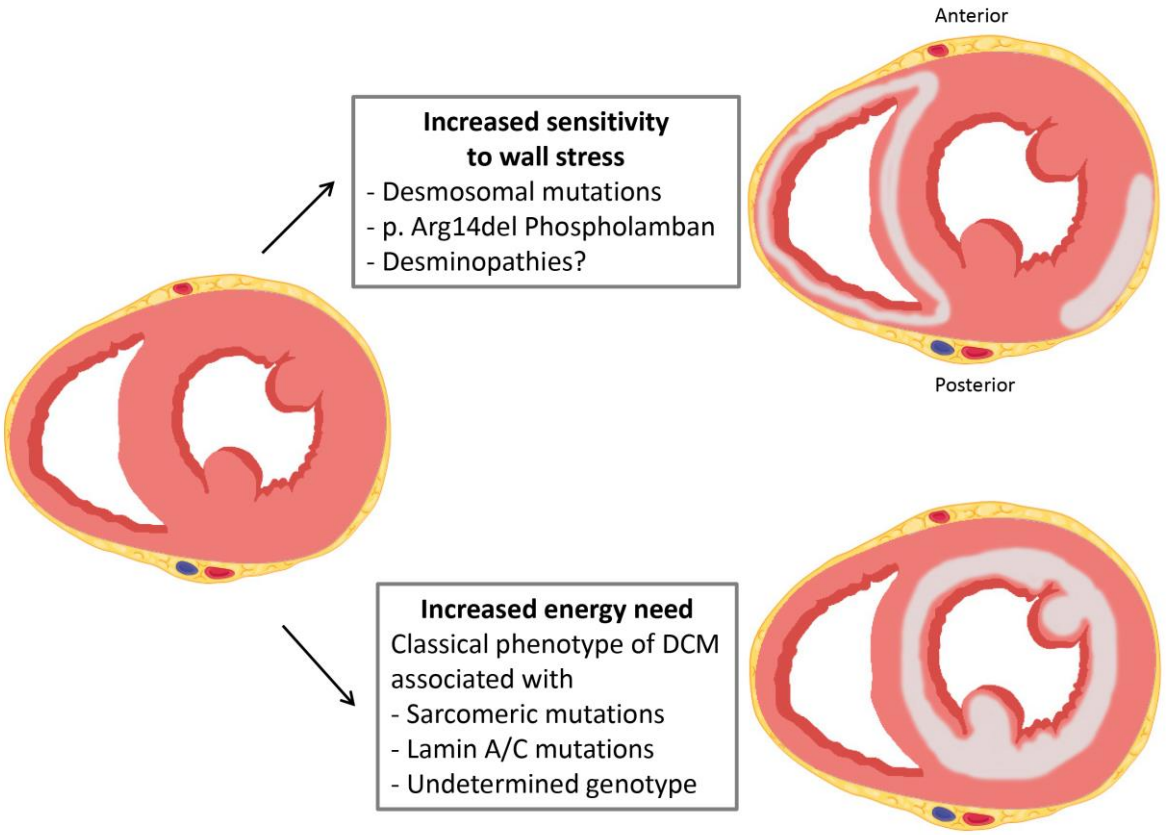

Figure 5. Hypothesis of pathophysiological mechanism.

The segregation in fibrosis patterns suggests that different groups of mutations make the cardiomyocyte vulnerable for different stressors with potential damaging mechanisms that are not evenly distributed over the various regions of the myocardium. We speculate that the pattern of predominantly RV and LV (posterolateral) epicardial fibrosis / fibrofatty replacement is induced by increased sensitivity to wall stress on the heart. In cardiomyopathies with fibrosis predominantly in the trabecular and the inner compact myocardial layer we postulate that there might be increased energy demand of cardiomyocytes. These layers are far away from the epicardial coronary arteries making them more vulnerable for shortness of energy. Figure prepared using templates from the Servier medical art website (http://servier.com/Powerpoint-image-bank).