A potential link between peroxisome proliferator-activated receptor signalling and the pathogenesis of arrhythmogenic right ventricular cardiomyopathy

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Received 6 February 2009; revised 5 May 2009; accepted 1 June 2009; online publish-ahead-of-print 4 June 2009

Time for primary review: 50 days

Aims Arrhythmogenic right ventricular cardiomyopathy (ARVC) is characterized by major fibro-fatty replacement of the right ventricle (RV). We hypothesized that changes in peroxisome proliferator-activated receptor (PPAR) signalling contributed to myocardium fatty accumulation and contractile dysfunction in ARVC.

Methods and results Real-time quantitative reverse transcriptase-polymerase chain reaction and western blotting were used to assess cardiac expression of PPARα and γ and two of their downstream target genes—medium-chain acyl-CoA dehydrogenase (MCAD) and phosphoenolpyruvate carboxykinase (PEPCK)—in both RV and left ventricle (LV) from five controls and five ARVC patients. In vitro motility assays were used to analyse functional properties of myosin. In the RV, sliding velocity was nearly two-fold lower in ARVC than in controls, whereas a 10% reduction in velocity values was noted between ARVC and non-failing myocardium in the LV. In controls, PPARα and MCAD mRNA and protein levels were higher in the RV compared with the LV. In ARVC, the expression of PPARα and MCAD mRNA and/or proteins was decreased in both RV and LV. RV from ARVC was also characterized by a dramatic activation of the PPARγ regulatory pathway, as attested by the increase in PPARγ mRNA and protein (500 and 270%, respectively, each P < 0.001) and by the induction of PEPCK gene. In contrast, the LV of ARVC heart exhibited no changes in the expression of the PPARγ regulatory pathway compared with control.

Conclusion ARVC is associated with major disturbances in the PPARα and PPARγ signalling pathway in the RV that may contribute to intracellular lipid overload and severe myosin dysfunction.

KEYWORDS
Cardiomyopathy; Contractile apparatus; Heart failure; Lipid signalling

1. Introduction

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a form of cardiomyopathy primarily affecting the right ventricle (RV) and characterized by major fibro-fatty replacement of the myocardium.1 ARVC is a clinically heterogeneous disease, which typically manifests as life-threatening ventricular arrhythmias in apparently healthy young adults. Progressive left ventricular (LV) dysfunction has been found to occur in at least 20% of cases,2 and in the severe forms, the disease may require heart transplantation.1

The pathogenesis of the disease is still largely unknown. Genetic studies have located 11 different loci associated with ARVC and mutations in eight genes have been identified, pointing out a high genetic heterogeneity.3 Understanding of ARVC pathogenesis mostly relies on patient heart examination.4 Histological findings suggest that the lipomatous replacement occurs in the context of cardiac myocyte loss due to metabolic, ultrastructural, inflammatory, or apoptotic injuries.5 Progressive lipid accumulation within the cardiomyocyte has been observed in ARVC6 and is thought to contribute to the pathogenesis. In fact, myocardial lipid accumulation is probably involved in contractile dysfunction and in the generation of cardiac arrhythmias in several other pathological states.7,8

The molecular events underlying myocardium fatty infiltration are presently unknown. In the last 10 years, the peroxisome proliferator-activated receptor (PPAR) family of ligand-activated transcription factors has been
shown to play a paramount role in regulating cellular lipid metabolism. It is now established that the alpha isoform of PPAR regulates cardiac fatty acid utilization pathways, through transcriptional control of numerous genes involved in fatty acid uptake and oxidation. Knockout mice studies have demonstrated that PPARα acts as a ‘lipostat’ to regulate cardiac lipid homeostasis, and impairments in heart PPARα gene regulatory pathways have been characterized in various mouse models. However, few data are available on PPARα expression and activity in the diseased human heart. PPARγ, another member of the PPAR family, is highly expressed in tissues involved in lipogenesis, in particular in the white adipose tissue in which it promotes cellular differentiation and fat storage. Consistent with the limited lipogenesis of cardiomyocytes, the mouse heart exhibits low levels of PPARγ. In the human heart, PPARγ gene expression has been detected in the coronary arteries, aorta, LV, and atrium, but there is little information on the expression and function of PPARγ under pathological conditions.

In the present study, we obtained cardiac samples from five cases of ARVC with severe RV and LV dysfunction, and we investigated histological, functional, and molecular features in both ventricles of ARVC heart. Our main objectives were to characterize possible alterations in cardiac myosin functional properties, and associated changes in the expression of PPARs and of PPAR target genes, in RV and LV of diseased heart.

2. Methods

The investigation conforms with principles outlined in the Declaration of Helsinki. The informed consent was obtained from all patients or relatives participating in the study according to the protocol approved by the Local Ethics Committee (Consultative Committee for the Protection of Human Subjects in Biomedical Research at the Pitie´-Salpeˆtrie`re Hospital, Paris, France).

2.1 Clinical characterization of ARVC patients and cardiac specimens

The ARVC study population comprised five patients (four males/one female) aged from 13 to 74 years (Table 1). Patients met the Task Force definition of ARVC (Table 1). In particular, ARVC cardiac specimens from left and right ventricular free wall were obtained at the time of heart transplantation. For the fifth patient, ARVC cardiac specimens from right ventricular free wall were obtained during cardiac surgery. Analysed ARVC samples typically presented RV myocardium with fatty infiltration (Figure 1A).

Table 1 Clinical characteristics of the patients

<table>
<thead>
<tr>
<th>Age (year)</th>
<th>Gender</th>
<th>LVEF (%)</th>
<th>RV dilation and/or reduced RVEF (%)</th>
<th>Task Force criteria</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Structural Depolarization Repolarization Arrhythmia Tissue/histology Family history</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>NA</td>
<td>Yes Major Major Minor None Major None</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>M</td>
<td>&lt;45</td>
<td>Yes Major Major Minor None Major None</td>
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</tr>
<tr>
<td>49</td>
<td>M</td>
<td>NA</td>
<td>Yes Major Major Minor None Major None</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>&lt;45</td>
<td>Yes Major Major Minor None Major Yes</td>
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<tr>
<td>48</td>
<td>M</td>
<td>&lt;45</td>
<td>Yes Major Major Minor None Major None</td>
<td></td>
</tr>
</tbody>
</table>

NA, not available.

Non-failing RV and LV specimens, obtained from five cardiac donors (three males/two females, mean age ± SD 41 ± 7 years) without previous medical history or any abnormalities in ECG and echocardiography (LV dimensions/contractility within normal ranges), were used as controls (Table 2). They were rejected for transplantation for other reasons than depressed cardiac function. All specimens were shock-frozen in liquid nitrogen immediately after heart removal and cryo-conserved at −80°C for further analysis.

2.2 Morphometry and immunolabelling

For histology, 5 μm paraffin sections of cardiac tissue were stained with haematoxylin and eosin and Mason’s trichrome. Frozen sections were stained with oil red O and counterstained with haematoxylin to detect neutral lipids. Anti-α-actin (Sigma, France), MLC1 antibodies (Biocytex, France), FITC-labelled anti-mouse IgG antibodies (Amersham, France), and 4',6-diamidino-2-phenylindole (Vector Laboratories, CA, USA) were used for immunolabelling. Intensity of myosin labelling was quantified using Metamorph. For each section, three to four fields were randomly selected. Segments representing muscle fibres were identified and a computer was programmed to calculate the average intensity of the stained regions. The results are given in arbitrary units. A minimum of five fields/heart were measured.

2.3 Preparation of human cardiac myosin and rabbit actin filaments

Myosin was isolated from the ventricular muscular specimens as described previously and used within 48 h of the experiments. Actin was prepared from rabbit back muscle and fluorescently labelled with tetramethylrhodamine-phalloidin (Molecular Probes).

2.4 In vitro motility assays

In vitro motility assays were performed at 30°C as described previously. Videotape recordings were made when >85% of all actin filaments over myosins were moving continuously within a visual field and analysed using RETRAC program (N. Carter, The Marie Curie Research Institute, UK).

2.5 ATPase assays

Actin-activated myosin ATPase activity was determined at 21°C. The maximal actomyosin ATPase activity (Kcat, in s−1) and the association constant of myosin for actin (K0) were determined from a double-reciprocal Lineweaver–Burk plot of the ATPase rate vs. actin concentration.

2.6 Real-time quantitative polymerase chain reaction

Total RNAs were isolated from quick-frozen heart samples using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA samples treated with RNase-free DNase I (Ambion, UK)
were reverse-transcribed into cDNA using the kit from Invitrogen and quantified using the SYBR Green I kit from Roche Diagnostics (Mannheim, Germany). Real-time quantitative polymerase chain reaction (RTQ-PCR) was performed using a Light Cycler instrument (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. RTQ-PCR primers (Table 3) were designed using the sequences available in GenBank and spanned an intron/exon boundary. The amounts of the various mRNAs were normalized to the amount of 18S ribosomal RNA measured by RTQ-PCR in each sample. The results of RTQ-PCR are given in arbitrary units.

2.7 Western blot analysis

Protein extracts of RV and LV samples from control or ARVC patients (fat free) were prepared in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 0.1% SDS, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride) according to standard methods. Protein concentration was determined using the Bradford method. Twenty to 40 μg of total protein per lane was resolved by 10% SDS-PAGE and transferred to Hybond-P PVDF membrane (Amersham Biosciences, Freiburg, Germany). The following antibodies were used for normalization: rabbit polyclonal anti-PPARα (Rockland, Gilbertsville, PA, USA), anti-PPARγ (Rockland), anti-medium-chain acyl-CoA dehydrogenase (MCAD) (kindly provided by D.P. Kelly, St Louis, MO, USA), anti-phosphoenolpyruvate carboxykinase (PEPCK) (Cayman Chemical, Ann Harbor, MI, USA), and calsequestrin (Affinity Bioreagent, St-Quentin-en-Yveline, France). The primary antibodies were used at a dilution of 1:1000 or 1:2000 and detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG, and the chemiluminescent reagent, ECL (Amersham Biosciences, Freiburg, Germany). Intensities of immunoreactive bands were measured by computerized densitometry. The results are characteristic of at least two independent experiments.

2.8 Statistical analysis

Data are expressed as mean ± SD. In each control or ARVC patient, the mean velocity was calculated from 50 actin filaments. Differences between groups were analysed by one-way ANOVA and the Fisher test. In addition, the Kolmogorov–Smirnov test was used to analyse potential difference between velocity distribution in control and ARVC myosins. A value of \( P < 0.05 \) was considered significant.

3. Results

3.1 Morphological analyses

Representative control and ARVC myocardium specimens are illustrated in Figure 1. In ARVC, the right myocardium showed extensive fatty replacement of the myocardium, whereas LV involvement was characterized by a diffuse increase in interstitial fibrosis (Figure 1A and B). Surviving RV myocardium exhibited severe fatty infiltration of myocardial cells that was not observed in the RV from control subjects (Figure 1B). In the LV, there was a trend for increased intramyocardial lipid deposition in ARVC patients, whereas sections from controls were negative for neutral lipid accumulation (Figure 1C).

<table>
<thead>
<tr>
<th>Age (year)</th>
<th>Gender</th>
<th>ECG</th>
<th>Contractility and ventricular dimensions at echocardiography</th>
<th>Diagnosis</th>
<th>Medicine before death</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>M</td>
<td>Normal</td>
<td>Normal</td>
<td>Head trauma</td>
<td>Dopamine 26 h</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>Normal</td>
<td>Normal</td>
<td>Cerebral stroke</td>
<td>Dobutamine–dopamine 4 h</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>Normal</td>
<td>Normal</td>
<td>Head trauma</td>
<td>Dopamine 1 h</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>Normal</td>
<td>Normal</td>
<td>Head trauma</td>
<td>Dopamine 24 h</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>Normal</td>
<td>Normal</td>
<td>Head trauma</td>
<td>Dopamine 72 h</td>
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</tbody>
</table>
3.2 Myosin properties and distribution in ARVC and control hearts

Mean velocities of actin filament sliding over myosins from control and ARVC myocardium are presented in Figure 2A. Sliding velocity was nearly two-fold slower in ARVC than in control RV (\(P < 0.0001\)). In the LV of diseased heart, a decrease of myosin velocity values was also observed, but it was more limited in amplitude (around \(2\%\), \(P < 0.001\)) than in the RV. The effects of actin on the myosin ATPase activity for human ventricular myosin isolated from control and dysplastic myocardium are presented in Table 4. In both ventricles, the \(V_{\text{max}}\) obtained from ARVC was significantly lower than in controls. \(K_m\) values did not differ between groups.

Because abnormalities in MLC1 may reduce \textit{in vitro} myosin sliding velocity,\textsuperscript{27} the distribution of MLC1 was assessed in both ARVC and control ventricles by using anti-MLC1 antibodies. As shown in Figure 2B, no changes were found in labelling intensity or distribution between ARVC and control cardiomyocytes.

3.3 Expression of PPARs and PPAR target genes in control heart ventricles

The expression levels of PPAR\(\alpha\) and PPAR\(\gamma\) genes in the RV and LV of control hearts are shown in Figures 3 and 4. PPAR\(\alpha\) mRNA and protein levels appeared higher in the RV compared with the LV. Consistent with this, gene expression and protein levels of MCAD, a typical PPAR\(\alpha\) target gene, were also found higher in RV compared with LV. Both ventricles exhibited comparable levels of PPAR\(\gamma\) transcripts and protein. Transcript levels of PEPCK, a known PPAR\(\gamma\) target gene, were quite low in both ventricles of control heart, and similar PEPCK protein levels were found in the RV and LV of control hearts.

3.4 Changes in the expression patterns of PPARs and PPAR target genes in cardiac ventricles from ARVC patients

PPAR\(\alpha\) gene expression level was markedly reduced (\(-50\%; P < 0.01\)) in the patient RV compared with controls, whereas the decrease observed in the LV was not significant (Figure 3). Marked decreases in PPAR\(\alpha\) protein levels were observed in patient RV (\(-50\%; P < 0.05\)) and LV (\(-60\%; P < 0.05\)), compared with control (Figure 4). Similarly, significant decreases in MCAD mRNA (Figure 3) and protein levels (Figure 4) were observed in both patient ventricles (RV \(-40\%, P < 0.05\); LV \(-60\%, P < 0.05\)). Altogether, these

Table 3 Primers used for RTQ-PCR

<table>
<thead>
<tr>
<th>Human genes</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR(\alpha)</td>
<td>TGAAGGCTGCAAGGGCTTTCTTT</td>
<td>CAGATCTTTGCATTGCTCCAAA</td>
</tr>
<tr>
<td>PPAR(\gamma)</td>
<td>TCTCAGAGTACACAAGGCTGACATC</td>
<td>AGGATCAAGAGTCTTCTCGG</td>
</tr>
<tr>
<td>PEPCK</td>
<td>GCTCTAGGAGGAGGAAATGG</td>
<td>TGCCTTGGAGGAGCAGTTAAC</td>
</tr>
<tr>
<td>MCAD</td>
<td>AACTGGTAGATCACTGCCATCCC</td>
<td>GGTTGACAGTCTATGGAGGG</td>
</tr>
<tr>
<td>18S</td>
<td>CGCTCAGAGGTTGAATCTCATCGG</td>
<td>GCATCGTATGGGGAACCT</td>
</tr>
</tbody>
</table>

Table 4 Enzymatic properties of myosin molecules in control and ARVC human hearts

<table>
<thead>
<tr>
<th></th>
<th>(V_{\text{max}}) (s(^{-1}))</th>
<th>(K_m) ((\mu\text{mol L}(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control RV</td>
<td>0.15 ± 0.02</td>
<td>12.6 ± 1.9</td>
</tr>
<tr>
<td>ARVC, RV</td>
<td>0.12 ± 0.02*,**</td>
<td>13.1 ± 1.4</td>
</tr>
<tr>
<td>Control LV</td>
<td>0.16 ± 0.02</td>
<td>12.2 ± 3.4</td>
</tr>
<tr>
<td>ARVC, LV</td>
<td>0.12 ± 0.02*,**</td>
<td>11.2 ± 1.8</td>
</tr>
</tbody>
</table>

Values are means ± SD. For each myosin preparation, ATPase activity measurements were made at least in duplicate. Only significant differences are figured.

\* \(P < 0.01\) vs. control RV.

\*\* \(P < 0.01\) vs. control LV.

Figure 2 Functional and structural analysis of human cardiac myosins. (A) Velocities of actin filament sliding over cardiac myosins extracted from RV and LV tissues of controls and ARVC patients  \((n = 5\) control and ARVC patients, \(n = 250\) filaments for each). \(\ast P < 0.05\), \(\ast\ast P < 0.01\), compared with the respective control group. (B) Merged images of transverse sections stained with antibodies for myosin light chain 1 (in green) and nucleus (in blue) in control and ARVC RV and LV. Scale bars 20 \(\mu\text{m}\). Mean intensities did not significantly differ between groups (RV 138 ± 25 and 144 ± 25 arbitrary units in control and ARVC, respectively; LV 142 ± 14 and 150 ± 22 arbitrary units in controls and ARVC, respectively, \(P = \text{NS}\)).
data reflected a global downregulation of PPARα regulation in patient LV and RV.

The pattern of changes observed for PPARγ in ARVC heart markedly differed from that found for PPARα. Indeed, dramatic increases in PPARγ mRNA (500%; P < 0.001) (Figure 3) and protein level (270%; P < 0.01) (Figure 4) were noted in patient RV, whereas no significant changes occurred in the LV. Upregulation of PPARγ in the RV went together with a robust induction of its target gene, as indicated by the increase in PEPCK gene expression (22.5-fold) (Figure 3) and protein level (2.8-fold) (Figure 4). In contrast, PEPCK mRNA and protein levels remained unchanged in patient LV, compared with control.

4. Discussion

Human ARVC is characterized by a progressive, fatty replacement occurring predominantly in the RV, possibly leading to severe cardiac failure. Although disease mechanisms remain poorly understood, the pathogenesis of ARVC is obviously reminiscent of a major disturbance in cardiac muscle lipid homeostasis. In the present study, we analysed a small series of ARVC heart biopsies to characterize structural and functional alterations, and to study possible changes in PPAR regulatory alterations, in patient RV and LV.

Our results point out that ARVC is associated with pronounced alterations in myosin function, revealed in particular by markedly reduced sliding velocity values in the dysplastic RV. Furthermore, a moderate decrease in sliding velocity values was noted in patient LV, in which histological observation revealed more fibrosis and larger muscular areas compared with the RV, in keeping with previous reports. Histological observation also showed fatty infiltration occurred predominantly in the RV, as reported previously. Thus, although there are a plethora of potential mechanisms of myocardial dysfunction apart from actomyosin dysfunction, myosin dysfunction was therefore clearly associated with abnormal myocardial lipid accumulation in the RV. It was shown in different animal models that alterations in lipid homeostasis can severely compromise contractile function of myocardial cells. Proposed mechanisms include direct toxic effects of lipid droplets or fatty acid species on myofibrillar function, or increased substrates for ceramide production possibly leading to the generation of reactive oxygen species and activation of the apoptotic pathways. Interestingly, it has been suggested that apoptosis might be involved in ARVC pathogenesis in children and adults. Activation of caspase-3, a key effector enzyme of myocardial apoptosis, could mediate the cleavage of several muscle cytoskeletal proteins, including the essential myosin light chain, MLC1. It was thus tempting to speculate that the reduced actin velocity observed in the right dysplastic ventricle was related to proteolytic cleavage of MLC1. However, we did not find evidence for morphological disruption of the organized MLC1 in ARVC. It remains possible that cardiac lipid accumulation induces other subtle structural changes in the myosin molecular motor that impaired its mechanical properties.

The PPAR signalling pathway clearly plays a pivotal role in the control of cardiac lipid metabolism in rodents, but there is little information on this regulatory pathway in the human heart. Our study documents the compared expression levels of PPARα and PPARγ in the RV and LV of the human heart and their possible changes under pathological conditions. In keeping with the high β-oxidation capacity, we found high expression levels of PPARα and of MCAD in both LV and RV of control heart. In contrast, PPARγ was expressed to low levels in both heart ventricles, consistent with data obtained in mice, and PEPCK mRNA levels were also faint in both ventricles of control heart.

Expression pattern of PPARs and their target genes was markedly modified in ARVC heart, compared with control. Interestingly, opposite changes in PPARα and PPARγ-dependent pathways were observed in the diseased heart. Indeed, mRNA and protein levels of PPARα and MCAD were downregulated in the RV and LV, suggesting a global impairment of fatty acid β-oxidation in both ventricles. In contrast, a dramatic increase in PPARγ and a strong induction of its target gene PEPCK were observed in the RV. Recent studies in transgenic mice showed that forced expression of PPARγ in heart resulted in severe cardiac dysfunction, possibly leading to premature death. Metabolic alterations in these animals included abnormal heart lipid accumulation, which was ascribed to constitutive stimulation of fatty acid uptake. Accordingly, cardiac lipid overload in PPARγ transgenic mice likely reflected imbalance between
fatty acid supply and mitochondrial fatty acid utilization, a situation known to promote lipotoxicity. Results obtained in our study indicate that upregulation of PPARγ and PEPCK in the patient RV occurred in the context of a decreased fatty acid β-oxidation, which could clearly favour the development of lipid accumulation. Indeed, in the white adipose tissue, upregulation of PEPCK increases the storage of fatty acids in the form of triglycerides, by promoting de novo synthesis of glycerol. This mechanism is strictly controlled by PPARγ, whose activation depends on the concentration of activating ligands, in particular fatty acids. In ARVC heart, the decreases in PPARα and β-oxidation might favour accumulation of fatty acids, which could then act as activators of PPARγ. The resulting induction of PPARγ target genes could, in turn, amplify the imbalance between fatty acid supply and their β-oxidation, creating conditions for overt lipid accumulation. Interestingly, mice knockout models of desmoplakin, one of the candidate genes for ARVC in human, exhibit a marked increase in cardiac expression of PPARγ and some of its target genes. Our results in ARVC heart also point out that the induction of PPARγ regulatory pathway was restricted to...
the RV, in which adipose phenotype was predominant. This could suggest that distinct pathogenic mechanisms operate in both ventricles, involving PPAR\(\alpha\) in the RV, but not in the LV. Alternatively, alterations in the LV, which appeared less pronounced than in the RV, might reflect milder stages of myocardium fibro-fatty conversion,\(^4\) in which expression of lipogenic markers is still efficiently repressed, as in normal myocardium.

In conclusion, compared analysis of RV and LV in a series of ARVC patients clearly suggested a decreased expression of PPAR\(\alpha\) and mitochondrial \(\beta\)-oxidation, indicating alterations in energy production in both heart ventricles. Functional studies of myosin revealed alterations in the RV, and to a lower extent in the LV, whereas PPAR\(\gamma\) overexpression was restricted to the RV. Accordingly, disruption of lipid homeostasis in the RV might be involved in the development of functional alterations. Altogether, our data appear consistent with a model of fat accumulation involving imbalance between fatty acid oxidation and fatty acid uptake. Finally, pathogenic alterations often displayed ventricular-specific patterns, pointing to the complexity of disease mechanisms in different heart areas, which will still require a lot of effort to be unravelled.

Acknowledgements
We thank the patients for their participation, and A.F.M., Dr R. Frank, and D. Charlemagne for their help in tissues collecting. We also thank Dr J. Quillard for helpful discussion.

Conflict of interest: none declared.

Funding
This work was supported by a grant from the Fondation de France. C.C. was the recipient of a ‘contrat d’interface Institut National Supérieur Enseignement et de la Recherche Médicale/Assistance Publique-Hôpitaux de Paris’, with the Service de Physiologie, Hôpital de Bicêtre, AP-HP.

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