



BASIC SCIENCE ARTICLE

Clinical utility of endomyocardial biopsies in the diagnosis of arrhythmogenic right ventricular cardiomyopathy in children

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BACKGROUND: Histomorphometry of endomyocardial biopsies is one component of arrhythmogenic right ventricular cardiomyopathy (ARVC) diagnosis, although there is a need for stricter diagnostic criteria for this disease in pediatrics. The clinical utility of biopsy analysis as a component of ARVC diagnosis was evaluated in pediatric patients.

METHODS: Histomorphometric analysis of fibrofatty infiltrate was completed on pediatric right ventricular endomyocardial biopsy samples. Myocardial replacement by fat and fibrosis was quantified. ARVC diagnosis was established using the 2010 ARVC Task Force criteria, with the biopsy measures compared across various ARVC diagnoses (definite, borderline, possible, or no ARVC). Receiver-operating characteristic (ROC) curve analysis was also completed using biopsy measures.

RESULTS: The greatest proportion of fat, fibrosis, and myocardial replacement was in the definite ARVC cohort, and was significantly larger than for the other diagnosis cohorts. ROC curve analysis (with the biopsy analysis removed from the diagnostic classification) produced cutoff values of 15 and 25% myocardial replacement, which is lower than current adult diagnosis criteria.

CONCLUSION: We propose modifications in pediatric major and minor biopsy diagnosis criteria to allow for improved sensitivity. This study suggests that biopsy analysis in children is most significant for subjects with a more severe disease presentation.

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INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a genetic form of cardiomyopathy associated with malignant arrhythmia, sudden death, and progressive heart failure. ARVC is characterized by fibrofatty infiltration of the myocardium affecting predominantly, although not exclusively, the right ventricle.^{1–4} The advent of genetic testing has significantly advanced the understanding of the pathophysiological mechanisms underlying the disease.⁵ Genetically confirmed ARVC is generally a result of mutations in the desmosomes within the intercalated disk, with numerous disease-causing mutations identified to date.^{6–11} ARVC is responsible for 11% of sudden deaths under the age of 65, and 10% of these ARVC-related deaths occur in individuals under the age of 18,¹² reinforcing the importance of accurate diagnosis in children.

An International Task Force proposed the diagnostic criteria for the diagnosis of ARVC that incorporated various clinical manifestations of ARVC.¹³ The 2010 Task Force Criteria (TFC) introduced modified criteria, with major and minor diagnostic parameters that included family history, genetics, electrophysiological findings, imaging, as well as the tissue characterization of the myocardium involving characterization of fibrofatty infiltration on endomyocardial biopsy samples.¹⁴ While characterization and quantification of the amount of fibrofatty replacement of the right ventricular myocardium in postmortem, explanted, or endomyocardial biopsy samples remained an important component of both the 1994 and 2010 ARVC TFC for diagnosis, the thresholds of myocardial replacement for major or minor criteria were derived

from adults with ARVC.^{13,14} Despite the fact that the 2010 revised TFC resulted in improved sensitivity and specificity, the diagnosis of ARVC during childhood remains difficult. Application of these adult criteria to pediatric patients may not result in appropriate specificity and sensitivity for pediatric diagnosis, supporting the need for specific criteria for the pediatric population.¹⁵ While genetic testing has resulted in an increased likelihood of fulfilling TFC in the absence of an endomyocardial biopsy, up to 2/3 of patients with ARVC remain gene-elusive.^{6,7} As such, biopsy continues to be an important consideration in the assessment of patients with possible ARVC. It is therefore important to assess the diagnostic utility of endomyocardial biopsy in children.

In this study, we completed a retrospective analysis of patients screened for suspected ARVC in whom right ventricular endomyocardial biopsy analysis was completed. We sought to quantify the degree of fibrofatty infiltration present in children undergoing biopsy analysis for the evaluation of suspected ARVC and assess the utility of histomorphometry analysis in the detection of disease. We hypothesized that pediatric biopsy samples will demonstrate lesser amounts of fibrofatty replacement than in adults.

METHODS

Patient diagnosis

The cohort was derived from patients evaluated at a single center, The Hospital for Sick Children, between 12 February 1981 and 16 March 2012. Patients were included in the study if an

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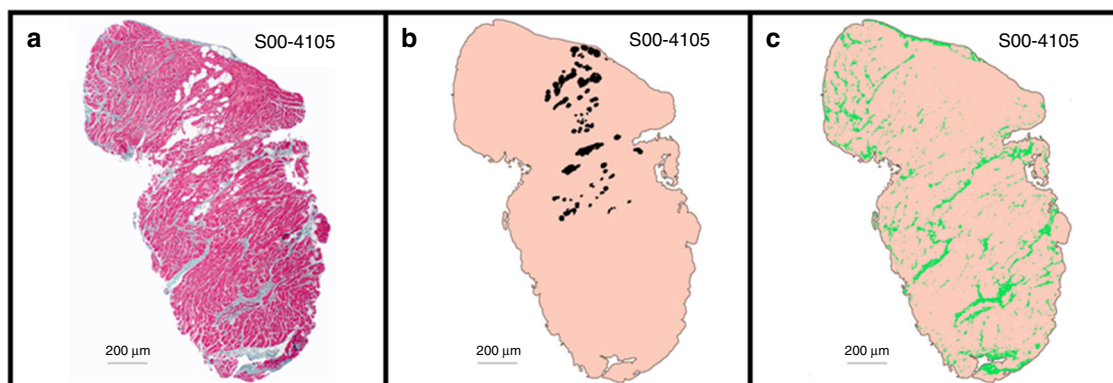


Fig. 1 Fibrofatty analysis of endomyocardial biopsies. A representative image of a biopsy sample (a), with pixels of fat in black (b), and fibrosis in green (c) colored for illustration. The total number of pixels

was counted for fat, fibrosis, and the whole biopsy, and the proportion of fat and fibrosis was expressed as a percentage

endomyocardial biopsy was performed, and morphometric analysis was completed retrospectively as outlined below. All described methods in this study were completed with research ethics approval from The Hospital for Sick Children's Research Ethics Board (REB # 0020010161).

Patients were classified into one of four ARVC diagnostic categories based on the 2010 TFC: definite ARVC diagnosis ("Definite"), borderline ARVC diagnosis ("Borderline"), possible ARVC diagnosis ("Possible"), or not characterized for ARVC ("No ARVC"). Diagnosis using the 2010 TFC was completed by using a combination of clinical assessments broadly grouped into the following categories: global or regional dysfunction and structural alterations (characterized using a combination of MRI, angiography, or echocardiography), biopsy histomorphometry analysis, repolarization abnormalities, depolarization/conduction abnormalities, arrhythmias, and family history including genetic testing. Major or minor criteria were described within each of these categories, and patients were classified into one of the four possible diagnoses following the diagnostic terminology outlined in the 2010 TFC. A definite diagnosis was determined if a patient met two major criteria or one major and two minor criteria or four minor criteria (from different categories). A borderline ARVC diagnosis was determined if a patient met one major and one minor criteria or three minor criteria (from different categories). Lastly, a possible ARVC diagnosis was determined if a patient met one major or two minor criteria (from different categories). Patients for ARVC prior to 2010 were assessed using the previously published 1994 ARVC TFC.¹³

Due to biopsy analysis being a component of the existing diagnostic criteria as per the modified 2010 TFC, biopsy major and minor criteria were removed from this diagnosis scoring when completing receiver-operating characteristic (ROC) curve analysis as described below. This was done as a means of avoiding channeling bias, in which incorporating biopsy analysis as one component of ARVC diagnosis could bias the diagnostic utility of the test via ROC curve analysis.

A separate cohort of eight control biopsies was collected from children who had prior cardiac transplantation. The first surveillance endomyocardial biopsy set was collected post transplant (to avoid any effects of rejection or previous biopsy), and was analyzed for histomorphometry as described below.

Histomorphometry

Right ventricular endomyocardial biopsies were obtained from patients who were identified as having suspected ARVC. Biopsy sections were stained with elastin trichrome and then screened to identify the individual specimen and section level that

demonstrated the highest proportion of combined fat and fibrosis based on visual assessment by the pathologist. Selected specimens were reviewed by an expert pathologist (GJW) at the Hospital for Sick Children to ensure agreement with the selection.

Images were taken at $\times 10$ power using a Nikon Eclipse E400 microscope and attached Nikon ACT III software (Nikon Canada Inc.; Mississauga, Ontario, Canada). When the entire biopsy specimen would not fit on a single photomicrograph, images were reconstructed from multiple overlapping biopsy images using Adobe Photoshop CS3 software (Adobe Systems Incorporated; San Jose, California). These images were then cropped appropriately to generate a single, complete image of the biopsy, and post processed to smooth any color variations.

Areas of fat were identified within the biopsy as clear (white) areas of the internal portion of the biopsy (with the outline of the adipocytes often visible) and these fat-related pixels were counted using Adobe Photoshop software. Using color detection for areas of fibrosis stained with trichrome (visible as green), fibrosis-related pixels were also counted. The total number of pixels of the biopsy image were counted and the relative proportions of fat (%Fat) and fibrosis (%Fibrosis) were each expressed (Fig. 1). The sum of %Fat and %Fibrosis (%Myocardial replacement), was also used for analysis.

Statistical analysis

Data are presented as mean \pm standard deviation (SD), and the threshold for statistical significance, α , was set at 0.05, unless otherwise stated. Statistical analysis was completed using GraphPad Prism 6 software (GraphPad Software Inc.; La Jolla, California). Biopsy measures of %Fat, %Fibrosis, and %Myocardial replacement were compared between the four diagnostic classifications (definite, borderline, possible, and no ARVC) and control using one-way analysis of variance (ANOVA). If a significant ANOVA was detected for a biopsy measure, pairwise multiple comparison Tukey post hoc testing was completed to distinguish significance between the diagnostic cohorts.

A ROC curve was constructed to assess the diagnostic ability of biopsy analysis (%Fat, %Fibrosis, and %Myocardial replacement). Patients were reclassified into the four previously described ARVC diagnoses (definite, borderline, possible, or no ARVC) after removing biopsy analysis (as outlined in 2010 TFC) from the diagnosis, as fibrofatty replacement on biopsy is a component of the TFC score. Individual ROC curves were constructed for each of the three biopsy measures, with a positive test result representing only patients classified as definite (i.e., borderline, possible, no ARVC, and control diagnoses were classified as a negative test result). The same ROC analysis was completed with both definite and borderline diagnoses defined to be a positive test result.

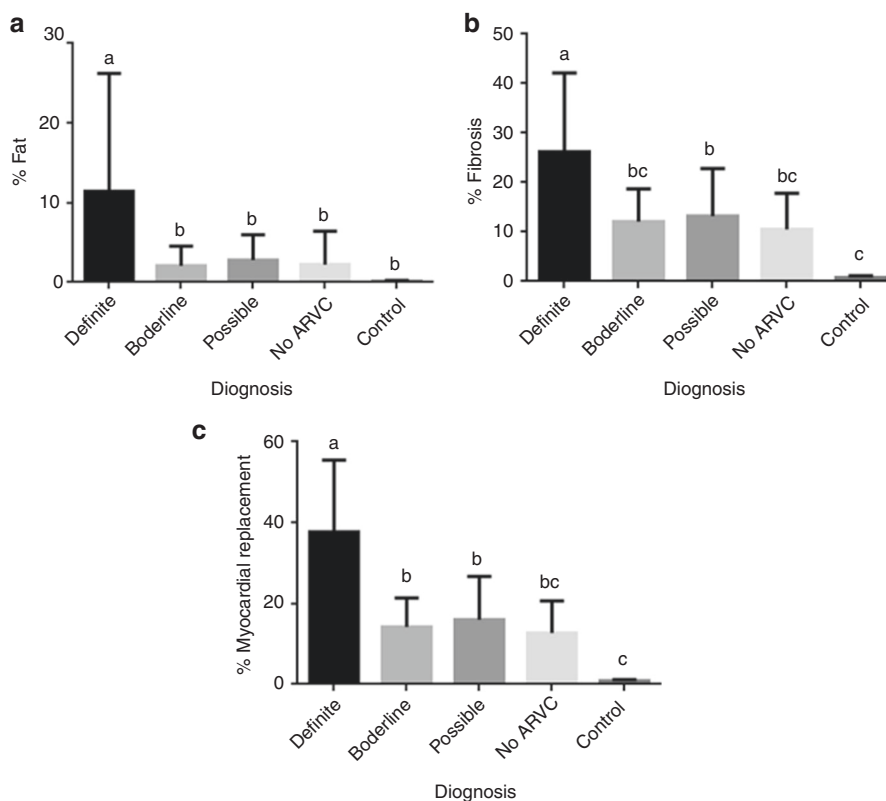


Fig. 2 Comparison of biopsy measurements between ARVC (arrhythmogenic right ventricular cardiomyopathy) diagnosis and control cohorts. Biopsy measures of %Fat (a) %Fibrosis (b), and % Myocardial replacement (c; a combination of both fat and fibrosis), were compared across ARVC diagnostic cohorts and control biopsy samples. Measurements were completed on pediatric endomyocardial biopsies, and diagnosis was assessed using the 2010 modified

Task Force Criteria. Error bars indicate standard deviation. Groups with the same letter code above the error bar are not significantly different (Tukey post hoc all-pairwise comparisons following significant one-way ANOVA; $p < 0.05$)

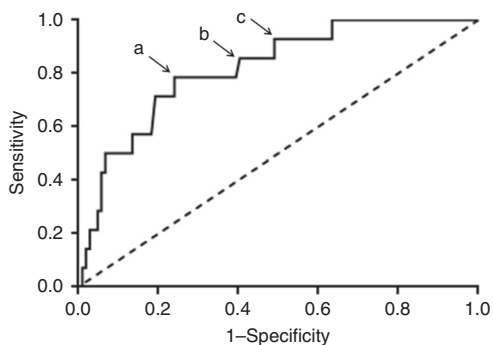


Fig. 3 Receiver-operating characteristic (ROC) curve of the percentage of myocardial replacement (fibrosis and fat replacement) of a healthy myocardium tissue) in pediatric endomyocardial biopsy samples. A positive test result is classified as a positive ARVC (arrhythmogenic right ventricular cardiomyopathy) diagnosis based on the modified 2010 Task Force Criteria with biopsy criteria removed from diagnostic classification. The area under the curve is 0.82 (95% CI: 0.71–0.93) and the p -value is 0.00012. The proposed major criterion of 25% (actual value of 22.54%) myocardial replacement (a) and minor criterion of ~15% (b) are indicated on the curve. An additional cut point of interest of ~12% myocardial replacement is also indicated on the curve (c). A 1:1 line of identity (–) is also shown for reference

RESULTS

A total of 110 patients, 41 females and 69 males, were included in this study and ARVC was diagnosed using the 2010 modified TFC.

The median age at the first visit was 12.6 years and the age range at the first visit was 0.9–18.4 years. Of the patients assessed using the 2010 TFC, 26 were classified as definite, 19 borderline, 27 possible, and 38 no ARVC. A complete summary of each patient’s major and minor criteria assessment following 2010 TFC is provided in Supplementary Table S1.

When comparing the three biopsy measures (%Fat, %Fibrosis, and %Myocardial replacement) across diagnostic cohorts, significant ANOVAs were detected for all three measures ($F_4 = 7.78$, $F_4 = 14.30$, and $F_4 = 27.56$ for %Fat, %Fibrosis, and %Myocardial replacement, respectively, and $p < 0.001$ for all three biopsy measures). For all three biopsy measures, the Definite cohort was the only diagnostic group that had a significantly greater proportion of each biopsy measure relative to all the other diagnoses and control cohorts (Fig. 2).

When the biopsy major (<60% residual myocytes) and minor (60–75% residual myocytes) criteria (from previously described 2010 TFC) were removed from the diagnostic score calculations, the diagnostic classifications changed for some patients. In total, 14 patients were classified as Definite when the biopsy component was removed (whereas 26 patients were classified as Definite with biopsy analysis included). Similarly, there were 25 patients classified as Borderline (19 when biopsy was included), 27 as Possible (27 when biopsy was included), and 44 as No ARVC (compared to 38 when biopsy was included). ROC curves were constructed for all three biopsy measures (%Fat, %Fibrosis, and % Myocardial replacement) with only the Definite diagnosis representing a positive test result. Of these three ROC curves, the myocardial replacement measure resulted in the ROC curve with the greatest area under the curve (Fig. 3; area under the curve =

Table 1. Descriptive statistics of constructed ROC (receiver-operating characteristic) curves for diagnosis of ARVC (arrhythmogenic right ventricular cardiomyopathy) in pediatric endomyocardial biopsy samples

Biopsy measure	Positive test result	Area under the curve	95% CI	p-value
% Fat	Definite	0.69	0.55–0.82	0.022*
	Definite, borderline	0.67	0.57–0.77	0.0030*
% Fibrosis	Definite	0.81	0.69–0.92	<0.001*
	Definite, borderline	0.69	0.59–0.78	<0.001*
% Myocardial replacement	Definite	0.82	0.71–0.93	<0.001*
	Definite, borderline	0.71	0.62–0.81	<0.001*

ROC curves were constructed for the three biopsy measurements (%Fat, % Fibrosis, and %Myocardial replacement or the sum of fat and fibrosis) with a positive test result being either only the Definite ARVC diagnosis or Definite and Borderline ARVC diagnostic cohorts as per the 2010 modified Task Force Criteria guidelines (with the biopsy component not included when assessing diagnosis)
*Significant p-value ($p < 0.05$)

0.82, 95% confidence interval (CI): 0.71–0.93). When both Definite and Borderline diagnoses were taken as representing a positive test result, the resulting ROC curves had lower areas under the curves, with the previously described Fig. 3 illustrating the ROC curve with the largest area under the curve. Statistical summaries of all six ROC curves are described in Table 1.

From the ROC curve in Fig. 3, the following cut-point values of interest were identified: 22.54% myocardial replacement (sensitivity = 78.6%, specificity = 76%), 14.75% myocardial replacement (sensitivity = 85.7%, specificity = 59.6%), and 12.59% myocardial replacement (sensitivity = 92.9%, specificity = 51%). Levels of 12.59% and 14.75% for myocardial replacement were selected as values with greater levels of specificity, and 22.54% myocardial replacement was selected as the value which optimized both sensitivity and specificity. Individual %Myocardial replacement values for each inflection point of the ROC curve in Fig. 3 are included in Supplementary Table S2.

DISCUSSION

The ROC curve analysis clearly demonstrated the potential value of modifying ARVC biopsy criteria for the pediatric ARVC population. Based on the adult 2010 modified TFC, the cutoff values of interest of 25% and 40% myocardial replacement (75% and 60% residual myocytes, respectively) would be biased toward diagnosis specificity based on the constructed ROC curve in this study (Fig. 3).¹⁴ These myocardial replacement values of 25% and 40% corresponded to 80.8% and 94.2% specificity, but only 71.4% and 42.9% sensitivity, respectively, on the constructed ROC curve from this study. Based on the identified cutoff values identified from this study population, we propose a modification from 25% and 40% myocardial replacement to 15% and 25%, respectively. This would result in a new major criterion being >25% myocardial replacement (or <75% residual myocytes) and the new minor criterion being 15–25% myocardial replacement (or 75–85% residual myocytes) by morphometric analysis in pediatric samples.

The proposed myocardial replacement cutoff values of 15% and 25% would allow for a greater sensitivity in pediatric diagnosis (with sensitivity/specificity of 71.4%/80.8% for proposed major and 85.7%/59.6% for proposed minor criteria) compared to applying adult criteria on pediatric patients as is currently done. Adoption of these modified diagnostic criteria for biopsy analysis in pediatrics would remove the bias toward specificity and involve a greater sensitivity when assessing children, which is of relevance as ARVC is a rare disease.

When comparing the biopsy measures across the ARVC diagnostic cohorts and control samples, it is evident that the measure of adipose tissue alone is not appropriate in pediatric endomyocardial biopsy samples (Fig. 2a). Although the fat measure resulted in the ability to significantly differentiate the Definite cohort, this measure had the largest variation and is further supported when using this measure to construct ROC curves, as it resulted in the ROC curves with the lowest area under the curve (Table 1). %Fibrosis and %Myocardial replacement (which was largely contributed toward by fibrosis rather than fat) presented better measurement variables, as demonstrated by more significant pairwise differences detected in the post hoc comparisons (Fig. 2b, c).

Fibrofatty replacement of the right ventricular myocardium has historically been reported to be one possible manifestation of ARVC. The earliest descriptions by Osler¹⁶ and Uhl¹⁷ of myocardial thinning may have in some instances related to ARVC, but it was not until Dalla-Volta et al.¹⁸ who first described the replacement of the right ventricular myocardium with a combination of fibrotic and adipose tissue (later confirmed to be a manifestation of ARVC). Fontaine et al.¹⁹ described the pathophysiological characterization of ARVC as involving one or more areas of the right ventricle where the myocardium is replaced with fibroadipose tissue. A dilated right ventricle with fatty replacement of the free wall is often described by the term “triangle of dysplasia” describing the tissue replacement occurring in the apex, infundibulum, and posterobasal inflow regions,²⁰ although apical replacement may be seen only rarely. Although there is usually no thinning or other macroscopic involvement of the left ventricle, left ventricular interstitial fibrosis may be seen microscopically. However, left ventricular involvement in the pathophysiology of ARVC is yet to be clearly characterized.

Endomyocardial biopsy histomorphometry analysis of fibrofatty infiltrate has been reported in other cardiac disorders other than ARVC. Peters et al.²¹ performed qualitative and semiquantitative analysis on right ventricular septal endomyocardial biopsies from 12 patients with ARVC compared to a cohort of 50 patients with idiopathic right ventricular outflow tract (RVOT) tachycardia. Pathological findings were reported in 11/12 ARVC patients with fibrolipomatosis in two cases, severe (>40% per biopsy) fibrosis in five cases, a finer form of fibrosis surrounding individual myocytes without lipomatosis in two cases, and a slight interstitial or subendocardial fibrosis in two cases. La Vecchia et al.²² assessed fibrosis quantitatively on endomyocardial biopsies from patients with ventricular tachycardia, normal left ventricular function, and no fatty replacement on histological analysis. Patients with reported late potentials on signal-averaged ECG (SAECG) had a significantly greater proportion of fibrosis ($7.6 \pm 1\%$) than patients without late potentials ($5.6 \pm 0.7\%$). Our morphometric findings of biopsies from young patients with varying degrees of ARVC demonstrated predominantly fibrotic replacement of the myocardium, which parallels the two studies described.

Although ARVC is classically described to be a disease of the right ventricular free wall, the clinical utility of right ventricular septal endomyocardial biopsies (although debated) has been recognized for decades in adults. Angelini et al.²³ performed histomorphometry analysis on right ventricular septal endomyocardial biopsies from 30 adults with ARVC, 29 with dilated cardiomyopathy (DCM), and 30 control adults. Fibrous tissue was

increased in both DCM and ARVC patients, whereas adipose tissue was predominantly a feature in ARVC, presenting in 67% versus 6% of DCM patients and control subjects. Others have questioned the clinical utility of septal endomyocardial biopsies in adult diagnosis of ARVC. For example, Chimenti et al.²⁴ assessed 30 patients with a diagnosis of ARVC based on available clinical criteria, and reported histological features of ARVC in only 30%, with myocarditis in the remaining 70% of the subjects.

Understandably, a consensus on the specific cutoff values of fibrofatty infiltration is needed by clinicians when assessing patients. The most recent 2010 modified TFC has reported cutoff values of <60% residual myocytes (i.e., replacement of >40% of the biopsy with fibrofatty tissue) to be a major criterion and 60–75% residual myocytes to be a minor criterion for diagnosis.¹⁴ It is important to note that the available 2010 TFC for histomorphometric analysis was based on biopsy samples obtained from the right ventricular free wall, in contrast to the interventricular septal biopsy samples (obtained on the right ventricular side) more routinely used in children, including in the present study. Pediatric institutions routinely perform endomyocardial biopsies from the interventricular septum to prioritize patient safety. Thus, in addition to providing biopsy criteria in pediatric patients relative to adult patients, our results also represent the differences related to the biopsy sample site.

For both fibrosis and myocardial replacement, the Definite cohort had the greatest percentage of each respective measure, and was significantly increased relative to all other comparisons between diagnostic cohorts. Again, these findings are further supported when considering the ROC curve analysis. When the Definite diagnostic cohort alone represented a positive test result, the resulting ROC curves for all three biopsy measures had a greater area under the curve compared to using both Definite and Borderline diagnoses as a positive test result (Table 1).

These results collectively support the conclusion that biopsy analysis in pediatric ARVC samples will only be clinically useful for more severe classifications of ARVC diagnosis. It should be noted however, that the ROC curve analyses illustrate that endomyocardial biopsy analysis is still far from ideal in this pediatric population. The ROC curve with the greatest area under the curve (%Myocardial replacement with Definite diagnosis indicating a positive test result) was still only 0.82 ± 0.055 SEM (with a theoretically 100% sensitive and specific test having an area under the curve of 1 and >80% generally indicating a strong test).

This study provided a retrospective histomorphometric analysis on the clinical utility of the right endomyocardial biopsy analysis as a component of the diagnosis of ARVC in children. Adult TFC values for myocardial replacement applied in this pediatric population provide poor sensitivity, and we therefore propose alternative values for myocardial replacement to be used when assessing pediatric samples. However, endomyocardial biopsy samples collected from children suspected to have ARVC were most positive in patients who met definite criteria even without biopsy data. In the era of genetic testing, which entails no physical risk to the patient, biopsy collection should usually be reserved for patients who remain indeterminate after all other testing, including genetic analysis is completed, unless there is a pressing need for immediate diagnosis. Should endomyocardial biopsy be required, such as in the case of a gene-elusive child with suspected ARVC short of definite criteria, this study indicates that the morphometric analysis should be adjusted for children to provide a trade-off between sensitivity and specificity. This study also suggests potential broader implications of applying 2010 TFC in pediatrics. The biopsy measure of fibrofatty infiltration is only one manifestation of the disease and only one component of the TFC. Electrical changes may precede structural alterations identified in the myocardium (such as from biopsy analysis).²⁵ It may be important to revisit 2010 TFC as a whole within the context of the pediatric population.

Limitations

There are a number of potential limitations in the present study. The non-ideal area under the curve for the constructed ROC curve could have been due to a number of limitations, including the fact that biopsies were collected from the interventricular septum rather than the right ventricular free wall (where the disease has classically been described to progress from the epicardium to the endocardium).⁴ An additional limitation in this study was the use of a small cohort ($n = 8$) of “control” biopsy samples from post heart transplant patients. While autopsy endomyocardial samples from children with various causes of death may be a different possible control cohort, it is unlikely that sampling that occurs during an endomyocardial biopsy could be reproduced. An additional limitation to the study was the minimal overlap between patients who had an endomyocardial biopsy set collected and ARVC genetic testing was completed. Owing to the prevalence of genetic testing in ARVC diagnosis in the present day, a comparison between these factors would be a potential subject of future studies.

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ADDITIONAL INFORMATION

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