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ORIGINAL RESEARCH

Influence of Pyroptosis Induced by NOD-Like Receptor Pyrin Domain-Containing 3 Inflammasomes on Viral Myocarditis in Children

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Purpose: Viral myocarditis (VMC) is a leading cause of heart failure and dilated cardiomyopathy in children. Pyroptosis, an inflammatory form of programmed cell death mediated by NOD-like receptor pyrin domain-containing 3 (NLRP3) inflammasomes, has been implicated in myocardial injury. However, the diagnostic significance of combined gene and protein expression analysis remains unclear. This study aimed to clarify the role of NLRP3 inflammasome-mediated pyroptosis in pediatric VMC and assess whether integrated gene–protein profiling could enhance diagnostic prediction.

Patients and Methods: A total of 100 children were enrolled, including a case group (n=50, children with viral myocarditis hospitalized between September 2023 and September 2024) and a control group (n=50, patients with dilated cardiomyopathy during the same time period). The expression levels of proteins interleukin-1β (IL-1β), NLRP3, Gasdermin D (GSDMD) and Caspase-1, and genes GSDMD, NLRP3, and Caspase-1 in myocardial tissues were measured through enzyme-linked immunosorbent assay, immunohistochemistry, real-time polymerase chain reaction, and Western blotting.

Results: No significant differences were found between the two groups regarding age, gender, or clinical history (P>0.05). Compared with the control group, the case group exhibited significantly higher expression of NLRP3, Caspase-1, GSDMD, and IL-1β at both protein and gene levels (P<0.05). Correlation analyses confirmed that these markers were positively associated with the presence of VMC. (P<0.05). Receiver operating characteristic (ROC) analysis revealed that the combined detection of gene and protein expression achieved a higher area under the curve (AUC) than any single indicator, demonstrating superior diagnostic accuracy.

Conclusion: NLRP3 inflammasome-mediated pyroptosis plays a crucial role in the pathogenesis of viral myocarditis in children. The combined assessment of gene and protein expression levels provides a novel and more reliable approach for early diagnosis and risk evaluation of pediatric VMC, highlighting the potential of inflammasome-related biomarkers as therapeutic targets.

Keywords: children, inflammasome, NLRP3, pyroptosis, viral myocarditis

Introduction

Despite differences among regions and populations, the incidence rate of myocarditis is estimated to be approximately 1–10/100,000 people/year globally, which may experience a significant elevation during some fulminant myocarditis epidemics. During the epidemic season of influenza viruses, viral myocarditis displays a raised incidence rate, which can also be induced by coxsackievirus, adenovirus, echovirus and other pathogens. Although it is difficult to accurately determine the incidence of viral myocarditis in children, it constitutes an important proportion of pediatric cardiovascular diseases and remains a major clinical concern.[1] Children of all ages are at risk of developing viral myocarditis, especially those in early childhood (1–3 years old) and preschool age (3–6 years old).[2] This susceptibility is largely due to an immature immune system, characterized by incomplete development of T and B lymphocytes and inadequate antiviral responses. Consequently, immune dysregulation may either trigger autoimmune injury of myocardial cells or permit

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persistent viral replication, both of which exacerbate myocardial damage. Moreover, children's myocardium is structurally fragile and metabolically active, making it more prone to injury from viral invasion. Clinically, mild cases may only present with fatigue or palpitations, whereas severe cases may progress to dyspnea, arrhythmia, heart failure, or even cardiogenic shock.[3] Because early symptoms are often nonspecific, early diagnosis remains difficult and misdiagnosis is common, highlighting the need for more reliable molecular biomarkers.

While clinical manifestations and imaging findings are important, the transition from clinical observation to understanding the molecular pathophysiology of viral myocarditis has been less thoroughly established. Recent evidence suggests that inflammatory programmed cell death, known as pyroptosis, may be a critical mechanism linking viral infection to myocardial injury. Pyroptosis is characterized by cell swelling, membrane pore formation, and release of proinflammatory contents, distinct from non-inflammatory apoptosis.[4]

Among several molecular mediators, the NOD-like receptor pyrin domain-containing 3 (NLRP3) inflammasome has emerged as a central regulator of pyroptosis.[5] Activation of NLRP3 leads to caspase-1 cleavage and subsequent activation of Gasdermin D (GSDMD), whose N-terminal fragments form membrane pores, culminating in cell lysis and inflammatory cytokine release.[6–8] The resulting increase in interleukin-1β (IL-1β) and other inflammatory mediators amplifies tissue injury. In pediatric viral myocarditis, viral infection–induced immune activation may trigger NLRP3 inflammasome assembly and pyroptosis, thereby aggravating myocardial inflammation and dysfunction.[9] However, the clinical relevance of this mechanism remains insufficiently characterized, and current diagnostic markers lack sensitivity for early-stage disease.

Therefore, this study was designed to explore the correlation between NLRP3 inflammasome-mediated pyroptosis and viral myocarditis in children, aiming to identify potential molecular predictors for improved diagnosis and disease assessment. We analyzed the expression of key genes (NLRP3, GSDMD, and Caspase-1) and proteins (NLRP3, Caspase-1, GSDMD, and IL-1β) to clarify their association with disease occurrence and their combined predictive value.

Materials and Methods

Subjects

Fifty children with viral myocarditis hospitalized in the Department of Pediatrics of our hospital between September 2023 and September 2024 were enrolled as the case group, and another 50 children with dilated cardiomyopathy admitted during the same period served as the control group. The diagnosis and classification of viral myocarditis were established according to the Guidelines for the Diagnosis and Treatment of Myocarditis in Children.[10] The recruited patients were in the acute or subacute stage of the disease, and none had progressed to end-stage heart failure at the time of enrollment. The severity of disease was assessed based on clinical symptoms, echocardiographic findings, and cardiac enzyme levels.

The sample size was determined based on feasibility and reference to previous studies with similar designs, which typically included 40–60 participants per group to detect medium effect sizes in protein and gene expression differences with a statistical power of approximately 0.80 at $\alpha = 0.05$. Although no formal a priori power calculation was performed, the current sample size was considered sufficient for preliminary exploratory analysis and hypothesis generation.

This study was approved by the Ethics Committee of the First Affiliated Hospital of Henan University of Chinese Medicine ethics committee (No. HNZZH2023910). All procedures followed the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from the parents or legal guardians of all participants prior to enrollment.

Inclusion and Exclusion Criteria

The following inclusion criteria were utilized: 1) Pediatric patients aged 0–18 years old, 2) diagnosed with viral myocarditis according to established diagnostic criteria, with confirmation of viral infection by serological testing or polymerase chain reaction (PCR) when available, 3) those who or whose family members were informed of and agreed to take part in this study, and 4) those able to cooperate with various examinations, including blood sample collection, cardiac ultrasound examination and endomyocardial biopsy.

The exclusion criteria involved: 1) other forms of myocardial inflammation (autoimmune, bacterial, or toxin-induced) , 2) severe systemic diseases illness or congenital heart disease 3) history of immunosuppressive therapy, corticosteroid use, or antiviral treatment within 4 weeks prior to sample collection, 4) received major surgery, trauma, or other conditions that might interfere with immune or inflammasome activity.

Collection of Samples

Each patient provided 5 mL of fasting venous blood at about 8 a.m. on the second day after admission. Blood collection was performed before any anti-inflammatory, antiviral, or immunomodulatory treatment was initiated to avoid confounding effects on cytokine or inflammasome expression. Myocardial tissue samples were obtained from both groups through endomyocardial biopsy under sterile conditions. Viral typing was performed in a subset of patients using PCR or enzyme-linked immunosorbent assay to identify common pathogens such as coxsackievirus B, adenovirus, or echovirus.

Detection of NLRP3 Protein Expression

Immunohistochemistry was adopted to measure the expression of protein NLRP.[11] Briefly, tissue samples were fixed in paraformaldehyde (Thermo Fisher Scientific) for 24 h. Afterwards, paraffin embedding and section slicing (thickness: four μm) were performed on the tissue samples. Sections were placed in sodium citrate buffer (Beyotime Biotechnology) and heated at 95°C for 20 min for antigen retrieval. Endogenous peroxidase activity was blocked using a peroxidase blocker (Roche Diagnostics), followed by incubation with blocking serum (Thermo Fisher Scientific) for 30 min at room temperature. The sections were then incubated with anti-NLRP3 primary antibody (Santa Cruz Biotechnology, diluted at 1:200) overnight at 4°C, followed by the incubation with horseradish peroxidase conjugated secondary antibody (Jackson Immunoresearch, diluted at 1:500) for 1 h at room temperature. Color development was performed using a diaminobenzidine (DAB), and counterstaining was conducted with hematoxylin (Sinopharm Chemical Reagent Co., Ltd.) for counterstaining. The staining intensity and percentage of positive cells were semiquantitatively analyzed using ImageJ software under identical exposure and magnification conditions. To ensure consistency, all slides were processed in parallel, and background correction was applied during image quantification.

Determination of Caspase-1 Protein Expression

Western blotting was implemented to determine Caspase-1 protein expression.[12] Tissue samples were lysed in RIPA lysis buffer (Shanghai Beyotime Biotechnology Co., Ltd)., homogenized and centrifuged at 12,000 rpm for 15 min at 4°C. Protein concentrations were measured using a BCA Protein Concentration Assay Kit (Thermo Fisher Scientific). N Equal amounts of total protein (40 μg per lane) were separated by SDS-PAGE and transferred to PVDF membranes. After blocking with 5% skim milk for 1 h, the membranes were incubated with anti-Caspase-1 primary antibody (Cell Signaling Technology, 1:1,000 dilution) overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibody (Abcam, diluted at 1:5,000) for 1 h at room temperature. β-actin (Cell Signaling Technology, 1:1,000 dilution) was used as the internal loading control for normalization. Signals were visualized using an ECL Kit (Millipore), and band intensity was quantified by ImageJ. Relative protein expression levels were calculated as the ratio of Caspase-1 to β-actin optical density.

Measurement of IL-1β Protein Expression

Serum IL-1β levels were determined using a commercial ELISA kit (Shanghai Enzyme-linked Biotechnology Co., Ltd.) according to the manufacturer's instructions. Blood samples were collected into anticoagulant tubes and centrifuged at 3,000 rpm for 10 min to isolate serum, which was stored at −20 °C until analysis. Serum samples and standards were added to plates pre-coated with anti-IL-1β antibody and incubated for 2 h at 37 °C, followed by biotinylated anti-IL-1β antibody for 1 h at 37 °C. Plates were washed three times with PBS and then incubated with horseradish peroxidase-labeled avidin for 30 min at 37 °C. After color development with substrate solution for 15 min, the reaction was stopped and absorbance was read at 450 nm using a microplate reader. Each sample was measured in triplicate, and the mean optical density (OD) value was used for analysis. The results were normalized to the corresponding standard curve to ensure quantitative accuracy. Blank wells and negative controls were included to correct for background absorbance.

Detection of GSDMD Protein Expression

Immunohistochemistry was employed as described above.[11] Myocardial tissue samples were fixed in paraformaldehyde (Thermo Fisher Scientific) for 24 h, paraffin-embedded, and sectioned at 4 μm. Antigen retrieval was performed in sodium citrate buffer (Beyotime Biotechnology) at 95°C for 20 min. Sections were incubated with anti-GSDMD primary antibody (Wuhan Sanying Biotechnology Co., Ltd., diluted at 1:200) overnight at 4°C, followed by horseradish peroxidase-labeled secondary antibody (Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd., 1:500 dilution) for 1 h at room temperature. DAB was used for color development, and hematoxylin for counterstaining. Image analysis for GSDMD followed the same semiquantitative normalization approach as for NLRP3.

Detection of Gene Expressions

Real-time polymerase chain reaction (PCR) was used to determine gene expression levels.[11] Total RNA was extracted from myocardial tissues using TRIzol lysis buffer [TIANGEN Biotech (Beijing) Co., Ltd.], and RNA integrity was assessed by agarose gel electrophoresis. Reverse transcription was performed using a Reverse Transcription Kit (Thermo Fisher Scientific). qPCR amplification was conducted with a Real-time PCR Kit (TaKaRa Biotech, Dalian) under the following conditions: 95°C for 30s, then 40 cycles of 95°C for 5s and 60°C for 30s. GAPDH was used as the internal reference gene, and the relative expression levels of target genes (NLRP3, CASPASE-1, and GSDMD) were calculated using the $2^{-\Delta\Delta Ct}$ method. Each sample was analyzed in triplicate to ensure reproducibility.

Statistical Analysis

Statistical analysis was performed using SPSS 26.0 software. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and the independent-samples *t*-test was used for intergroup comparisons of continuous variables after testing for normality. The count data were compared by the chi-square (χ^2) test. The statistically significant difference was denoted as P<0.05.

Results

Age, gender, history of preceding infections, history of allergies and history of hereditary diseases were of no significant differences (P>0.05) (Table 1).

Statistically significant intergroup differences were found in the expressions of selected proteins (P<0.05) (Table 2).

The expressions of selected genes in the two groups showed differences of statistical significance (P<0.05) (Table 3).

The occurrence of viral myocarditis in children was positively correlated with proteins (IL-1β, NLRP3, GSDMD, and Caspase-1) and genes (NLRP3, Caspase-1 and GSDMD) at the expression level, which was statistically significant (P<0.05) (Table 4).

According to logistic regression analysis on protein (IL-1β, NLRP3, GSDMD, and Caspase-1) and gene (NLRP3, Caspase-1 and GSDMD) expressions plus viral myocarditis in children, the OR [95% confidence interval (CI)] values

Table 1 General Data of Two Groups [$x \pm s, n(\%)$]

Item		Case Group (n=50)	Control Group (n=50)	Statistical Value	P
Gender	Male	28 (56.00)	26 (52.00)	χ^2=0.161	0.688
	Female	22 (44.00)	24 (48.00)		
Age (year)		7.5±2.3	7.2±2.1	*t*=0.6811	0.497
History of preceding infections	Yes	4 (8.00)	2 (4.00)	χ^2=0.177	0.673
	No	46 (92.00)	48 (96.00)		
History of allergies	Yes	6 (12.00)	4 (8.00)	χ^2=0.444	0.505
	No	44 (88.00)	46 (92.00)		
History of hereditary diseases	Yes	5 (10.00)	3 (6.00)	χ^2=0.136	0.712
	No	45 (90.00)	47 (94.00)		

Table 2 Protein Expressions in Case and Control Groups ($\bar{x} \pm s$)

Protein	Case Group (n=50)	Control Group (n=50)	t	P
NLRP3	46.98±14.04	34.56±11.54	4.832	0.001
Caspase-1	37.25±16.87	27.18±10.47	3.586	0.001
GSDMD	27.43±15.64	20.46±8.89	2.740	0.007
IL-1β	26.46±10.38	18.24±9.64	4.103	0.001

Table 3 Expressions of Genes in Case and Control Groups ($\bar{x} \pm s$)

Gene	Case Group (n=50)	Control Group (n=50)	t	P
NLRP3	54.23±10.24	45.21±9.84	4.491	0.001
Caspase-1	42.14±12.47	34.53±11.02	3.234	0.002
GSDMD	42.24±10.01	32.44±10.65	4.741	0.001

Table 4 Correlations of Protein and Gene Expressions with Occurrence of Viral Myocarditis in Children

	Expression of Protein				Expression of Gene		
	NLRP3	Caspase-1	GSDMD	IL-1β	NLRP3	Caspase-1	GSDMD
Pearson correlation coefficient	0.439**	0.341**	0.267**	0.368**	0.413**	0.310**	0.432**
P	0.001	0.001	0.007	0.001	0.001	0.002	0.001

Notes: **Significant correlations at the 0.01 level.

were 1.084 [1.026–1.146], 1.136 [1.044–1.236], 1.084 [1.010–1.163], 1.131 [1.045–1.223], 1.150 [1.052–1.258], 1.122 [1.036–1.216], and 1.121 [1.026–1.226], respectively, indicating that NLRP3-mediated pyroptosis exerts a significant effect on viral myocarditis in children ($P<0.05$) (Table 5).

With viral myocarditis in children as the dependent variable (viral myocarditis in children =1, control =0) and NLRP3, Caspase-1, GSDMD and IL-1β protein expressions, NLRP3, Caspase-1 and GSDMD gene expressions and their combination as independent variables, receiver operating characteristic (ROC) curves were constructed, of which the Y-axis was set as sensitivity and the X-axis was determined as "1-specificity" (Figure 1). According to the curves, the area under curve (AUC) values of proteins Caspase-1, IL-1β, GSDMD and NLRP3 protein, and genes NLRP3, Caspase-1 and GSDMD at the expression level for predicting viral myocarditis in children were 0.759, 0.668, 0.635, 0.710, 0.739, 0.656 and 0.739, respectively, signifying that the aforesaid indicators have good predictive value for the occurrence of viral myocarditis in children. However, the AUC value of their combination was 0.954, higher than that of any single indicator, indicating better prediction effect of protein expressions plus gene expressions (Table 6).

Table 5 Results of Regression Analysis on Expressions of Selected Proteins and Genes and Viral Myocarditis in Children

		B	Standard error	Wald	Degree of Freedom	Significance	EXP (B)	95% CI
Protein	NLRP3	0.081	0.028	8.273	1	0.004	1.084	1.026–1.146
	Caspase-1	0.128	0.043	8.740	1	0.003	1.136	1.044–1.236
	GSDMD	0.081	0.036	5.062	1	0.024	1.084	1.010–1.163
	IL-1β	0.123	0.040	9.431	1	0.002	1.131	1.045–1.223
Gene	NLRP3	0.140	0.046	9.406	1	0.002	1.150	1.052–1.258
	Caspase-1	0.115	0.041	7.998	1	0.005	1.122	1.036–1.216
	GSDMD	0.114	0.045	6.355	1	0.012	1.121	1.026–1.226
Constant		−27.75	6.407	18.759	1	0.001		

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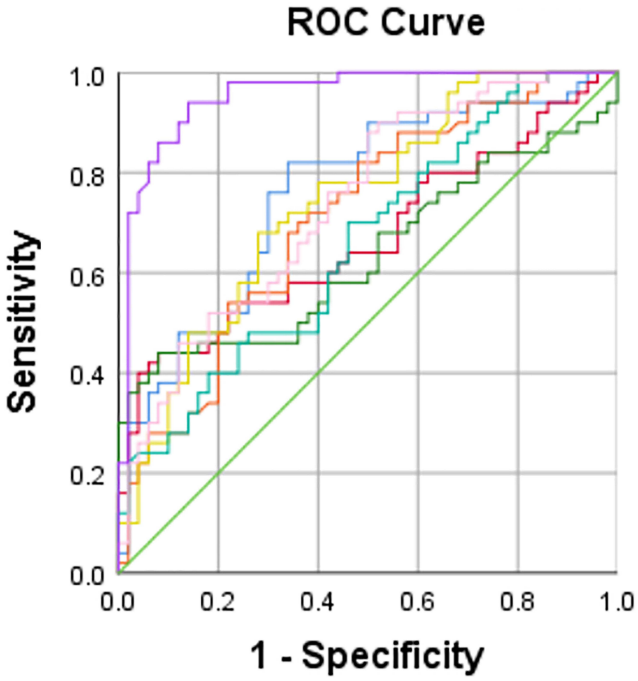

Figure 1 ROC curves showing the diagnostic performance of NLRP3, caspase-1, GSDMD, and IL-1β proteins and genes. The purple line represents the combined indicators.

Discussion

The pathogenesis of viral myocarditis in children has always been the focus concerned by the medical field, and, at present, the known causes of myocarditis are complicated and diverse. On the one hand, viral infections are the most common etiological factors. Respiratory and gastroenteric viruses such as coxsackievirus, echovirus, adenovirus, and enterovirus can initially infect the respiratory or digestive tract, enter the bloodstream through viremia, and subsequently reach myocardial tissues. Once these viruses invade cardiomyocytes, they replicate by hijacking the host's metabolic machinery, leading to direct cytopathic damage, mitochondrial dysfunction, and structural disintegration of myocardial cells.[13,14] On the other hand, the autoimmune response may also exert crucial effects on myocarditis development and progression. Viral antigens may share molecular mimicry with cardiac proteins, prompting the immune system to misidentify myocardial tissue as foreign and initiate an autoimmune attack. Furthermore, viral infection can release intracellular antigens that trigger chronic immune activation, resulting in prolonged inflammation even after viral clearance.[15]

Table 6 Predictive Values of Protein Expressions Plus Gene Expressions for Occurrence of Viral Myocarditis in Children

Factor		AUC	Cut-Off	95% CI	P	Specificity	Sensitivity	Youden Index
Protein	NLRP3	0.759	0.048	0.664–0.854	0.001	0.100	0.940	0.040
	Caspase-1	0.668	0.055	0.560–0.775	0.004	0.140	0.960	0.100
	GSDMD	0.635	0.057	0.523–0.746	0.020	0.200	0.840	0.040
	IL-1β	0.710	0.051	0.609–0.811	0.001	0.180	0.940	0.120
Gene	NLRP3	0.739	0.049	0.643–0.836	0.001	0.360	0.880	0.240
	Caspase-1	0.656	0.054	0.550–0.762	0.007	0.220	0.940	0.160
	GSDMD	0.739	0.049	0.643–0.835	0.001	0.260	0.960	0.220
Combination		0.954	0.020	0.914–0.994	0.001	0.560	0.980	0.540

In addition to infection and immune dysregulation, host susceptibility factors also contribute to the development of myocarditis. Genetic predispositions, such as polymorphisms in immune-regulatory genes (eg, NLRP3, IL1B, CASP1), may amplify inflammatory responses. Environmental factors, including viral exposure, pollution, or nutritional deficiencies, and certain pharmacological agents that alter immune balance, can further increase vulnerability to myocardial injury.[16–18]

During the in-depth study of the pathogenesis of myocarditis, a specific inflammatory cell death process known as pyroptosis has drawn increasing attention. Activation of the NLRP3 inflammasome triggers caspase-1-dependent cleavage of GSDMD, forming membrane pores and promoting the release of inflammatory cytokines such as IL-1β and IL-18. This cascade amplifies local inflammation, increases vascular permeability, and exacerbates cardiomyocyte injury.

In the present study, we found that NLRP3 inflammasome–induced pyroptosis is closely associated with the occurrence of viral myocarditis in children. Elevated protein and gene expression levels of NLRP3, Caspase-1, GSDMD, and IL-1β were observed in affected children, indicating inflammasome activation and enhanced inflammatory cell death. The rupture of cell membranes during pyroptosis leads to the release of intracellular components, further intensifying immune responses and tissue damage. Such a self-perpetuating inflammatory loop may underlie the transition from acute viral infection to chronic myocardial injury.[19–21]

According to our findings, these inflammasome-related molecules showed strong positive correlations with disease presence, and logistic regression confirmed their potential as predictive biomarkers. Moreover, the combined assessment of gene and protein expression achieved higher diagnostic sensitivity, specificity, and AUC values than individual markers, underscoring the diagnostic advantage of integrating multiple molecular indicators.

From a therapeutic perspective, our results also suggest potential clinical implications. The NLRP3/IL-1β axis represents a promising target for intervention. Anti-IL-1 therapies such as anakinra and canakinumab have demonstrated efficacy in other inflammatory cardiac conditions, and NLRP3 inhibitors like MCC950 or OLT1177 are under investigation for their cardioprotective effects. These findings indicate that modulation of the inflammasome pathway could help attenuate excessive inflammation and myocardial injury in pediatric viral myocarditis, opening new avenues for precision treatment.

This study has several limitations. The relatively small sample size may limit the robustness and generalizability of the findings. Although standard statistical analyses (*t*-test, chi-square, and logistic regression) were applied, adjustments for potential confounding factors were limited, and unmeasured clinical or environmental variables may have influenced the observed associations. Future investigations with larger cohorts and more comprehensive multivariate models are warranted to confirm these results and better define the independent role of inflammasome-related markers. Only myocardial tissues were examined, and expression patterns in peripheral blood or other tissues were not evaluated. The use of DCM patients as pathological controls was based on ethical and practical considerations, as myocardial samples from healthy children could not be obtained. This approach allowed comparison within a clinically relevant disease context while avoiding invasive procedures in healthy subjects. However, because DCM is accompanied by myocardial remodeling and low-grade chronic inflammation, it may have altered baseline inflammasome activity and introduced residual confounding despite matching for age and sex. Furthermore, the high AUC value observed, together with relatively narrow confidence intervals, may suggest possible model overfitting given the modest sample size. Therefore, future multicenter studies incorporating non-diseased myocardial samples, larger validation cohorts, longitudinal follow-up, and mechanistic experiments are needed to verify the stability and clinical relevance of NLRP3 inflammasome activation in pediatric viral myocarditis.

Conclusion

In conclusion, the present study suggests a potential involvement of NLRP3 inflammasome–mediated pyroptosis in the pathogenesis of pediatric viral myocarditis rather than confirming a disease-specific mechanism. The integrated analysis of gene and protein expression provides preliminary insight into inflammasome-related inflammatory injury in the myocardium and indicates possible diagnostic and mechanistic relevance. However, given that NLRP3 activation is a common feature of various infectious and inflammatory conditions, the observed association may reflect a broader

inflammatory response rather than a myocarditis-specific effect. The relatively small sample size and limited tissue scope further restrict the interpretation of causality.

Despite these limitations, this study contributes to understanding inflammasome activation in pediatric myocardial inflammation and highlights the need for larger, multicenter investigations to determine whether targeting the NLRP3/IL-1β axis could provide clinical benefit in viral myocarditis.

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Disclosure

The authors declare no conflict of interest.

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