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CLINICAL RESEARCH ARTICLE

Pediatric and adult dilated cardiomyopathy are distinguished by distinct biomarker profiles

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BACKGROUND: Emerging evidence suggests that pediatric and adult dilated cardiomyopathy (DCM) represent distinct diseases. Few diagnostic tools exist for pediatric cardiologists to assess clinical status and prognosis. We hypothesized that pediatric DCM would have a unique biomarker profile compared to adult DCM and controls.

METHODS: We utilized a DNA aptamer array (SOMAScan) to compare biomarker profiles between pediatric and adult DCM. We simultaneously measured 1310 plasma proteins and peptides from 39 healthy children (mean age 3 years, interquartile range (IQR) 1–14), 39 ambulatory subjects with pediatric DCM (mean age 2.7 years, IQR 1–13), and 40 ambulatory adults with DCM (mean age 53 years, IQR 46–63).

RESULTS: Pediatric and adult DCM patients displayed distinct biomarker profiles, despite similar clinical characteristics. We identified 20 plasma peptides and proteins that were increased in pediatric DCM compared to age- and sex-matched controls. Unbiased multidimensionality reduction analysis suggested previously unrecognized heterogeneity among pediatric DCM subjects. Biomarker profile analysis identified four subgroups of pediatric DCM with distinguishing clinical characteristics.

CONCLUSIONS: These findings support the emerging concept that pediatric and adult DCM are distinct disease entities, signify the need to develop pediatric-specific biomarkers for disease prognostication, and challenge the paradigm that pediatric DCM should be viewed as a single disease.

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IMPACT:

- Pediatric and adult DCM patients displayed distinct biomarker profiles, despite similar clinical characteristics and outcomes.
- Our findings suggest that pediatric DCM may be a heterogeneous disease with various sub-phenotypes, including differing biomarker profiles and clinical findings.
- These data provide prerequisite information for future prospective studies that validate the identified pediatric DCM biomarkers, address their diagnostic accuracy and prognostic significance, and explore the full extent of heterogeneity amongst pediatric DCM patients.

INTRODUCTION

Dilated cardiomyopathy (DCM) is an important cause of childhood mortality and is the most common indication for heart transplantation (HTx) in the pediatric population across all age groups.[1] Despite efforts to improve patient outcomes, pediatric DCM remains a challenging disease with an estimated 50% 5-year transplant-free survival.[2] A recurring theme in pediatric cardiomyopathy studies is that critical clinical differences exist between pediatric and adult DCM. Children with DCM display higher rates of cardiac recovery and lower rates of sudden cardiac death compared to adults.[3–7] Some evidence also suggest that medical therapies for adult heart failure may be less effective in children.[8] Recent analyses of myocardial tissue from pediatric and adult DCM patients also revealed differences in tissue remodeling and gene expression. Pediatric DCM subjects displayed less extensive cardiomyocyte hypertrophy and myocardial fibrosis compared to adults and distinct gene expression signatures including decreased expression of genes associated with adverse remodeling.[9,10] Outside HTx few options exist for children with DCM and heart failure, exemplifying the clinically unmet need to define the pathogenesis of pediatric DCM and develop targeted treatment strategies.

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Currently, there is a paucity of biomarkers, prognostic tools, and therapies available for children with DCM and heart failure. While the genetic basis for pediatric DCM is increasingly recognized, available genetic data do not yet provide clear insight into prognosis or therapeutic selection.[11–13] Discovery of candidate biomarkers represents a promising approach to explore pathophysiologic mechanisms that contribute to pediatric DCM and has previously been successfully applied to the adult heart failure population.[14–17] In this study, we employed an innovative biomarker discovery platform (SOMAscan®, Somalogic, Boulder, CO) capable of simultaneously measuring 1310 individual peptides and proteins to investigate differences in the biomarker profiles between pediatric controls and subjects with pediatric and adult DCM.

METHODS

Subjects

Pediatric DCM subjects. Pediatric DCM subjects were enrolled from the National Heart Lung and Blood Institute (NHLBI)-funded Pediatric Cardiomyopathy Registry (PCMR) Biomarkers study, a previous prospective cohort study of both incident and prevalent pediatric cases of DCM enrolled between August 2013 and January 2016 at 12 pediatric cardiology centers in the USA and Canada. The PCMR has been funded by the NHLBI since 1994. The original aims of the PCMR were to establish a robust database of clinical, imaging, and outcome data for children with cardiomyopathy and to determine the incidence and descriptive epidemiology of pediatric cardiomyopathies in the USA.[18] The pediatric DCM cohort used in our study was identified from the previously completed PCMR Biomarker study[15] to include pediatric patients with idiopathic DCM with a goal of 40 subjects. To be eligible for the PCMR Biomarker study, children had to be <21 years of age with a diagnosis of DCM determined by echocardiographic or cardiac magnetic resonance imaging evidence of DCM, defined by at least two of the following criteria: (1) a left ventricular (LV) shortening fraction or ejection fraction (EF) >2 standard deviations below normal mean for age, (2) an LV end-diastolic thickness-to-dimension ratio <0.12, and (3) an LV end-diastolic dimension or volume >2 standard deviations above normal mean for body surface area. Children with specific secondary causes of myocardial abnormalities were excluded, which included associated congenital heart disease, endocrine disorders known to cause myocardial damage, a history of chemotherapy or pharmacology-associated cardiotoxicity, chronic arrhythmia, pulmonary parenchymal or vascular disease, and chronic inflammatory or immunologic disease. The diagnostic inclusion criteria reflected that of the PCMR Biomarker study enrollment criteria with the exception that subjects with a diagnosis of myocarditis were excluded from our study cohort.[15] Plasma samples were collected as part of the PCMR Biomarker study, and our study aimed to include subjects with samples collected within 2 years of DCM diagnosis.

Adult DCM subjects. Adult DCM subjects were matched based on the approximate time from diagnosis to sample collection and sex to pediatric DCM subjects. Subjects were selected from the Washington University Heart Failure (WUHF) Registry, which prospectively enrolls patients >21 years old with a heart failure diagnosis.[17] Registry participants undergo the collection of demographic and clinical data as well as the collection of plasma samples. For our study, inclusion criteria required a diagnosis of DCM with an EF ≤50% and an available archived research plasma sample. Exclusion criteria included ischemic cardiomyopathy, chronic inflammatory conditions, chronic infections, active malignancies, myocarditis, liver failure, and chronic kidney disease greater than stage 3 to avoid possible confounding effects on detectable biomarkers. Inclusion and exclusion criteria were similar to the pediatric cohort with the exception that liver failure and greater stage 3 chronic kidney disease were not exclusion criteria for the pediatric DCM patients due to limited information regarding liver and renal function within the PCMR, although pediatric patients with uremia were excluded.

Pediatric control patients. Pediatric sex- and age-matched healthy control subjects were enrolled at the time of elective outpatient procedures through the same-day surgery unit at St. Louis Children's Hospital between January and July 2019. Examples of elective procedures included eye muscle surgery, hypospadias repair, orchiopexy, inguinal hernia repair, etc. Plasma samples were collected at the time of routine care after parental consent was obtained. Exclusion criteria included diagnosis of heart failure or congenital heart disease (except previous/resolved patent ductus arteriosus, atrial septal defect, and/or ventricular septal defect in patients with normal findings on most recent echocardiogram), genetic or neuromuscular disease/syndrome, immune or inflammatory disease, use of immune-modulating therapy within 6 months of enrollment, or known recent infection within 3 months of enrollment. All research was approved by the Institutional Review Board at Washington University in Saint Louis. All patients included in this study from the PCMR and WUHF registries had provided written informed consented previously for use of data and specimens for future research use. All control pediatric control subjects underwent written informed consent at the time of study enrollment.

Protocol

The PCMR Biomarker study and WUHF Registry provided the plasma samples as well as the following clinical data for each subject: patient demographic information, anthropomorphic measurements, HF etiology, cardiac family history, heart failure class, echocardiographic results, cause of death, need for mechanical circulatory support, and most recent clinical status (alive without a transplant, listed/transplanted, death). Enrolled subjects were chosen based on those with a specimen sample time nearest to the time of diagnosis (time point 0) with the aim to be within 2 years of diagnosis. This time point was chosen to standardize as much as possible comparison between groups given the small subject numbers in this study. EF measured by echocardiogram was compared by absolute percent as well as severity categories: mildly decreased (EF 45–55%), moderately decreased (EF 30–45%), and severely decreased (EF < 30%). Available clinical information and echocardiographic data were limited to that available from the PCMR and did not include genetic testing results.

Initially, 40 pediatric control, 41 pediatric DCM, and 42 adult DCM samples were collected for analysis. The previously collected registry plasma samples were stored according to the protocols for each respective registry.[15,19] Blood samples were obtained, transferred to sodium citrate tubes, and plasma prepared. Plasma aliquots were frozen at −80 °C. Control samples were stored in a −80 °C freezer until processing. One adult subject was excluded due to sample clotting. Two pediatric DCM subjects were excluded from the final analysis due to incorrectly reported diagnosis (hypertrophic cardiomyopathy) and failure to meet echocardiographic inclusion criteria at the time of PCMR plasma sample collection. The final cohort for data analysis comprised 39 pediatric control, 39 pediatric DCM, and 40 adult DCM subjects for biomarker analysis (Fig. 1a, b).

SOMAScan®

The Biomarker core laboratory at Washington University Genome Technology Access Center (GTAC) performed the processing of plasma samples. The details of SOMAscan processing have previously been described.[20] Briefly, plasma samples were mixed with beads conjugated to one or more of the 1310 validated aptamer probes. Protein–aptamer complexes were then purified, and the number of bound aptamers was quantified using a custom Agilent microarray. The SOMAmer reagent was used to quantify hybridized material. The microarray readout, in relative fluorescent units, is directly proportional to the amount of target protein in the initial sample for each protein–SOMAmer reagent pair. A pooled control sample was used as the bridging sample to account for batch effect between grouped plasma samples.[21] See Supplemental Material for further details regarding the SOMAscan® platform.

Data analysis

SOMAscan® analysis. The off-scanner raw signal values are processed through SomaLogic's SOMAscan® standardization procedures, including hybridization normalization, plate scaling, median scaling, and final SOMAmer calibration, each of which generates a SOMAscan® ".adat" data file. R package "limma" was utilized for differential expression analysis, and it is subjected to a linear model fitting of the signal data and an empirical Bayesian statistic (a moderated *t* test) for group comparison.

Clinical analysis. Groups were compared using Student's *t* tests or analysis of variance (ANOVA) for normally distributed data, Mann–Whitney *U* test, or Kruskal–Wallis test for nonparametric data, logistic regression, and hierarchical clustering. Logarithm base 2-fold change (FC), a common measure in the field of genomics, represents the log 2 ratio of two quantities. Biomarker pairs with FC > 2 and p value <0.05 were considered statistically significant. Principal component analysis (PCA) plots were used to visualize the proteomic data across the different groups. This is a statistical technique that uses an orthogonal transformation to convert a

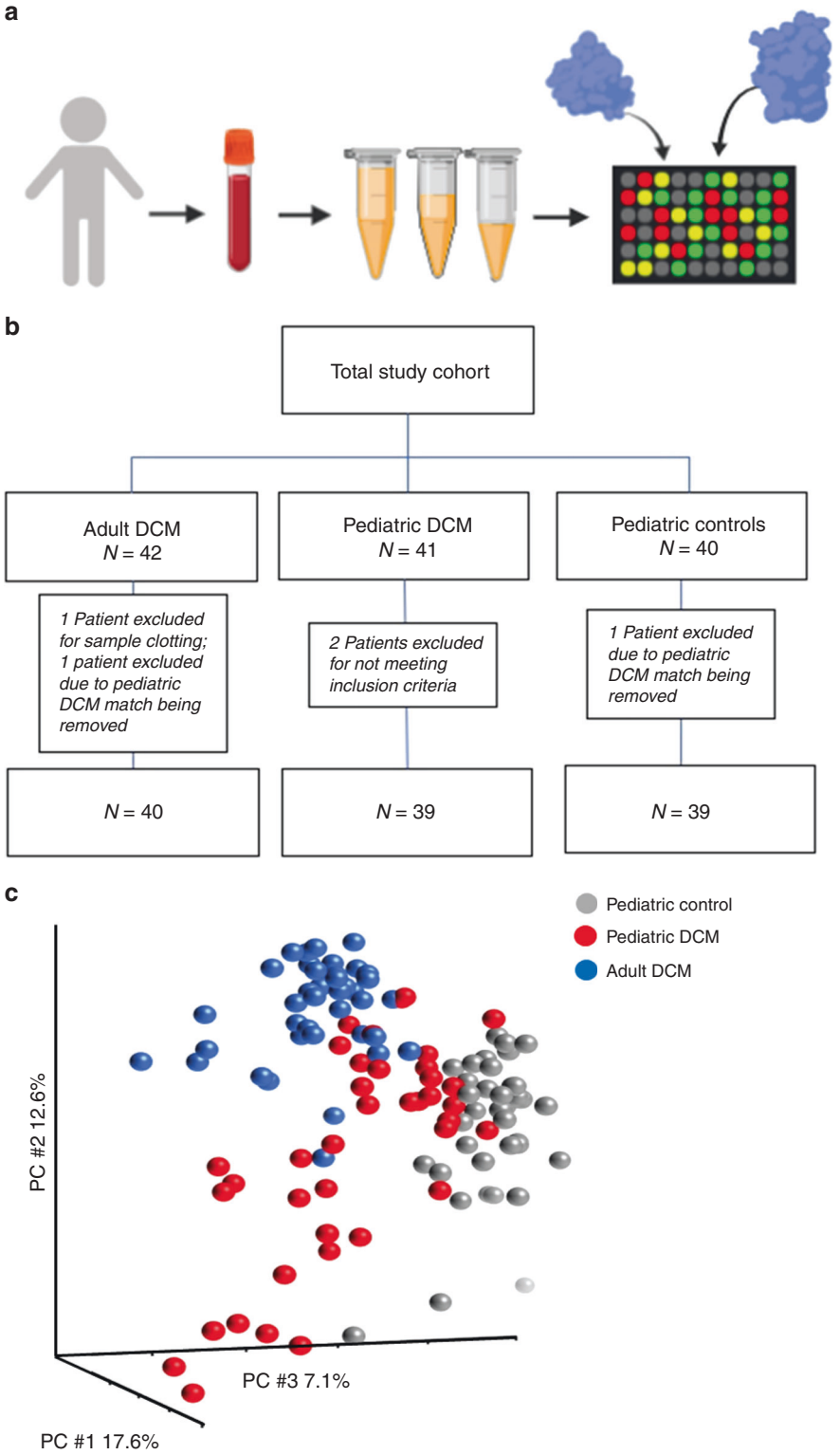


Fig. 1 Details of final study cohort. a Schematic of study design. **b** Flow chart of patient enrollment. **c** PCA plot comparing all three groups showing distinct heterogeneity between groups.

set of correlated variables into a fixed number of components or dimensions. Each component or dimension describes the variance or heterogeneity of the data. Additionally, volcano plots are a form of scatterplot that displays statistical significance (p value) versus magnitude of change (FC). T-distributed stochastic neighbor embedding (tSNE) clustering was performed in R. This analysis was used to provide an unbiased means to identify substructures or subgroups within the larger dataset. Intergroup differences were then assessed using the nonparametric Kruskal–Wallis one-way ANOVA test with pairwise comparisons between subgroups to determine significance. Biomarker analysis was performed using Partek Genomics Suite (version 7.0) and PRISM (version 7.0). Gene Ontology (GO) was utilized for pathway analysis. The remainder of the statistical analyses were performed using SPSS (version 27).

RESULTS

Subject characteristics

Patient demographic information for the pediatric and adult DCM cohorts are detailed in Tables 1 and 2. The majority (22/39) of pediatric DCM subjects had their study sample obtained within 6 months of diagnosis and within 1 month of enrollment (34/39). Only two subjects were enrolled beyond 24 months of diagnosis. Complete echocardiographic parameters were available for 35 pediatric DCM subjects with LV EF, EF severity group, LV end-diastolic volume (EDV) *z*-score, and LV end-systolic volume (ESV) *z*-score. Based on the PCMR enrollment standard requirements, 38 pediatric subjects met the EF criteria, 30 met the LVEDD criteria, 26 met the LVEDV criteria, and 3 met LV end-diastolic thickness-to-dimension ratio criteria. For pediatric DCM subjects, 7 (20%) had mildly decreased EF, 10 (29%) had moderately decreased EF, and 18 (51%) had severely decreased EF. Pediatric controls were age- and sex-matched at enrollment to pediatric DCM subjects with no statistical differences in median age (2.4 versus 2.7 years, $p = 0.897$) or sex (49% female versus 49% female, $p = 1.0$) between pediatric control and DCM subjects. Due to insufficient racial demographic data available for the PCMR subjects, controls could not be matched based on this characteristic. There was no difference between pediatric and adult DCM patients with respect to sex, EF at enrollment (median 31 versus 29%, $p = 0.36$), or time from diagnosis to plasma sample (median 3 versus 4 months, $p = 0.84$) (Table 2). Pediatric and adult DCM patients had similar rates of LV assist device (LVAD) implantation (5.1 versus 5.0%, $p = 0.98$) and HTx (5.1 versus 5.0%, $p = 0.98$) during the follow-up period after enrollment. Pediatric and adult DCM subjects had a similar status at last follow-up, including a number of subjects alive without heart transplant (85 versus 90%, $p = 0.13$). The median time to last follow-up was longer in adults (median pediatric 1.9 versus adult 2.4 years, $p = 0.001$).

SOMAscan analysis of pediatric DCM biomarkers

PCA revealed that pediatric control, pediatric DCM, and adult DCM groups clustered independently highlighting underlying differences between these patient populations (Fig. 1c). Differential expression analysis identified plasma biomarkers enriched in pediatric control and DCM groups (Fig. 2a, b). We identified 45 plasma peptides and proteins that markedly differed between pediatric DCM and pediatric control groups (FC >2, FDR $p < 0.05$) (Supplementary Table 1).

Table 1. Clinical characteristics of pediatric DCM cohort and subgroups.

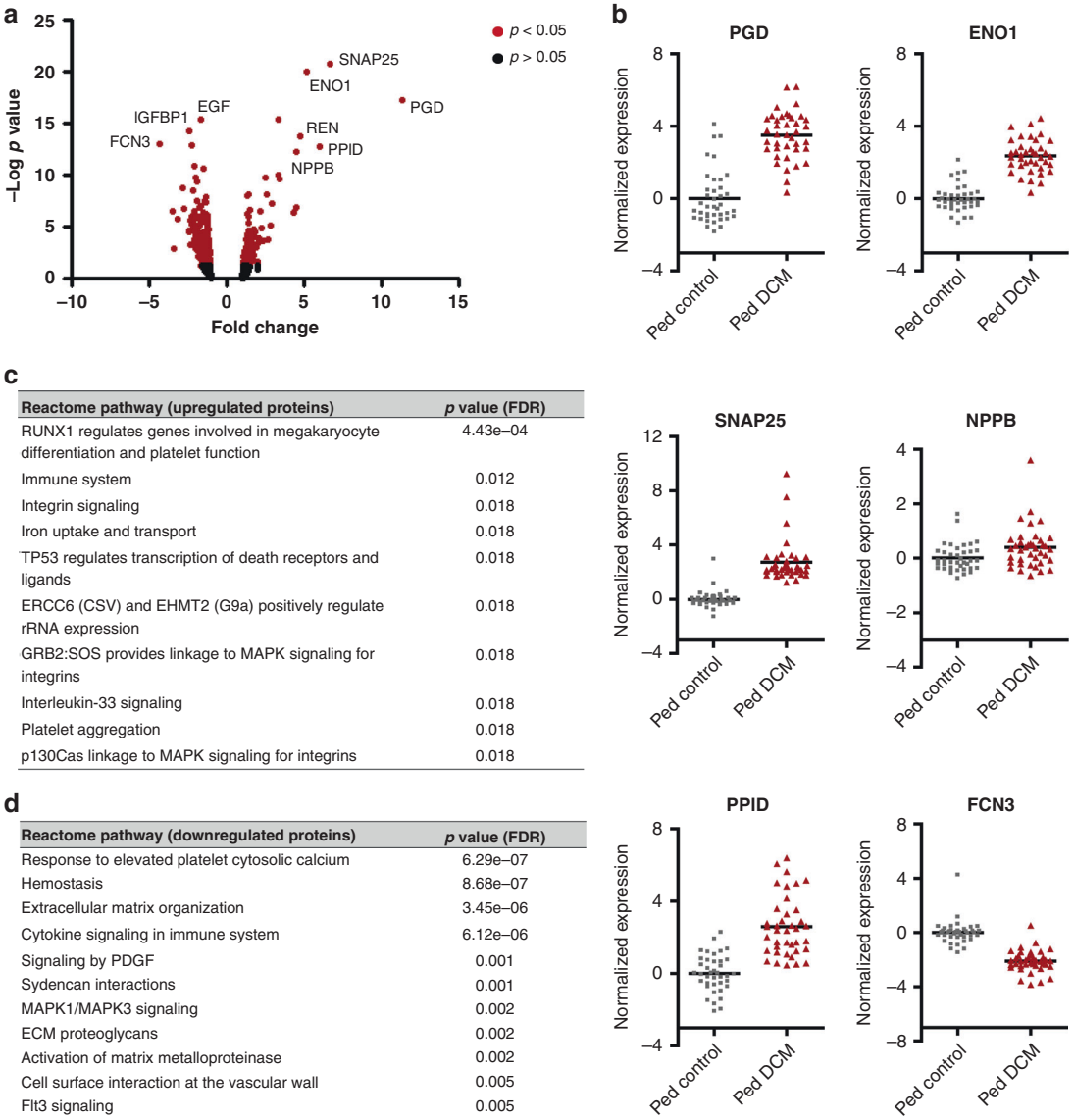
	All pediatric DCM ($n = 39$)	Group 1 ($n = 15$)	Group 2 ($n = 7$)	Group 3 ($n = 6$)	Group 4 ($n = 11$)	*P* value
Age at sample (years)	2.8 (1–13)	2. 8 (0.9–15)	10 (4.1–18.3)	4.4 (1–14.5)	2 (0.5–2.9)	0.19
Sex, female, *n* (%)	19 (49)	6 (40)	4 (57)	4 (67)	5 (45)	0.69
Weight (kg)	13 (8–50)	15.4 (7.1–69.1)	32.5 (16.2–82)	13 (9.3–52.1)	10 (7.9–13)	0.25
Time from diagnosis to sample (ms)	3 (1–14)	1.7 (0.9–2.2)	2.4 (1.9–5.4)	2.1 (1.7–3.6)	1.3 (1.1–2.1)	0.07
EF (%)	31 (22–40)	27.6 (19.6–38.5)	50 (38.7–52.7)[a]	41.7 (30.8–52.8)	21.6 (20.2–32.3)[b]	0.005
LVESV *z*-score	5.4 (3.9–8.2)	5.7 (3.9–8.4)	1.6 (0.4–5)[c]	4.4 (2.4–5.3)[d]	8.2 (5.3–8.7)	0.004
LVEDV *z*-score	3.1 (1.9–5.0)	3.1 (2.4–5.2)	1.2 (−0.6–3.2)[e]	2.2 (0.1–3.5)[f]	5.5 (2.9–7.6)	0.02
Ventricular assist device, *n* (%)	2 (5)	0	0	0	2 (18)	0.15
Alive without transplant, *n* (%)	33 (85%)	15 (100)	7 (100)	4 (67)	7 (64)	0.03

Values are given as median and interquartile range unless otherwise noted. *P* value < 0.05 was considered significant.
EF ejection fraction, *LVESV* left ventricular end-systolic volume, *LVEDV* left ventricular end-diastolic volume.
[a]Group 2 had a statistically higher EF compared to group 1 ($p = 0.01$) and group 4 ($p = 0.01$).
[b]Group 4 also had a statistically lower EF compared to group 3 ($p = 0.03$).
[c]Group 2 had a lower ESV *z*-score compared to group 1 ($p = 0.006$) and group 4 ($p = 0.001$).
[d]Group 3 had a lower ESV *z*-score compared to group 4.
[e]Group 2 had a lower EDV *z*-score compared to group 1 ($p = 0.045$) and group 4 ($p = 0.003$).
[f]Group 3 had a lower EDV *z*-score compared to group 4.

Table 2. Demographic information for adult and pediatric DCM.

	Adult DCM ($n = 40$)	Pediatric DCM ($n = 39$)	*P* value
Age at sample (years)	53 (46–63)	2.8 (1–13)	<0.001
Time diagnosis to sample (months)	4 (1–9)	3 (1–14)	0.841
Time to last follow-up (years)	2.4 (2.2–2.8)	1.9 (1.1–2.3)	0.001
Female sex, *n* (%)	17 (43)	19 (49)	0.58
EF at enrollment	29 (20–42)	31 (22–40)	0.357
Severely decreased EF at enrollment, *n* (%)[a]	20 (51)	25 (64)	0.37
Heart transplant, *n* (%)	2 (5)	6 (17)	0.98
Ventricular assist device, *n* (%)	2 (5)	2 (5)	0.98
Alive without transplant at last follow-up	36 (90)	33 (85)	0.128

Values are given as median (interquartile range) unless otherwise noted. A *p* value < 0.05 was considered significant.
DCM dilated cardiomyopathy, *EF* ejection fraction.
[a]Defined as ejection fraction < 30%.



c

Reactome pathway (upregulated proteins)	p value (FDR)
RUNX1 regulates genes involved in megakaryocyte differentiation and platelet function	4.43e–04
Immune system	0.012
Integrin signaling	0.018
Iron uptake and transport	0.018
TP53 regulates transcription of death receptors and ligands	0.018
ERCC6 (CSV) and EHMT2 (G9a) positively regulate rRNA expression	0.018
GRB2:SOS provides linkage to MAPK signaling for integrins	0.018
Interleukin-33 signaling	0.018
Platelet aggregation	0.018
p130Cas linkage to MAPK signaling for integrins	0.018

d

Reactome pathway (downregulated proteins)	p value (FDR)
Response to elevated platelet cytosolic calcium	6.29e–07
Hemostasis	8.68e–07
Extracellular matrix organization	3.45e–06
Cytokine signaling in immune system	6.12e–06
Signaling by PDGF	0.001
Sydencan interactions	0.001
MAPK1/MAPK3 signaling	0.002
ECM proteoglycans	0.002
Activation of matrix metalloproteinase	0.002
Cell surface interaction at the vascular wall	0.005
Flt3 signaling	0.005

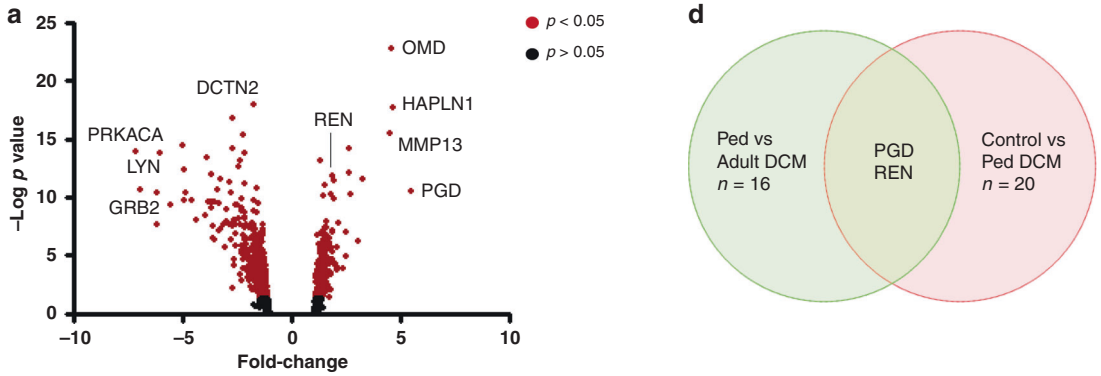
Fig. 2 Pediatric DCM versus pediatric controls. a Volcano plot portraying proteins downregulated in pediatric DCM compared to pediatric controls (FC < 0) and proteins upregulated in pediatric DCM compared to pediatric controls (FC > 0). **b** Differential expression analysis revealed upregulation of 20 plasma analytes with those shown as most significant. **c** List of most significant upregulated pathways in pediatric DCM. **d** List of most significant downregulated pathways in pediatric DCM. FCN3 ficolin 3, IGFBP1 insulin-like growth factor-binding protein 1, EGF epidermal growth factor, NPPB natriuretic peptide B, PPID peptidylprolyl isomerase D, REN renin, PGD 6-phosphogluconate dehydrogenase decarboxylating, ENO1 enolase 1, SNAP25 synaptosome-associated protein 25, RUNX1 RUNX family transcription factor 1, TP53 tumor protein P53, ERCC6 excision repair 6, chromatin remodeling factor, EHMT2 euchromatic histone lysine methyltransferase, GRB2 growth factor receptor-bound protein 2, SOS Ras/Rac guanine nucleotide exchange factor 1, MAPK mitogen-activated protein kinase, p130Cas breast cancer anti-estrogen resistance 1 scaffold protein, cas family member, PDGF platelet-derived growth factor, ECM extracellular matrix, Flt3 Fms-related receptor tyrosine kinase 3.

6-Phosphogluconate dehydrogenase (PGD), SNAP2, PPID, ENO1, renin (REN) and B-type natriuretic peptide were among the biomarkers most elevated in pediatric DCM. Pathway analysis demonstrated that pediatric DCM samples displayed enrichment of proteins implicated in RUNX1 signaling, immunity, integrin signaling, iron uptake, TP53 signaling, megakaryocyte differentiation and platelet aggregation, and p130/Cas signaling. Proteins reduced in pediatric DCM samples were implicated in hemostasis, extracellular matrix organization, cytokine signaling, PDGF signaling, MAPK signaling, MMP activation, and Flt3 signaling (Fig. 2c, d).

Pediatric and adult DCM biomarker profiles

Differential expression analysis revealed marked differences in the biomarker profiles between pediatric and adult DCM (Fig. 3a), with 113 biomarkers having an expression FC >2, FDR p value <0.05 (Supplementary Table 2). Pathway analysis revealed that FGFR2 receptor signaling, FOXO-mediated transcription, TRKA signaling, cytokine signaling, IGF1 signaling, and PI3K signaling were enriched in pediatric DCM compared to adults. In contrast, interleukin signaling, apoptosis, platelet aggregation, KIT, NTRK, FLT3, VEGFA, ERBB2, and CD28 signaling pathways were upregulated in adult DCM compared to pediatric DCM (Fig. 3b, c).

To identify biomarkers that might be unique to pediatric DCM, we calculated the intersection between proteins upregulated in pediatric versus adult DCM (FC >2, FDR $p < 0.05$, $n = 16$) and proteins upregulated in pediatric DCM versus pediatric controls (FC > 2, FDR $p < 0.05$, $n = 20$). This analysis revealed PGD (5.5-FC compared to adult DCM) and REN (2.1-FC compared to adult DCM) as candidate



b

Reactome pathway (upregulated in pediatric DCM)	*p* value (FDR)
FGFR2 mutant receptor activation	4.62e–04
Diseases associated with glycosaminoglycan metabolism	0.002
FOXO-mediated transcription	0.006
Activation of TRKA receptors	0.008
Regulation of TLR by endogenus ligand	0.011
PI3K cascade	0.011
Cytokine signaling in immune system	0.013
IRS-mediated signaling	0.015
IGF1R signaling cascade	0.019

c

Reactome pathway (upregulated in adult DCM)	*p* value (FDR)
Signaling by interleukins	3.49e–14
Intrinsic pathway for apoptosis	8.36e–14
Platelet aggregation signaling and activation	9.28e–14
Signaling by SCF-KIT	7.81e–12
Signaling by NTRKs	1.17e–10
FLT3 signaling	9.11e–10
VEGFA-VEGFR2 pathway	2.03e–09
Signaling by ERBB2	2.69e–09
CD28 co-stimulation	3.37e–09

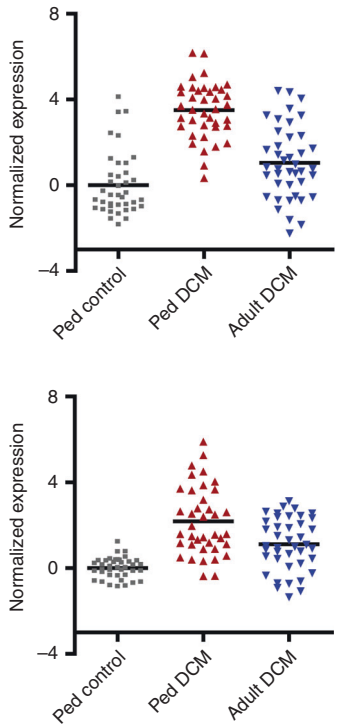
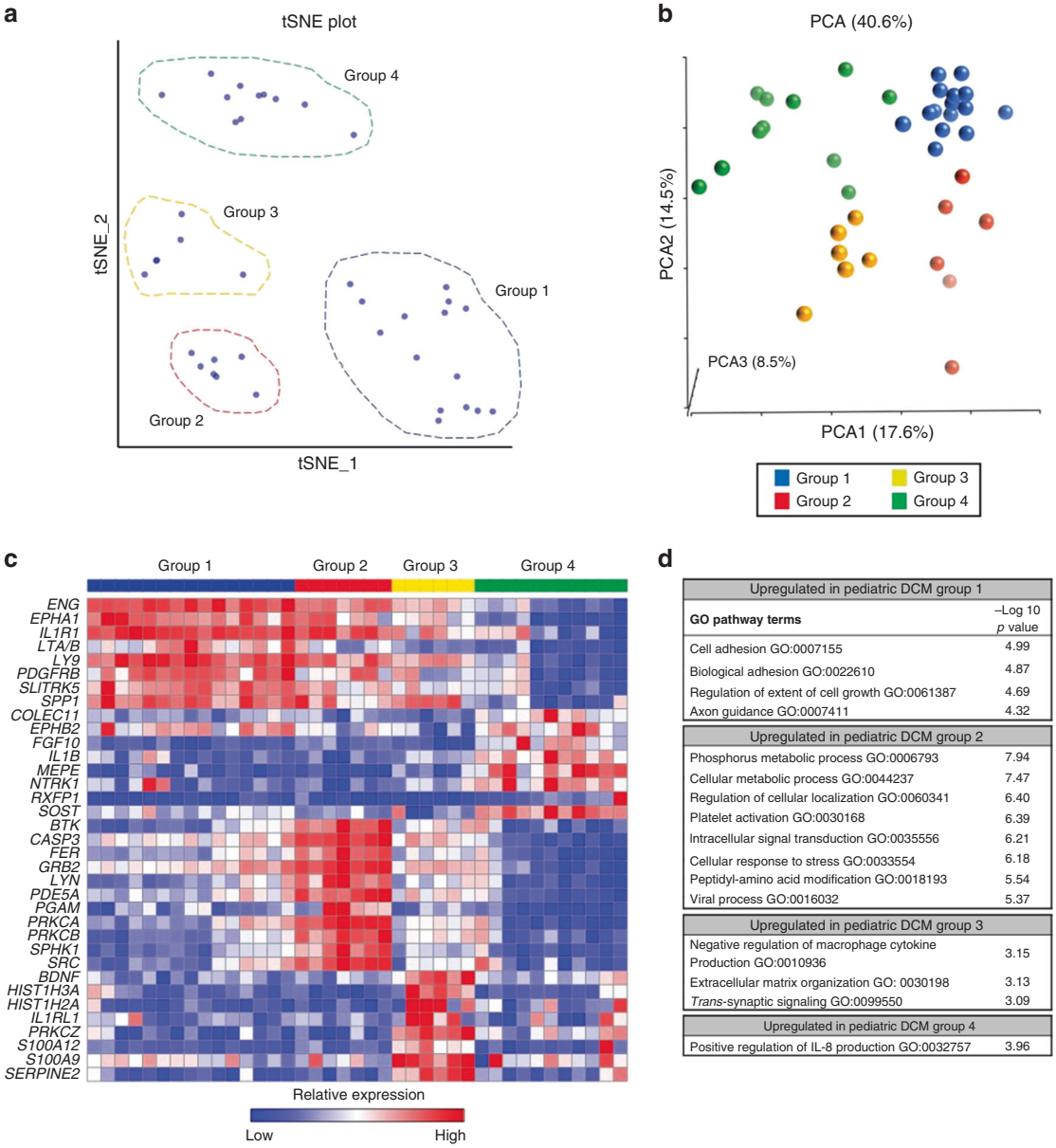


Fig. 3 Pediatric DCM versus adult DCM. a Volcano plot portraying proteins downregulated in pediatric DCM compared to adult DCM (FC < 0) and proteins upregulated in pediatric DCM versus adult DCM (FC > 0). **b** List of most significant pathways upregulated in pediatric DCM compared to adult DCM. **c** List of most significant pathways upregulated in adult DCM compared to pediatric DCM. **d** Venn diagram showing significance of PGD and REN after including comparison with pediatric controls (*n* signifies the number of significant biomarkers for each initial analysis). **e** Differential expression analysis for PGD and renin. GRB2 growth factor receptor-bound protein 2, LYN tyrosine protein kinase Lyn, PRKACA protein kinase CAMP-activated catalytic subunit alpha, DCTN2 dynactin subunit 2, REN renin, PGD 6-phosphogluconate dehydrogenase decarboxylating, MMP13 matrix metallopeptidase 13, HAPLN1 hyaluronan and proteoglycan link protein 1, OMD osteomodulin, FGFR2 fibroblast growth factor receptor 2, FOXO class O of forkhead box transcription factors, TRKA neurotrophic receptor tyrosine kinase 1, TLR toll-like receptor, PI3K phosphatidylinositol-4,5-bisphosphate 3-kinase, IRS insulin receptor substrate, IGF1R insulin-like growth factor 1 receptor, SCF-KIT tyrosine protein kinase Kit, NTRK neutrotrophic receptor tyrosine kinase, FLT3 Fms-related receptor tyrosine kinase 3, VEGFA vascular endothelial growth factor A, VEGFR2 vascular endothelial growth factor receptor 2, ERBB2 Erb-B2 receptor tyrosine kinase 2.

pediatric DCM biomarkers (Fig. 3d, e). Linear regression analysis of available patient clinical variables did not show any significant correlation with REN or PGD levels in the pediatric DCM cohort (Supplementary Table 3).

Pediatric DCM biomarker sub-phenotypes

tSNE-based clustering and PCA of pediatric DCM subjects revealed evidence of heterogeneity amongst analyzed subjects. We identified four subgroups with distinct biomarker profiles (Fig. 4a, b, Supplementary Table 4). Group 1 ($n = 16$) displayed differential expression of ENG, EPHA1, IL1R1, LTA, LTB, LY9, SPP1, and, PDGFRB. Significant pathways enriched included cell adhesion, cell growth, and axon guidance. Group 2 ($n = 7$) was characterized by elevated expression of LYN, SPHK1, PRKCA, PRKCB, BTK, CASP3, PDE5A, GRB2, and SRC. Pathways enriched included signal transduction, response to stress, and platelet activation. Group 3 ($n = 8$) demonstrated increased expression of HIST3H2A, HIST1H3A, S100A8, S100A12, SERPNE2, BDNF, IL1RL1, and PRKCZ. Pathway analysis revealed enrichment of cytokine production, extracellular matrix organization, and trans-synaptic signaling.



d

Upregulated in pediatric DCM group 1	
GO pathway terms	–Log 10 *p* value
Cell adhesion GO:0007155	4.99
Biological adhesion GO:0022610	4.87
Regulation of extent of cell growth GO:0061387	4.69
Axon guidance GO:0007411	4.32
Upregulated in pediatric DCM group 2	
Phosphorus metabolic process GO:0006793	7.94
Cellular metabolic process GO:0044237	7.47
Regulation of cellular localization GO:0060341	6.40
Platelet activation GO:0030168	6.39
Intracellular signal transduction GO:0035556	6.21
Cellular response to stress GO:0033554	6.18
Peptidyl-amino acid modification GO:0018193	5.54
Viral process GO:0016032	5.37
Upregulated in pediatric DCM group 3	
Negative regulation of macrophage cytokine Production GO:0010936	3.15
Extracellular matrix organization GO: 0030198	3.13
Trans-synaptic signaling GO:0099550	3.09
Upregulated in pediatric DCM group 4	
Positive regulation of IL-8 production GO:0032757	3.96

Fig. 4 Pediatric DCM subgroups. a tSNE plot demonstrating segregation of pediatric DCM subjects into four subgroups. **b** PCA plot similarly showing four distinct subgroups among pediatric DCM subjects. **c** Heat map portraying differential gene expression by groups. **d** List of most significant pathways upregulated in each subgroup. RUNX1 RUNX family transcription factor 1, FOXP3 forkhead box P3, TNF tumor necrosis factor, TNFR2 tumor necrosis factor receptor 2, NF-κB nuclear factor kappa-light-chain of activated B cells, TFAP2 transcription factor AP-2 alpha-enhancer, EPH erythropoietin-producing hepatoma, BAD BCL2-associated agonist of cell death, NTRK neurotrophic tropomyosin receptor kinase, SCF stem cell factor, KIT tyrosine kinase receptor, FZD frizzled class 1 receptor, NRH2 nucleoside *N*-ribohydrolase 2, NR1H3 nuclear receptor subfamily 1 group H member 3, TRKA neurotrophic receptor tyrosine kinase 1, CLEC7A C-type lectin domain containing 7A, PI3K phosphoinositide 3-kinase, PI5P phosphatidylinositol 5-phosphate, PP2A protein phosphatase 2A, IER3 immediate early response 3, AKT protein kinase 3.

Group 4 ($n = 8$) displayed selective expression of SOST, NTRK1, FGF10, IL1B, MEPE, and RXFP1. Pathway analysis revealed upregulation of interleukin-8 signaling (Fig. 4c).

Clinically, there was no significant difference in median sample age ($p = 0.19$), median patient weight ($p = 0.25$), time from diagnosis to plasma sample acquisition ($p = 0.07$), or sex ($p = 0.69$) between pediatric DCM subgroups (Table 1 and Fig. 5). Group 4 had the highest number of subjects with severely decreased EF (63%), while groups 1, 2, and 3 had 53%, 33%, and 25% of subjects with severely decreased EF, respectively ($p = 0.015$). Group 2 had significantly higher EF and lower LV EDV and ESV *z*-scores (less dilated) compared to other groups. During a median 1.9-year follow-up period, HTx was only performed in subjects belonging to group 3 (2/6 subjects, 33%) and group 4 (4/11 subjects, 36%), with group 4 least likely to be alive without transplant at follow-up ($p = 0.03$). LVAD support was only required within the group 4 cohort, with 2 of 11 patients undergoing VAD implantation, although this comparison did not reach statistical significance. There were no deaths in the pediatric DCM cohort during the follow-up period. Additionally, there was no significant difference in REN or PGD levels between the pediatric subgroups.

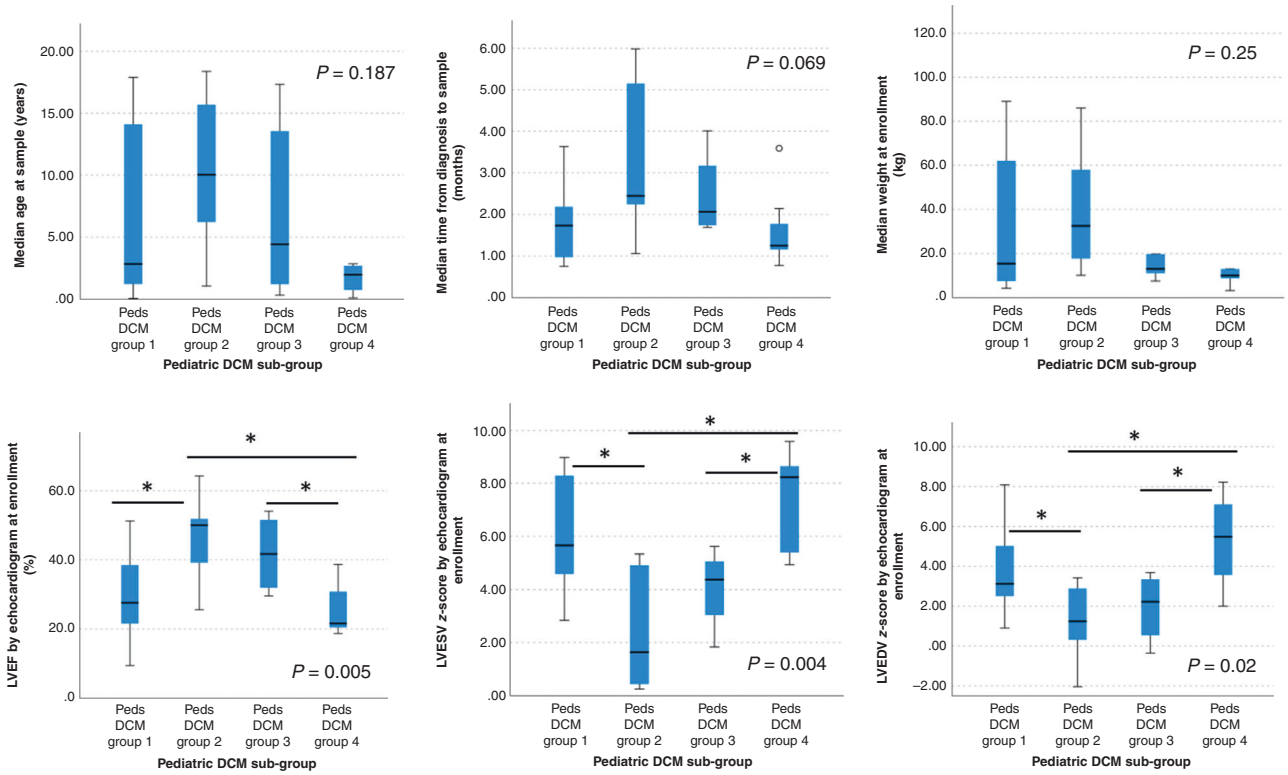


Fig. 5 Comparison of Patient Characteristics at Enrollment by Pediatric DCM Subgroup. Comparison of patient characteristics at enrollment by pediatric DCM subgroup. *P value < 0.05. LV EF: group 4 versus group 3 ($p = 0.03$), group 4 versus group 2 ($p = 0.01$), group 1 versus group 2 ($p = 0.01$); LV ESV: group 2 versus group 1 ($p = 0.006$), group 2 versus group 4 ($p = 0.001$), group 3 versus group 4 ($p = 0.04$); LV EDV: group 1 versus group 2 ($p = 0.045$), group 2 versus group 4 ($p = 0.003$), group 3 versus group 4 ($p = 0.049$).

DISCUSSION

The goal of this proof of principle study was to evaluate patterns of pediatric DCM biomarkers using a proteomic discovery platform. By comparing age- and sex-matched pediatric controls with DCM subjects, we observed significant dysregulation of numerous plasma peptides and proteins amongst subjects with pediatric and adult DCM. We further uncovered evidence for important differences between pediatric and adult DCM, identifying two putative biomarkers (PGD and REN) that were specifically upregulated in pediatric as compared to adult DCM when accounting for pediatric control data. Exploratory analysis suggested possible distinct subgroups or sub-phenotypes of pediatric DCM with distinguishing echocardiographic characteristics and clinical outcomes. Collectively, these findings provide initial evidence for pediatric-specific DCM biomarkers and lay the groundwork for larger prospective validation studies examining diagnostic performance and prognostic implications.

We identified 45 peptides and proteins with >2-FC between the control and pediatric DCM groups. Consistent with prior studies we observed increased B-type natriuretic and C-reactive protein levels in the DCM group.[22–27] However, many of the dysregulated species have not previously been investigated in clinical pediatric heart failure. As prior studies have proposed that biomarkers identify pathways involved in the pathogenesis of cardiomyopathy and heart failure, it is likely that our analysis will provide insights into the pathogenesis of pediatric DCM.[14,22–24,28–32] Many of the serum proteins increased in pediatric DCM subjects are implicated in the regulation of inflammation, angiogenesis, cell growth, and remodeling. GO pathway analysis further implicated RUNX1 and TP53 mechanisms previously implicated in animal models of myocardial remodeling and heart failure pathogenesis.[33,34] RUNX1-deficient mice display reduced LV remodeling and preserved LV systolic function following myocardial infarction.[35] Examination of TP53-deficient mice revealed contributions to LV remodeling and LV systolic function in myocardial infarction and pressure overload models.[36] Deletion of TP53 from mice harboring a Lamin A/C mutation implicated in DCM resulted in reduced myocardial fibrosis, LV dilation, and improved LV systolic function.[37]

This study provides further support for the hypothesis that pediatric and adult DCM are distinct clinical entities. Prior publications yielded evidence of pathological differences, including less extensive cardiomyocyte hypertrophy and fibrosis in pediatric DCM compared to adult DCM patients.[7,9,10] These studies also demonstrated distinct transcriptional signatures in pediatric and adult DCM. Our findings are complementary as they establish that pediatric and adult DCM have distinct biomarker profiles including the existence of pediatric-specific biomarkers. PGD and REN were specifically increased in pediatric DCM subjects compared to pediatric controls and adult DCM subjects. PGD functions in the pentose phosphate pathway and catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO_2, with concomitant production of NADPH. Excess NADPH may contribute to the production of reactive oxygen species (ROS) by providing additional substrate capacity to the NADPH oxidase complex. ROS is a known determinant of LV remodeling and progression to congestive heart failure.[29,38–42] REN is a protease that cleaves angiotensinogen to generate angiotensin I, a key component of the REN–angiotensin––aldosterone pathway (RAAS). Prolonged RAAS activation has devastating effects on cardiac structure and function.[43] In the adult population, the treatment of DCM with beta-blockade versus RAAS inhibition is a point of discussion, but these data suggest that a bias towards RAAS inhibition may be beneficial in the pediatric cohort.

The existence of distinct sub-phenotypes of heart failure is increasingly appreciated across patient populations. Among

children with LV non-compaction cardiomyopathy, different clinical phenotypes were associated with significant differences in clinical outcomes, including risk for death or heart transplant.[44] Children with restrictive cardiomyopathy also have had noted differences in transplant-free survival based on clinical phenotype.[45] Prior PCMR analysis reported that individuals with increased LV end-diastolic dimensions were associated with increased risk of HTx.[46] Within the PCMR, younger age at diagnosis and lower LV end-diastolic dimension *z*-score were associated with increased likelihood of recovery of normal LV size and systolic function over time.[47] More recently, Adamo et al. demonstrated distinct biomarker profiles and corresponding clinical subgroups among adults with heart failure using the SOMAscan platform.[17] The authors found that adults with preserved, moderate range, and severely reduced EF had unique biomarker clustering suggesting differences in disease pathophysiology, with differences also noted based on changes in function over time and heart failure etiology.

Consistent with these concepts, secondary analysis of pediatric biomarker profiles suggested four distinct groups or subphenotypes. Although available demographic data were limited within the registries, we observed strong trends and significant differences among the pediatric DCM subgroups. Subjects in group 4 were younger, had more severely decreased EF, a greater degree of LV dilation, and a higher frequency of transplant or LVAD. Conversely, subjects in group 2 displayed a less severe clinical phenotype with smaller LV volumes, higher EF, and no adverse clinical outcomes. It is not clear whether genetic determinants, including the presence of pathological DCM mutations, contributed to these subgroups as genetic information was not available for the majority of pediatric subjects. While all samples were obtained in the ambulatory setting, it is important to note that sample acquisition relative to heart failure exacerbations may account for some observed differences in clinical and biomarker data. However, our findings are an important initial step in defining the heterogeneity among pediatric DCM and provide insight for future investigation. Larger prospective studies will be required to fully evaluate the extent of differences among pediatric DCM patients, establish relationships with clinical characteristics and genetic information, and clarify prognostic relevance.

Limitations of this proof of concept study include small sample size, absence of genetic information, absence of medication history (including angiotensin-converting enzyme inhibitor), and utilization of historically collected plasma samples and registry data. Despite these limitations, we identified biomarker profiles for subjects with pediatric DCM and provide further evidence that pediatric and adult DCM are distinct pathological entities. Our observations suggest that pediatric DCM may be a heterogeneous disease with various sub-phenotypes including differing biomarker profiles and clinical findings. We recognize that the pediatric intergroup *p* values may over- or underestimate differences between groups due to the small subject numbers and this further supports the need for a larger-scale study. These findings provide the requisite information for future prospective studies that validate the identified pediatric DCM biomarkers, the relationship of biomarker profile to genetic data, address diagnostic accuracy and prognostic significance, and explore the full extent of heterogeneity amongst pediatric DCM patients.

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AUTHOR CONTRIBUTIONS

All authors listed above have met at least one of the requirements for authorship. These authors contributed equally: M.R.F.G., S.E.L., K.J.L., C.E.C., and K.E.S. J.D.W, J.A.T. and S.D.C jointly supervised this work.

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COMPETING INTERESTS

The authors declare no competing interests.

CONSENT STATEMENT

All adult and pediatric registry patients included had provided written informed consented previously for use of data and specimens for future research use at the time of registry enrollment. All control pediatric control subjects underwent written informed consent at the time of study enrollment.

ADDITIONAL INFORMATION

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