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Original Research Article

Study of New Biomarkers as Potential Predictors in Pediatric Dilated Cardiomyopathy

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Abstract

***Background*:** Dilated cardiomyopathy (DCM) poses a significant health risk in pediatric populations, yet its pathophysiological mechanisms remain unclear. This study aims to explore new biomarkers as potential predictors in pDCM. ***Methods*:** A total of 84 pediatric patients diagnosed with dilated cardiomyopathy (DCM) and 34 age-matched healthy controls were prospectively recruited. Inclusion criteria were based on clinical diagnosis, echocardiographic findings, and relevant exclusion criteria for other cardiac or systemic conditions. Serum levels of total carnitine, procollagen type III N-terminal propeptide (PIIINP), cystatin C (Cys-C), β2-microglobulin (β2M), and haptoglobin (Hp) were quantitatively assessed using enzyme-linked immunosorbent assay (ELISA). Expression of acylcarnitine was assessed via qRT-PCR. ***Results*:** There were significantly higher plasma levels of total carnitine (p=0.001), PIIINP (p=0.016), Cys-C (p=0.001), β2M (p=0.009), and haptoglobin (p=0.001) in pDCM compared to matched controls. Total carnitine, PIIINP and β2M at cut-off points 65 umol/ml &3 & 2 mg/L showed 73&79% & 75% sensitivity and 91& 56% & 81% specificity respectively for predicting risk of pDCM. Haptoglobin at cutoff point 164 mg/L has highest specificity (100%) but with low sensitivity 56%). Combined β2M, PIIINP, and total carnitine demonstrated the best accuracy (83.5%) with 75% sensitivity, 92% specificity, 90% PPV, and 79% NPV for the presence of pDCM. A significant upregulation of the acylcarnitine expression gene was also observed in the DCM group compared to controls. ***Conclusion*:** Acylcarnitine, PIIINP, Cys-C, β2M and heptoglobin are potential emerging predictors for pDCM and might have a pathogenic role in pDCM with mechanistic associations.

Keywords: β_2-Microglobin, Cystatin-C, Pediatric Cardiomyopathy, Predictors, Fibrosis.

INTRODUCTION

Pediatric cardiomyopathies are a global public health burden, accounting for a considerable risk of death and comorbidities, and are the main indication for pediatric heart transplantation. The overall annual mortality in pediatric population with dilated cardiomyopathy (DCM) has been reported to be 2-3%.

The natural history of many forms of cardiovascular diseases involves persistent inflammation and oxidative stress with consequent cardiac interstitial fibrosis. Pediatric DCM (pDCM) is heterogeneous and has a variety of etiologies, including genetic, metabolic, inflammatory, valvular, and neuromuscular factors [1]. Despite the value of conventional and cardiac-specific markers in predicting adverse consequences, early risk reclassification and discrimination of pDCM remains a major challenge. As a result, the identification of new biomarkers is of great interest.

Cardiac muscle metabolism is mostly dependent on mitochondrial fatty acid β-oxidation (FAO), accounting for 40–60% of total cardiac ATP production [2]. L-carnitine (levocarnitine) and various short-, medium-, and long-chain acylcarnitines constitute the endogenous carnitine pool and are involved in mitochondrial fatty acid transport and are important agents for the normal function of mitochondria [3].

In DCM of various etiologies, pathologic stimuli to the myocardium cause a complex series of compensatory mechanisms, including metabolic imbalances and triggering of the renin-angiotensin-aldosterone hormonal axis and adrenergic systems, resulting in myocardial fibrosis and myocyte impairment. This process of cardiac remodeling is progressive and harmful, frequently resulting in ventricular dysfunction and decompensated heart failure (HF). Because of the complexities of cardiac fibrosis pathophysiology and related pathways, numerous clusters of fibrosis-related indices have been identified, such as biomarkers of collagen synthesis and degradation, fibrosis-specific microRNAs, extracellular matrix (ECM) proteins, cytokines, and growth factors [4]. However, among children with DCM, little was known on the fibrosis-related molecules levels.

Procollagen type III N-terminal propeptide (PIIINP) is a collagen III derived product that can be detected in the bloodstream and is the most promising candidate biomarker of myocardial fibrosis. Deposition of PIIINP in the ECM is an integral feature of cardiac remodeling [4,5]. Earlier studies have shown that increased PIIINP levels are closely reflected collagen synthesis at disease site and served as an indicator of a reparative process, regardless of etiology [6, 7]. Nevertheless, the predictive performance of PIIINP in pediatric DCM is poorly assessed.

Cystatin-C (Cys-C) and β2-Microglobulin (β2M) are low-molecular weight non-glycosylated polypeptides that are expressed on nearly all nucleated human cell membranes and are considered marker of renal function [8-10].

Under pressure overload, for example, β2M enhances myocardial fibrosis and stimulation of myocardial fibroblasts [11, 12]. An imbalance of cysteine proteinases such as cathepsins B, H, and L and Cys-C can lead to connective tissue remodeling [13].

Increasing evidence supports the involvement of β2M and Cys-C in the pathogenesis of the cardiovascular disease (CVD) both in general population researches and in individuals with kidney diseases and can correlate with disease severity independently of many other risk factors [14, 15].

The primary hemoglobin (Hb) binding glycoprotein, haptoglobin (Hp), is mostly secreted by the liver. Additionally, it is an acute-phase protein whose expression rises in response to inflammation. Its primary function is to protect the body from oxidative stress by capturing free Hb released from erythrocytes. Aside from its antioxidant function, Hp also plays a role in the immune response and the acute phase response [16].

Predicting cardiomyopathy is one of the most critical clinical approaches when treating children with cardiovascular diseases. The prototype marker of cardiac mechanical stretch and neurohormonal activation (cardiac natriuretic peptides) and the chief marker of cardiac cellular injury is cardiac troponin. Although established biomarkers are widely used in clinical practice, there remains a significant need for supplementary markers, particularly in pediatric dilated cardiomyopathy (pDCM). Given this gap, our study aims to investigate the potential role of novel biomarkers, including PIIINP, Cystatin C (Cys-C), β2-microglobulin (β2M), and haptoglobin (Hp), as predictors of disease progression in pediatric DCM.

PATIENTS AND METHODS

The children enrolled in this case-control study were sequentially admitted to coronary care unit (CCU). An informed written consent was obtained from the parents or guardians of all participants prior to their inclusion in the study. The study was approved by the ethical committee of Faculty of Medicine and according to The Code of Ethics of the World Medical Association (IRB no: 17300761**)** and according to Good Clinical Practice guidelines developed by the International Conference on Harmonization. The laboratory portion of the study was conducted at the Medical Research Centre and the Department of Medical Biochemistry, Faculty of Medicine.

The patients were selected based on the following inclusion criteria: children who were first diagnosed as DCM patients and receiving no treatment, either males or females up to 18 years of age. Exclusion criteria were other forms of cardiomyopathy (restrictive or hypertrophic), congenital heart disease, patients on regular conventional treatment (i.e., digoxin, diuretics, and ACE inhibitors), and those with any chronic illness e.g., chronic kidney disease.

All patients enrolled in the study had a full medical history taking and careful clinical examination. Two-dimensional transthoracic echocardiography was performed to assess left ventricle (LV) dimensions and fractional shortening percent (FS %) by the modified Simpsons Method. Z-score of echocardiography calculation was considered for measurements of cardiac performance factors such as left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and interventricular septum (IVS). The Z-score is the standard deviation unit, that is applied for standardization and normalization.

In addition, 34 healthy children were invited to participate in the study. They are subjected to careful clinical history, examination and echocardiography.

Sample Collection

4 ml of blood was collected in EDTA tubes that allowed to stand in room temperature for few minutes then centrifuged at 3000g for 15 min. Plasma was collected in Eppendorf tubes and kept at -80 °C until batch analysis for determination of PIIINP, Cys-C, β2M, and Hp levels.

Biochemical Assay

Measurements of the plasma levels of total carnitine, PIIINP, Cys-C, β2M, and Hp in all participants were performed by using commercially available sandwich enzyme-linked immunosorbent assays (ELISA) kits, supplied by Cloud Clone Corporation, USA with the catalog numbers: SED087Hu, SEA896Hu, SEA260Hu, and SEA817Hu, respectively and following the guidelines supplied with each kit.

Real-Time Quantitative PCR Analysis (RT-PCR) of Acylcarnitine

The genomic RNA was extracted from whole blood specimens using a Gene JET RNA purification kit (Thermo Scientific, Catalog No. #K0731) in accordance with the operational instructions. Purified RNA was quantitated using Nanodrop® (Epoch Microplate Spectrophotometer, Biotek, USA). The High-Capacity cDNA synthesis kit (Applied Biosystems, CA, USA) was used to obtain complementary DNA (cDNA) as per manufacturer's instructions. Next, cDNA was collected after transcription.

Real-time PCR reactions were done with Maxima SYBR Green qPCR master mix (2X) kit supplied by Thermo Fisher Scientific, USA, Catalog #K0251). Performance of PCR was done using Step one 7500 fast real-time PCR machine (Applied BioSystem, CA, USA). Each amplification cycle comprised of initial denaturation step for 5 min at 95° C, followed by 40 cycles of denaturation (95° C for 45 sec), annealing (60° C for 45 sec), and extension (72° C for 1 min) then final extension (72°C for 10 min).

The 5′-3′ primer sets used for the detection of acylcarnitine were forward: CTTCATTGGTATGGAATCTGCTGG and reverse: CATTGTTGGCATACAGGTCCTTG.

Housekeeping gene *GADPH* primers used were forward: CAAGG CTGAGAACGGGAA and reverse: GCATCGCCCCACTTGATTTT. Primers sequences were obtained from (Invitrogen, UK) according to primer 3 algorism. Acylcarnitine expression levels were normalized to *GAPDH* as the endogenous control and results were expressed as fold change by the 2-ΔΔCT equation [23]. Non-template blank wells were used to guarantee the accuracy of the expression.

Statistical Analysis

Data were verified, coded by the researcher, and analyzed using IBM_SPSS version 24. Descriptive statistics: Means, standard deviations, medians, and percentages were calculated. Normality was tested for the main parameters using Shapiro Wilk test. Test of significances: Student t-test/Mann Whitney U test was calculated to test the mean/median differences of the data according to normality. Pearson product-moment correlation coefficient was calculated for the univariate correlations between biomarkers levels with each other and clinical data. ROC curve was depicted for the disease biomarkers, analyzed as area under the curve (AUC), standard error (SE), and 95% confidence interval (CI). The following validity statistics were computed: sensitivity, specificity, and positive and negative predictive values (PPV and NPV). A p-value of less than 0.05 was deemed significant.

RESULTS

The Demographic and Echocardiographic Data of the Study Cohort:

The demographic and echocardiographic data of the cases and controls are presented in Table 1. The was no differences regarding ages or sex ratio. However, there was a high mean level of LVEDD, LVESD, IVS, and a reduced mean level of FS.

Table 1: Baseline demographic and echocardiographic data of the study cohort

Parameter	Case (n=84)	Control (n=34)	P-value
Age/years			= 0.480
• Mean ± SD	6.96 ± 3.7	7.5 ± 4.6	
• Median (Range)	6.7 (1.2 – 18)	6.6 (1.3 – 18)	
Sex			=0.872
• Female	32 (38.1%)	12 (35.3%)	
• Male	52 (61.9%)	22 (64.7%)	
LVEDD (mm)			
• Mean ± SD	50.02 ± 11.98	26 ±4.1	**< 0.001***
• Median (Range)	48 (33.5 – 74.6)	25 (22 – 30.5)	
LVESD (mm)			
• Mean ± SD	39.2 ± 11.74	15.0±3.5	**< 0.001***

• Median (Range)	38.5 (23 – 65)	17.5 (12 – 21)	
IVS (mm)			= 0.215
• Mean ± SD	6.58 ± 1.5	7.0 ± 2.0	
• Median (Range)	7 (3.5 – 8.8)	6.5(4 – 9.0)	
FS (%)			**< 0.001***
• Mean ± SD	22.23 ± 3.94	33 ± 4.9	
• Median (Range)	20 (7 – 26)	35 (28– 44)	

FS: fractional shortening, IVS: interventricular septum, LVEDD: left ventricular end diastolic diameter, LVESD: left ventricular end systolic diameter, SD: standard deviation. P value significant if <0.05%.

The Levels of Biochemical Parameters among the Study Groups:

Levels of biochemical parameters in DCM children versus controls as shown in Table 2 revealed significant increase in the plasma level of PIIINP (p = 0.016), Cys-C (p = 0.001), β2M (p =0.009), and Hp (p < 0.001) in the DCM group relative to the control group. In addition, a significant upregulation of the acylcarnitine expression gene was observed in the DCM group compared to controls (Fig.1,2).

Table 2: Levels of the studied biomarkers among the study cohort

Parameter	Case (n=84)	Control (n=34)	P-value
Total Carnitine (umol/ml)			**< 0.001***
• Mean ± SD	98.09 ± 51.2	52.68 ± 13.1	
• Median (Range)	82.5 (30 – 229)	55 (23 – 77)	
Total Carnitine (umol/ml)			**< 0.001***
• Mean ± SD	98.09 ± 51.2	52.68 ± 13.1	
Procollagen type III N-terminal propeptide (U/ml)			**= 0.016****
• Mean ± SD	3.82 ± 2.5	2.73 ± 2.1	
• Median (Range)	3.6 (0.5 – 7.3)	3.2 (0.2 – 6.1)	
Cystatin-C			**= 0.001***
• Mean ± SD	1.11 ± 0.7	0.86 ± 0.1	
• Median (Range)	0.8 (0.6 – 2.9)	0.9 (0.6 – 1.1)	
β2-Microglobulin (mg/L)			**= 0.009****
• Mean ± SD	2.98 ± 1.7	1.20 ± 0.6	
• Median (Range)	2.8 (0.5 – 8.6)	0.9 (0.7 – 2.8)	
Haptoglobin (mg/dl)			**< 0.001***
• Mean ± SD	187.49 ± 105.5	142.85 ± 6.3	
• Median (Range)	195 (39 – 396)	145 (129 – 155)	

***Student t-test was used to compare the difference in the mean among groups**
****Mann Whitney U test was used to compare the difference in the median among groups**

Figure 1: Agarose gel electrophoresis. The PCR product of carnitine palmitoyl transferase 1was visualized via UV light on 2% agarose gel. Lane 1 contains 100 bp DNA ladder; lane 2-10 for amplified fragment 200 bp

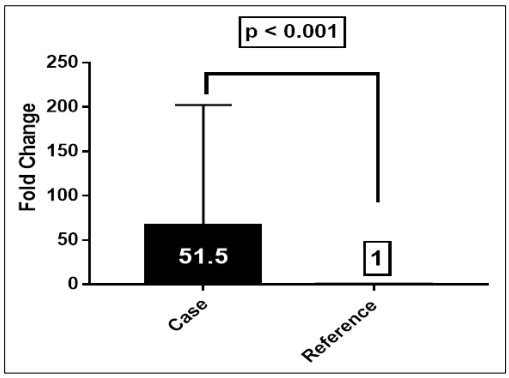

Figure 2: Carnitine palmitoyl transferase 1fold change among the study cohort

Diagnostic Criteria of the Studied Biomarkers for Prediction of pDCM:

To determine whether the levels of biochemical markers had diagnostic accuracy for DCM discrimination, the receiver operator characteristic (ROC) curve analysis was used to define biomarkers sensitivity, specificity, accuracy, area under the ROC curve (AUC), positive predictive value (PPV), negative predictive value (NPV), and Cutoff (Table 3, and Fig. 3).

Total carnitine, PIIINP and β2M at cut-off points 65 umol/ml &3 & 2 mg/L showed 73&79% & 75% sensitivity and 91& 56% & 81% specificity respectively for predicting risk of pDCM. Haptoglobin at cutoff point 164 mg/L has highest specificity (100%) but with low sensitivity 56%). See table 3.

Table 3: Diagnostic criteria of the studied biomarkers for prediction of pDCM

Parameter	Total Carnitine	PIIINP	Cystatin-C	β2-Microglobulin	Haptoglobin	β2M + PIIINP + total carnitine
• AUC	0.771	0.642	0.512	0.869	0.565	0.786
• Cut-off	65	3	0.9	2	164	2 & 3 & 65
• Accuracy	82%	72%	49.5%	78%	78%	83.5%
• Sensitivity	73%	79%	50%	75%	56%	75%
• Specificity	91%	65%	49%	81%	100%	92%
• PPV	89%	69%	49.5%	80%	100%	90%
• NPV	77%	75.5%	49.5%	76%	70%	79%
• 95% CI⁺	0.689 - 0.853	0.527 - 0.757	0.411 - 0.612	0.803 - 0.935	0.460 - 0.670	0.707 - 0.866
• SE**	0.042	0.059	0.051	0.034	0.054	0.040
• P-value***	< 0.001	= 0.016	= 0.842	< 0.001	= 0.272	< 0.001

AUC: area under the curve; PPV: positive predictive value; NPV: negative predictive value; β2M: β2-Microglobulin; PIIINP: procollagen type III N-terminal propeptide
****SE = Standard Error +CI = Confidence Interval**
*****Null hypothesis: true area = 0.5**

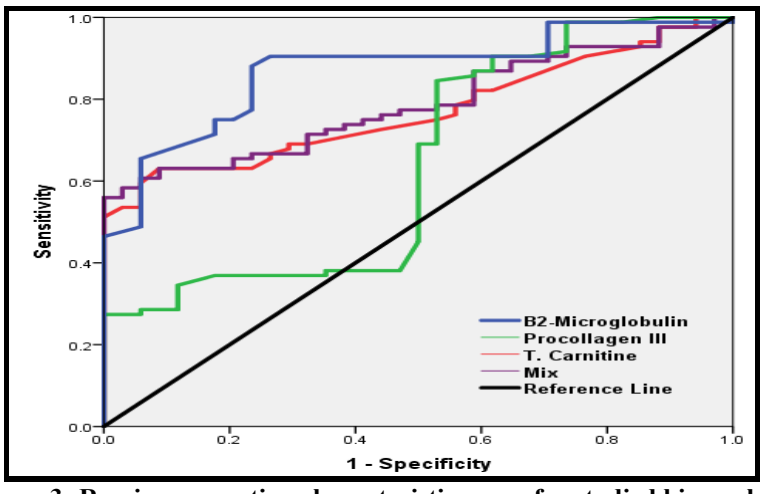

Figure 3: Receiver operating characteristic curve for studied biomarkers

Correlation Coefficients (r) of the Studied Biochemical Markers

Significant positive correlations were found between plasma β2M with Cys-C (r = 0.408, p< 0.001) and PIIINP biomarkers (r = 0.278, p=0.005). Also, haptoglobin showed significant positive correlation with β2M (r = 0.699, p< 0.001), Cys-C (r = 0.452, p< 0.001) and PIIINP biomarkers (r = 0.362, p< 0.001). See Table 4, Fig. 4&5.

Table 4: Correlations between studied Biomarkers

	Haptoglobin	B_2-Microglobin	Cystatin-C
	r* (p-value)		
• **Haptoglobin**			
• **B_2-Microglobin**	**0.699 (< 0.001)**		
• **Cystatin-C**	**0.452 (< 0.001)**	**0.408 (< 0.001)**	
• **Procollagen III**	**0.362 (< 0.001)**	**0.278 (= 0.005)**	0.169 (= 0.062)

***Pearson's correlation coefficient**
****Spearman Rank Correlation**
-Correlation is significant at the 0.05 level (-tailed), based on normal approximation

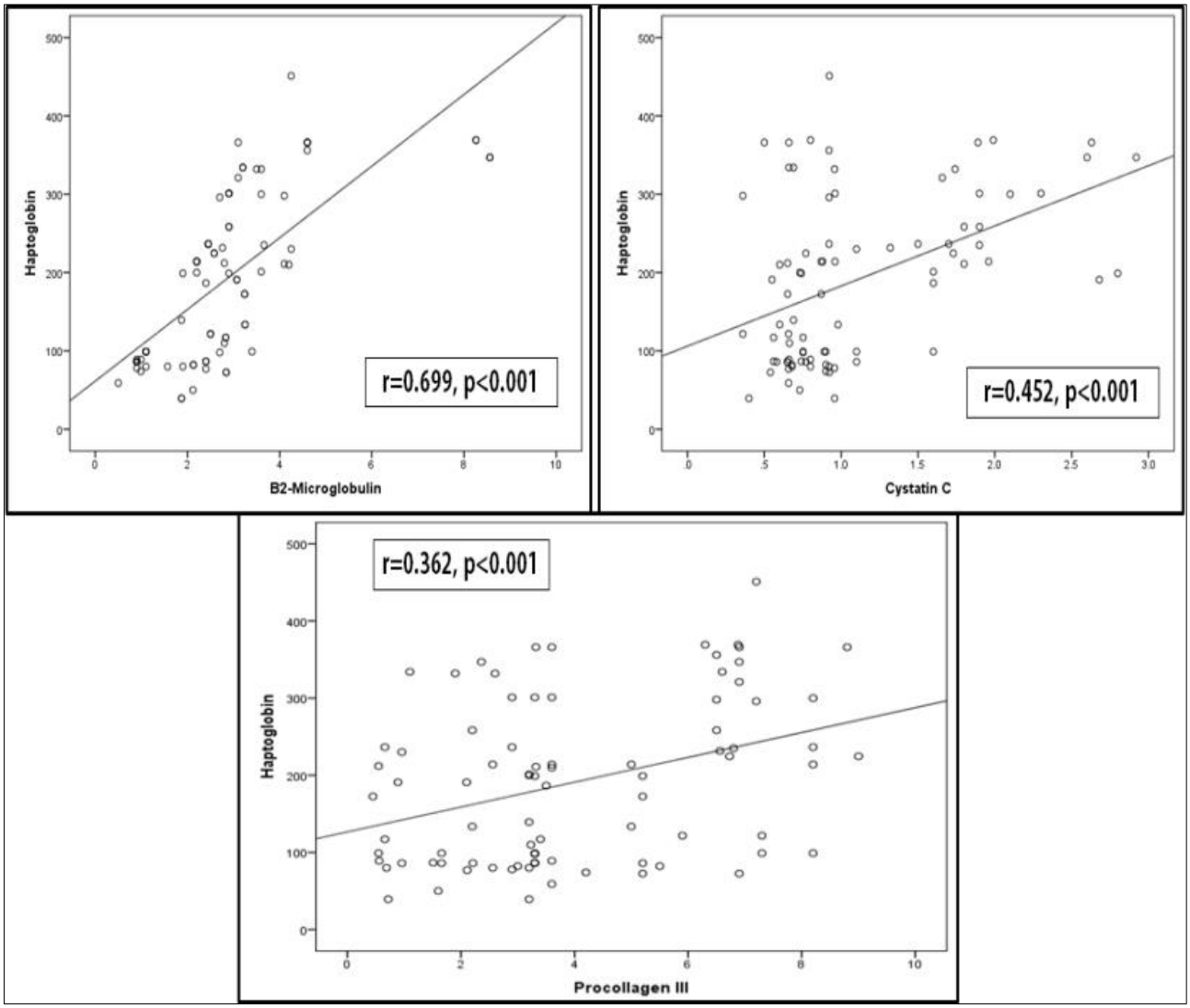

Figure 4: Correlations of Haptoglobin, B2-Microglobin, Cystatin-C and Procollagen III Biomarkers

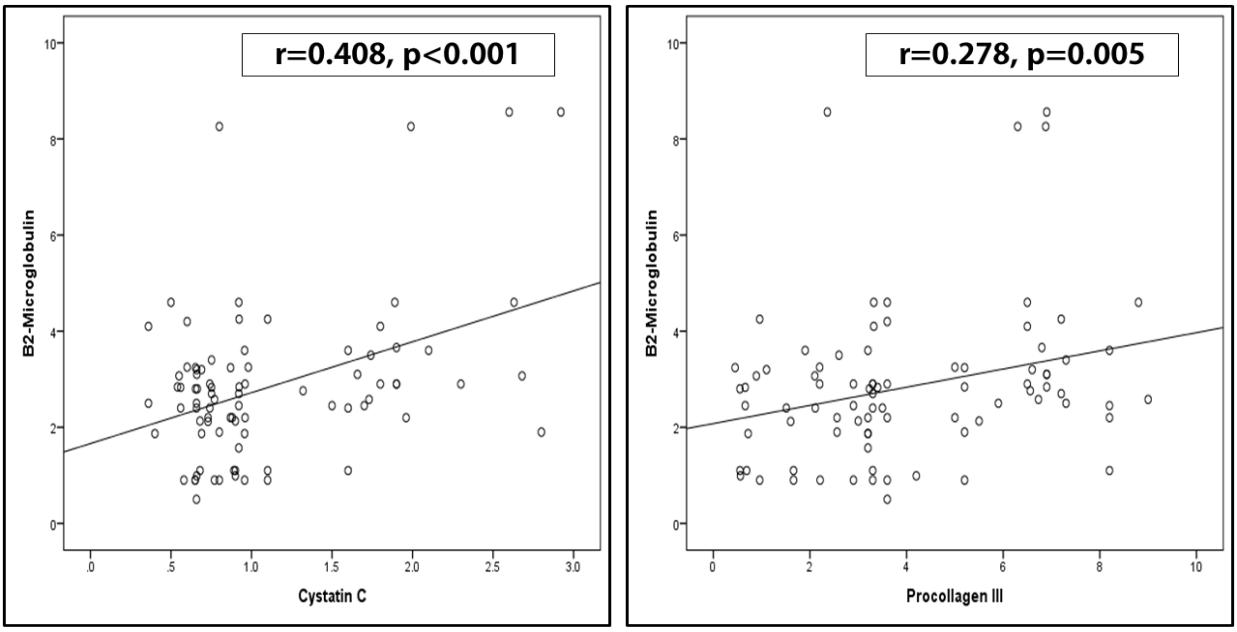

Figure 5: Correlation of B2-Microglobin, Cystatin-C and Procollagen III Biomarkers

DISCUSSION

Cardiomyopathy is the most serious disorder and the leading cause of disability in children worldwide. Pediatric cardiomyopathy has different pathophysiological characteristics and includes a hidden early phase of cell and metabolic dysfunction, compensated middle phase, where cell apoptosis starts and decompensated late phase, which is characterized by systolic and diastolic impairment. It usually manifested as chronic systolic heart failure leading to comorbidities, arrhythmias, and sudden death [17]. Hence, identifying the underlying mechanism and novel non-invasive biomarkers are essential for developing new therapeutic strategies to prevent the progression of myocardial remodeling, enhance the quality of life, avoid the endomyocardial biopsy (EMB), and reduce mortality.

Energy metabolism deterioration in DCM patients is established as lipotoxicity and reduced metabolic flexibility [18]. Carnitine is crucial in the transport of fatty acids with lengths greater than 10 carbon atoms throughout mitochondrial membranes for oxidation, detoxification of organic acids, and amino acid catabolism. Disruption in carnitine shuttle enzymes and an ineffective beta-oxidation as increased CPT I activity and decreased CPT II activity resulting in an accumulation of cellular long-chain acylcarnitines [19]. A high acylcarnitine to free carnitine ratio indicates insufficient free carnitine to eliminate organic acids and acyl compounds out of the mitochondria.

In this study, a significantly higher plasma level of total carnitine was found in pediatric DCM relative to control. Acylcarnitine expression level was significantly elevated in DCM children with a higher level in severe cases and those who had low left ventricular fractional shortening. Moreover, the biological significance of total carnitine as a diagnostic and predictive biomarker in pediatric DCM was demonstrated using the ROC curve, indicating that accumulation of acylcarnitine plays a key pathogenic role, and enhances a mismatch in fuel burn of the myocardial cells. These results support the obtained findings of McHugh *et al*., who found significant plasma long-chain acylcarnitine (LCAC) overexpression in inherited FAO disorders that evident with cardiac dysfunction (amongst other symptoms), like CPT II deficiency [20]. It has been reported that high LCAC levels have been linked to important CVD such as HF, CAD, and cardiac arrhythmias [21, 22].

Kukharenko *et al*., found that medium-chain acylcarnitines distinguish patients with the cardiovascular disorders from clinically healthy individuals [23]. Also, long-chain acylcarnitine was found to be positively associated with cardiovascular death and reduced cardiac function [24], and is capable of predicting cardiovascular events in elderly people with a past history of CAD [25]. It has been reported that acylcarnitine species are highly correlated to the degree of affected coronary arteries that predict the adverse outcomes in CAD. This suggests that circulating acylcarnitine levels may reflect the degree of severity of cardiomyopathy.

A buildup of intramitochondrial long-chain acylcarnitines can disturb numerous regulatory mechanisms [18-26]. First, it impedes pyruvate and lactate oxidation resulting in the inability to switch between substrates for energy production based on availability. Second, it impairs membrane function, causes electrophysiological changes via calcium and potassium channel signaling (contributing to heart arrhythmias), enhances inflammation, hinders oxidative phosphorylation, and enhances the generation of reactive

oxygen species [27]. As a result, the heart is subjected to excessive strain, resulting in structural and functional changes that eventually lead to DCM [28]. Excess acylcarnitine can then be released through the blood and urine, detoxifying mitochondria of excess carbons [29, 30]. Thus, assessing the dysregulation of acylcarnitine expression may be used as the potential indices to predict DCM in children. Another potential mechanism for acylcarnitines-induced tissue damage is characterized by pro-inflammatory signaling pathways that cause cardiomyocyte membrane damage through induction of cyclooxygenase-2, proinflammatory cytokines, phosphorylating ERK/JNK/NF-kB, and reactive oxygen species production [31].

Exogenous carnitine supplementation is not routinely used in cardiomyopathy. However, pharmacotheraputic use in DCM should be considered since the anti-inflammatory impacts of carnitine supplementation on restoring mitochondrial metabolic activity in cardiomyocytes and lowering oxidative stress and inflammation via cytokine reduction have been identified [32]. Additionally, a meta-analysis study conducted by Weng *et al*., revealed that the addition of L-carnitine to the treatment of DCM patients may have an additional improvement in heart functioning [33].

Fibrosis of the cardiac ECM is among the main characteristics of DCM, specifically reactive fibrosis, and is observed in 40–60% of patients. Cardiac fibrosis contributes significantly to the development of HF symptoms and re-entrant ventricular arrhythmias, which leads to augmented morbidity and mortality in DCM [34]. Endomyocardial biopsy and microscopic evaluation of cardiac samples have lengthy been regarded as the gold standard in estimation of fibrosis [7]. Non-invasive easily-accessible biomarkers that allow for measurement of replacement and interstitial fibrosis, on the other hand, are gaining traction. ECM proteins and/or fibrosis-related biomarkers are released into the circulation during the fibrosis process. The analysis of circulating fibrosis-related indices can provide information about end-organ fibrosis [5].

In previous studies, Yang *et al*., revealed links between collagen biomarkers and matrix metalloproteinase-2 with collagen volume fraction in hypertrophic cardiomyopathy patients [35]. Likewise, Ravassa *et al*., reported an association between the ratio of collagen and matrix metalloproteinase-1 and biopsy-proven fibrosis among hypertensive HF patients [36]. Furthermore, Osokina *et al*., demonstrated a high prognostic probability of serum PIIINP in predicting progression of cardiac fibrosis 1 year after myocardial infarction among preserved systolic function patients [37]. This goes with our findings where plasma levels of PIIINP were significantly higher in the DCM children compared to free control values ($P = 0.016$). It has been reported that raised PIIINP level is associated with lower survival rates in both HF and DCM and it is an independent predictor of HF death [38]. In contrast to our findings, Rubiś *et al*., found a dearth of associations between PIIINP levels and interstitial fibrosis in a cohort of DCM patients [39]. This incoherence could be attributed to differences in sample size, methodology, and ethnicity. So, additional evaluation is warranted.

B2-Microglobin and Cys-C have gained broad international attention in the pathogenesis of poor health problems, including kidney disease, inflammation, atherosclerosis, cardiovascular diseases, immunologic, metabolic, neoplastic disorders, and overall death [10,11].

Plasma β2M and Cys-C displayed significantly higher levels among the pediatric DCM group relative to the control group. B2-Microglobin showed the best AUC of 0.869 with 75% sensitivity and 81% specificity at cut-off point 2 mg/L. In keeping with our findings, Wojciechowska *et al*., found that elevated β2M levels are characteristic of non-ischaemic DCM patients versus healthy controls. Circulating B2M has been also stated to be an independent predictor of death in adults with CVD, possibly better compared to other inflammatory cytokines such as C-reactive protein and Cys-C [40, 41].

In large and diverse populations with normal renal function, meta-analysis studies demonstrated a significant association between raised Cys-C and the risk of cardiovascular incidents or mortality and found that Cys-C was a good independent predictor of adverse outcomes in acute coronary syndrome (ACS) patients [15-42].

The link between β2M, Cys-C, and pediatric DCM may be partly related to renal function. β2M and Cys-C have been identified as renal function markers and their circulating levels increase when the glomerular filtration rate GFR falls. Inflammatory response has also been proposed as a possible mechanism that linking β2M, Cys-C, and CVD. Evidence suggested that the increased β2M and Cys-C levels were positively related to inflammatory markers. This is supported by the positive correlation between β2M and Cys-C and between β2M, PIIINP, and Hp. Zhang *et al*., demonstrated that β2M is elevated in patients with left atrial remodeling due to atrial fibrillation (AF) as AF is closely linked to myocardial strain, fibrosis, and inflammation [43].

We found that mean level of Hp was significantly increased in DCM children compared with controls ($P < 0.001$). Elevated plasma Hp concentration perhaps because of its acute-phase response nature and role in removing oxidative species from bloodstream. Hp showed a significant positive correlation with β2M, Cys-C, and PIIINP ($P < 0.001$ each). These findings support the mechanistic relationships of these biomarkers and favoring their role in the pathogenesis of myocardial remodeling. In an agreement, previous studies proposed

that high Hp plasma concentrations predict the risk of cardiovascular disease, representing an important risk factor of acute MI, stroke, and HF [44, 45]. Chiang *et al*., identified that raised levels of Hp were aligned with poor overall survival in MI patients [46]. On the other hand, a previous study determined lowest level of Hp in the most clinically severe group of HF patients [47].

CONCLUSION

The novel contribution of this study lies in examining the impact of elevated levels of acylcarnitine, total carnitine, β2-microglobulin (β2M), cystatin C (Cys), haptoglobin (Hp), and procollagen type III N-terminal propeptide (PIIINP) on the risk and future clinical outcomes of pediatric dilated cardiomyopathy (DCM). These findings suggest that these biomarkers could serve as early potential predictors of pediatric DCM. However, further research is needed to determine the optimal timing for these tests, evaluate their advantages over other clinical indices, and conduct a comprehensive investigation into the molecular mechanisms underlying their pathogenic roles. Larger sample sizes are recommended to validate their clinical utility.

Limitations:

Single center study and small sample size are limitations for our study. Also, further correlation of these new biomarkers with disease severity and prognosis needs further studies.

Data Availability Statement:

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent and Ethical Approval:

An informed written consent was obtained from the parents or guardians of all participants prior to their inclusion in the study. The study was approved by the ethical committhee of Faculty of Medicine and according to The Code of Ethics of the World Medical Association (IRB no: 17300761**)** and according to Good Clinical Practice guidelines developed by the International Conference on Harmonization.

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Author Contributions

Marwan S. Mahmoud: Conception and design of the study, collection of patient data, and critical review of the manuscript. **Naglaa K I**: Conducted laboratory experiments, performed biomarker analysis, and contributed to the revision of the manuscript for important intellectual content **Blann AD**: Analyzed and interpreted clinical data, performed statistical analysis, and drafted the manuscript. **Marwa AG**: Interpreted molecular findings and contributed to manuscript writing. **Reham IE**: Conducted statistical analyses and provided technical assistance throughout the study. **Sally A S, Mohamed GE& Mohammed M M**: Provided oversight of the experimental design, offered critical feedback on the manuscript. **Mahmoud A, Asmaa MI& Duaa MR**: Assisted in data collection, provided pediatric cardiology insights, and contributed to data validation & **Amr A**: Conducted statistical modeling, and assisted in the recruitment of pediatric samples. All authors contributed to the writing, editing, and final approval of the manuscrip**t.**

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