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Interleukin 1 receptor-like 1 protein (ST2) is a potential biomarker for cardiomyopathy in Duchenne muscular dystrophy

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Abstract

Background: Duchenne muscular dystrophy (DMD) is a rare, fatal X-linked disorder characterized by the lack of dystrophin, a key sarcolemma muscle protein. Cardiac failure is a significant cause of death in DMD subjects.

Objective: The purpose of our research was to identify potential cardiac serum biomarkers associated with DMD cardiomyopathy.

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Conflict of Interest: Julia Andersen declares that she has no conflict of interest. Haeri Seol declares that she has no conflict of interest. Heather Gordish-Dressman declares that she has no conflict of interest. Yetrib Hathout declares that he has no conflict of interest. Christopher Spurney declares that he has no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Ethical approval: This article does not contain any studies with animals performed by any of the authors.

Informed consent: Informed consent was obtained from all individual participants included in the study.

Methods: This is an observational, case-controlled study using subjects from the CINRG DMD Natural History study with cardiomyopathy (ejection fraction (EF)<55%; shortening fraction (SF)<28%), subjects without cardiomyopathy (EF ≥55%; SF ≥28%) compared to normal healthy volunteer subjects.

Results: The DMD with cardiomyopathy group had significantly lower average EF and SF (EF=45±10% / SF=25±2%) than the DMD without cardiomyopathy group (EF=58±5% and SF=32±3%; p<0.01). Among a selected set of potential biomarkers for cardiomyopathy (MMP9, BNP, GAL3, CRP, LEP, TNC, TLR4 and ST2) we validated ST2 as significantly elevated in the serum of DMD cardiomyopathy group (35798±4884 pg/mL) compared to normal controls (9940 ± 2680 pg/mL; p<0.01; n=6). Matrix metalloproteinase 9 (MMP9) levels were found significantly increased in both DMD groups compared to controls (p<0.01). No significant differences were seen in BNP, GAL3, CRP, LEP, TNC or TLR4 levels.

Conclusions: Increased ST2 levels were found in serum of DMD subjects compared to healthy volunteers and further elevated in DMD subjects with cardiomyopathy. Future studies correlating cardiomyopathy with ST2 levels may allow for improved non-invasive monitoring of cardiac disease in DMD subjects.

Keywords

Duchenne muscular dystrophy; cardiomyopathy; biomarkers; ST2

Introduction:

Duchenne muscular dystrophy (DMD) is a rare, fatal X-linked disorder characterized by the lack of dystrophin, a key sarcolemma muscle protein, leading to membrane fragility and muscle cell death [1]. Due to improvements in the treatment of skeletal and respiratory muscular systems, the majority of DMD subjects now die from cardiac related causes [2]. Therefore, early recognition and treatment of cardiac disease is imperative. Recent advances in non-invasive imaging have demonstrated the presence of early myocardial disease in pediatric patients with DMD prior to the development of systolic dysfunction [2]. These include echocardiographic measures including tissue Doppler and myocardial strain imaging [3]. Cardiac magnetic resonance (CMR) is becoming an important imaging modality providing myocardial characterization using T1 imaging and late gadolinium enhancement to aid in the early diagnosis of myocardial fibrosis in DMD and guide early therapeutics [4].

Another useful approach to early diagnosis and monitoring of musculoskeletal disease is the analysis of serum biomarkers [5]. Serum biomarkers are becoming an important and non-invasive measure of DMD disease progression and disease milestones [6,7]. Recently, Nadarajah et al. showed that serum levels for the matrix metalloproteinase 9 (MMP9) are associated with skeletal muscle pathogenesis and progression in DMD [8]. Serum biomarkers are also used to monitor cardiac disease. Proven cardiomyopathy biomarkers include brain natriuretic peptide (BNP), tenascin C (TNC), thrombospondin-4 (TSP4), interleukin 1 receptor-like 1 protein (ST2) and galectin-3 (Gal3) [9]. Of these, ST2 is involved in CD4⁺ T cell activation and stimulates production of Th2 cytokines. Elevated ST2 levels are emerging as a significant cardiac biomarker predicting heart failure in adult

subjects with cardiac fibrosis, a common pathological finding in DMD cardiomyopathy [10]. The purpose of our research was to identify potential cardiac serum biomarkers associated with DMD cardiomyopathy.

Methods:

The study protocol was approved by the Institutional Review Board at Children's National Health System (PR#02004). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study or their parent/ legal guardian. This is an observational, case-controlled study. All subjects were male. Serum samples were collected from consenting DMD (n=24) and healthy sex matched control (n=6) subjects enrolled in the Cooperative International Neuromuscular Research Group (CINRG) DMD Natural History Study at 5 clinical sites (clinicaltrials.gov: NCT00468832) from 2011 to 2013. Cases were randomly selected from subjects with documented echocardiogram results to form appropriate age matched groups. Data recorded at the time of serum collection included age, steroid use, date of last echocardiogram, measure of ejection fraction (EF) and/or shortening fraction (SF) and cardiac medication use. Cardiomyopathy was defined as a reported EF < 55% and/ or SF < 28% [11].

Serum Sample Preparation:

Serum extraction from blood samples included inverting blood samples, allowing samples to clot for 30 minutes at 21 °C and centrifuging tubes at 1000-1300 g for 10 minutes. Serum was then carefully collected and aliquoted equally into polypropylene Nalgene Cryogenic vials (ThermoScientific, Waltham, MA).

SOMAscan Assay:

The SOMAscan proteomic assay (SomaLogic, Boulder, CO) is designed to measure concentrations of different amount of proteins in a small volume of serum sample [12]. In brief, each of the 1,125 proteins measured in serum by the version of the SOMAscan assay performed in this study has its own targeted SOMAmer reagent, which is used as an affinity binding reagent and quantified on a custom Agilent hybridization chip. SOMAmer reagents include modified nucleotides which form complexes with the proteins in solution, and the concentration of the hybridized protein and reagent are quantified by microarray or quantitative polymerase chain reaction (qPCR) [12]. DMD and control serum samples were prepared at collection sites. In the SOMAscan assay, samples were randomly assigned to plates within each assay and run along with a set of calibration and normalization samples. No identifying information was available to the laboratory technicians operating the assay. Intra-run normalization and inter-run calibrations were performed according to SOMAscan v3 assay data quality control procedures as defined in the SomaLogic good laboratory practice quality system.

ELISAs:

Target markers were analyzed via enzyme-linked immunosorbent assays (ELISAs) (R&D Systems, Minneapolis, MN) in duplicate using an xMark spectrometer (Bio-Rad Laboratories, Hercules, CA). ST2, MMP9, Gal3, LEP, and CRP antibodies were purchased from R&D Systems and TLR4, TNC and BNP antibodies were purchased from [Antibodies-Online.com](#) (Atlanta, GA). Samples were stored in -80°C freezers and diluted with ELISA kit specific reagents in phosphate buffered saline (PBS) or PBS Tween in specified dilutions ranging from 1:250 to 1:50. They were plated in duplicate and run in conditions specified in the kit instructions, and washed with either PBS or PBS Tween (as required by the kit protocol). Streptavidin conjugated horse radish peroxidase was used for coloration and 2N sulfuric acid stopped the colorimetric reaction (R&D Systems).

Statistical methods:

All continuous outcomes (protein expression levels, serum levels, EF%, SF%) evaluated in this study were assessed for normality using both the Shapiro-Wilk normality test and a visual inspection of distributions. Log transformations were applied to any outcome not normally distributed and normality of the log-transformed data was verified.

Comparisons of continuous outcomes between patient groups were performed using a student's t-test or analysis of variance (ANOVA) as appropriate. For any ANOVA yielding a significant overall F-test, post-hoc pairwise comparisons were performed and the resulting p-values adjusted for multiple comparisons using the Sidak method. Comparisons of categorical characteristics (steroid use, cardiac medication use) were compared between groups using a Fisher's exact test.

Further analysis evaluated the relationship between serum levels and age and compared that among the three groups using linear regression models. In each model, serum level was the dependent variable, age was the independent variable and group was a categorical factor. For each model a group x age interaction term was tested. If significant, the interaction term was retained in the model. The healthy control group was included as a comparison group for all proteins except Gal-3, TNC, BNP, and C-reactive protein (CRP) for which the number of control samples available for analysis was inadequate (2 or 3).

All analyses were performed using Stat V14 (College Station, TX) and a p-value of 0.05 was considered statistically significant. No adjustment for multiple testing was done for the proteins tested in ELISAs.

Results:**Patient Characteristics:**

A subset of 12 subjects with DMD and cardiomyopathy, 12 subjects with DMD and normal cardiac systolic function and 6 normal healthy controls were selected from our natural history DMD cohort [13]. The average age of the DMD with cardiomyopathy group was 18.2 ± 6.7 years (mean $\pm$ SD), DMD without cardiomyopathy group 18.3 ± 6.3 years and normal controls 12.2 ± 4.2 years. While the control groups mean was younger, there was no

significant difference in age between these groups. The DMD with cardiomyopathy group had significantly lower average EF and SF (EF=45±10% / SF=25±2%) than the DMD without cardiomyopathy group (EF=58±5% and SF=32±3%; $p<0.01$). Glucocorticoids were used in 9 of 12 DMD subjects with cardiomyopathy and 8 of 12 DMD subjects without cardiomyopathy. There were 9 of 12 DMD cardiomyopathy subjects taking cardiac medications: all 9 used ACE inhibitors or angiotensin-receptor blockers, 3 used beta-blockers, 2 used diuretics and 1 used digoxin. There were 7 of 12 DMD subjects without cardiomyopathy taking medications: 5 used ACE inhibitors or angiotensin-receptor blockers and 4 used beta blockers. One DMD subject with cardiomyopathy reported a hospitalization for heart failure symptoms; all others reported no cardiac symptoms.

SOMAscan Assay:

From a panel of over 1,125 proteins in the SOMAscan assay, we selected 8 proteins known to be related to cardiac and skeletomuscular disease: MMP9, ST2, CRP, Toll-like receptor 4 (TLR4), Leptin (LEP), TNC, BNP, and Gal3 [6]. Of these proteins, ST2, MMP9, LEP and CRP showed elevated, upward trends (not significant) in DMD subjects compared to controls and were selected for validation (Table 1).

ELISAs:

We validated protein levels of MMP9, ST2, CRP, TLR4, LEP, TNC, BNP, and Gal3 using ELISAs based on SOMAscan results and literature review (Table 2) [9,14]. Of this panel, only ST2 and MMP9 showed significantly different levels among groups. DMD subjects with cardiomyopathy showed a significantly greater ST2 level than normal controls ($p=0.008$) (Table 2; Figure 1). DMD subjects without cardiomyopathy also exhibited greater mean ST2 levels than healthy controls, however the difference showed a trend but did not reach statistical significance ($p=0.1$). There was no statistical difference between ST2 levels in DMD cardiomyopathy and DMD without cardiomyopathy group ($p=0.27$). ST2 levels showed a significant increase with age ($p=0.01$) with an increase of 1177 pg/mL per year of age regardless of group (Table 3; Figure 2). In addition, the DMD subjects with cardiomyopathy showed consistently greater and significantly increased ST2 levels over all ages as compared to normal controls ($p=0.01$; Table 3; Figure 2). MMP9 levels were found to be significantly greater than normal controls in both the DMD subjects with cardiomyopathy ($p=0.001$) and the DMD subjects without cardiomyopathy ($p=0.002$) (Table 2; Figure 3). MMP9 levels showed a significant increase with age ($p=0.004$) with an increase of 0.05 ng/mL per year of age regardless of group (Table 3; Figure 4). In addition, both DMD subjects with cardiomyopathy and without cardiomyopathy showed consistently greater and significantly increased MMP9 levels over all ages as compared to normal controls (Table 3; Figure 4). No significant differences between groups were seen in BNP, Gal3, CRP, LEP, TNC or TLR4 levels (Table 2) nor were any significant group/ age interactions observed when the relationship between biomarker levels and age were assessed.

Discussion:

This study demonstrated that the serum biomarker ST2 was increased in subjects with DMD cardiomyopathy compared to subjects with DMD and normal cardiac systolic function and healthy controls. We also validated MMP9 as a serum biomarker in DMD, indicative of skeletal muscle disease as it was elevated in all subjects with DMD, regardless of cardiac status, compared to normal controls.

Increased ST2 levels associated with DMD cardiomyopathy is a novel finding and could improve diagnosis and management of cardiac disease in DMD. We also demonstrate increased ST2 levels in DMD subjects without cardiomyopathy compared to controls. ST2 levels are known to increase significantly with age in normal pediatric and adult male subjects, approximately 500 pg/ml per year in pediatric males [15,16]. The etiology of this increase is not yet known. This study showed an increase of 1177 pg/ml per year in DMD cardiomyopathy subjects (Table 3). These increased ST2 levels and rate of increase in DMD subjects could reflect early myocardial fibrosis related to the loss of dystrophin. Cardiac magnetic resonance imaging is becoming the preferred imaging in DMD, but with the current concerns regarding gadolinium contrast, cost and limited accessibility of MRI, ST2 levels may provide an easier, quicker minimally invasive means of following the progression of cardiac fibrosis undetectable by echocardiography in young DMD boys [17,18].

ST2 is known to be elevated in adults with chronic heart failure and used as a biomarker to track the prognosis and mortality in chronic heart failure patients with high sensitivity [19,20]. Bayes-Genis et al. also showed ST2 was more sensitive than Gal3 in detecting myocardial fibrosis [21]. Pathological specimens demonstrate the presence of myocardial fibrosis in DMD [22,23]. Therefore, as shown in this study, ST2 levels should correlate with the progression of cardiac fibrosis and development of systolic dysfunction in DMD. ST2 levels were also shown to predict sudden death in patients with chronic heart failure, a significant concern and area of limited understanding in DMD [24]. ST2 may aid cardiologists with the monitoring of therapies and the progression of cardiomyopathy in DMD patients. However, further studies are necessary to validate ST2 and better determine threshold and prognostic levels for DMD.

As seen in Table 2, Leptin serum levels were three fold greater in DMD cardiomyopathy and non-cardiomyopathy subjects than the normal control group, but the difference was not significant between DMD groups with and without cardiomyopathy. Leptin is a known biomarker for cardiovascular disease related to obesity and oxidative stress, both seen in DMD, and further study is needed to better understand its role in DMD cardiomyopathy [25].

We did not demonstrate any significant differences in the serum levels of the other known cardiac biomarkers BNP, Gal3, LEP, CRP and TLR4 between DMD with cardiomyopathy, DMD without cardiomyopathy and normal control groups. Due to the age range of our subjects, the biomarkers displayed a very wide range of values leading to high variability and low statistical power (Table 2). Given these limitations, we did not test for interactions between age and group as we felt the data was not adequate to test that hypothesis. These

biomarkers may prove useful in DMD if studied in a larger cohort. In addition, BNP, TNC and Gal3 have been proven to be significantly elevated in end-stage heart failure patients, suggesting these levels may become significant in DMD patients if heart failure were to progress further [26].

We also confirmed MMP9 as a biomarker for DMD skeletal muscle disease [8]. We found MMP9 was significantly elevated in the DMD cardiomyopathy and DMD non-cardiomyopathy groups compared to controls. In addition, we found an increase in serum protein concentration as subjects increased in age (Figure 4), presumably due to increased inflammation resulting from disease progression. This mirrors the results published by Nadarajah et al. in which MMP9 is significantly elevated in the DMD population and can be a predictor of the progression in skeletal muscle disease [8].

Further study may also correlate ST2 levels with other non-invasive imaging modalities. Echocardiography can be used to determine early myocardial changes using tissue Doppler and Speckle tracking strain imaging, both shown to be decreased in DMD subjects with normal cardiac systolic function [3]. In addition, CMR imaging also demonstrates decreased myocardial strain and increased late gadolinium enhancement in DMD patients with normal systolic function [4,27,28].

This study is limited by a small number of samples. The CINRG DMD Natural History study was not designed to primarily measure cardiac function; therefore, echo results were retrospectively obtained and controls were not required to obtain echo data.

In conclusion, we identified ST2 as a potential serum biomarker for cardiomyopathy in DMD and confirmed previously published findings of MMP9 as a biomarker for DMD skeletal muscle disease. Further clinical study is warranted to determine the use of ST2 levels in the management of DMD cardiomyopathy.

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Figure 1: ST2 Serum Levels of DMD with cardiomyopathy, DMD without cardiomyopathy and control groups from ELISA testing.

Serum ST2 levels are significantly greater in DMD subjects with cardiomyopathy than in normal controls. The dots represent data points outside of 1.5 times the inter-quartile range.

* $p < 0.01$ compared to the normal control group.

Figure 2: ST2 Serum Levels. Linear regression analysis showed that overall serum ST2 levels increase with age in DMD and normal controls.

The DMD with cardiomyopathy group showed a statistically significant difference in ST2 levels compared to normal controls over all ages ($p=0.024$).

Figure 3: MMP9 Serum Levels of DMD with cardiomyopathy, DMD without cardiomyopathy and normal control groups from ELISA testing.

Serum MMP9 levels are significantly greater in DMD subjects than normal controls. The dots represent data points outside of 1.5 times the inter-quartile range. * $p < 0.01$ compared to normal controls.

Figure 4: MMP9 Serum Levels. Linear regression analysis showed that overall serum MMP9 levels increase with age in DMD and normal controls.

The DMD with cardiomyopathy and the DMD without cardiomyopathy groups showed a statistically significant difference in logMMP9 levels compared to normal controls over all ages ($p=0.006$ and $p=0.005$, respectively).

Table 1:

SOMAscan expression levels in DMD subjects with and without cardiomyopathy compared to normal controls.

Biomarker	DMD cardiomyopathy		DMD non-cardiomyopathy		Normal controls		Overall p-value
	N	Mean±SD	N	Mean±SD	N	Mean±SD	
ST2 (pg/ml)	7	5326±3701	7	5726±3824	6	1825±807	0.09
log MMP9 (ng/ml)	7	6.98 ± 0.64	7	6.85 ± 0.52	6	6.39 ± 0.28	0.13
LEP (ng/ml)	7	1579±682	7	2286±1053	6	1260±244	0.07
CRP (ng/ml)	7	56850±40772	7	55792±40569	6	15264±17607	0.09

Table 2:

Serum biomarker concentrations from ELISA testing in DMD subjects with and without cardiomyopathy compared to normal controls.

Biomarker	DMD cardiomyopathy		DMD non-cardiomyopathy		Normal controls	
	N	Mean±SD	N	Mean±SD	N	Mean±SD
ST2 (pg/ml)	12	35798±16918 *	12	28837±17304	6	9940±6565
log MMP9 (ng/ml)	12	6.17 ± 0.60 *	12	6.12 ± 0.58 *	6	5.06 ± 0.41
Gal3 (ng/ml)	12	11.5±3.3	12	10.0±2.8	2	10.3±1.1
log TLR4 (ng/ml)	12	2.16±0.371	12	2.29±0.44	4	2.01±0.51
log LEP (ng/ml)	12	9.84±1.09	12	9.64±1.07	4	8.80±0.30
TNC (ng/ml)	12	54.0±31	12	35.5±9.2	3	38.6±31.2
log BNP (pg/ml)	12	5.62±0.59	12	5.26±0.42	3	5.69±0.38
CRP (ng/ml)	12	1870±1541	12	1859±1232	2	672±938

* p<0.01 compared to normal controls

Table 3:

Linear regression models evaluating the relationship between serum biomarker level from ELISA testing and age.

Biomarker	Age		Significant group differences
	$\beta \pm \text{SE}$	p-value	
ST2 (pg/ml)	1177 $\pm$ 425	0.01	DMD CM vs. controls (p=0.024)
log MMP9 (ng/ml)	0.05 $\pm$ 0.01	0.004	DMD CM vs. controls (p=0.006) DMD non CM vs. controls (p=0.005)
Gal3 (ng/ml)	-0.1 $\pm$ 0.1	0.33	none
log TLR4 (ng/ml)	-0.02 $\pm$ 0.01	0.13	none
log LEP (ng/ml)	0.04 $\pm$ 0.03	0.24	none
TNC (ng/ml)	-0.9 $\pm$ 0.7	0.20	none
log BNP (pg/ml)	0.03 $\pm$ 0.02	0.10	none
CRP (ng/ml)	7.1 $\pm$ 45	0.88	none

CM – cardiomyopathy; SE – standard error