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Integrated genomic, proteomic and cognitive assessment in Duchenne Muscular Dystrophy suggest astrocyte centric pathology

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ABSTRACT

Introduction: Documented Duchenne Muscular Dystrophy (DMD) biomarkers are confined to Caucasians and are poor indicators of cognitive difficulties and neuropsychological alterations.
Materials and methods: This study correlates serum protein signatures with cognitive performance in DMD patients of South Asian origin. Study included 25 DMD patients aged 6–16 years. Cognitive profiles were assessed by Wechsler Intelligence Scale for Children. Serum proteome profiling of 1317 proteins was performed in eight DMD patients and eight age-matched healthy volunteers.
Results: Among the several novel observations we report, better cognitive performance in DMD was associated with increased serum levels of MMP9 and FN1 but decreased Siglec-3, C4b, and C3b. Worse cognitive performance was associated with increased serum levels of LDH-H1 and PDGF-BB but reduced GDF-11, MMP12, TPSB2, and G1B. Secondly, better cognitive performance

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in Processing Speed (PSI) and Perceptual Reasoning (PRI) domains was associated with intact Dp116, Dp140, and Dp71 dystrophin isoforms while better performance in Verbal Comprehension (VCI) and Working Memory (WMI) domains was associated with intact Dp116 and Dp140 isoforms. Finally, functional pathways shared with Alzheimer's Disease (AD) point towards an astrocyte-centric model for DMD.

Conclusion: Astrocytic dysfunction leading to synaptic dysfunction reported previously in AD may be a common pathogenic mechanism underlying both AD and DMD, linking protein alterations to cognitive impairment. This new insight may pave the path towards novel therapeutic approaches targeting reactive astrocytes.

1. Introduction

There is ample evidence suggesting a variability of presenting learning and behavioral difficulties and their severity in Duchenne Muscular Dystrophy (DMD) patients [1–4]. Similarly, variable presentation of psychiatric and behavioral problems and intellectual difficulties have been identified in Becker Muscular Dystrophy (BMD) [5–8].

Over the past 15 years, published studies that correlated DMD genotype with IQ and cognitive performance data were mostly from developed countries, including the United Kingdom, Netherlands, Italy, Australia and the USA [2,9–11]. The Indian subcontinent is represented by only a few studies, all from India [12–14]. Most importantly, none of the published papers to date have integrated patient IQ data, dystrophin gene mutation sites and serum proteomic signatures to seek novel biomarkers of cognitive function in DMD.

To address this, we have carried out a detailed study of 25 DMD patients. To the best of our knowledge, this is one of the most comprehensive studies from the Indian subcontinent correlating FSIQ scores, WISC-IV subscale scores, potential missing dystrophin isoforms and serum proteomic profiles in a group of patients with genetically confirmed DMD. The research findings reported on the serum proteomic profile and its correlation with cognition can be seen as a stepping stone for future research on this clinically important topic.

At a minimum, the dystrophin gene encodes seven major isoforms from seven recognized promoters, where they demonstrate differential expression in the central nervous system and cell-type specificity [9]. The production of dystrophin protein variants (Dp) arises from unique promoter usage, alternative splicing, and/or alternative polyadenylation signals [15]. Three full-length isoforms are derived from unique upstream promoter/first exon sequences, Dp427 m, Dp427b/c, and Dp427p, which are expressed in skeletal and cardiac muscles, neurons in the cortex, and cerebellar Purkinje cells, respectively. At least four shorter mRNA products, namely, Dp260 (retina), Dp140, Dp116 (Schwann cells), and Dp71, are transcribed from more distal promoters located within downstream introns of dystrophin. Of these, the most predominant in the brain is Dp71, followed by Dp140, which is expressed throughout the

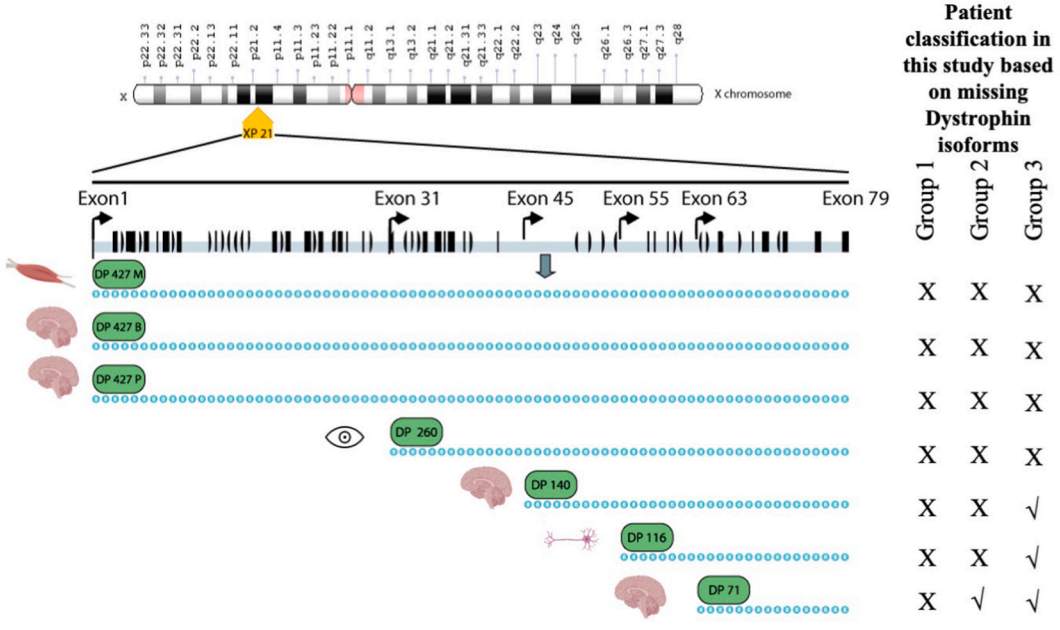

Fig. 1. The DMD isoforms, their expression patterns and the patient classification used in this study based on the missing dystrophin isoforms. The patients were categorized as follows: [01] Group 1: all isoforms are absent; [02] Group 2, only Dp71 isoform is present; and [03] Group 3: only Dp140, Dp116, and Dp71 isoforms are present.

central nervous system [16]. This is illustrated in Fig. 1.

As reported in the literature, dystrophin gene mutations resulting loss of specific dystrophin isoforms are associated with the cognitive impairment observed in DMD. Studies have suggested that mutations in the distal region (downstream of exon 44) are more often associated with cognitive impairment than proximal-region mutations (upstream of exon 44) [17]. Many reports demonstrate that about 30% of DMD patients experience a cognitive impairment (IQ < 70), where a correlation has been observed in Full-Scale IQ (FSIQ) scores with the number of missing dystrophin isoforms [9]. In this regard, the Wechsler Intelligence Scale for Children (WISC-IV), an individually administered measure of cognitive abilities intended for children aged 6–16 years, is reportedly a reliable tool where the FSIQ score represents a combination of performances across the core subtests [18].

2. Material and methods

2.1. Patient population

This study meets the ethical guidelines of the Ethics Committee, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka (Ethical Approval Nos. 449/09 and 38/19), and is in compliance with the Helsinki Declaration. The inclusion criteria were as follows: [01] males clinically evaluated by a consultant pediatric neurologist/consultant neurologist and genetically confirmed for DMD through Multiplex Ligation Dependent Probe Amplification (MLPA) for deletions/duplications in the dystrophin gene; [02] aged 6–16 years; [03] uncomplicated term delivery, with gestation from 37 completed weeks (259 days) up to and including 41 completed weeks and 6 days (293 days) [19], to avoid neurocognitive sequelae due to preterm birth; and [04] consent to participate, where written informed consent was obtained from every proband where applicable. For patients incapable of providing consent on their own, consent was obtained from a proxy. This study enrolled 25 DMD patients aged 6–16 years (mean age 10 years and 4 months).

2.2. Patient categorization and cognitive assessment

Based on the mutation location of the patient cohort and published literature [2,9,16], missing dystrophin isoforms (Dp427m, Dp427c, Dp427p, Dp260, Dp140, Dp116 and Dp71) were predicted. The patients were categorized as follows: [01] Group 1, $n = 2$ (all isoforms are missing); [02] Group 2, $n = 11$ (only Dp71 isoform is present); and [03] Group 3, $n = 12$ (Dp140, Dp116, and Dp71 isoforms are present). The patient classification is illustrated in Fig. 1.

Cognitive profiles were assessed using the WISC-IV [18] for children, and information on developmental milestones was obtained from the Child Health Development Record (CHDR) of the Family Health Bureau, Ministry of Health Nutrition and Indigenous Medicine, Sri Lanka, and a standard questionnaire.

2.3. Serum proteomic analysis; a pilot study

Serum proteome profiling was performed on a subset of samples collected from eight glucocorticoid-naïve DMD patients (age range 6–16 years old) and eight age-matched school children volunteered from the general population were recruited as controls for proteomic study. School assessment grades and teacher's reports were considered to exclude any cognitive impairment of the controls. The patient samples for serum protein biomarker discovery were selected to include approximately equal numbers of samples from patients having distal and proximal mutations in the dystrophin gene.

Proteomic analysis was performed using aptamer-based proteomic technology pioneered by SomaLogic to screen 1317 proteins as previously described by others [20,21]. Proteomic studies were carried out with support from the Houston Omics Collaborative (https://hoc.bme.uh.edu/), with institutional IRB approval from the University of Houston, Houston, TX. Of the 1317 analyzed proteins, the expression of 249 proteins was significantly different ($p < 0.05$) between DMD patients and healthy volunteers. Of the 249 proteins, the top 20 upregulated proteins (fold change > 2.0) and top 20 downregulated proteins (fold change < 0.6) were selected for further correlation analysis with WISC-IV cognitive data and missing dystrophin isoforms (Supplementary Table).

Pathway analysis was performed using Gene ontology and KEGG/DAVID platforms, and all data are displayed ordered by statistical significance (Fig. 3a–c). Dysregulated or upregulated processes in DMD were enriched for various signaling cascades (MAPK, HIF-1, JAK-STAT and protein phosphorylation) and cell proliferation. Protein–protein interaction networks were also created for the top 249 proteins (DMD vs. control, p value < 0.05) through the Cytoscape.

2.4. Data analysis

The normality of the distribution of the dataset was confirmed by Shapiro–Wilk and Kolmogorov–Smirnov tests. To assess the variations in FSIQ composite scores and subset scores, the Verbal Comprehension Index (VCI), Perceptual Reasoning Index (PRI), Working Memory Index (WMI), and Processing Speed Index (PSI) of the WISC-IV were determined and then analyzed in terms of the mutation regions and predicted missing dystrophin isoforms. One-way ANOVA tests and multivariate analysis were performed. To analyze the effect of dystrophin isoforms on FSIQ scores, subset scores, and trends, ANOVA and pairwise Student's t tests were performed. Pearson's correlation test was performed to identify the significance of the impacts of the variables (20 upregulated proteins, 20 downregulated proteins, and 15 WISC subscale scores) on one another. Statistical analysis was performed using R Statistical software version 4.2.

Pathway analysis was performed using Gene ontology and KEGG/DAVID platforms, including the readxl and ggplot2 packages, and

Table 1
DMD patient groups based on Dystrophin isoforms and Clinical Assessment.

DMD ID	Mutation	In frame/Out Frame	Development Delay	Patient groups based on affected dystrophin isoforms	Predicted Dystrophin isoform[a]							WISC-IV Score					Proteomics performed[b]
					Dp427m	Dp427b	Dp427p	Dp260	Dp140	Dp116	Dp71	FSIQ	VCI	PRI	WMI	PSI	
1	Del.Exon 8-44	Out Frame	–	Group 3	–	–	–	–	+	+	+	75	87	92	74	62	X
2	Del.Exon 48-52	Out Frame	–	Group 2	–	–	–	–	–	–	+	50	59	73	50	50	X
3	Del.Exon 51-55	Out Frame	+	Group 2	–	–	–	–	–	–	+	78	87	94	62	80	✓
4	Del.Exon 3-7	Out Frame	–	Group 3	–	–	–	+	+	+	+	88	102	88	91	78	X
5	Del.Exon 45-50	Out Frame	–	Group 2	–	–	–	–	–	–	+	75	87	96	62	68	X
6	Del.Exon 61-62	Out Frame	+	Group 2	–	–	–	–	–	–	+	56	65	63	56	70	✓
7	Del.Exon 61-62	Out Frame	+	Group 2	–	–	–	–	–	–	+	56	65	63	56	70	✓
8	Del.Exon 45-52	Out Frame	–	Group 2	–	–	–	–	–	–	+	67	71	86	62	68	X
9	Del.Exon 3-11	Out Frame	+	Group 3	–	–	–	+	+	+	+	89	83	90	91	109	X
10	Del.Exon 64-67	Out Frame	+	Group 1	–	–	–	–	–	–	–	45	57	59	50	50	X
11	Del.Exon 45-54	Out Frame	+	Group 2	–	–	–	–	–	–	+	62	65	82	56	70	X
12	Del.Exon 8-25	Out Frame	–	Group 3	–	–	–	+	+	+	+	89	93	100	91	80	X
13	Dup.Exon 8–9 & 11	Out Frame	–	Group 3	–	–	–	+	+	+	+	88	87	102	86	88	X
14	Del.Exon 8-10	Out Frame	–	Group 3	–	–	–	+	+	+	+	87	95	112	80	65	X
15	Del.Exon 46-47	Out Frame	–	Group 2	–	–	–	–	–	–	+	59	61	86	59	56	X
16	Del.Exon 3-6	Out Frame	–	Group 3	–	–	–	+	+	+	+	78	83	90	71	85	X
17	Del.Exon 12-19	Out Frame	+	Group 3	–	–	–	+	+	+	+	89	99	98	91	73	X
18	Del.Exon 45-49	In Frame	–	Group 2	–	–	–	–	–	–	+	68	71	90	62	68	X
19	Del.Exon 20-42	Out Frame	–	Group 3	–	–	–	–	+	+	+	78	81	100	91	56	X
20	Del.Exon 1-42	Unpredictable	+	Group 3	–	–	–	–	+	+	+	85	96	94	97	62	✓
21	Del.Exon 1-42	Unpredictable	–	Group 3	–	–	–	–	+	+	+	94	102	98	110	68	✓
22	Dup.Exon 52-67	In Frame	+	Group 1	–	–	–	–	–	–	–	42	55	49	54	50	✓
23	Del.Exon 8-11	Out Frame	–	Group 3	–	–	–	+	+	+	+	74	79	98	74	62	✓
24	Del.Exon 45-52	Out Frame	–	Group 2	–	–	–	–	–	–	+	83	81	98	94	73	X
25	Del.Exon 45-52	Out Frame	+	Group 2	–	–	–	–	–	–	+	83	81	98	94	73	X

[a] "-" Absence and "+" Presence.
[b] Proteomics has been performed in 8 DMD patients of which 7 patients marked in "√" consented to perform WISC-IV cognitive assessment.

all data are displayed ordered by statistical significance. Cytoscape 3.9.0 was used to generate protein-protein interaction networks. Nodes that were most tightly related to each other were identified using MCODE..R programming was used to generate 2D correlation plots, PCA plots and Volcano plots. The classification potential of different biomarkers was also elucidated using Random Forest in R, and the best performing proteins are ordered in terms of statistical significance (GINI coefficient).

3. Results

3.1. DMD patient groups based on dystrophin isoforms and clinical assessment

In our patient cohort, 40% (10/25) of patients had developmental delay, of whom 70% (7/10) possessed a distal mutation predicted to be affecting Dp140 brain isoform. (downstream of exon 44). Patient number 22 in our cohort had a rare in-frame duplication of exons 52–67 and had the lowest WISC-IV scores. Patient numbers 6 & 7 are identical twins with rare out-frame deletions of exons 61 and 62, and they had the same WISC scores (FSIQ = 56). These results are summarized in Table 1.

3.2. Groupwise comparison of WISC-IV composite scores and core subset scores

As summarized in Table 2.

1. When Group 1 vs. Group 3 and Group 2 vs. Group 3 were compared for the VCICORE_S, VCICORE_V and VCICORE_C, statistically significant differences were observed. Intriguingly, no significant difference in performing these activities was observed between Group 1 and Group 2. This observation may reflect that intact Dp116, and Dp140 brain dystrophin isoforms result in better performance on verbal comprehension activities.
2. When PRICORE_M was considered, statistically significant differences were observed among all three groups compared, which indicates the possible cumulative involvement of Dp140, Dp71 and Dp116 brain dystrophin isoforms in better performance of activities related to PRICORE_M.

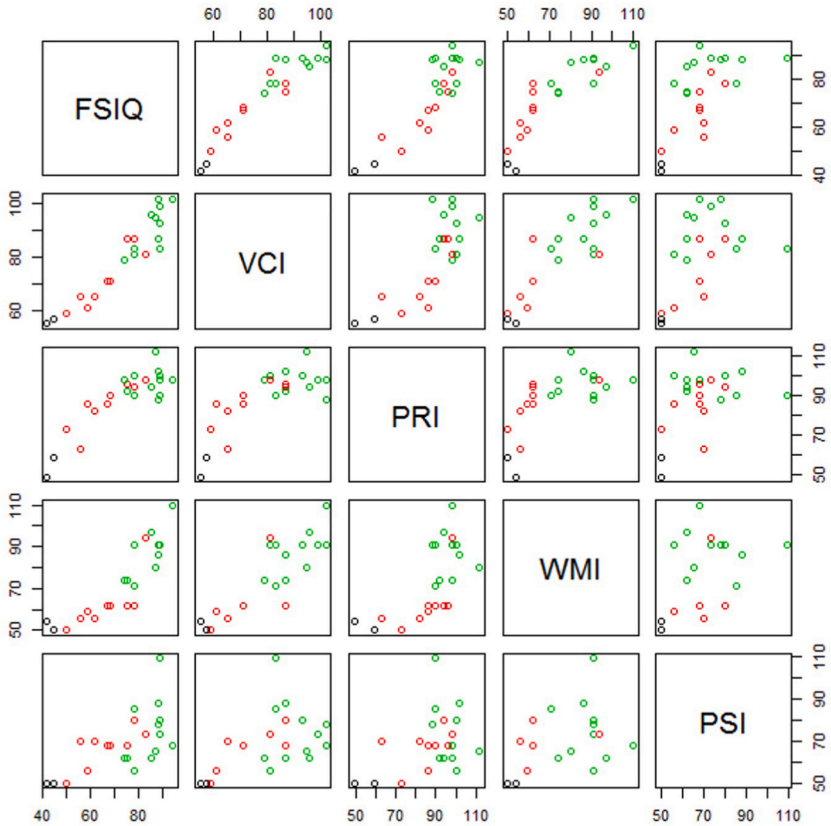

Fig. 2. Scatter plot matrix. The correlation and distribution of WISC-IV FSIQ, VCI, PRI, WMI, and PSI composite scores as a function of predicted missing brain isoforms. Black dots, Group 1 patients; red dots, Group 2 patients; green dots, Group 3 patients. Numbers on X and Y axis represent percentile distributions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.In the WMI core subsets, namely, WMICORE_D (Digit Span) and WMICORE_L (Letter Number Sequencing), statistically significant differences were observed in Group 1 vs. Group 3 and Group 2 vs. Group 3. However, no significant difference was observed in performing these activities between Group 1 and Group 2. These findings show a possible involvement of the Dp116 and Dp140 brain dystrophin isoforms in the better performance activities related to working memory.
4.In the PSI core subset PSICORE_S (Symbol Search), a significant difference was observed in Group 1 vs. Group 3, which indicates the cumulative role of the Dp140, Dp116 and Dp71 brain dystrophin isoforms in the better performance of activities related to processing speed.

As represented in Fig. 2, in Group 1, cumulative WISC-IV subscale scores were at the lower limit, suggesting that the predicted loss of the Dp71 brain dystrophin isoform may result in a worse performance of WISC-IV activities related to verbal comprehension, perceptual reasoning, working memory and processing speed. Interestingly, in Group 2, cumulative WMI subscale scores were toward the lower limit, indicating that the predicted loss of the Dp140 and Dp116 brain dystrophin isoforms may result in worse performance, especially in activities related to working memory.

3.3. Serum proteomic analysis

As shown in Fig. 3d, the top Reactome pathways associated with the dysregulated proteins in DMD included cytokine signaling, extracellular matrix organization and receptor tyrosine kinases. The top transcription factor regulator of these pathways was identified using iRegulon to be SMAD1, while the top signaling molecule regulator was identified to be ASCL2 (Fig. 3e–f).

As shown in Fig. 4a, Principal Component Analysis demarcated DMD patients from healthy controls (Fig. 4a). Fig. 4b presents a volcano plot representation of changes in all 1317 proteins, illustrating that 21 proteins were increased at fold changes >2, including 6

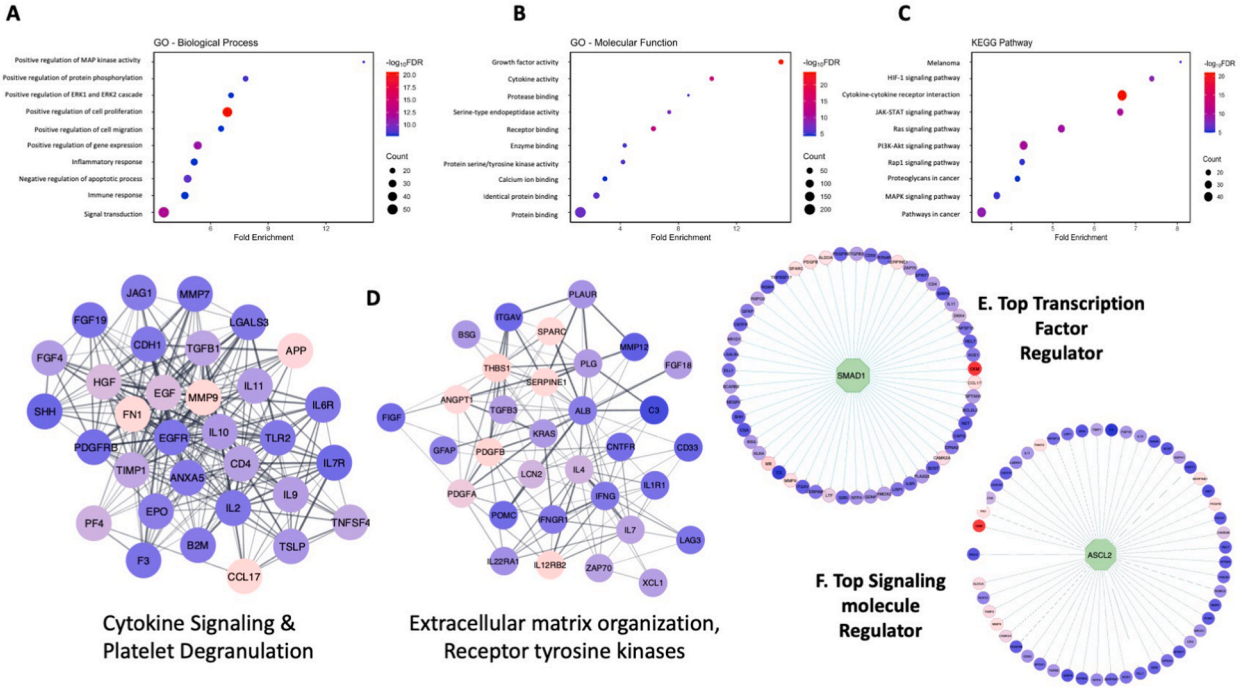

Fig. 3. Serum proteomic analysis. (a), (b) and (c)- All 249 proteins with a Mann–Whitney p value < 0.05 (DMD versus HC) were used for functional pathway enrichment. The top 10 Gene Ontology biological processes, molecular functions and KEGG pathways obtained through DAVID are plotted based on p value significance in order of fold enrichment. The size of the dots represents the count of genes belonging to the annotation term, and the color of the dots is representative of the $-\log_{10}$FDR value. (d) Protein–protein interaction networks were created for the top 249 proteins (DMD vs. control, p value < 0.05) through the Cytoscape string App with a confidence cutoff of 0.4. MCODE clustering was performed, and the top three clusters are displayed. The color of each node corresponds to the fold change. Nodes with a fold change less than one range in color from blue/purple, while those with a fold change greater than one range from pink to red. The confidence score of each interaction is displayed as the edge thickness and opacity. The top reactome pathways associated with each cluster are displayed below each cluster in order of significance. (e) The top transcription factor regulator was identified for the top 249 proteins (DMD vs. control, p value < 0.05) through the iRegulon plugin available for Cytoscape. The figure is presented in a circular layout. Nodes with a fold change less than one range in color from blue/purple, while those with a fold change greater than one range from pink to red. (f) The top signaling molecule regulator was identified for the top 249 proteins (DMD vs. control, p value < 0.05) through the iRegulon plugin available for Cytoscape. The figure is presented in a circular layout. Nodes with a fold change less than one range in color from blue/purple, while those with a fold change greater than one range from pink to red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

proteins that were most highly increased in DMD (5-fold increase, $p < 0.05$). Fig. 4c is a heatmap representation of the results of the 249 proteins significantly expressed in DMD compared to controls (p value < 0.05), following hierarchical clustering. A machine learning algorithm, Random Forest analysis (Fig. 4d), was also used to independently identify the best performing protein discriminators of DMD, ordered by their GINI coefficient. Fig. 4e is a correlation plot displaying the expression profiles of the top 20 and bottom 20 proteins in DMD ordered by fold change with p value < 0.05. This figure clearly displays several clusters of proteins that are differentially expressed in DMD, including clusters centered on proteins that have previously been implicated in the DMD literature, including CK-MB, CK-MM. Myoglobin, Troponin I, Troponoin T, etc. As represented in the Supplementary Figure, the correlation analysis comparing the upregulated and downregulated proteins of Distal Vs Proximal mutated patients identified strong negative and positive correlations among the proteins of DMD patients with Proximal mutations. Interestingly in the patients with Distal mutations all the upregulated and down regulated proteins had positive correlations. However, since the sample size is small, the effects were hard to conclude.

3.4. *Mining for biomarkers of cognition in DMD*

The availability of comprehensive serum proteomic data together with patient cognitive status allowed us to mine for biomarkers of cognition in DMD. Fig. 5 is a correlation plot displaying the relationship between serum proteins and cognitive function. In this context, our proteomic analysis revealed that when better cognitive performance was considered:

01) increased levels of FN1 and decreased levels of Siglec-3 and C4b were associated with better performance of WISC-IV activities related to FSIQ and all core subsets, namely, VCI, WMI, PRI, and PSI.

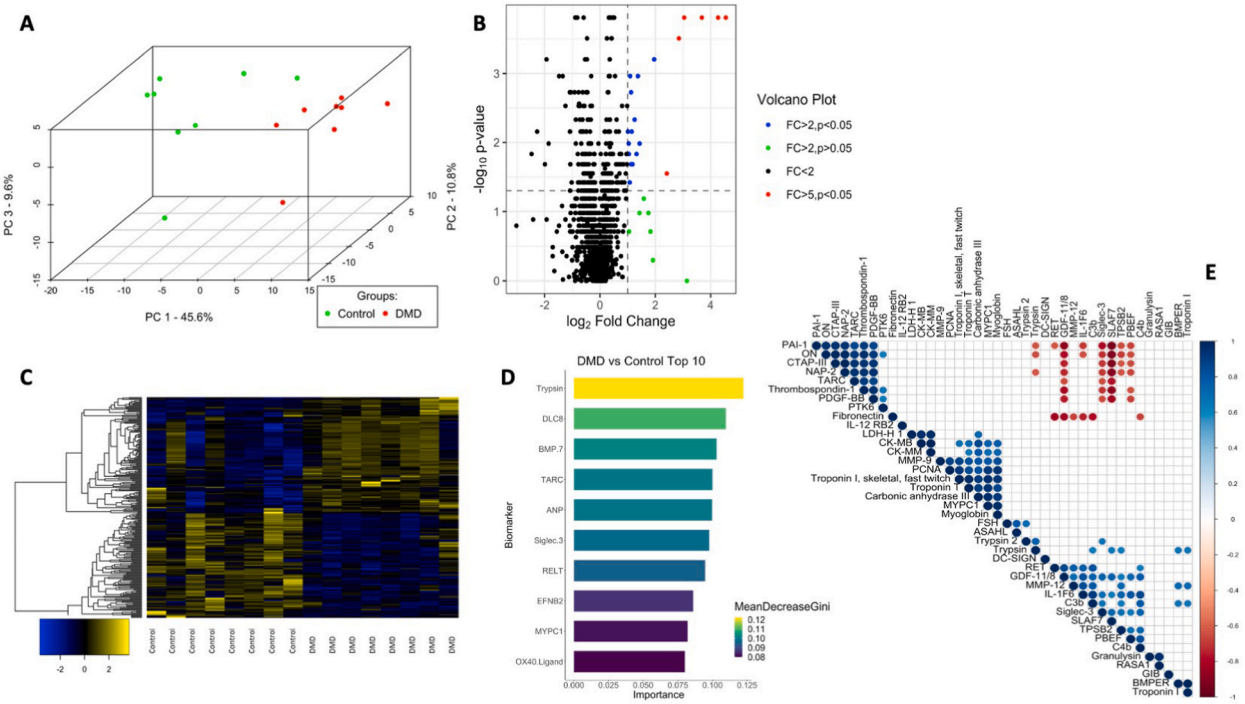

Fig. 4. Serum proteomic analysis. (a) A 3D PCA plot using the 249 proteins that were differentially expressed with $p < 0.05$. DMD is represented by a red circle, while controls are represented by a green circle. The first three principal components are displayed on each axis of their respective plots. (b): A volcano plot representation of the results of 1317 proteins. Data was log transformed and analyzed. Each dot represents one of the 1317 proteins. The x-axis plots the log2 transform of the fold change. The y-axis displays the –log10 transform of the p-value. Of the 249 proteins that were differentially expressed between the groups, 21 proteins were elevated at fold change >2 and $p < 0.05$, 6 proteins were elevated at a fold change >5 and $p < 0.05$ in DMD versus control. (c) A heatmap representation of the results of the 249 proteins significantly expressed in DMD compared to controls (p value < 0.05). Hierarchical clustering was performed. Proteins above the mean value for each protein are shaded yellow. Proteins below the mean are shaded blue. Those that are comparable to the mean are shaded black. (d) Random forest analysis of the top 249 proteins (p value < 0.05). The top 10 proteins are ordered by their GINI coefficient and their importance in discriminating between DMD and controls. (e) Correlation plot displaying the expression profiles of the top 20 and bottom 20 proteins ordered by fold change with p value < 0.05. Pearson's correlation coefficient was determined for each protein pair. Correlations identified as significant with $p < 0.01$ are displayed. The proteins were ordered based upon hierarchical clustering. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

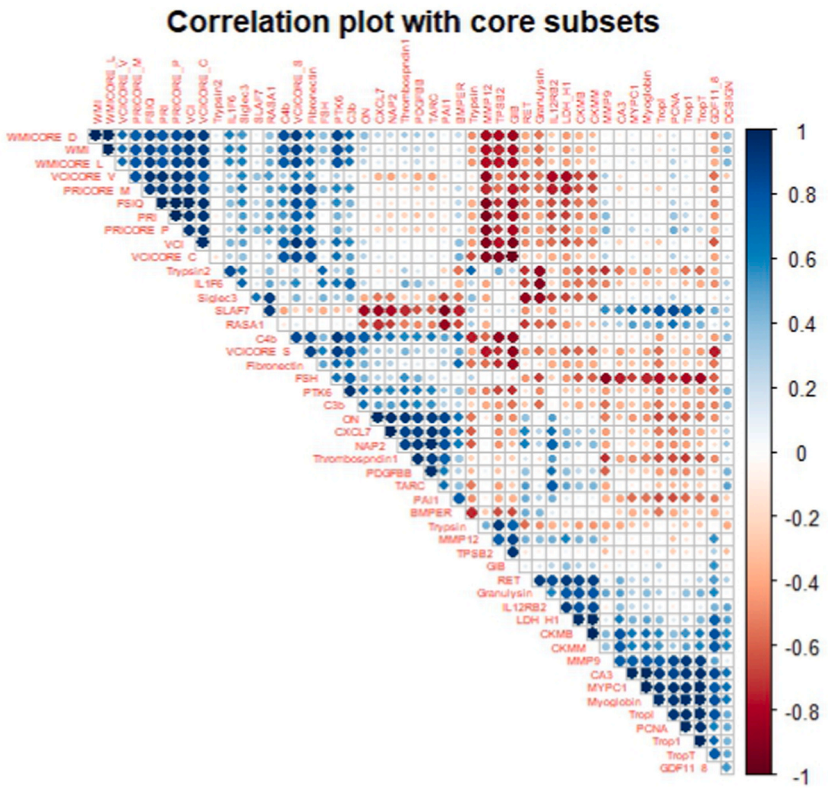

Fig. 5. Correlation plot of WISC-IV cognitive assessment scores with the expression profiles of the top 20 and bottom 20 serum proteins ordered by fold change with p value < 0.05. Pearson's correlation coefficient was determined, and the proteins were ordered based upon hierarchical clustering. Blue denotes positive correlation while red denotes negative correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

02) An increased level of MMP9 was associated with better performance of activities related to PSI and PRI. [03] A decreased level of C3b was associated with better performance of activities related to WMI and VCI. Moreover, when worse cognitive performance was considered,
01) increased levels of LDH-H1 were associated with worse performance of WISC-IV activities related to FSIQ and core subset scores, namely, VCI, PRI, and PSI.
02) Increased levels of PDGF-BB were associated with worse performance of activities related to the core subsets scores PRI and PSI.
03) Decreased levels of MMP12, TPSB2, G1B, and GDF-11 were associated with worse performance of WISC-IV activities related to FSIQ and core subset scores, namely, VCI, WMI, and PRI.

4. Discussion

The WISC scores reported in our study are in line with the previous studies [2,9,14,22]. The observed deficit in the FSIQ composite scores (Table 1) ranging from 42 to 94 (below the average level; 90 to 109) in all the patients of this study, may be due to the involvement of Dp427c. Dp427c is predicted to be absent in all patients with a dystrophin mutation regardless of the mutation site and expressed in cortical and hippocampal pyramidal neurons [2,9,11]. Moreover, it has been previously reported that Dp427c play a role in anchoring GABAA receptors to the postsynaptic membrane of GABAergic neurons. Displacement of GABAA receptors may be a consequence of the absence of full length Dp427c [23–25].

According to our results (Table 2), intact Dp116 and Dp140 brain dystrophin isoforms result in better performance of cognitive activities related to VCI and WMI. Moreover, intact Dp71 results in better performance of cognitive activities related to PSI and PRI. As highlighted by Matsuo et al., 2017, the individual or cumulative effect of Dp116 on the development of DMD non-muscle symptoms is ambiguous. Thus it is suggested that Dp116 deficiency in DMD may be characterized by abnormalities in motor and/or sensory neurons [26]. It is noteworthy that activities related to PSI and PRI subsets of WISC-IV comprise of motor demanding subtests namely Coding, Symbol Search and Block Design suggesting a confounding effect of processing speed and motor output speed in performing these activities. Therefore, it is uncertain that whether the identified weaknesses in performing activities related to PSI and PRI in DMD reflects actual cognitive and motor processing speed difficulties, or a combination. As summarized in Table 2, the study by Glascher et al., 2009 indicated that for VCI, the Broca's area is mainly involved; for PRI, the supramarginal and posterior inferior gyri and dorsal bank of the middle superior temporal sulcus are involved; WMI involves the central sulcus and postcentral gyrus; and finally, PSI

Table 2
Association of WISC-IV FSIQ, VCI, PRI, WMI, and PSI composite scores and core subset scores with predicted missing brain dystrophin isoforms according to dystrophin mutation.

WISC subsets and Core subsets	Assessment[4]	Brain Regions involved [27]	Group comparisons	Significance[a]
FSIQ	Full Scale IQ (Cumulative score of core subsets)		Group 1 Vs Group 2	0.038
			Group 1 Vs Group 3	0.035
			Group 2 Vs Group 3	0.001
VCI	Verbal Comprehension	Pars opercularis and pars triangularis of the left inferior frontal cortex (Broca's area)	Group 1 Vs Group 2	0.036
			Group 1 Vs Group 3	0.035
			Group 2 Vs Group 3	0.001
VCICORE_S (Similarities)	Verbal reasoning and concept formation		Group 1 Vs Group 2	NS
			Group 1 Vs Group 3	0.030
			Group 2 Vs Group 3	0.000
VCICORE_V (Vocabulary)	Word knowledge and concept formation		Group 1 Vs Group 2	NS
			Group 1 Vs Group 3	0.035
			Group 2 Vs Group 3	0.034
VCICORE_C (Comprehension)	Verbal reasoning and conceptualization		Group 1 Vs Group 2	NS
			Group 1 Vs Group 3	0.033
			Group 2 Vs Group 3	0.007
PRI	Perceptual Reasoning	Supramarginal gyrus, posterior inferior frontal gyrus, posterior part of the superior temporal sulcus, and dorsal bank of the middle superior temporal sulcus	Group 1 Vs Group 2	0.037
			Group 1 Vs Group 3	0.035
			Group 2 Vs Group 3	0.011
PRICORE_B (Block Design)	Analysis and synthesis, nonverbal concept formation		Group 1 Vs Group 2	0.03
			Group 1 Vs Group 3	NS
			Group 2 Vs Group 3	NS
PRICORE_P (Picture Concepts)	Scanning ability, Planning, organization, visual motor sequential processing		Group 1 Vs Group 2	NS
			Group 1 Vs Group 3	0.033
			Group 2 Vs Group 3	NS
PRICORE_M (Matrix Reasoning)	Scanning ability, planning and organization		Group 1 Vs Group 2	0.036
			Group 1 Vs Group 3	0.034
			Group 2 Vs Group 3	0.003
WMI	Working Memory	Anterior and posterior bank of the central sulcus and postcentral gyrus	Group 1 Vs Group 2	NS
			Group 1 Vs Group 3	0.032
			Group 2 Vs Group 3	0.004
WMICORE_D (Digit Span)	Short-term memory, registration, attention, and concentration		Group 1 Vs Group 2	NS
			Group 1 Vs Group 3	0.035
			Group 2 Vs Group 3	0.000

(*continued on next page*)

Table 2 (*continued*)

WISC subsets and Core subsets	Assessment[4]	Brain Regions involved [27]	Group comparisons	Significance[a]
WMICORE_L (Letter Number Sequencing)	Sequencing ability, mental manipulation, and attention		Group 1 Vs Group 2	NS
			Group 1 Vs Group 3	0.034
			Group 2 Vs Group 3	0.002
PSI	Processing Speed	Postcentral gyrus, anterior precentral gyrus, postcentral sulcus, inferior parietal gyrus, lingual gyrus, and right middle frontal gyrus	Group 1 Vs Group 2	NS
			Group 1 Vs Group 3	0.03
			Group 2 Vs Group 3	NS
PSICORE_C (Coding)	Short-term visual memory and learning ability		Group 1 Vs Group 2	NS
			Group 1 Vs Group 3	NS
			Group 2 Vs Group 3	NS
PSICORE_S (Symbol Search)	Visual-motor integration and perceptual Speed		Group 1 Vs Group 2	NS
			Group 1 Vs Group 3	0.033
			Group 2 Vs Group 3	NS

NS- Not significant.

activities involve the precentral gyrus, postcentral sulcus, lingual gyrus and right middle frontal gyrus [27]. Therefore, it is noteworthy to further investigate the specific expression of Dp71, Dp140 and Dp116 brain dystrophin isoforms in the aforementioned brain regions and their relationship to various cognitive activities.

Similar to our findings, Chamova et al., 2013 and Tyagi et al., 2020 reported that DMD patients lacking Dp140 scored significantly worse not only on full-scale intelligence and verbal intelligence (VIQ) [14,28] but also on verbal learning/memory; and higher order cognitive functions Dp140 may therefore have a function in episodic memory processes and could indeed be related to normal cognitive functioning. Even though mutations affecting the Dp71 brain dystrophin isoform have been reported to be rare, Ricotti et al., 2016 studied DMD patients from the UK, Italy, and Belgium and reported that dystrophin gene mutations affecting the Dp71 brain isoform tend towards a higher incidence of intellectual disability [2] and most severely affected working memory, which is in line with our findings. A similar trend has also been reported in DMD patients from Australia [9] and the Netherlands [11]. Moreover, in Indian DMD patients, the association of DMD mutations affecting the Dp140 isoform and cognitive performance has been reported by Tyagi et al., 2020; unfortunately, the effect of Dp71 was not investigated [14]. In this context, the reported relationship between mutation-specific loss of brain dystrophin isoforms and the scoring abilities of patients for activities in core WISC-IV subsets may provide insight toward developing a DMD specific diagnostic test battery, which is yet to be established, as highlighted by Doorenweered et al., 2020 [4].

It has been observed that delayed on most language and motor milestones in children with DMD compared to their siblings [29,30]. It is noteworthy that 50% (5/10) of the patients in our cohort who presented with a delay in motor milestones were admitted to the hospital followed by a diagnosis for DMD before the age of 3 years. In this context, motor and language developmental delays may be the earliest signs of DMD that often go unnoticed since most clinicians still do not associate [31]. Therefore, the amendment of the recommendation for global developmental delay by the American Academy of Neurology and Child Neurology Society [32] is warranted to provide more opportunities for earlier diagnosis of DMD patients with motor and language developmental delays.

With emerging proteomic technologies, numerous DMD biomarkers have been reported and validated across different laboratories and patient cohorts [20,21,33–35]. Nonetheless, none of these published papers to date have correlated cognitive impairment, dystrophin gene mutation sites and serum proteomic signatures to seek novel cognitive biomarkers in DMD. Most importantly, some of the potential biomarker candidates for DMD identified in previous studies have also been reported in our study, which confirms the validity of our proteomic assay. As displayed in Fig. 5, most of the previously reported candidate biomarkers of DMD, including myosin binding protein C1 (MYPC1), myoglobin (MB), troponin, proliferating cell nuclear antigen (PCNA), carbonic anhydrase 3 (CA3), troponin 1, and troponin T are poor indicators of cognition. Interestingly, creatine kinase MM (CKMM) and creatine kinase MB (CKMB) are modestly associated with poor cognitive function. Therefore, there is a clear need for identifying improved serum biomarkers of cognition in DMD.

Supported by the findings of two human studies [36,37] and one mdx mouse study [38], the amyloidogenic pathway is becoming an increasingly recognized mechanism providing novel insight into cognitive impairment in DMD. The pathophysiological consequences of the absence of brain dystrophin isoforms and its impact on proteome-wide alterations in the central nervous system and changes in brain function remain largely unresolved [39–42]. Table 3 summarized the reported relationship of cognitive impairment including AD with the upregulated and downregulated proteins correlated with cognitive statues of DMD, as identified in this study.

Even though MMP-9 levels in DMD patients have been previously studied [54], our study is the first report of a positive correlation between serum MMP-9 levels and cognitive performance in DMD.

Although the molecular mechanism of Sialic Acid-Binding Ig-Like Lectin 3 (Siglec-3) is not fully discovered to date, recent studies suggest that Siglec-3 expressed on microglia combine with sialylated amyloid plaques than consequently reduces the phagocytose of amyloid plaques by microglia [55,56]. It is tempting to speculate that Siglec-3 levels of DMD patients affecting the Dp140 brain isoform may have been reduced to facilitate phagocytosis of misfolded Dp140 protein, similar to the reported role of Siglec-3 in AD. Moreover, what is critical is to determine the expression levels of C3 and C4 within the brain in DMD patients, as this will shed further insights on the role of complement proteins in DMD.

In line with our findings, increased levels of Lactate Dehydrogenase (LDH) in the serum of DMD patients have been previously reported by Zhu et al., 2015, where all patients with BMD and up to 97% of patients with DMD had elevated LDH values [57]. Interestingly, the activity of the LDHA isoform has been previously shown to be elevated in the frontal and temporal cortices of patients with AD [58]. However, the relationship of LDH with the cognitive function of DMD has not been previously studied. This is the first report of increased LDH being associated with worse cognitive function. Similarly the level of sPDGFRβ in the CSF is reported to predict dementia and other neurodegenerative diseases, such as AD [59]. Hence, it is tempting to speculate that PDGF-BB may play similar roles in DMD-associated dementia. In line with our findings, Hathout et al., 2016 reported decreased levels of GDF11 in the serum of DMD patients; however, the possible relationship of serum GDF-11 levels and cognition in DMD patients has not been previously reported [20]. Findings are suggestive for the enhancement of neurogenesis by GDF11 through vascularity and blood flow in the neurogenic niche [60]. Further studies are clearly required to determine whether and how GDF11 in blood and CSF may influence brain health in aging and neurodegenerative disease, both in the context of AD and DMD.

Intriguingly, as reported by Keeney et al., 2015 and Conejero-Goldberg et al., 2014, reduced expression of complement components in the presence of the Apolipoprotein E2 (APOE ε2) allele may considered to be neuroprotective [61,62]. This idea is consistent with separate evidence of neuroprotective effects of APOE ε2 with respect to AD [63], which calls for genetic characterization of DMD patients for APOE alleles. Unfortunately, reports on the genetic characterization of DMD patients for APOE alleles are lacking to date, opening novel avenues for future research.

The role of astrocytes in DMD, neurodegenerative disorders and neuropsychiatric disorders is an emerging area of research [64,65]. Importantly, Lange et al., 2021 revealed morphological and molecular changes of DMD astrocytes and their altered response to inflammatory stimuli and oxidative stress in comparison to controls. These findings shed light on the role of astrocytes in the observed brain comorbidities in DMD [66]. In this context, as evident by Mahyoob Rani et al., 2019, and Matsuo et al., 2017, Dp71 was the main upregulated isoform in astrocytes, whereas Dp140 and Dp116 were also identified in neurons [26,67]. Patel et al., 2019 revealed that DMD genotypes that disrupt the expression of full-length dystrophin isoform will consequently disrupt the homeostatic activity of astrocytes and affect neuronal health [65].

Thus, it is worth hypothesizing that the incapacity of DMD astrocytes to counteract the chronic inflammatory stimuli resulted due to astrocyte impaired control of Blood Brain Barrier (BBB) tightness, may contribute to functional impairment in DMD brains [68]. In this context, although recent reports describe a role of impaired cerebral perfusion in DMD, studies on BBB function in DMD patients are lacking to date, and future studies will provide pivotal and novel insight into the understanding of DMD neural pathology [16].

In this context, our serum proteomic analysis (Figs. 3 and 4) identified several KEGG pathways as being dysregulated in DMD patients. Similar KEGG pathways have been identified by Lang et al., 2019, in their study on human astrocytes cultured from the pluripotent stem cells of DMD patients [66]. Intriguingly, the KEGG pathways identified in these two studies on DMD overlap significantly with the dysregulated molecular pathways of reactive astrocytes reported to be associated with the pathology of AD. Thus, our findings show a common disease mechanism underlying the cognitive impairment associated with DMD and AD involving

Table 3
The reported relationship of cognitive impairment including AD with the upregulated and downregulated proteins correlated with cognitive statues of DMD, as identified in this study.

Protein	Our findings	Findings in Literature		
		Disease	Finding	References
Fibronectin	A significant increase- fold change 2.1, $p = 0.03$	AD	higher amounts in the plasma of patients with AD	[43]
		Autism	Increased in the serum	[44].
MMP-9	A significant increase- fold change 2.260	AD	increases in MMP-9 expression levels were found in AD plasma,	[45]
		AD	in vivo activation of endogenous MMP-9 has been suggested to be neuroprotective	[46,47]
Siglec-3	Decreased compared to those in controls (fold change 0.5, $p = 0.0001$	AD	expression of Siglec-3 (CD33) was significantly increased	[48]
serum C3 and C4 levels	(fold change 0.2, $p = 0.0006$) and C4 levels (fold change 0.2, $p = 0.02$)	AD	mRNAs for C3 and C4 in healthy and AD brains revealed that the expression levels were threefold higher in AD brains	[49].
PDGF-BB levels	Significant increase compared to controls (fold change 2.1, $p = 0.004$)	AD	leakage of the BBB in AD patients starts at the hippocampus, resulting in an increase in soluble PDGFRβ (sPDGFRβ) in the CSF.	[50,51]
GDF11	Significant decrease compared to controls (fold change 0.5, $p = 0.000$)	AD	twice daily treatment (totaling 0.1 mg/kg) with rGDF11 in mice with Alzheimer's disease improved cognition.	[52]
		Schizophrenia	positively associated with immediate memory and delayed memory in schizophrenia patients	[53]

dysregulated molecular pathways in reactive astrocytes. We have compiled these relationships into a novel astrocyte-centric model for DMD and AD (Fig. 6) [69–87], which may further pave the way toward novel therapeutic approaches targeting reactive astrocytes [74] to overcome the cognitive dysfunction associated with DMD.

It is noteworthy that to effectively restore the functional dystrophin in the brain, compounds crossing the BBB are crucial, which is not the case for all compounds [16], except tricyclo-DNA antisense molecules, which are associated with exon skipping in the brain [88]. Moreover, it is argued that clinical trials need to monitor the effect on the CNS [16], where traditional prognostic measurements should be combined with fluid biomarker signatures (i.e., Siglec-3, fibronectin, and MMP-12). In this scenario, natural products (e.g., cinnamon, green tea) [89,90] targeting the brain would be an innovative approach in developing natural product-based nutraceuticals that nourish the brain (i.e., "neuro-nutraceuticals") to address the cognitive impairment associated with DMD [91].

We acknowledge as limitations of our study that the size of the sample for WISC assessment was limited (n = 25). Moreover, the limited size of the sample utilized for serum proteomic analysis rendered this analysis a pilot assessment for proteomic analysis. Despite the limited sample size, salient biomarkers identified have already been validated by independent proteomic studies (26, 46, 47). Regardless, we believe the reported findings are relevant for clinical and research practices and may contribute to improving the understanding of the cognitive clinical markers and serum protein markers involved in the cognitive impairment of DMD. We could not systematically assess behavioral abnormalities or other neuropsychiatric parameters through the standard clinical battery that we used in our study, which we acknowledge is another limitation. All of these limitations could be addressed in future longitudinal studies.

5. Conclusions

Among the several novel observations we report, better cognitive performance in DMD was associated with increased serum levels of MMP9 and FN1 but decreased Siglec-3, C4b, and C3b. Worse cognitive performance was associated with increased serum levels of LDH-H1 and PDGF-BB but reduced GDF-11, MMP12, TPSB2, and G1B. Secondly, better cognitive performance in PSI and PRI domains was associated with intact Dp116, Dp140, and Dp71 dystrophin isoforms while better performance in VCI and WMI domains was associated with intact Dp116 and Dp140 isoforms. Finally, functional pathways shared with AD point towards an astrocyte-centric model for DMD. Astrocytic dysfunction leading to synaptic dysfunction reported previously in AD may be a common pathogenic mechanism underlying both AD and DMD, linking protein alterations to cognitive impairment. This new insight may pave the path towards novel therapeutic approaches targeting reactive astrocytes.

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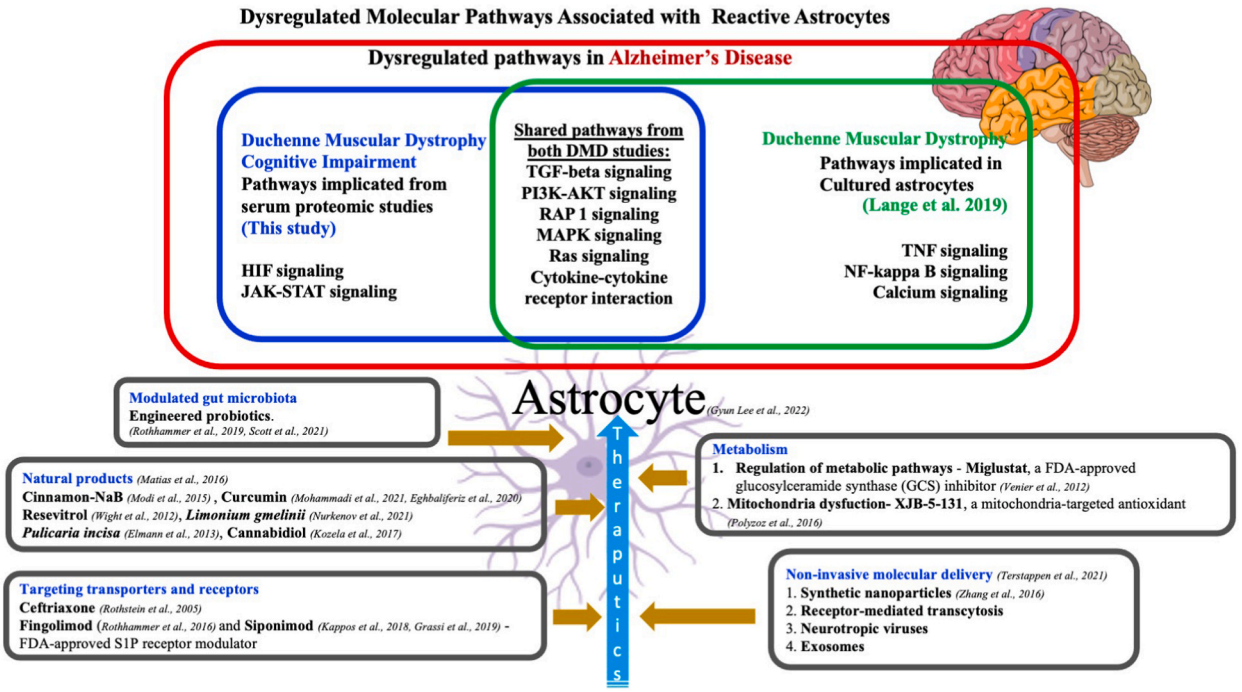

Fig. 6. Shared pathogenic pathways underlying cognitive impairment in DMD and AD and therapeutic opportunities.

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Authors' contributions

Nalaka Wijekoon, Lakmal Gonawala: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Pulasthi Dissanayaka, Kamala Vanarsa, Jessica Castillo: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Dhammika Amaratunga, Isuru Gunarathne: Analyzed and interpreted the data; Wrote the paper.

Pyara Ratnayake, Sunethra Senanayaka, Saraji Wijesekara, Hemal H Gunasekara, Roshan Liyanage, Yetrib Hathout, Ashwin Dalal, Harry WM Steinbusch, Eric Hoffman: Contributed reagents, materials, analysis tools or data.

Chandra Mohan: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

K. Ranil D. de Silva: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability

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

Ethics approval and consent to participate

This study meets the ethical guidelines of the Sri Lankan institutional review board, which is in compliance with the Helsinki Declaration (Ethical Approval No. 449/09 and 38/19 from The Ethics Committee, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka). Written informed consent was obtained from every proband where applicable. For patients incapable of providing consent on their own, consent was obtained from a proxy.

Consent for publication

Not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18530.

List of abbreviations

DMD	Duchenne muscular dystrophy
AD	Alzheimer's disease
Dp	Dystrophin protein variants
FSIQ	Full Scale IQ
MLPA	Multiplex Ligation Dependent Probe Amplification
PRI	Perceptual Reasoning Index
PSI	Processing Speed Index
VCI	Verbal comprehension Index

WISC-IV Wechsler Intelligence Scale for Children
WMI Working Memory Index
BBB Blood Brain Barrier

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