LCMS-based Validation of Glycosylation Variants in Transferrin – A Diagnostic Approach for Congenital Disorders of Glycosylation

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KEYWORDS
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ABSTRACT
Congenital disorders of glycosylation (CDG) are rare genetic diseases caused by defects in the glycosylation pathways. Early and accurate diagnosis of congenital disorders of glycosylation (CDG) is crucial for timely initiating appropriate therapies, which can significantly improve clinical outcomes. Our research aims to develop a fast and reliable diagnostic test for CDG Carbohydrate-deficient transferrin (CDT) is a key biochemical marker in the diagnosis of CDG. Transferrin (TF) is an iron-transport protein that contains N-glycans at Asn432 and Asn630 in humans. It is important to note that CDT represents a group of transferrin sialoforms, including asialo-, monosialo-, disialo-, and rarely trisialo-TF. In this study, we developed and validated a targeted proteomics assay using liquid chromatography-mass spectrometry (LC/MS/MS) to measure transferrin glycosylation variants in human serum. We selected the tryptic peptides 421CGLVPVLAENYNK433 and 622QQQHLFGSNVTDCSGNFCLFR642 as analytes for quantification, each containing glycosylation variants at N-432 and N-630, respectively. Additionally, we evaluated the feasibility of directly quantifying peptides for transferrin sialoforms using selected reaction monitoring (SRM) – Mass Spectrometry (MS) analysis

ABBREVIATIONS:

CDG: Congenital Disorders of Glycosylation; CDT: Carbohydrate-deficient transferrin; DTT: Dithiothreitol; ESI-MS: Electro Spray Ionization – Mass Spectrometry; IEF: Iso-Electric Focusing; hTRF: Human Transferrin; IDA: Iodoacetamide; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; PTM: Post Translational Modifications; SRM-MS: Selected Reaction Monitoring – Mass Spectrometry; TF: Transferrin; TOF-MRM: Time of Flight – Multiple Reaction Monitoring

INTRODUCTION:

Human transferrin (hTRF) is the primary protein responsible for transporting iron in the body. Its key function is to bind circulating iron and deliver it to various cell types [1]. Several studies have suggested that hTRF in serum could serve as a potential biomarker [2] for the early detection of CDG. These findings have driven the development of an accurate and reliable CDG diagnostic assay. Consequently, hTRF quantification is routinely used in clinical diagnosis and helps differentiate between CDG-I and CDG-II types [3]. CDG-I includes defects in the formation of the lipid-linked oligosaccharide (LLO) chain and its subsequent transfer to proteins. The enzyme

defects associated with CDG-I are found in the cytosol (CDGIa, CDG-Ib) and the endoplasmic reticulum (ER) (CDG-Ic, CDG-Id, CDG-Ie). On the other hand, CDG-II involves defects in the processing of the glycan structures attached to proteins, occurring either late in the ER (CDG-IIb) or within the Golgi apparatus (CDG-IIa, CDG-IIc) [4].

CDG diagnosis is challenging not only due to the large number of disorders but also because of the significant clinical heterogeneity. The prevalence of most CDG forms is unknown. The true incidence of CDG in India remains unknown due to a lack of comprehensive studies [5]. The traditional screening test, serum transferrin iso-electro focusing, is only positive in approximately 60% of CDG cases and can even yield negative results in some types of CDGs [6]. TF has two N-glycosylation sites in humans at asparagine 432 and asparagine 630 [7]. Glycosylation plays a critical role in determining the structure and function of glycoproteins. The types and abundance of glycans on glycoproteins can change in response to diseases. Given the essential role of glycans in modulating protein function, analyzing glycosylation could reveal novel biomarkers [8]. Transferrin glycosylation (Fig.1), specifically carbohydrate-deficient transferrin (CDT), is widely used as a biomarker for human CDGs [9]. However, it's important to note that CDT does not represent a single molecular form but rather a group of transferrin (TF) sialoforms (such as asialo-, monosialo-, disialo-, and occasionally trisialo-TF) [10].

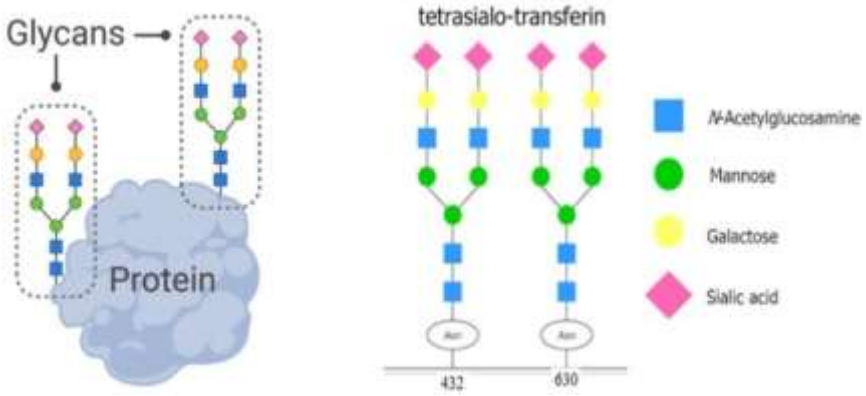


Figure 1: Glycosylation pattern at N-432 and N-630 of transferrin protein in a healthy individual (Ref -11)

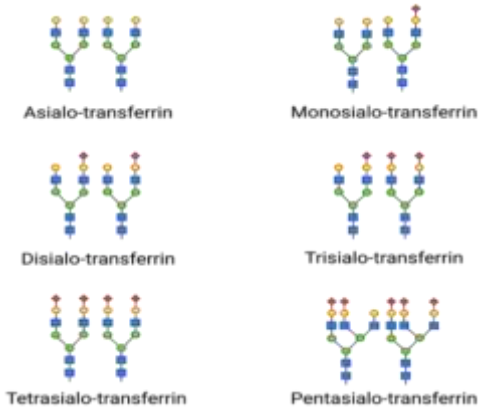


Figure 2: Different sialoforms of Transferrin

Here, we describe a targeted proteomics assay using liquid chromatography-tandem mass spectrometry (LC/MS/MS) to quantify transferrin sialoforms (Fig.2) at Asn432 and Asn630 [11]. The glycosylated peptides 421CGLVPVLAENYNK433 and 622QQQHLFGSNVTDCSGNFCLFR642 [12] are used as analytes for quantification and can be detected with high sensitivity and specificity.

This method involves enzymatically digesting the transferrin protein with trypsin to generate smaller peptides, followed by selecting specific peptides for detection and their quantification [13]. By employing LC/MS/MS, we can accurately identify and quantify these peptides, as mass spectrometry detects peptide ions based on their mass-to-charge ratio (m/z) and fragmentation patterns, ensuring reliable peptide identification [14]. SRM-MS (Selected Reaction Monitoring-Mass Spectrometry) is a targeted mass spectrometry technique that is becoming increasingly popular in proteomics for the quantification of proteins and peptides, even at low concentrations [15]. SRM is particularly valuable in the diagnosis of Congenital Disorders of Glycosylation (CDG), where predefined sets of peptides identified as potential biomarkers must be measured with consistency, reproducibility, and quantitative accuracy across multiple samples [16]. One of the key advantages of targeted mass spectrometry methods is their ability to quantify multiple biomarkers simultaneously (multiplexing) with high accuracy and specificity [17].

Since serum biomarkers are highly relevant and easily accessible for CDG diagnosis and subtyping, our research focused on developing proteome-wide analyses of glycopeptides to identify protein-specific biomarkers for CDG [18]. This methodology would be particularly useful for precise transferrin measurement in both clinical and research settings, offering exceptional specificity and sensitivity. The development and validation of such an assay involved optimizing critical parameters, such as sample preparation, chromatographic conditions, and mass spectrometer settings, to ensure the results are robust and reproducible.

MATERIAL AND METHODS:

This prospective study involves 27 serum samples from human participants and it has been approved by the S2J Independent Ethics Committee (S2J IEC – ECR/284/Indt/AP/2017/RR-20) and with the informed consent of the participants. Children with seizures or stroke-like episodes, non-progressive ataxia, stable mental retardation, or peripheral neuropathy are included in this study. Different age groups, 3 months to 15 years, are included in the study, and a few samples of the parents of the affected children are also taken for comparison of the profiles. All these samples are received for transferrin-IEF testing from various hospitals across Hyderabad and have been used for research with the informed consent of the participants. Out of 27 serum samples, 16 were from male participants, and another 11 were from female participants and were collected over a period of 3 years from 2022 onwards. A control sample was taken from a healthy individual with no complications.

5.1: LCMS Analysis for Specific Peptide Identification and Quantification

LC-MS enables the characterization of various aspects of sialoglycoprotein structure, including the identification and quantification of the peptide backbone, the associated glycan structure, the speciation of sialic acid, and its subsequent modifications. Relative quantification of sialoglycopeptides using ESI-MS signal intensities has been proven to be precise through meticulous optimization of sample preparation and mass spectrometry parameters [19].

Transferrin purification and sample preparation for LC-MS/MS were performed following a standardized protocol [20]. After trypsin digestion, desalting was done using C18 ZipTip pipette tips from Millipore. The desalted peptides were then subjected to reverse-phase separation on a Waters Acquity UPLC BEH C18, 1.7µm, 2.1x150mm column. A 10µL injection volume was used, with buffer-A as 0.1% formic acid in water and buffer-B as 0.1% formic acid in acetonitrile. The chromatographic separation was achieved through a short 20-minute gradient run with a flow rate of 0.3mL/min, column temperature set to 40°C, and a gradient starting with 2% B at 0 minutes, increasing to 50% B at 10 minutes, 80% B at 13.5 minutes, and returning to 2% B at 20 minutes.

The peptides separated on the column were directed to the Waters Xevo-G2 XS Q-TOF instrument for detection. The TOF-MRM method was employed to detect specific peptides in positive resolution mode, with electrospray (ES) as the ionization source and leucine enkephalin (m/z 556.2771 Da) as the lockspray reference standard. TOF-MS was conducted within the m/z range of 300-1600 Da, with a scan time of 0.5 seconds in continuum data format mode. The +3 and +4 charge states of different sialoforms of the peptide are used for quantification at both the N-432 and N-630 sites [21]. CDG-I has elevated asialo- and disialo forms, whereas CDG-II has elevated monosialo and trisialo forms of transferrin [22]. The peptide masses used for the MRM study are listed in Table 1, with each m/z corresponding to a specific function in the method.

Transferrin Isoforms (m/z)	**432 Peptide**		**630 Peptide**	
	3+	**4+**	**3+**	**4+**
Asialo	1034.1190	775.8412	1380.2432	1035.4344
Monosialo	1131.1508	848.6151	1477.2750	1108.2082
Disialo	1228.1826	921.3889	1574.3068	1180.9821

Table 1: Peptide m/z's taken for TOF-MRM analysis

The raw data was processed using MassLynx software version 4.1 from WATERS. Quantitative analysis of the peptides was carried out using QuanLynx. The method was developed by assigning specific acquisition functions for each m/z, with the base peak ionization (BPI) chromatogram serving as the quantification trace for calculating the area response based on the predicted retention time.

RESULTS AND DISCUSSION:

Isoelectric focusing was performed on all the samples using the established method [19], and the results are displayed in Figure 3. IEF of serum transferrin serves as a primary screening method for detecting abnormalities in protein N-glycosylation. Various transferrin protein variants lead to shifts in isoelectric point (pI), complicating interpretation as these variants may co-migrate with transferrin glycoforms.

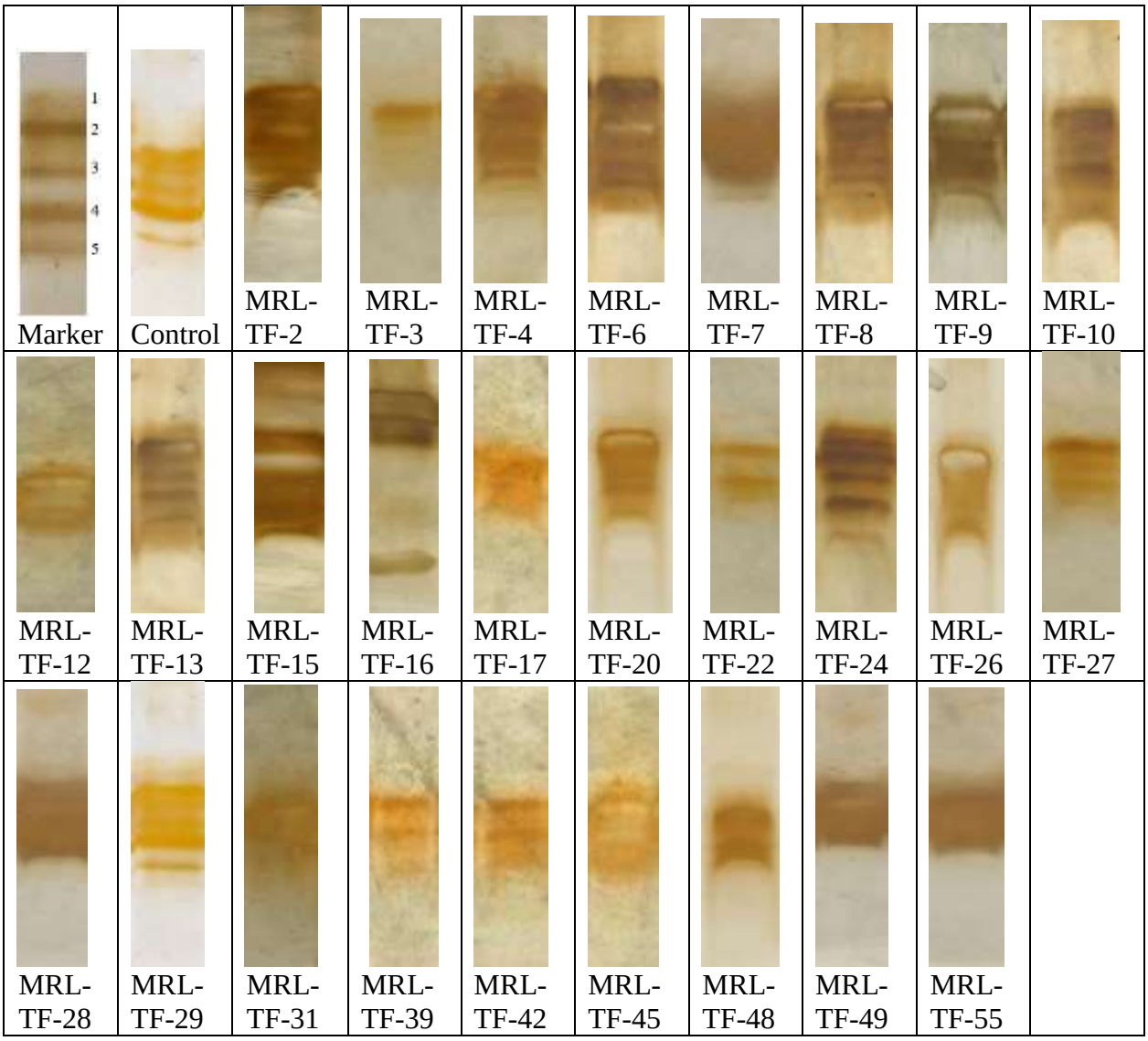


Figure 3: Represents the IEF profiles of all the samples along with marker and control

To address the issue, this study developed and validated a simple yet sensitive targeted proteomics assay using LC-MS/MS. This assay enabled the detection and relative quantification of hTRF sialoforms in clinical samples. After IEF analysis, samples with both positive and negative results were specifically selected to establish TOF-MRM method for detecting various sialoforms. The TOF-MRM method was done with +3 and +4 charge states, after observing the response of peaks with both charge states, the +4 charge states was fixed as quantification trace. A QuanLynx method was developed by freezing the retention times for +4 charge states; table 2 shows the m/z, RT, and representative TOF-MRM peaks for the sialoforms under study. Table 3 shows the response for each sialoform in all the samples.

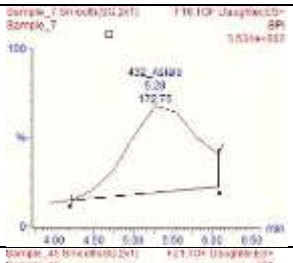
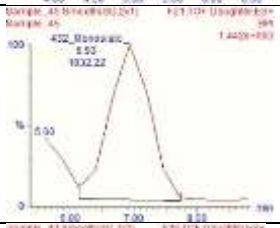
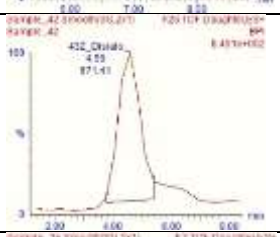
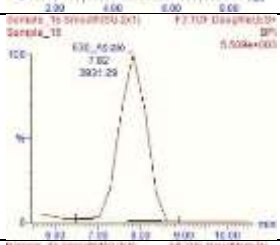
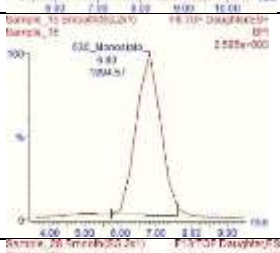
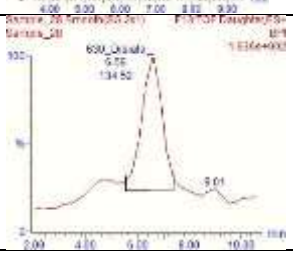
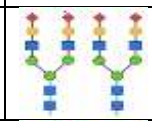
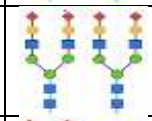
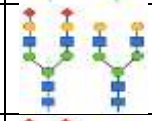
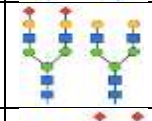
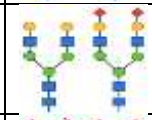
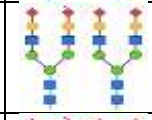
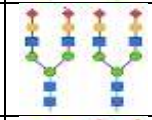
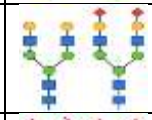
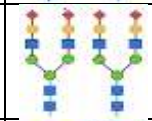
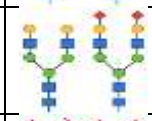
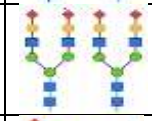
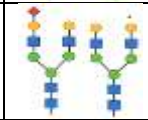
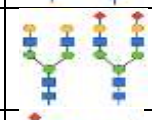
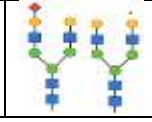
Transferrin Isoforms (m/z)	RT	m/z	Representative Peak
432 - Asialo	5.28	775.8412	
432 – Monosialo	6.63	848.6151	
432 - Disialo	4.58	921.3889	
630 - Asialo	7.82	1035.4344	
630 - Monosialo	6.8	1108.2082	
630 - Disialo	6.59	1180.9821	

Table 2: Depicts the m/z, RT, and representative TOF-MRM peaks for transferrin sialoforms

	Peptide - 433N			Peptide - 630N				
Sample ID	**Asialo**	**Monosialo**	**Disialo**	**Asialo**	**Monosialo**	**Disialo**	**CDG Type**	**Glycan Structure**
Control	-	11.23	406.03↑	24.67	-	112.14↑	Normal	
MRL-TF-2	-	49.74	213.55↑	24.91	-	195.92↑	Normal	
MRL-TF-3	-	-	492.61↑	105.14↑	-	58.02	CDG-I	
MRL-TF-4	161.89	76.10	405.51↑	178.18↑	-	112.60	CDG-I	
MRL-TF-6	176.72↑	-	74.63	48.16	69.85	112.35↑	CDG-I	
MRL-TF-7	72.75	-	200.24↑	-	-	198.23↑	Normal	
MRL-TF-8	-	-	117.48↑	54.25	58.25	126.32↑	Normal	
MRL-TF-9	254.52↑	-	106.17	23.99	-	113.11↑	CDG-I	
MRL-TF-10	-	-	223.99	15.32	-	132.32	Normal	
MRL-TF-12	118.63↑	-	77.26	36.09	-	120.44↑	CDG-I	
MRL-TF-13	-	-	191.86↑	-	55.70	120.38↑	Normal	
MRL-TF-15	-	3826.46↑	382.70	3931.29↑	1994.57	-	CDG-II	
MRL-TF-16	418.71↑	92.18	252.53	121.94	138.79	167.88↑	CDG-I	
MRL-TF-17	-	1094.72↑	265.80	1574.00↑	356.45	80.44	CDG-II	

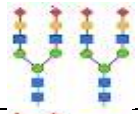
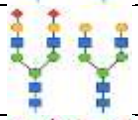
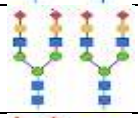
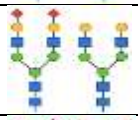
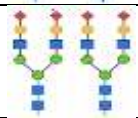
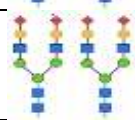
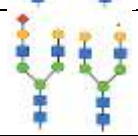
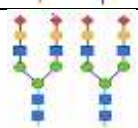
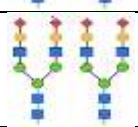
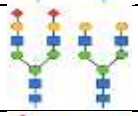
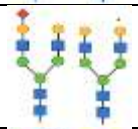
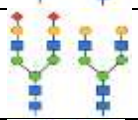
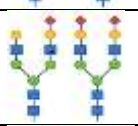
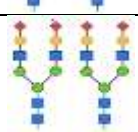
MRL-TF-20	-	14.62	176.82↑	7.25	14.52	122.97↑	Normal	
MRL-TF-22	-	21.32	115.20↑	49.85↑	-	-	CDG-I	
MRL-TF-24	-	-	120.76↑	-	-	159.64↑	Normal	
MRL-TF-26	-	-	123.56↑	121.52↑	-	-	CDG-I	
MRL-TF-27	-	-	233.49↑	-	-	171.95↑	Normal	
MRL-TF-28	109.95	-	176.15↑	-	-	134.52↑	Normal	
MRL-TF-29	-	538.48↑	225.81	349.50↑	169.45	89.19	CDG-II	
MRL-TF-31	-	-	177.81↑	8.15	-	105.89↑	Normal	
MRL-TF-39	-	34.55	196.08↑	70.89	89.47	186.45↑	Normal	
MRL-TF-42	-	597.18	671.41↑	372.94↑	155.05	90.19	CDG-I	
MRL-TF-45	-	1032.22↑	194.44	538.79↑	198.77	72.21	CDG-II	
MRL-TF-48	-	22.94	166.46↑	115.22↑	-	-	CDG-I	
MRL-TF-49	-	335.02↑	158.84	76.63	114.64	123.49↑	CDG-II	
MRL-TF-55	-	34.08	200.91↑	24.45	-	194.79↑	Normal	

Table 3: Response of each sialoform in all the samples. Upwards arrow represents the transferrin sialoform that is abundant for each peptide in the sample. CDG Type-I: Increased Disialo forms and CDG Type-II: Increased trisialo and monosialo forms.

A strong correlation was found between the LC-MS/MS method and the isoelectric focusing approach. While the IEF results allowed for distinguishing normal and abnormal profiles, the LC-MS/MS method provided greater clarity. The results highlighted the effectiveness of the LC-MS/MS assay in identifying both the type of CDG and the sialoform of transferrin in various clinical samples, as shown in Table 4. Furthermore, this targeted proteomics method offers enhanced sensitivity.

Sample ID	**IEF Interpretation**	**LCMS Interpretation**	**Glycosylation by LCMS**
Control	Normal Profile	Normal	Tetrasialo TF
MRL-TF-2	Normal Profile	Normal	Tetrasialo TF
MRL-TF-3	Abnormal Profile	CDG-I	Disialo TF
MRL-TF-4	Normal Profile	CDG-I	Disialo TF
MRL-TF-6	Abnormal Profile	CDG-I	Disialo TF
MRL-TF-7	Normal Profile	Normal	Tetrasialo TF
MRL-TF-8	Abnormal Profile	Normal	Tetrasialo TF
MRL-TF-9	Normal Profile	CDG-I	Disialo TF
MRL-TF-10	Normal Profile	Normal	Tetrasialo TF
MRL-TF-12	Abnormal Profile	CDG-I	Disialo TF
MRL-TF-13	Abnormal Profile	Normal	Tetrasialo TF
MRL-TF-15	Abnormal Profile	CDG-II	Monosialo TF
MRL-TF-16	Abnormal Profile	CDG-I	Disialo TF
MRL-TF-17	Abnormal Profile	CDG-II	Monosialo TF
MRL-TF-20	Normal Profile	Normal	Tetrasialo TF
MRL-TF-22	Abnormal Profile	CDG-I	Disialo TF
MRL-TF-24	Normal Profile	Normal	Tetrasialo TF
MRL-TF-26	Abnormal Profile	CDG-I	Disialo TF
MRL-TF-27	Normal Profile	Normal	Tetrasialo TF
MRL-TF-28	Normal Profile	Normal	Tetrasialo TF
MRL-TF-29	Abnormal Profile	CDG-II	Monosialo TF
MRL-TF-31	Normal Profile	Normal	Tetrasialo TF
MRL-TF-39	Normal Profile	Normal	Tetrasialo TF

MRL-TF-42	Normal Profile	CDG-I	Disialo TF
MRL-TF-45	Abnormal Profile	CDG-II	Monosialo TF
MRL-TF-48	Abnormal Profile	CDG-I	Disialo TF
MRL-TF-49	Normal Profile	CDG-II	Trisialo TF
MRL-TF-55	Normal Profile	Normal	Tetrasialo TF

Table 4 depicts the comparative interpretation of IEF and LCMS results

Out of 27 samples, a deviation in results was observed between the IEF and LCMS methods for 6 samples. Specifically, samples MRL-TF-4, 9, 42, and 49 showed Out of 27 samples, a deviation in results was observed between the IEF and LCMS methods for 6 samples. Specifically, samples MRL-TF-4, 9, 42, and 49 showed normal results with IEF but exhibited abnormal peptides with LCMS. Samples MRL-TF-8 and 13 showed abnormal profiles in IEF but normal peptides in LCMS. This could suggest that IEF may not be as sensitive or might be picking up certain artifacts, whereas LCMS can identify the peptides more clearly. Given that only 6 samples out of 27 exhibited this discrepancy, the deviation is minimal, which supports the effectiveness of the developed TOF-MRM method. However, to further validate these findings, additional molecular genetic testing or enzymatic assays are recommended. This observation supports the potential of the TOF-MRM method, but further validation is crucial to ensure its reliability and consistency across a broader range of samples.

CONCLUSION

Validation and relative quantification of disease-related protein biomarkers are crucial in the biomarker development process. A rapid and efficient diagnostic test for CDG was developed using the LC-MS/MS method. Comparing the IEF results with those from LC-MS/MS allowed us to conclude that LC-MS/MS provided a clearer identification of the transferrin isoforms present in the sample, ultimately helping to determine the CDG form. In our study involving 27 samples, we observed elevated levels of asialo and disialo transferrin forms in CDG-I, while in CDG-II, elevated levels of monosialo and trisialo transferrin forms were noted. The excellent specificity and multiplexing capabilities of MRM offer significant advantages over isoelectric-focusing assays. With enhanced sensitivity and high-throughput capabilities, this method has the potential to be widely adopted in clinical settings. Our subsequent research aims to validate this method in a larger cohort of samples to develop it into a rapid diagnostic test.

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