N- and O-glycan analysis for the detection of glycosylation disorders

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Abstract

Background: Congenital disorders of glycosylation (CDGs) are defined as a group of several rare autosomal recessive inborn errors of metabolism that affect the glycosylation of many proteins and/or lipids. Variable clinical presentation is very characteristic for all types of CDGs; symptoms include severe neurological manifestations that usually start in the neonatal period and cause aggressive irreversible neurological damage. These disorders are usually misdiagnosed as other non-inheritable disorders or remain undiagnosed for a long time, leading to severe neurological complications. The diagnosis of CDGs is quite tedious due to their diverse clinical presentation. In Egypt, there is still no available screening programme to detect CDGs in patients at a young age. Therefore, the need for a reliable rapid test that uses a small sample size has emerged.

This study included 50 suspected subjects and 50 healthy controls with matching age and sex. Western blotting and liquid chromatography-tandem mass spectrometry were used for the analysis of N- and O-glycans, respectively.

Results: The study detected 9 patients with hypoglycosylation (18%). Eight of the nine patients showed abnormal separation of N-glycoproteins using Western blotting indicative of reduced glycosylation (16% of the study subjects and 89% of the subjects with hypoglycosylation). Only one of the nine patients showed a decreased level of sialyl-T-antigen with a normal T-antigen level leading to an increased T/ST ratio (2% of study subjects and 11% of the subjects with hypoglycosylation).

Conclusion: Although N- and O-glycan analysis did not determine the underlying type of CDG, it successfully detected hypoglycosylation in 9 clinically suspected patients (18% of the studied subjects). All detected CDG cases were confirmed by molecular analysis results of mutations causing 4 different types of congenital disorders of glycosylation.

Keywords: Congenital disorders of glycosylation, Inborn errors of metabolism, Glycans, Western blotting, Liquid chromatography-tandem mass spectrometry, T-antigen
neurological presentations. As a result of this resemblance and physician unawareness of the existing CDGs, many CDGs remain under or misdiagnosed [2].

Defects in N-glycosylation can be detected by Western blotting (WB), where glycosylated proteins are electrophoretically separated in a specific gel according to their molecular weights prior to transfer to nitrocellulose. Patients with type I CDG tend to separate additional low molecular masses of different hypoglycosylated protein forms in addition to the normally glycosylated protein band, while patients with type II CDG tend to separate only one band corresponding to the hypoglycosylated protein at a lower level than that of the normally glycosylated protein [3].

The CDGs due to mucin-type O-glycan biosynthesis defects are the most common among O-glycosylation defects. The most common mucin forms in humans are the T-antigen and its sialylated form sialyl T (ST)-antigen. Both forms can be detected using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [4].

The aims of this study are as follows:

- Analysis of N- and O-glycans in clinically suspected subjects in comparison to normal healthy controls.
- Determination of the reference range for T-antigen and ST-antigen.
- Emphasis of the need for early detection of CDGs in suspected patients to avoid irreversible neurological complications.

**Methods**

This study included 50 subjects and fifty healthy controls to determine the reference range of O-glycans. The study was conducted during the period from July 2016 until February 2020.

The inclusion criteria were as follows:

- Subjects with a positive family history of CDGs and had one or more unexplained neurological manifestations with or without other organ disease.
- Subjects with a negative family history of CDGs but had unexplained neurological manifestations with or without other organ disease.
- Apparently normal neonates having a sibling previously diagnosed with a CDG.

The exclusion criteria were as follows:

- Subjects with galactosemia, hereditary fructose intolerance, cancer, and inflammatory diseases were excluded from the study.

The whole study group was subjected to the following analyses:

- Full patient anamnesis and physical examination.
- Qualitative analysis of N-glycan profiles in dried blood spots using WB technique.
- Quantitative analysis of O-glycan profiles in serum using LC-MS/MS.

All patients were referred for molecular analysis to confirm the biochemical results and to determine the CDG type.

**Qualitative analysis of N-glycan profiles in dried blood spots using WB technique**

**Samples and materials used**

Dried blood spot samples collected on S&S grade 903 filter paper (Schleicher and Schuell) from 50 subjects and 50 controls were used.

Donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase, enhanced chemiluminescent (ECL) substrate, and Hyperfilm-ECL were purchased from Amersham (Les Ulis, France). Rabbit antihuman protein antibodies were obtained from Behring (Rueil-Malmaison, France). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) standards, SDS-PAGE cells, and nitrocellulose membranes were purchased from Bio-Rad (Ivry S/Seine, France).

**Procedure**

The electrophoretic separations of 4 glycosylated proteins (transferrin, α-1-antitrypsin, hepatoglobin and α-1-acid glycoprotein) were performed according to the method of Seta et al. [3] where 100 μL of distilled water was added to the dried blood spot, mixed by vortexing and left for 20–30 min before removal of the eluate. Dilution of the eluate was performed as follows: 1:5 for transferrin and α-1-antitrypsin and 1:2 for hepatoglobin and α-1-acid glycoprotein and then 40 μL of Coomassie blue dye and 8 μL of reducing agent were added to 40 μL of diluted sample and heated for 10 min in a water bath at 70 °C.

Samples and a positive control (sample from a previously confirmed CDG patient) were applied on the gel and left for 80 min at 200 volts for the samples to reach the bottom of the gel. Proteins in the samples were then electro-transferred to nitrocellulose membranes in 25 mmol/L Tris, 132 mmol/L glycine and 20% methanol at 100 V for 1 hour.

After the transfer was completed, membranes were kept in 10% (w/v) non-fat milk for at least 1 h at room temperature with agitation to block the membrane. The membrane was then washed 4 times for 10 min each
with washing solution (20 mmol/L Tris, 0.1% Tween 20, pH 7.5)

The protein bands were detected with a 1:2000 dilution of the corresponding rabbit antihuman-specific protein antibodies followed by a 1:5000 dilution of antirabbit IgG antibodies conjugated to horseradish peroxidase. The glycan profiles were acquired using a Chemidoc XRS camera system (Bio-Rad®).

Quantitative analysis of O-glycans by LC-MS/MS

Samples and materials used

Serum from 50 subjects and 50 controls were used.

The internal standard raffinose (1250 pmol/5 μL) and disodium tetraborate were purchased from Lobachemie (Mumbai, India). Ion exchange AG 50 W-X8 resin, T-antigen standard, ST-antigen standard, anhydrous dimethyl sulfoxide (DMSO), and iodomethane (CH3I) were purchased from Sigma-Aldrich (St Louis, USA). Ion exchange AG 50 W-X8 resin, T-antigen as well as the T/ST ratio. The internal standard raffinose (1250 pmol/5 μL) and disodium tetraborate were purchased from Lobachemie (Mumbai, India). Ion exchange AG 50 W-X8 resin, T-antigen standard, ST-antigen standard, anhydrous dimethyl sulfoxide (DMSO), and iodomethane (CH3I) were purchased from Sigma-Aldrich (St Louis, USA). C18 stage tips were purchased from Waters (Milford, USA).

Procedure

The O-glycan chains were released from the protein by β-elimination according to the method of Liu et al. [5], whereby 25 μL of internal standard and 65 μL of water were added to 10 μL of serum. Then, 100 μL of freshly prepared sodium borate in sodium hydroxide solution was added, and the mixture was incubated for 16 h in a water bath at 45°C. Then, 1.6 mL acetic acid in methanol was added dropwise to neutralize the reaction and the solution was desalted using ion-exchange AG 50 W-X8 resin. The separated glycans were vacuum evaporated until completely dry. The glycan chains were then permethylated according to the method of Faid et al. [6], whereby four NaOH pellets were crushed in 10 mL of anhydrous DMSO and 0.5 μL of water to make a slurry. Then, 0.5 mL of the slurry was added along with 0.2 mL of CH3I to the lyophilized glycan and vigorously shaken for 1 h. The mixture was then extracted 5 times using a mixture of 200 μL of water and 600 μL of chloroform, and then, the chloroform phases were pooled and dried under nitrogen for 30 min.

To quantify the free glycans in each sample, 20 μL of serum was diluted to 500 μL and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was then separated and processed as a normal sample. The concentration of the free glycans was then subtracted from that of the sample.

Dried permethylated patient and control samples were reconstituted with 50 μL of methanol, purified through C18 stage tips, and finally analysed on a Waters® Xevo TQD triple quad mass spectrometer using a 3-μm C18 column (2 mm × 100 mm) and a 10 μL sample volume in positive ion mode. The mobile phase consisted of 2 buffers: buffer A (1:0.199 acetonitrile: formic acid: water) and buffer B (990:0.1 acetonitrile: formic acid: water) with a flow rate of 0.25 ml/min. Gradient elution was used as follows: from 0–20 min, 50 to 80% buffer B; from 20–28 min, 98% buffer B; and from 28–39 min, 50% buffer B. Calibration curves were constructed using 6 concentrations of T-antigen and ST-antigen each. The results are expressed as the concentration of T-antigen and ST-antigen as well as the T/ST ratio.

Statistical analysis

GraphPad Prism® software was used for the analysis of data. All quantitative data are presented as the mean ± standard deviation. Unpaired Student’s t test was performed to analyse the statistical significance of the studied group. A level of significance of 0.05 was used, below which the results were considered statistically significant.

Results

The current study included a total of 50 subjects during the period from July 2016 until February 2020. Samples from 50 normal healthy individuals were used as controls. The data of the study group (Table 1) showed 58% males (n = 29) and 42% females (n = 21). Their age at presentation ranged from 0.5 to 11 years with a mean age of 3.59 ± 2.6 years. More than half of the subjects presented between 1 and 4 years of age (54%, n = 27) (Table 2). Consanguinity accounted for 74% of the subjects (n = 37) who were children to consanguineous marriages while only 26% were children to non-consanguineous marriages (n = 13). Forty-eight percent of the studied subjects had a positive family history (n = 24) (Table 1). However, if we compare the subjects from the consanguineous group to those from the non-consanguineous group, 56% of subjects coming from consanguineous marriages had a previous family history while only 23% of subjects coming from a non-

| Table 1 | Data of the study group (n = 50) |
|---------|------------------|
| Age (years) | Mean ± STD | Range |
| Number % (n = 50) |
| Age (years) | 3.59 ± 2.6 | 0.5–11 |
| Sex | | % |
| Male | 29 | 58 |
| Female | 21 | 42 |
| Parental consanguinity | | |
| Consanguineous | 37 | 74 |
| Non consanguineous | 13 | 26 |
| Family history | | |
| Positive | 24 | 48 |
| Negative | 26 | 52 |
consanguineous family had a previous family history. Three subjects in the study group were siblings to the same family. Subjects in the study presented with a wide range of symptoms with hypotonia and psychomotor retardation being the most common symptoms (66 and 40% respectively) (Fig. 1).

The qualitative analysis of N-glycosylation of 4 different proteins (transferrin, hepatoglobin, α-1-acid glycoprotein and α-1-antitrypsin) using WB was carried out for all the subjects and controls. Eight patients (16%) showed a separation of N-glycoproteins indicative of hypoglycosylation in one or more of the studied proteins. Examples of the WB separation of the four proteins in 13 suspected subjects and 10 healthy controls are shown in Figs. 2 and 3, respectively.

Seven out of the 8 patients with hypoglycosylation showed separation of N-glycoproteins indicative of hypoglycosylation of transferrin (87.5% of positive WB subjects, 14% of total subjects), 7 out of the 8 patients with hypoglycosylation showed separation of N-glycoproteins indicative of hypoglycosylation of α-1-antitrypsin (87.5% of positive WB subjects, 14% of total subjects), all patients with hypoglycosylation showed separation of N-glycoproteins indicative of hypoglycosylation of α-1-acid glycoprotein (100% of positive WB subjects, 16% of total subjects), and 7 out of the 8 patients with hypoglycosylation showed separation of N-glycoproteins indicative of hypoglycosylation of hepatoglobin (87.5% of positive WB subjects, 14% of total subjects).

The use of LC-MS/MS for the analysis of T-antigen and ST-antigen revealed 1 patient (2%) with an abnormal O-glycans profile when compared to the levels of normal healthy controls. Two calibration curves were constructed using 6 concentrations of each T-antigen and ST-antigen. The 50 control samples were used to determine the normal range to which the subjects were compared (Table 3). The calibration curves along with the mean ± SD of subjects and controls in both tests are shown in Fig. 4.

The only patient with an abnormal O-glycans profile had a normal level of T-antigen (0.99 μM) but a decreased level of ST-antigen (5.76 μM), thus resulting in an elevation of the T/ST ratio (0.173) when compared to the normal range of controls. The chromatogram of this patient in comparison to the control is shown in Fig. 5.

The results of molecular testing of the study group matched the biochemical results of this study. The 9 patients with hypoglycosylation showed mutations causing 4 different types of CDGs. The most common CDG type was PMM2 deficiency (4 cases, 8% of the study group) followed by DPM1 deficiency (3 cases, 6% of the study group). One patient had SRD5A3 deficiency and another had a COG5 CDG (each 2% of the study group). Most cases (6 out of 9 patients, 67%) were detected in patients between 1 and 4 years of age (Table 4).

**Discussion**

The study showed a close distribution of symptoms between boys and girls (Table 1) in the study group in contrast to previous studies that showed male predominance in many IEMs in the Egyptian community [7, 8]. The close distribution of the disease is homogenous with
the autosomal recessive inheritance pattern of most types of CDGs.

The study also revealed the absence of a distinct age of presentation in the study group. The subjects of this study presented in a very wide range of ages (Table 1). The mean age of presentation was 3.59 years. It was also noted that the highest presentation was in the 2nd group (Table 2) with 54% of subjects presenting between 1 and 4 years of age while only 7 subjects (14%) presented in the first year of their lives, 3 of them already had affected siblings (6% of studied subjects, 42% of group I). It was noted from the history of the subjects that they remained undiagnosed or were previously misdiagnosed with other non-inheritable disorders.

Group II had the highest number of diagnosed cases (Table 4) while group I had only one diagnosed case who had 2 previously affected siblings. These data support the need for a CDG screening programme to decrease the age of presentation and diagnosis and therefore decrease the complications of these aggressive diseases.

The delayed diagnosis of these patients can be attributed to lack of specific symptoms of CDG, multi-system involvement, the limited number of cases reported for
Fig. 3 Separation of a transferrin, b α-1-antitrypsin, c α-1-acid glycoprotein and d hepatoglobin of 10 controls using WB technique.

| Glycan          | Predicted component | m/z  | Reference level (μM) | Mean±SD (controls) | Mean±SD (Subjects) | P value  |
|-----------------|---------------------|------|-----------------------|--------------------|--------------------|----------|
| T-antigen       | HexHexNAc           | 534  | ≤1.2                  | 0.44±0.35          | 0.6±0.27           | 0.0102   |
| ST-antigen      | NeuAcHexHexNAc      | 895  | ≥14.2                 | 22.06±5.41         | 18.88±4.5          | 0.0019   |
| T/ST            |                     |      | ≤0.041                | 0.02±0.01          | 0.03±0.02          | 0.0066   |

Table 3 O-glycan analysis using LC-MS/MS technique showing the 2 O-glycans measured for subjects and controls.
Fig. 4 Analysis of T-antigen and ST-antigen by LC-MS/MS with 

- **a** calibration curve of T-antigen standard,
- **b** calibration curve of ST-antigen standard,
- **c** mean ± SD of T-antigen and
- **d** mean ± SD of ST-antigen

Fig. 5 Chromatograms obtained from LC-MS/MS analysis.

- **a** Normal healthy control
- **b** A case of COGS-CDG. Types of CDG are determined after molecular analysis
individual types of CDGs, lack of awareness by clinicians about the symptoms that raise suspicion of CDGs and/or the lack of screening programmes for IEMs in general and CDGs in particular [9].

A correlation with parental consanguinity was very strong in the study group (Table 1). The number of affected family members was much higher in consanguineous families than in non-consanguineous families. These findings point to the presence of a genetic disorder in patients born to consanguineous marriages especially those with diverse unexplained symptoms or with symptoms unassigned to a specific disease [10].

Subjects in this study presented with variable neurological and/or non-neurological signs and symptoms (Fig. 1). This was also noted by many other studies such as the study conducted by Goreta et al. [9], who recommended that CDGs should be suspected in any case with unexplained neurological manifestations, particularly in cases where the neurological manifestations are accompanied by other organ diseases. Additionally, CDGs should be suspected in cases with any unexplained syndrome without obvious neurological manifestation.

The diversity of clinical presentations can be explained in many ways, first by the complexity of various glycosylation pathways. Second, knowledge about CDGs started to develop in the 1980s and, as a result, experience with disease symptoms is still very limited. Moreover, the small number of patients diagnosed with different CDGs makes it very difficult to determine the phenotype of each type of disease [11].

In this study, we studied the N-glycosylation profile for subjects and controls using WB, while the O-glycosylation profile was studied by LC-MS/MS techniques.

Due to its convenience, WB was used to detect the hypoglycosylated form of 4 different serum proteins (transferrin, hepatoglobin, α-1-acid glycoprotein and α-1-antitrypsin) in all subjects and controls. The 4 proteins were analysed in parallel, and the results depended on the interpretation of the 4 profiles together. The study showed separation of molecular masses lower than those found in healthy individuals in at least one of the studied proteins in 8 of the studied subjects (16% of the study group).

We choose WB because it avoids some of the disadvantages of isoelectric focusing (IEF). WB separates glycans depending on their molecular weight; thus, any change will be due to defects in the synthesis of glycans, while in IEF, any change leading to alteration of sialic acid groups attached to glycans will result in a false-positive result. WB also provides a chance to analyse many N-glycosylated proteins not only transferrin as in the case of IEF [3].

Alpha 1-acid glycoprotein is an acute-phase plasma alpha-globulin that acts as a carrier for basic and neutral lipophilic compounds. Normally it has 5 N-glycan chains that are bi-, tri-, and tetra-antenna capped by terminal sialic acid residues. In our study, the separation of the affected subjects and positive controls showed separation of only 3 bands and not 6 corresponding to the normally present form with 5 glycan chains along with 5 abnormal forms with tetra-, tri-, di-, and mono-glycan chains as well as no glycan chains. In this study, the 2 most hypoglycosylated forms were not detectable. This is similar to the results obtained by Denecke et al. [12] and Seta et al. [3], who recommended further studies to detect the reasons for the absence of those 2 least glycosylated forms.

The study emphasized the importance of using more than one protein in WB analysis for patients suspected to have CDGs to avoid false results. The results of the 4 proteins were analysed in parallel for every sample, and the separation was repeated when the results were questionable. The selection of these proteins provided a range of glycosylation sites (transferrin: 2, α-1-antitrypsin: 3, hepatoglobin: 4 and α-1-acid glycoprotein: 5). The presence of only 2 glycan chains in transferrin guaranteed a very clean WB band compared to the more diffuse larger bands of hepatoglobin and α-1-acid glycoprotein; however, when the phenotypic expression of underglycosylation is low, the last 2 proteins could more easily give abnormal patterns than transferrin.

Although there was no significant difference in the number of patients with abnormal profiles of each individual protein (87.5% of patients with positive WB profiles showed hypoglycosylation of transferrin, alpha-1-antitrypsin and hepatoglobin compared to a 100% of the patients with positive WB profiles showing hypoglycosylation of alpha 1-acid glycoprotein), not all patients with abnormal WB profiles showed separation of N-
glycoproteins indicative of hypoglycosylation of the 4 proteins together. Only 56% of abnormal patients in our study group showed hypoglycosylation of all 4 proteins together.

In our study, α-1-acid glycoprotein had a higher sensitivity than the other tested proteins (100% compared to 87.5%). This is different from the work done by Seta et al. [3], which showed a relatively high sensitivity of hepatoglobin, and the work done by Yussa et al. [13], which showed similar sensitivity of both α-1-acid glycoprotein and transferrin.

The choice of a mass spectrometric method for the analysis of O-glycan profiles depends mainly on the size of the analysed glycan; the LC-MS/MS method is sensitive to analyse the small T-antigen with an MRM transition of the analysed glycan; the LC-MS/MS method is sensitive to the size of glycoprotein is important in the assignment of the N- or O-glycan to their specific glycosylation sites, the analysis of the glycopeptides is complicated for many reasons. According to various reports [14, 15], the medium, microheterogeneity, macroheterogeneity, and protease resistance of O-glycoprotein can make it difficult to analyse these by MS/MS. They also added that peptide sequencing is very difficult to determine when the protein is heavily glycosylated; this peptide sequence is the main factor that assigns glycan chains to their glycosylation sites.

As an alternative, in this study, we chose to analyse the released O-glycans and not the whole set of O-glycopeptides. This was achieved by chemical cleavage using β-elimination in which the glycopeptide is subjected to alkali treatment, leading to breaking of the bond between GalNAc and Thr/Ser of the polypeptide chain with concomitant reduction of GalNAc to N-acetylgalactosaminitol by borohydride. The reduction of GalNAc prevented further degradation of the released glycan chain (peeling reaction) and facilitated fragmentation during MS/MS.

After the separation of the glycan chains from the polypeptide, derivatization of the glycan chain was performed to overcome the weakness of the glycosidic linkage and increase the glycan stability during ionization. We chose permethylation as a method for derivatization. After permethylation, all the free OH groups of the glycan are converted to methyl ethers, and the labile sialic acid terminals were esterified into more stable sialyl esters leading to successive separation of both the sialylated and nonsialylated T-antigen (T-antigen at m/z = 534 and ST-antigen at m/z = 895) (Table 3 and Fig. 5).

The reference ranges obtained from the analysis of O-glycans from 50 controls were close to the reference range of the study conducted by Xia et al. [4], who investigated 10 patients affected with different types of CDGs and compared their results to 150 normal healthy controls. Our reference ranges also matched those of another study conducted by Liu et al. [5], who investigated the glycosylation profiles of 19 galactosemia patients and compared them to 150 normal healthy controls.

The analysis of per methylated O-glycans for the study group by LC-MS/MS revealed only one patient (2%) with an abnormal O-glycan profile compared to the reference ranges of normal healthy controls (Fig. 5). The subject showed a normal T-antigen level and a low ST-antigen level with concomitant elevation of the T/ST ratio. The same patient showed a normal N-glycosylation pattern with WB separation, and this result suggests a disorder of O-glycosylation. However, the 2-dimensional electrophoresis of this sample showed an abnormal N-glycosylation pattern of the four proteins which is common in type II CDGs. This result raised our suspicion of an N-glycosylation disorder accompanied by disturbance of the O-glycosylation mechanism. These mixed findings highlight the importance of analysing both O-glycans and N-glycans together in any patient suspected to have a glycosylation defect, with 2-dimensional electrophoresis being necessary for the detection of CDGs II hypoglycosylation patterns. This conclusion matches the conclusion of Xia et al. [4], who recommended a combined N- and O-glycan profile analysis for better detection of different types of CDGs II as well as CDGs due to Golgi dysfunction or defects in nucleotide transfer. The molecular analysis of this subject showed a COG5 gene mutation.

This study added to the studies performed by Prien et al. [16] and Ahn et al. [17] that the analysis of N- and O-glycans can effectively detect changes in the glycan profile from the normal profile. However, they cannot determine the underlying cause of the change in this glycan profile. Molecular analysis to detect gene mutations is required to determine the specific type of CDG.

Similar to WB and mass spectrometric analysis, samples from the study group were sent for molecular analysis. The DNA results matched the biochemical results of our study. The 9 patients with abnormal N- and O-glycans, detected by WB and LC-MS/MS, showed mutation of genes involved in protein glycosylation (Table 4), with PMM2 deficiency having the highest incidence among the studied study groups (44.4% of the positive cases and 8% of total subjects).
Conclusion

In this study, we concluded that the combined analysis of N- and O-glycans using WB and LC-MS/MS can provide a solid foundation towards the detection of hypoglycosylation in subjects suspected to have CDG. Both tests should be performed to avoid missing the diagnosis of mixed N- and O-glycan disorders such as the CDGs resulting from COG deficiency.

Abbreviations

CDG: Congenital disorders of glycosylation; DMSO: Dimethyl sulfoxide; ECL: Enhanced chemiluminescence; IEF: Isoelectric focusing; IEM: Inborn errors of metabolism; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; LLO: Lipid-linked oligosaccharide; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; STDF: Science and Technology Development Fund; WB: Western blotting

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Authors’ contributions

ASG was responsible for the conception and design of the study, analysis and interpretation of data and critical revision of the submitted protocol for important intellectual content, statistical analysis and contents. AFE was responsible for the conception and design of the study as well as acquisition, analysis and interpretation of data. She was also responsible for critical revision of the submitted protocol for important intellectual content, statistical analysis and contents. TD was responsible for the conception and design of the western blot analysis of all samples in the study group. He was also responsible for the interpretation of western blot results. OSMA contributed in the conception and design of the study as well as revision of the submitted protocol for important intellectual content, statistical analysis and contents. MSZ was responsible for selection of subjects for the study, their clinical evaluation, history taking as well as revision of the clinical data of the submitted work. EMF was responsible for the conception and design of the study and critical revision of the submitted protocol for important intellectual content, statistical analysis and contents. All authors have read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Medical Research Ethics Committee of the National Research Centre (no: FWA 00014747), and a written informed consent was taken from the patients parents. The work has been performed in accordance with Helsinki Declaration consent for publication.

Consent for publication

Consent for publication data was taken from the patients’ parents, and they agree for publication.

Competing interests

The authors declare that they have no competing interests.

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