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METABOLIC ANALYSES OF NODDING SYNDROME IN UGANDA: A PILOT STUDY IS A BIOTINIDASE AND ACETYL CARNITINE DEFICIENCY; A METABOLIC DISORDER. AN OBSERVATIONAL STUDY DESIGN

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ABSTRACT

Background: Nodding Syndrome (NS) is a childhood neurological disorder which presents with a "head nodding", cognitive decline, wasting, stunting and school dropout. We conducted a metabolic analysis on NS children receiving treatment at Hope for HumaNs (HfH) rehabilitation centre in Gulu, Uganda. **Methods:** We conducted a biochemical analysis on 48 NS children's blood and urine samples collected in 2014 as part of a pilot study. Ethical approval was obtained from Lacor IRB and STATA was used for data analysis. A p-value of <0.05 was considered statistically significant. **Results:** Most 37/47(78.7%) had low mean biotinidase (95% CI) of 1.98(1.62, 3.92). Similarly, mean acetyl carnitine level was low 4.68(5.77, 9.49), and BMI-for-age, 16.9(16.1, 17.7), MUAC 19.89(19.02, 20.76), normal urate concentration 0.23(0.20, 0.25), normal urate/creatinine ratio 0.25(0.20, 0.30). All NS children with data on propionyl carnitine and butyryl carnitine levels were high and 43/47(91.5%) had normal free plasma carnitine level. **Conclusion:** NS is a childhood neurological disorder whose cause is not known. This study demonstrated biotinidase and Acetyl carnitine deficiency and elevated levels of proprionyl carnitine (C3) and Butyryl carnitine (C4) suggesting a possibility of a probable metabolic aetiology. Result of our interventional study suggests biotin supplementation improves symptoms of NS.

KEYWORDS: Nodding Syndrome, Gulu University, biotinidase and acetyl carnitine deficiency, metabolic disorder, Hope for HumaNs (HfH).

1.0 INTRODUCTION

Nodding Syndrome (NS) arose exclusively in children who were resident in Internally Displaced Peoples' camps (IDPs) as an outbreak during war, initially in the 1990's in South Sudan and in Northern Uganda,[1,2,3] NS represents a progressive neurological disorder characterised by clusters of head nodding, cognitive decline, thermal dysfunction, muscle weakness, wasting, stunting and cerebral/cerebellar atrophy.[1,2,3,4,5] Nodding Syndrome in Uganda appeared in a manner that was clustered in person (onset mainly at the age of 5-15years); in time (IDP camp stay) and in space (geographically clustered on either side of Aswa and Pager rivers and their tributaries) whose water sources originate from mountainous areas where there are reported open mining of gold, phosphate and uranium.[5] NS in this region increased in incidence and reached a peak between 2008-2009, then rapidly decreased in incidence, which has been attributed to the resolution of war, resettlement of those living in IDPs, feeding on home grown foods, setting up of supportive Ugandan Ministry of Health (MOH) treatment centres and mass insecticide spray programs to eliminate the black flies.[2,6,7] As of 2012, there has been no new NS cases reported by the Ugandan MOH or WHO but the disorder has so far affected between 3,000-4,000 children in Northern Uganda.[6,7] However, there is uncertainty regarding accuracy of the Ugandan Government and non-governmental reports on Northern Ugandan NS cases, the number of displaced persons and mortality rates[6,7] and the Sudanese NS epidemiology is lacking due to ongoing regional instability.[8] The syndrome encompasses several geographical areas of Eastern Africa i.e. South Sudan, Northern Uganda and Southern Tanzania but the common factors are internal displacement and eating of relief food provided by relief agencies.[1,2,3,9,10] In addition, there has been extensive

search for infectious agents with no uniform identifiable link.[11,12,13,14,15] NS aetiology has been researched by several authors and of note the WHO and CDC have organised several seminal observational and small case control studies[2,3] and several studies on NS remain either unpublished or partially published.[2,3,5,7,12,16] Interestingly, there have been statistically significant associations established between NS and the following variables: Malnutrition, high anion gap acidosis, low serum bicarbonate levels, *Onchocerca volvulus (OV)*, *Mansonella perstans*, exposure to munitions, exposure to gun raids, eating relief foods at weaning, consumption of crushed roots, consumption of serena, emergency food supplies and mouldy maize.[1,2,5,7,10,11,12,13,16,17] In addition, recent data suggests an association with cerebrospinal fluid (CSF) VGKC antibodies and serum leiomidin-1 antibody, suggesting a neuro-inflammatory cause.[18] Additionally, several studies have demonstrated equivocal associations with vitamin B6 deficiency,[12] *Onchocerca Volvulus* infestation and previous measles exposure.[1,2,11,20,21,22,23] Some of the unpublished data on NS in South Sudan has revealed unremarkable urinary mercury, thiocyanate and arsenic results.[17] Interestingly, it is a widely held belief among the communities in Northern Uganda that NS has possibly originated from contaminated relief food provided by relief agencies or exposure to war munitions/chemicals during the protracted 20 year old war.[14,15] Several studies have reported that NS children consumed spoiled relief foods during IDP camps but there is no mention of the proportions of NS children that ate it.[11,17,19,20,26] The recent findings in a case control study, case series and case reports conducted by Kitara *et al*. (2013) at Gulu in Northern Uganda are the studies that identify high anion gap metabolic acidosis among NS children compared to their sex and age matched controls.[1,10,13,19] These suggested perhaps that NS could be secondary to a metabolic disorder and perhaps a mitochondrial disorder.[1,5,10,13,19] This researcher argues that nodding episodes are triggered by sights of local food, starvation, exposure to cold weather/temperatures or cold water, physical exercises and that there is a statistically significant association with high anion gap acidosis.[1,10,13,19] These observations perhaps points towards a disease process that affects the energy pathway in NS Children. It is therefore important to note that there has not been any published data that carries out metabolic analysis on affected individuals and we suggest that NS is an emerging neurological disorder in East Africa likely due to factors that were experienced during the Internal displacement into IDPs that secondarily precipitates disturbance in the metabolic pathway.[1,5,7,10,13]

We aimed in this study to answer the hypothesis that NS is perhaps a metabolic disorder and that we conducted metabolic analysis on urine and blood to determine urinary, plasma organic acids and enzymes for disorders of metabolism on the affected NS children in Gulu, Northern Uganda.

2.0 MATERIALS AND METHODS

2.1 Study Design
This was a prospective observational study in which we conducted clinical and biochemical analysis on blood and urine samples on 48 NS children.

2.2 Study site
This study was conducted at Odek sub County, Gulu district; an area in the epicentre of NS epidemic in Northern Uganda.[1,2,7,13,23] This was largely a rural community which has one of the highest levels of poverty, inadequate water and sanitation and with significant disease burden.[2,7,23,24,25,26] Hope for HumaNs (HfH) centre for NS children treatment and rehabilitation at Aromowang lobo is 5 kilometres East of Awere Trading Centre-Gulu district off the road from Gulu to Pader district (Figure 1). This centre was built in 2012 by two American founders from St. Antonio, Texas, USA. It is a well built facility with classrooms for teaching basic education; a medical clinic which is run by qualified medical staff (nurses, clinical officer; Community Health Workers (CHWs); visiting psychologist; occupational therapist, nutritionist, psychiatric clinical officer and a volunteer clinician (DKL); a refectory and cooking place for the food rehabilitation, a play field for soccer; a piggery for livelihood project and a medical staff quarter. There was a daily schedule of activities for NS children beginning with travel from home, registration, administration of medication, physical exercises, feeding, bathing, hygiene, toiletry and sometimes physiotherapy. This was a comprehensive centre which was efficiently and effectively run by the administrators of this organization.[1,2,23]

2.3 Study population
We recruited forty eight (48) NS children from Aromowang lobo, in Odek Sub County, Gulu district. They were undergoing rehabilitation at HfH centre and the others were part of the outreach services of the centre, Ugandan MoH and Gulu District Health Department. NS children were undergoing a multidisciplinary management using Sodium Valproate (200-400mg BD); multivitamins (1 tablet BD); The composition of each coated vitamin tablet contained; Vitamin A [as acetate BP 2500IU]; [Cholecalciferol BP 300IU]; [Thiamine Hydrochloride BP 1mg]; [Ascorbic Acid BP 15mg]; [Riboflavin 5D Phosphate 500mg]; [Nicotinamide BP 7.5mg]; folic acid (5mg/day); Carbamazepine (200mg BD); high energy local food for nutritional rehabilitation; social care; hygiene and basic educational skills. Each NS child was assessed before enrolment into care by a multidisciplinary team from the Ugandan MoH, Gulu District Health Department, Gulu University and other research institutions in Uganda. Each NS child was individually screened and examined by the research team to confirm conformity to the inclusion criteria.

2.4 Recruitment methods
We recruited NS children consecutive into this study.

2.5 Inclusion criteria
We recruited NS children as participants in accordance with WHO epidemiological and surveillance case definition of probable Nodding Syndrome.[1,2,10,13,19,23] Informed consent from parents/Guardians and assent for children 14 years and above were obtained.

2.6 Exclusion criteria
We excluded those children aged 2 years and below with reported history of abnormal physical, cognitive and social development prior to onset of nodding and lack of consent from their parents/guardians.

2.7 The study instruments
We used a questionnaire to investigate the current and past physiological, psychosocial and mental health conditions of NS children. These questions were directed towards the parents/guardians. They included information on the socio-demographic characteristics of the child and family; the environmental and dietary history, the child and the mother's history, the pattern of NS child's growth and development and the present physical, psychological and nutritional status of the affected child.

In addition, more information was obtained about the caregiver, school attendance, when nodding episodes were first observed, the birth order, when and where the onset of NS; the year of onset; the month; relationship between onset of nodding with IDPs, nodding trigger factors, the number of nodding episodes that occurred per day, the observed time when it most frequently occurred; Other symptoms such as epileptic fits, uncoordination of limb movements, disorientation, drooling of saliva, tongue biting, urinary and stool incontinence. In addition, a whole section of the questionnaire explored the psychological and social assessment of a wide range of inquiry into the sleep pattern, changes in appetite, emotions, predominant thoughts/worries, perceptual disturbances before and after nodding, history of mental illness in the family, the number of other siblings with NS and extent to which social support was being offered to the NS child.

Further to that, each NS child underwent a neurological assessment using a 5 criteria methods to determine whether there is any mental impairment, visual impairment, Gait ataxia, inability to walk and features of Parkinsonism (resting tremours, bradykinesia and rigidity). In addition, each NS child underwent disability assessment using a four scale criteria; motor disability using Gross Motor Function Classification System (GMFCS) which was Graded as; I, II, III, IV and V; feeding and swallowing difficulties, behavioural difficulties, cognitive and learning difficulties, emotional problems, malnutrition and growth failure, speech difficulties, personal care and daily activities and injuries

and/or burns that may have been experienced over the period.

We further conducted a mental state examination of each NS child using a nine step assessment methods including awareness of the environment; concentration and attention; ability to recall (memory) for recent and past events; general appearance; interaction with caregivers and others; Speech and language ability whether appropriate or not; the mood and affect whether normal, low or elated; the thought process whether appropriate or inappropriate with emphasis on obtaining whether they had suicide ideation or persistent fears/worries; and finally assessment of the patient's perceptions including knowing whether the patient had hallucinations or any illusions.

Additionally, the questionnaire explored the suspected risk factors of the syndrome among the NS children including IDP camp life, maternal factors during pregnancy; maternal vaccination, maternal illnesses during pregnancy; maternal medications with adverse effects during pregnancy; mode of delivery of affected NS child and postnatal care. It further looked into the breastfeeding habits of the NS child, its duration, when the supplementary foods were introduced, what were the weaning foods and the weaning age. In addition, the types of illnesses suffered by the NS child before NS onset were explored including diseases which could present similarly such as cerebral malaria, meningitis, measles, head trauma and others that were required to be specified by the parents/Guardians. Furthermore, the pattern of growth and development of the affected NS child was probed to obtain information on family history of epilepsy and Nodding Syndrome and the number of family members affected by the two conditions.

Finally, the last part of the questionnaire described the treatment history after the onset of the Nodding Syndrome symptoms. The questions explored the treatment being received for the condition; whether modern or traditional medicine and to establish whether each of them was providing improvement to the condition of NS child. This was followed by a thorough physical, psychosocial, mental health and anthropometric assessments.

2.7.1 Anthropometric measurements
Each NS child was measured clothed and barefoot for height (cm) and body weight (kg). Weight was measured using a calibrated digital scale which was standardised before use while height was measured in centimetres using a stadiometer. The mid upper-arm-circumference of the left arm was measured using a MUAC tape approved by the WHO for the assessment of malnutrition. The findings were recorded in centimetres (cm).

2.7.2 Sample collections

After completing filling and checking the questionnaires by the Principal Investigator, blood samples was obtained from the anterior left cubical fossa using aseptic technique. A vacutainer was used to collect approximately 5mls of whole blood. It was labelled using an indelible ink and immediately stored in a cool box packed with dry ice ready for transportation to the main laboratory at Gulu University. In order to obtain early morning urine, each NS patient and their parents were advised to hold the early morning urine until they reached the study site. Each NS child was then helped by the researchers to obtain the early morning midstream urine which was collected into a sterile container with a lockable lid. Each urine container was labelled with an indelible marker and placed in a sterile plastic sheath and stored in a cool box well covered with dry ice and ready for transportation to the Gulu University laboratory.

2.7.3 Analysis of Samples

Preparation of samples: Whole blood samples obtained were centrifuged at 3000 r.p.m for five minutes and serum obtained were transferred and stored into 4ml cryo vials and labelled. Each urine sample was placed in a test tube with lockable lid and placed in a plastic sheath to prevent any possible leakages and contamination to other samples. The samples were then refrigerated for 30 minutes at -80^0C at Gulu University laboratory and later removed and transferred into a cold box packed with dry ice at -20^0C for transportation to Glasgow, Scotland, United Kingdom by one of the researchers where they underwent metabolic analysis. It was within 24 hours from sample collection to sample reaching the analytical laboratory. The nature of specimens, quantity collected and transportation to the site for analysis were as follows: serum (4ml) and urine (4ml) and transported frozen at -20^0C.

Sample analysis: Quantitative analysis of serum and urine samples was processed by a metabolic consultant for Greater Glasgow & Clyde NHS Trust, using a metabolic analyser. These included; amino acid analyser with ion-exchange column, a High Performance Liquid Chromatography (HPLC) system with a reverse phase column and capillary gas chromatography. Further analysis was conducted using proton NMR Spectroscopy. These techniques measured the following: Urine (organic acids, creatinine and Urate); serum (amino acids, carnitine esters, pyruvate and biotinidase). It was planned that with resources available, further metabolic/biochemical tests would be carried on NS children's parents/Guardian, subject to their informed consent.

2.8 Ethical Considerations

This study was approved by the Local IRB; Lacor Hospital Institutional, Review and Ethics Committee (LHIREC No. 065/10/14). The research team worked in close collaboration with the administration of HfH centre, Gulu District Health Department, the local councillors and the village health teams. Parents/Guardians of the NS children gave informed consent on behalf of the NS child participants but for those who were above 14 years but below 18 years, assent was obtained. Two medical students from Gulu University Medical School were the research assistants (Dr. Akite Sarah and Dr. Lucy Akello Emma) together with a senior clinician DLK (author) and OAM (Researcher) supervised the data collection. The majority of the parents/Guardian of the study participants could not read and write and so we used the placement of inked thumbprints on the position for signature onto the questionnaires.

In addition, the study was conducted according to good clinical practice and confidentiality of NS medical records was maintained with the patients' identity anonymized and only accessible to the main investigator of the research. We obtained informed consent from parents/guardians for this information to be published.

2.9 Data analysis

The data collected was analyzed using STATA version 12 (Stata Corp LP, Texas, USA) where parametric data was presented as mean ± Standard Deviation (SD), median, maximum and minimum values. We used two medical students from Gulu University medical school to extract information from the questionnaires independently and we compared the data consistency and resolved any inconsistencies in consultation and mutual agreement. We used Chi Square (χ^2) and Fisher's exact tests for bivariate analysis to identify differences between variables by age, sex, birth order, biotinidase and Acetyl carnitine levels, plasma and urine organic acid levels in NS patients. In addition, we used logistic regression to screen 74 potential explanatory variables (5 continuous, 45 ordinal and 24 dichotomous) for associations with NS. Formal adjustments for the multiple testing were done because the investigation sought to identify associations and correlations of specific interest with pre-specified lists of targets.

We fitted Ordinary Least Squares (OLS) regression models to identify trends in the low biotinidase and Acetyl carnitine activity in relation to NS during the time period. Stepwise regression was used as an exploratory tool to guide the introduction of covariates in our modeling approach. Finally, a multivariable logistic regression analysis was conducted to identify the variables that correlated with the occurrence of NS among the participants. A p-value less than 0.05 was considered statistically significant.

3.0 RESULTS

The descriptive statistics showed that the mean age was 14.1SD±2.8 years with a minimum of 6 and maximum of 19 years. The male to female ratio was 1.5:1. The mean Body Mass Index (BMI) was 16.9 SD±2.7 with a minimum of 11.4 and maximum of 23.2; meanwhile the mean Mid-Upper-Arm-Circumference (MUAC) was

19.9SD$^{\pm}$2.8 with a minimum of 13.1 and maximum of 25.4cm. The majority of NS children (77%) had dropped out of school; 20% had never attended school while 3% were still in primary school (these were mainly children in the outreaches). The head of the households were exclusively peasant farmers and the majority of children was 1st, 2nd and 3rd in birth order and constituted 62.2%

of all study participants. The number of siblings with NS were notably higher in families with the NS child as; 1st (10/45), 2nd (9/45) and 3rd (6/45) born respectively in that descending birth order. All NS children lived in IDP camps and experienced life in IDPs at varying durations (Table 1& 2).

Table 1: The bivariate analysis of factors observed in the NS children.

Variables	Chi square (χ^2)	p-value	Fisher's test
Year of nodding onset	10.22	0.511	0.477
Length of IDP camp stay	7.50	0.277	0.277
Birth order of NS child	9.68	0.377	0.270
School Attendance	0.76	0.683	1.000
Mother was the Caretaker	**6.39**	**0.041**	**0.140**
NS child was in IDP camps	**22.15**	**0.005**	**0.156**
NS child had other siblings with NS	**9.68**	**0.004**	**0.267**
>50 nodding episodes since the onset	**22.15**	**0.005**	**0.296**
Number of nodding episodes per day	5.10	0.825	0.664
Epileptic fits experienced by NS child	**4.64**	**0.099**	**0.180**
NS child had fixed gaze/staring	3.14	0.208	0.199
Uncoordinated movement of limbs	0.01	0.923	0.721
Drooling of Saliva (open mouth)	0.68	0.411	0.567
Disorientation	1.91	0.385	0.327
Loss of consciousness	**5.76**	**0.056**	**0.155**
Localized Tonic clonic seizures	0.60	0.742	1.000
Generalized Tonic-clonic seizures	4.19	0.123	0.151
Urine incontinence	3.14	0.208	0.367
Stool incontinence	1.16	0.561	1.000
Tongue biting	0.95	0.331	0.471
Sleeping after nodding episodes	3.22	0.200	0.252
Confusion after fits/Nodding	4.43	0.107	0.327
Rhythmic jerking during nodding episodes	2.62	0.270	0.236
Recent injuries, burns and scars on NS patients	0.01	0.923	0.721
Good sleep pattern	1.53	0.675	1.000
Aggressive behavior after fits/nodding	2.19	0.139	0.233
Foaming in the mouth after nodding	**3.45**	**0.063**	**0.137**
Perceptual disturbances before/after nodding	1.16	0.283	0.410
Presence of visual hallucinations	3.45	0.486	0.384
History of mental illness in the family	**3.21**	**0.073**	**0.212**
Good family social support to NS child	**10.59**	**0.005**	**0.088**

Table 1 shows that NS was a statistically and significantly associated with the following variables; IDP camp life (χ^2)=22.15, p=0.005; NS child had other NS siblings (χ^2)=9.86, p=0.004; NS child received better social support from the mother (χ^2)=10.59, p=0.005; Caretaker of NS child was the mother (χ^2)=6.392, p=0.041; NS child had more than 50 nodding episodes since onset (χ^2)=22.15, p=0.005; In addition, some variables nearly reached a statistically significant associations with NS at 95% CI: NS child experienced epileptic fits (χ^2)=4.64, p=0.099; NS child had an episodes of loss of consciousness (χ^2)=5.76, p=0.056; NS child experienced foaming in the mouth during nodding episodes (χ^2)=3.447, p=0.063; and NS child had history of mental illness in the family (χ^2)=3.205, p=0.073.

Figure 1: The study site, Aromowang lobo, Odek Sub County, Gulu District, Uganda.

Figure 1 shows the study site and that NS children in Northern Uganda were from Acholi and Lango tribes from the districts of Oyam, Lira, Gulu, Amuru, Pader, Kitgum and Lamwo. All NS children lived in the IDP camps and were fed on food provided by the relief agencies during the internal displacement. Since 2012 when the communities of Northern Uganda were returned to their villages and feed on their own home grown foods, the Ugandan Ministry of Health and World Health Organization have reported no new cases of NS in the region.
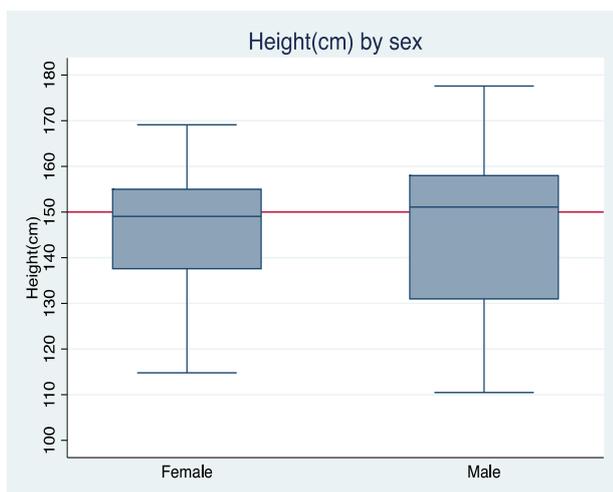
Figure 2: The heights by sex for the NS children.

Figure 2 shows that the mean height for female NS children were below the 150 cm mark while male were just above the 150 cm mark.
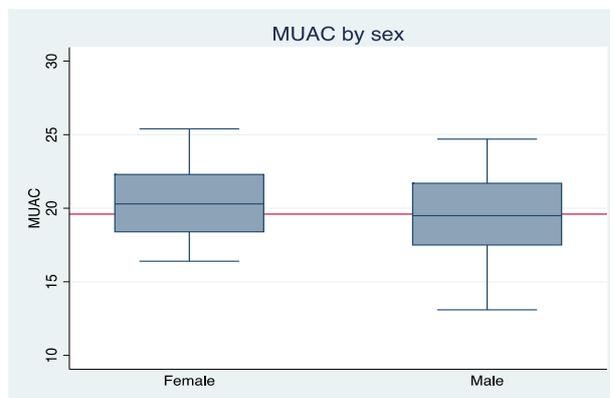
Figure 3: The Mid-Upper-Arm-Circumference (MUAC) by sex of NS children.

Figure 3 shows the mean Mid-Upper-Arm-Circumference (MUAC) of NS children. The female patients had mean MUAC just above the 20 cm mark while the males were just below the 20 cm mark. The male NS children were much thinner compared to their female counterparts.
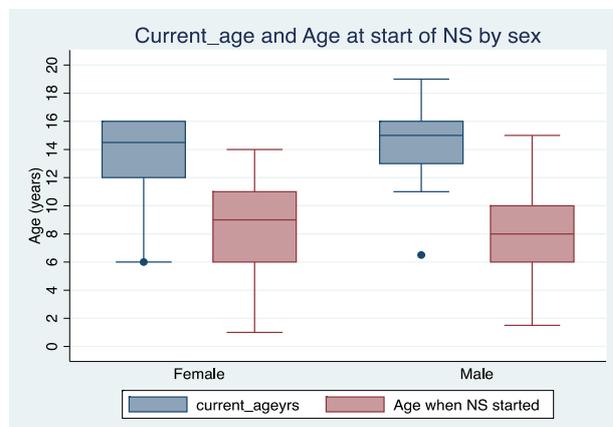

Figure 4: The age of the NS children and the age of onset of nodding by sex.

Figure 4 shows that female NS children began nodding at an older age (9 years) compared to their male counterparts at 8 years.
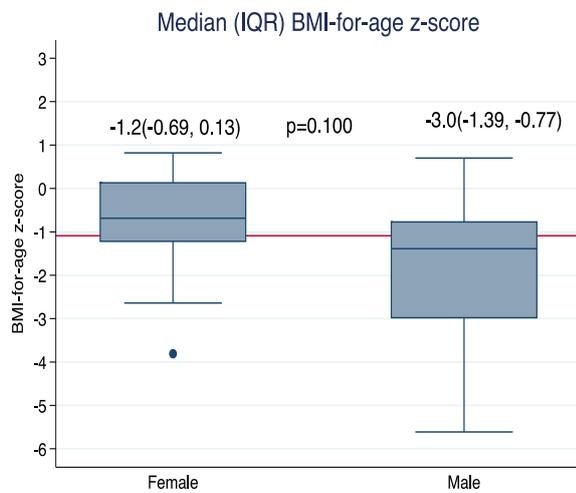

Figure 5: The BMI for age (z-scores) by sex of NS children.

Figure 5 shows the BMI for age (z-scores) by sex of NS children and shows that all NS children were below the expected BMI for age however, females had better z-scores of -0.5 while the males had about -1.5 however, there was no statistically significant difference in BMI for age (z-scores) between the two sexes (p=0.100).
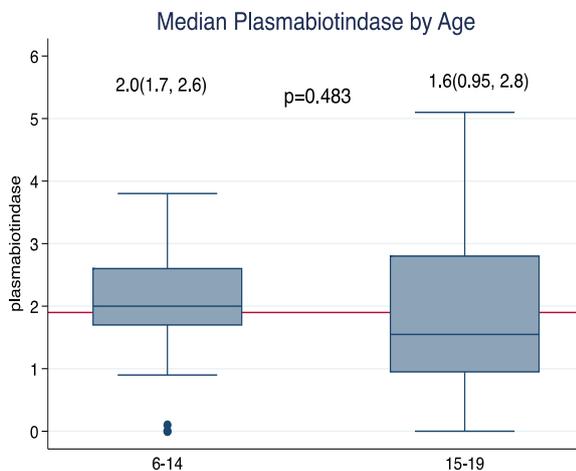
Figure 6: The median Plasma Biotinidase level by age of NS children.

Figure 6 shows the median plasma biotinidase level by age of NS children (normal range=2.5-7.0IU/L).Those between 6-14 years had low serum biotinidase level and was just minimally above 2.0IU/L while for the 15-19 years, the mean was less than 2.0IU/L. There was however no statistically significant difference between the two age groups (p=0.483).
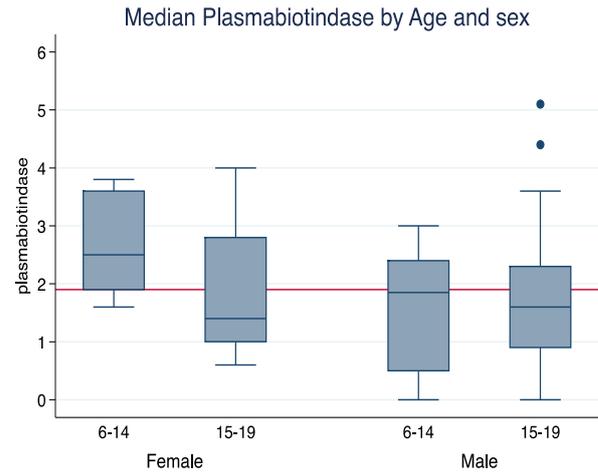

Figure 7: The Median plasma Biotinidase level by age and sex.

Figure 7 shows that females between 6-14 years had better serum biotinidase level which averaged 2.5IU/L while those in 15-19 years had an average of 1.5IU/L. For the males; 6-14 years had plasma biotinidase level just below 2.0IU/L while those 15-19 years had a much lower plasma biotinidase levels. Overall, the average plasma biotinidase was below 2.0IU/L adjusted for patient's characteristics. However, age, sex, duration with NS and nutritional status as measured by BMI-for-age (z-scores) and poor growth were not associated with low plasma biotinidase levels.
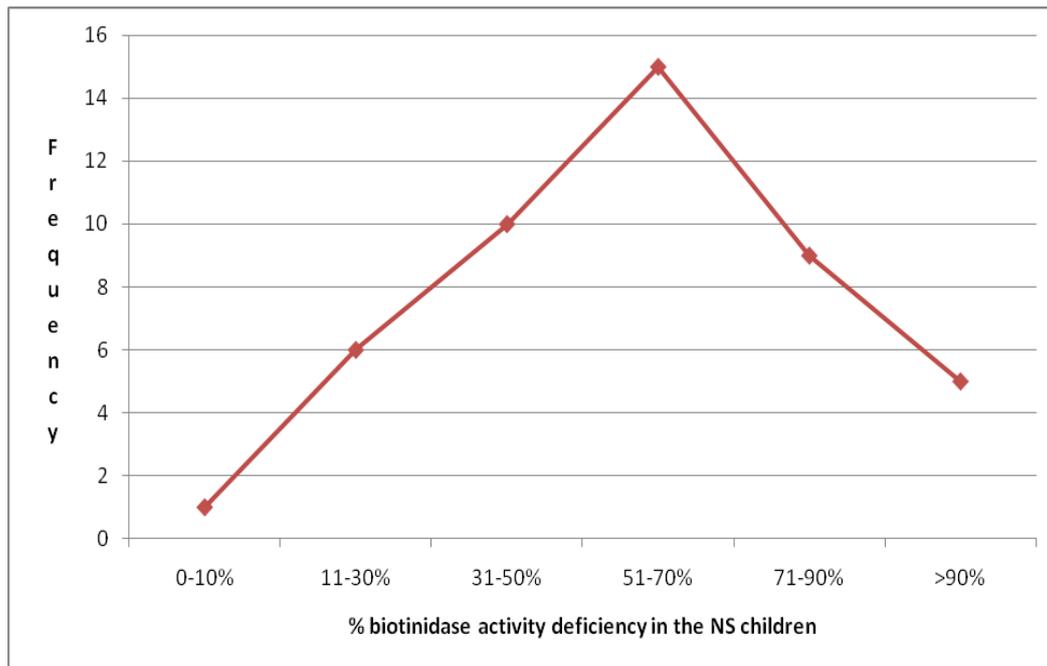
Figure 8: The pattern of % Biotinidase activity deficiency in the NS children studied.

Figure 8: shows a line graph begining at 0-10% biotinidase activity deficiency then rising to 31-50% deficiency and reaching a maximum at 51-70% biotinidase activity deficiency. (10-30% activity deficiency is classified as Partial biotinidase activity deficiency). The graph slopes from 51-70% deficiency and gradually to 71-90% deficiency and to a minimum of >90% biotinidase activity deficiency (<10% biotinidase deficicency is classified as profound biotinidase activity deficiency). In this scenario, the study suggests that we observed partial to profound serum biotinidase percentage activity deficiency in 48 Nodding Syndrome children at the centre in Gulu. This indicates perhaps that NS children occur in a spectrum ranging from the most severe (profound) to the moderate (Partial).

Figure 9 shows the Acetyl carnitine level between the two sexes [normal ranges=25-54μmol/L in male Children ≤17 years and 19-51μmol/L in female children ≤17 years]. The levels were generally low although higher among females 5.9(4.9, 6.8) compared to their male counterparts 4.2(3.7,4.9) at 95% CI and the differences in levels of acetyl carnitine between the two sexes were statistically significant (p=0.009). The average acetyl carnitine was 7.6μmol/L after adjusting for patient's characteristics. In the adjusted analysis the mean acetyl carnitine was significantly lower among males compared to females, β=-1.47(-2.77,-0.16), p=0.027. Long duration with NS was associated with lower mean acetyl carnitine, β= -0.37(-0.65, -0.09), p=0.010. However, current age, nutritional status as measured by BMI-for-age (z-scores) and experience with poor growth were not significantly associated with serum acetyl carnitine deficiency.
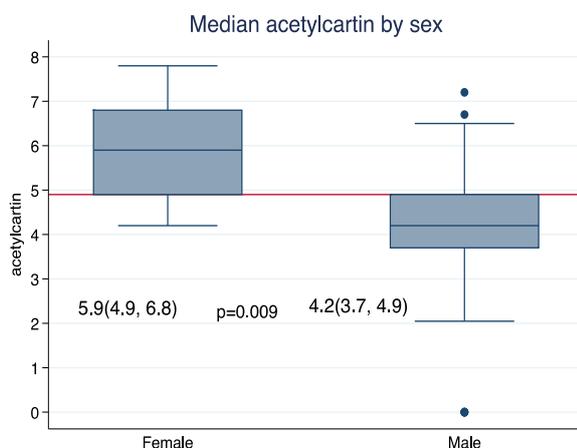

**Figure 9: The Median Acetyl Carnitine level between the two sexes.**
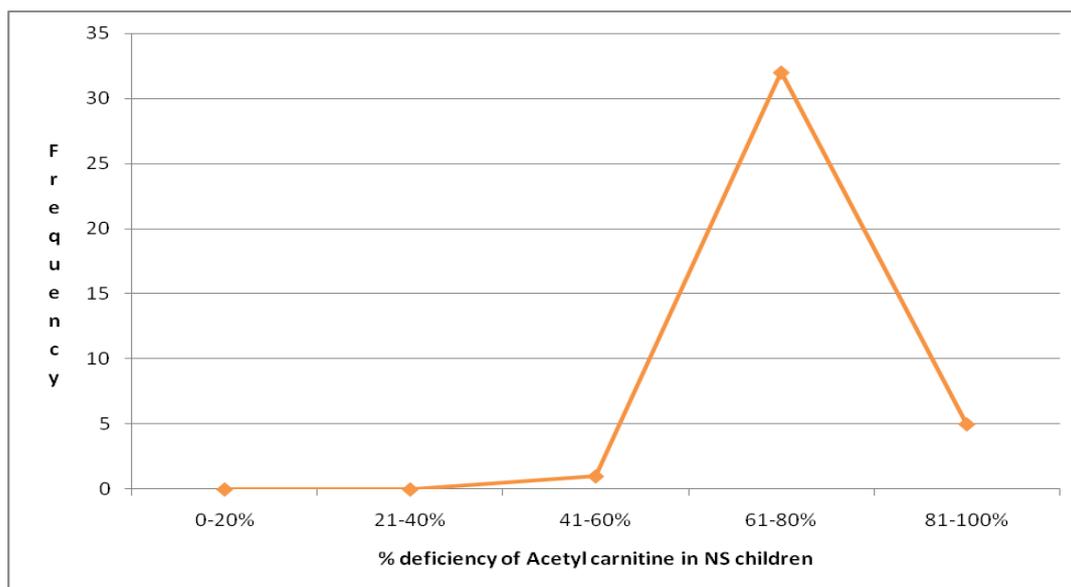
Figure 10: The percentage deficiency of acetyl carnitine level in NS children.

Figure 10 shows a line graph beginning at 0-20% and 21-40% Acetyl carnitine deficiency and rising steeply at 41-60% deficiency and peaking at 61-80% then slopes down to 81-100% deficiency. In this case, we suggest that there is a spectrum of deficiency of Acetyl carnitine occurring in NS children.

3.1 Serum Carnitine analysis

The serum carnitine analysis resuts were as follow: 7 samples were tested for proprionyl carnitine (C3) [normal range <0.9μmol/L] and found a mean 1.80 SD$\pm$0.01μmol/L; maximum 4.90μmol/L, minimum 0.98μmol/L and all were high 7/7 (100.0%). Similarly, we tested 9 samples for Butyryl Carnitine (C4) [normal range <0.45μmol/L] and we found a mean 0.81 SD$\pm$0.23μmol/L; maximum 1.15μmol/L, minimum 0.45μmol/L and all were high 9/9 (100.0%). Serum carnitine analysis demonstrated high levels of Proprionyl (C3) and Butyryl carnitine (C4). This is probably a reflection of increased fatty acid oxidation associated with malnutrition/stress seen in NS children.

3.2 The urine organic acid analysis

The other organic acids measured were: Oxalate, isovalerate, methylmalonate, isobutyrate, p-oHphenylacetate and valproic acid, which were generally high. Of the total of 47 NS children tested for organic acids, 8 had abnormalities detected; out of these four had prominent oxalate peak and four cases with isovalerate. Some samples consisted of methylmalonate but without methylcitrate. One other case had prominent hippurate level, some had isobutyrate, others traces of 3-OH proprionate or p-OHphenylacetate present. In general the result showed there was no specific disease that could be ascribed to the pattern of urine organic acids observed. These organic acids may have perhaps been a contributory factor in the high anion gap metabolic acidosis observed in the previous clinical studies.[1,10,13,19]

3.3 Urine Amino acid analysis

Urine amino acid analysis was performed on one NS child, the finding was unremarkable and perhaps excluded pheny ketonuria. There were 28 different types of amino acids tested in this patient. Low to normal concentrations were seen in 3/28(10.7%); normal in 17/28(60.7%) and high to normal in 8/28(28.6%).

3.4 Serum sodium valproate analysis

The serum sodium valproate level (normal range <13mg/L) was measured in 10 NS children samples and the mean was 63.7mg/L; median 74.5mg/L; mode of 87mg/L; lowest value 13mg/L; highest value 87mg/L with a range, 74; interquantile range, 43.5 and first quartile, 39.75. It was interesting to note that previous studies had noted that sodium valproate decreased biotin levels [27,28] and the result of this study showed that 9/10(90%) of the sodium valproate levels measured in NS children were above the normal reference ranges; with a mean of 63.7mg/L which were far above the upper limits.

Table 2: The anthropometric measurements and clincal findings in NS children.

Variables	Female	Male	Total	Mean(SD)	Ranges
Number of NS children	18	27	45		
Age(yrs)	13.4(3.3)	14.5 (2.4)	45	14.1(2.8)	6, 19
Weight(Kg)	38.4(12.1)	35.7(10.8)	45	36.8(11.3)	17, 58
Height(cm)	145.6(14.9)	145.6(16.9)	45	145.6(15.9)	110.5, 177.6
Age at onset (yrs)	8.1(3.5)	7.9(3.3)	45	8.0(3.3)	1, 15
Duration with NS (yrs)	5.3(1.9)	7.9(3.3)	45	8.0(3.3)	1, 15
Duration in the IDP camps (yrs)	4.7(1.4)	4.9(1.1)	45	4.8(1.2)	0, 7
% of family members with Epilepsy	33.4	29.6	45	31.1	
% of family members with NS	72.2	48.1	45	57.8	
Number of siblings with NS	22.0	26.0	48		
NS started before the IDP camps	0.0	0.0	0		
NS started during the IDP camps	6.0	16.0	22		
NS started after the IDP camps	12.0	11.0	23		

NS: Nodding Syndrome; yrs: years; SD: Standard Deviation; IDP: Internally Displaced peoples camps.
Table 1 shows that there was no NS child that developed Nodding Syndrome before displacement into the IDP camps and that the majority developed the syndrome during and immediate after the IDP camps.

Table 3: The clinical chemistry results for the NS children.

Variables	Female (n=18)	Male (n=27)	Mean (SD)	p-value
Serum biotinidase level	2.3(1.1)	1.8(1.3)	2.0(1.2)	<0.001
Serum Acetyl carnitine level	5.8(1.1)	3.8(2.2)	4.7(2.1)	<0.001
Free Plasma carnitine (CO) level	21.6(7.5)	18.1(7.9)	19.5(7.8)	<0.001
Urate level	0.24(0.05)	0.22(0.09)	0.22(0.07)	0.447
Urate/creatinine ratio	0.30(0.22)	0.21(0.14)	0.25(0.18)	0.075

The normal ranges for serum biotinidase was [2.5-7.5IU/L]; mean 2.0 SD$\pm$1.2IU/L; maximum 5.1IU/L, minimum 0.0 IU/L] and this was categorized as: majority were Low 37/47(78.7%) and few were normal 10/47(21.3%); Normal ranges for serum acetyl carnitine [25-54µmol/L in male children $\leq$17years and 19-51µmol/L in female children $\leq$17years]; The mean was 4.7SD$\pm$2.1 µmol/L; maximum 7.8µmol/L, minimum 0.0µmol/L] and this was categorized as: All 37/37(100.0%) were Low; Free Plasma Carnitine (CO) [normal ranges 10-50 µmol/L; mean 19.5SD$\pm$7.80µmol/L; maximum 37.5µmol/L, minimum

0.00µmol/L; and this was categorized as: majority were normal 43/47 (91.5%) and rest low 4/47(8.5%); Urate [Normal ranges 0.11-0.3mmol/L; The mean was 0.22SD$\pm$0.07mmol/L; maximum 0.37mmol/L; minimum 0.0mmol/L; categorized as high 5/47(10.64%); majority as normal 39/47(82.98%); and low 3/47(6.38%); Urate/creatinine ratio [Normal ranges 0.3-0.8mmol/L]; The mean 0.25SD$\pm$0.18mmol/L; maximium 1.00µmol/L, minimum 0.00µmol/L; and categorized as high 1/47(2.13%); normal 15/47(31.91%); and majority as low 31/47(65.96%).

Table 4: The correlation between plasma biotinidase level and other variables.

Serum Biotinidase	Coef.	Std. error	z	p>|z|	(95% Confidence Intervals)
Sex	-0.403	0.423	-0.95	0.341	-1.232, 0.426
Current age	0.023	0.397	0.06	0.953	-0.755, 0.801
Duration of NS	-0.065	0.086	-0.76	0.448	-0.233, 0.103
Nutritional status	-0.263	0.386	-0.68	0.496	-1.019, 0.303
BMI for age (z- scores)	0.022	0.143	0.15	0.878	-0.259, 0.303
Average plasma biotinidase	2.769	0.586	4.73	0.000	1.621, 3.917

Table 4 shows that the average plasma biotinidase was 2.0IU/L adjusted for patient's characteristics. However, age, sex, duration with NS and nutritional status measured by BMI-for-age (z scores) and experience with poor growth were not associated with low serum biotinidase levels.

4.0 DISCUSSIONS

This pilot study was conducted at HfH centre (Figure 1) and it demonstrates that NS children were malnourished as shown by the anthropometric measurements with respect to age and onset of nodding (Figure 2,3,4,5,6); (Table 2) and there was a demonstrable association with partial to profound biotinidase activity deficiency (Figure 7 & 8); (Table 2 & 3). In addition, most NS children showed a deficiency of biotinidase enzyme activity ranging from 0.0% to 100.0% (Figure 8). The mean percentage deficiency was 78%(78 SD±13.36%), an indication that the biotinidase deficiency was perhaps a spectrum which varied considerably from one NS child to another (Figure 8) and was not depended on the sex, current age, duration of NS and nutritional status of the NS child but rather perhaps as an independent occurrence (Table 4).

It is important to note that biotinidase functions by recycling the vitamin biotin (vitamin B7) and it is bound to amino groups of lysine residues of apoenzymes.[29,30,31,32,33] If levels of serum biotinidase are low then biotin cannot be broken down and released from proteins in the diet.[29,30,31,32] In addition, biotin serves as a coenzyme for four carboxylases enzymes; propionyl-CoA carboxylases & β-methyl crotonyl-CoA carboxylases (important in protein catabolism); pyruvate carboxylases (essential for gluconeogenesis) and acetyl CoA carboxylases (involved in the first step in fatty acid synthesis).[30,33,34,35] The clinical presentation of biotinidase activity deficiency varies depending on its percentage deficiency.[29,30,31,32,36] Interesting, the severity and clinical presentations of NS varied from one child to another and were more severe among those that had a longer duration with the disease, delayed diagnosis and intervention. In addition, biotinidase deficiency has commonly been classified as partial or profound whereby the clinical presentations and occurrence depended on whether it was profound (<10% activity deficiency) or partial (10-30% activity deficiency) and the presence of stressor factors.[29,30,31,32,37] Profound biotinidase deficiency is mainly considered a treatable autosomal and recessively inherited genetic neurocutaneous disorder which result in a multitude of presentations including multiple seizure types, ataxia, sensory defects, hypotonia, hearing/visual loss, ataxia, cognitive deterioration, skin rash, alopecia and recurrent infections including candidiasis.[29,30,31,32] The commonest features experienced by sufferers are seizures and hypotonia, however sometimes several central nervous symptoms may be present.[29,30,31,32] The seizure types are usually reported to be myoclonic or tonic clonic and can be focal.[30,35] In addition, untreated biotinidase deficiency is commonly complicated by lactic acidosis, organic aciduria and mild hyperammonia; however it is reported that their absence doesn't exclude the diagnosis of profound biotinidase deficiency.[30] Partial biotinidase deficiency on the other hand is a milder form of this condition in which without treatment with biotin, the affected children may experience hypotonia, skin rashes and hair loss but these problems may appear only during illnesses, infections or other times of stress.[29,30,31,32,36,37,38,39] In addition, partial biotinidase deficiency may be present with any of the symptoms described in profound deficiency, which only occurs to a milder degree when the child is stressed.[30,33] There are only anecdotal reports regarding clinical presentation in children with untreated partial biotinidase deficiency and there are no short or long term studies exploring the effects of partial biotinidase deficiency which have been clearly documented.[30,31,32] However, treatment is recommended for those with >1%-10% biotinidase activity deficiency and it is reported that the symptoms can be successfully treated with biotin.[30,31,32] Further to this, there is no known neurotoxic effect of biotin to children with partial deficiency who were treated with 1-5mg of oral biotin per day.[29,30,31,32,33] It had been previously reported that biotin deficiency could reduce the seizure threshold of those affected.[30,31,32] Similarly, a previous study had noted a near significant association between NS and Pyridoxine deficiency (Bunga's study, unpublished (p=0.06).[16] This is important as seizures are associated with abnormal pyridoxine metabolism.[16]

We suggest that malnutrition, *Onchocerca volvulus* infestation or other unknown toxin agents present in environment could have perhaps depleted biotin levels, either by way of enhancing degradation or impairing the biotinidase structure and/or function, respectively. Perhaps NS occurs as a spectrum too just like biotinidase deficiency and those NS children with partial to profound biotinidase deficiency manifest with multiple seizure types and cognitive deterioration as seen in the natural history of NS. We suggest that the cause of biotin deficiency could have arisen from chronic morbidities (such as *Onchocerca Volvulus* infection) which afflicted nearly 80% of all NS children,[1,2,19] malnutrition associated with the IDP camp environment, dietary factors and other unknown toxins,[1,2,5,6,7,10,11,20-25] Interestingly, the clinical presentations of NS children examined in 2012 and again in 2014 are similar to some of the clinical presentations seen in partial to profound biotinidase deficiency. However, most NS children in this study which was conducted in 2014, didn't exhibit features of profound biotinidase deficiency. Perhaps the supportive treatment and rehabilitation received at HfH, which included treatment of co-morbidities, local food supplementation, seizure treatment, multivitamin supplementations and psychosocial rehabilitation may have reduced the likelihood of a typical clinical manifestation of profound biotinidase deficiency.[29,30,31,32]

There are potential limitations in the validity of these low biotinidase results observed in these 48 NS children who had been undergoing a multidisciplinary management at the HfH centre for two years (from June 2012 to October 2014). The biotinidase deficiency results could be incorrectly low due to enzyme degradation from inappropriate storage of samples in transit from Uganda

to Glasgow, United Kingdom, where the metabolic analysis was conducted. However, the overall transit time from sample collection to samples analysis was about 24 hours (NB: all samples were stored and transported in dry ice at -20^{0}C and transported by one of the researchers).

Secondly, previous studies had shown that patients being treated with sodium valproate had decreased level of biotinidase in their serum.[27,28] Indeed, our study showed that 9/10 (90%) of the NS samples tested, the level of sodium Valproate were all above the normal reference ranges, with a mean of 63.6 mg/L. However, these were random levels of sodium valproate and at the time of blood draw, it was two hours after NS children had taken their morning sodium valproate dosage, so we could only interpret compliance from these results and we were therefore unable to make associations with low biotinidase levels. In addition, more than half of the NS children in the outreaches who were not on regular treatment with sodium valproate also had low level of biotinidase and furthermore the remaining half had just started treatment with sodium valproate for less than 2 weeks and they too had low biotinidase level. Interestingly, the two groups of NS children at the HfH centre and outreaches had different diets and the frequency of feeding. This could perhaps mean that the food rehabilitation at the HfH centre could not be blamed for the low biotinidase levels since both groups had low levels. Therefore the researchers came to a conclusion that the low level of biotinidase seen in NS children may have perhaps not been the effect of treatment with sodium valproate nor diet but perhaps a pathological finding in NS children. Encouragingly, we have conducted a pilot interventional study in which five NS patients have received biotin for over 4 weeks and have yielded tremendous improvement. We look forward to publishing our results in the near future.

In addition, this study demonstrated a statistically significant association between NS and acetyl carnitine deficiency (Table 3; Figure 9 & 10). Acetyl carnitine is known to function by transferring long chain fatty acids into the mitochondria for metabolism.[40] Interestingly, the serum carnitine analysis demonstrated high levels of Proprionyl Carnitine (C3) and Butyryl Carnitine (C4), which is probably a reflection of the increased fatty acid oxidation associated with malnutrition seen in NS children. However, this could also represent a disorder of fatty acid entry to the mitochondia due to low acetyl carnitine levels, resulting in raised levels of Proprionyl Carnitine (C3) and Butyryl Carnitine (C4) (Table 3).

Additionally, the serum urate levels were overall unremarkable, which provides supporting evidence that NS isn't associated with abnormalities involving purine or pyrimidine metabolism. Furthermore, the urate/creatinine ratios were lower than the normal ranges, possibly suggesting that NS may be associated with rhabdomyolysis. This finding could mean that NS is associated with vitamin D deficiency which was reported in 7 out of 8 cases in another pilot study undertaken in Northern Uganda by one of the authors.[13]

In addition, several studies had observed wasting and stunting to be associated with Nodding Syndrome.[1,2,12,13,16,17,19,26] These authors suggest that these findings were perhaps due to severe malnutrition experienced during the IDP camps or a metabolic disorder secondary to a mitochondrial pathology.

The analysis of the organic acids, fatty acids and urine organic acids in most parts were not consistent with any specific metabolic abnormality. In addition, the anthropometric findings suggest that in spite of the good feeding program at HfH centre, NS children were still generally malnourished although there were no prevailing factors that could be attributed to; an indication that perhaps there is a biochemical factor that prevents them from fully absorbing the food nutrients being provided at the HfH centre (Figure 2,3,4,5). Here we suggest that it was because of the deficiency in biotinidase and acetyl carnitine that were in parts responsible for this observation in NS children.

Treatment and rehabilitation outcomes of NS children at Odek in Uganda
The comprehensive multidisciplinary rehabilitation approach of NS children by correcting protein-energy using local food supplement and vitamin-related malnutrition, de-worming, use oral fungicide, anti-seizure medications (sodium valproate with/or without Carbamazepine), close monitoring, tailored dosing and adjustments, special needs education program and counseling pioneered by Hope for HumaNs (HfH) at their Odek care center has proven clinically transformative (with steady growth) (Figure 2,3,5). There was an improved emotional and marked seizure reduction status-though greater among males than females for unknown reasons.[1,2,7] However, the cognitive and behavioral problems and social difficulties (both requiring formal evaluation) still afflicted NS children at this HfH centre[2,7] (Table 1).

Limitations and strengths of this study
1. We were unable to perform a case-control study due to difficulty in transporting the matched controls for the study at the time.
2. We were not able to complete all the metabolic analyses due to service constraints and resource limitations.
3. The socio-demographic and natural history data depended on the accurate information of the recall of the caretakers therefore it was subject to recall bias. All the caretakers were reportedly living with the NS children at the time of nodding onset.

Strengths of the study

1. This is one of the first few studies that have analyzed the metabolic status of NS children in an area in which NS occurred at epidemic proportion.
2. We reduced the selection bias due to differential participation among outreach NS patients by visiting the homes of the affected NS children and making sure their physical disabilities weren't a barrier for inclusion into this study.
3. We reduced recall bias from parents/guardians of NS children by cross checking the records they provided at HfH centre and comparing with that at Government Health centres in the area. Interestingly, they were consistently the same and therefore believed it was true information.
4. We conducted a logistic regression analysis while controlling for possible confounders in the study and we identified factors that were correlated with Nodding syndrome.

CONCLUSION

Nodding Syndrome is a childhood neurological disorder affecting thousands of children and young people in East Africa. As Nodding Syndrome in South Sudan and Northern Uganda emerged in the context of war, internal displacement and feeding on food ration provided by relief agencies, the study suggests that the aetiology is perhaps infectious, dietary or environmental. This pilot study demonstrates an association between NS and biotinidase and Acetyl carnitine deficiency, suggesting a possible metabolic cause. We recommend further investigations with confirmatory quantitative determination of biotinidase and acetyl carnitine activity in serum in a larger study group. In addition, we suggest a research exploring the possibility of environmental toxins in NS pathogenesis and this should be published and prioritised.[2,11,23] Encouragingly, our pilot study with biotin supplementation with five NS children has shown remarkable results. We hope this small pilot study serves as an impetus to publish and undertake more studies exploring possible metabolic/toxicological aetiologies of Nodding Syndrome.

ABBREVIATIONS

HfH: Hope for HumaNs (a nongovernmental organization that has been rehabilitating children with NS for the last five years with transformative results); NS: Nodding Syndrome; IDP: Internally Displaced Peoples camps; BMI: Body Mass Index; MUAC: Mid-Upper-Arm-Circumference; CDC: Centre for Disease Control and Prevention; WHO: World Health Organization; OV: *Onchocerca Volvulus;* CSF: Cerebrospinal fluid; MOH: Ugandan Ministry of Health

Ethical Approval and Consent to participate

All authors hereby declare that this study was approved by the local Ethical Review Committee (LHIREC No. 065/10/14) and the study was performed in accordance with good ethical standards laid down in the 1964 Declaration of Helsinki. We obtained informed consent from the participants and parents/Guardians of the NS children in order for them to participate in the study. Where it was appropriately required, assent was obtained from the NS child participant.

Consent for Publication

All authors declare that written informed consent was obtained from the participants to publish this information.

Availability of data and materials

The datasets generated and analysed during the current study have been submitted to Editor in chief of this Journal. This dataset can be made available for use on reasonable request from the corresponding author.

COMPETING INTEREST

All authors declare no conflict of interests.

AUTHORS' CONTRIBUTIONS

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