Article

Nodding Syndrome, a Case-control Study in Mahenge, Tanzania: *Onchocerca volvulus* and not *Mansonella perstans* as Risk Factor

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Abstract: In South Sudan, three case-control studies found a positive association between Nodding Syndrome (NS) and a *Mansonella perstans* infection. To investigate whether *M. perstans* could play the primary and sole causal role in the development of NS, we examined blood films of persons with and without epilepsy in rural villages affected by NS in Mahenge, Tanzania, to identify *M. perstans* infections. Study participants were also examined for onchocercal nodules and skin lesions and tested for the presence of anti-*O. volvulus* antibodies (Ov16 ELISA). One hundred and thirteen epilepsy cases and 132 controls were enrolled, with a mean age of 28.3 years (interquartile range 22 – 34 years); 125 (51%) were females. Of the persons with epilepsy, 43 (38.1%) met the probable NS criteria. *M. perstans* infection was absent in all participants, but Ov16 prevalence and onchocerciasis-associated skin manifestations were positively associated with epilepsy, including probable NS. Onchocercal nodules in the mothers of cases were also positively associated with epilepsy. In conclusion, in contrast with *O. volvulus*, *M. perstans* is likely not endemic in Mahenge and therefore cannot be a co-factor for NS in the area. Hence, this filaria cannot be the primary and sole causal factor in the development of NS.

Keywords: *Mansonella perstans*; *Onchocerca volvulus*; onchocerciasis; Nodding Syndrome; epilepsy; Tanzania; Nakalanga Syndrome

1. Introduction

Since the first description of Nodding Syndrome (NS) in 1960 [1], a link between NS and onchocerciasis has been suspected. More recent epidemiological studies suggest that NS is only one of the clinical manifestations of onchocerciasis-associated epilepsy (OAE) [2]. Onchocerciasis is caused by the filarial nematode *Onchocerca volvulus* and spread by blackflies (*Simulium* spp.), also leading to skin and eye disease. OAE appears in previously healthy children between the ages of 3 and 18 years, with a peak age of onset around 8–11 years [2]. The epidemiology of epilepsy in onchocerciasis-endemic areas differs from the non-endemic regions in Africa, where most epilepsy develops in children before the age of 5 years [3] due to perinatal causes and genetic antecedents or in adulthood due to brain tumours, strokes and neurocysticercosis [4]. OAE presents with a broad spectrum of clinical manifestations, including generalised tonic-clonic seizures, absences and head nodding seizures (NS), stunting and delayed puberty (Nakalanga Syndrome) [2]. Despite a large number of epidemiological studies showing the association between onchocerciasis and epilepsy, there is a reluctance to consider this association causal, mainly because there is little evidence that *Onchocerca volvulus* microfilariae are able to penetrate the brain [5] and because, so far, no indirect mechanism has been identified to explain the pathogenesis of OAE. Co-factors could play a role in the pathogenesis of OAE. A study in South Sudan suggested that certain human leukocyte antigen (HLA) types could be a risk factor for

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developing NS, while other HLA types could be protective [6]. It has also been suggested that the presence of parasitic co-infections could increase the risk of developing epilepsy [7].

Another filarial infection potentially associated with NS is *Mansonella perstans* [8]. In three case-control studies conducted between 2001 and 2002 in Mundri, Western Equatoria State in South Sudan, not only a positive association was found between NS and an *O. volvulus* infection (odds ratio (OR) 9.2, p-value (p) < 0.001) but also between NS and *M. perstans* infection (OR 3.2, p = 0.005) [8]. However, in a case-control study conducted in 2014 in Titule, an onchocerciasis-endemic area in Bas-Uéle in the Democratic Republic of Congo, an M. *perstans* infection was found in 13 (68.4%) of 19 persons with epilepsy and 13 (65.0%) of 20 healthy controls (p = 1.0) [9].

Despite being the least studied, *M. perstans* is considered the most prevalent human filaria [10]. The infection is spread by midges (*Culicoides* spp.) and is believed to be widespread in Western, Eastern and Central Africa and neotropical regions in South America [10]. The spectrum of clinical symptoms and signs remains ill-defined for *Mansonella* infections. Various clinical manifestations have been reported, including itching, swelling, joint pain, enlarged lymph nodes, vague abdominal symptoms, and eosinophilia, but mansonellosis is often asymptomatic [10]. Moreover, it is often difficult to attribute clinical manifestations to an *M. perstans* infection due to the high frequency of co-infections with other parasites [10,11].

The diagnosis of *M. perstans* relies on detecting blood circulating microfilariae, real-time PCR or loop-mediated isothermal amplification assays (LAMP) testing [12]. Antibody-detecting enzyme-linked immunosorbent assay tests are available but not specific for *Mansonella* spp. [12].

During the International Scientific Meeting on NS organised in 2012 by WHO in Kampala, it was recommended that further investigation was needed to study *Mansonella* and *O. volvulus* infections as potential risk factors for NS [13]. Therefore we investigated whether *M. perstans* could play a causal role in the development of NS in villages affected by NS in Mahenge, Tanzania.

This analysis was a sub-study of a case-control study to identify potential genetic markers of OAE, namely to explore the findings of the study from South Sudan describing specific HLA haplotypes that may impact the susceptibility to develop NS [6]. The results of these genetic studies will be published elsewhere. This paper reports the prevalence of *M. perstans* and *O. volvulus* infection in epilepsy cases and controls. We also investigated the role of an *O. volvulus* infected mother as a potential risk factor for epilepsy.

2. Materials and Methods

2.1. Study settings

Between September 2021 and March 2022, we conducted a case-control study in Mahenge, Ulanga District, Tanzania. Persons with epilepsy identified in previous door-to-door surveys in 2017, 2018 and 2021 were selected from two suburban villages (Isongo and Vigoi) and eight rural villages in the Mahenge area, known to have a high prevalence of epilepsy: Mzelezi, Mdindo, Sali and Msogezi, included in previous epilepsy studies [14,15], and Ebuyu, Euga, Isyaga and Mgolo villages (Figure 1).

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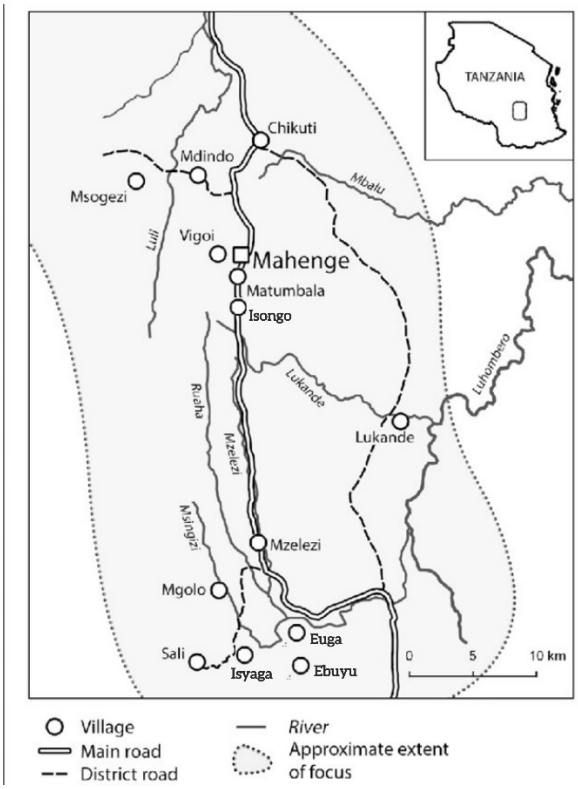

Figure 1. – Map of the Mahenge area and surveyed villages.

The Mahenge Mountains are of high altitude, abundant in banana trees and rich in fast-flowing rivers, the latter providing suitable breeding sites for blackflies (locally known as *vifuna* in *Kiswahili*) [16]. The area was one of the most onchocerciasis endemic foci in Tanzania before the implementation of community-directed treatment with ivermectin (CDTI) in 1997 [17,18,19]. In 2019, the CDTI was strengthened from annual to biannual. Most of the local population belongs to the Wapogoro ethnicity and subsists on agriculture, livestock (chickens, goats and pigs, the latter mostly kept indoors and in suburban villages) and gem mining. *Loa loa* and *Wuchereria bancrofti* filarial infections are not believed to be prevalent in the area [20].

2.2. Study design

One hundred and thirteen epilepsy cases and 132 controls were enrolled in the study. Persons with epilepsy, including NS and Nakalanga Syndrome, were matched with controls by age, sex and village. When a matching control was not available in the village of a case, one was sought in neighbouring villages. Epilepsy cases and controls were people who screened positive or negative for epilepsy in the previous door-to-door surveys, respectively.

A case of probable NS was defined according to the 2013 modified consensus case definition of NS [21]. In brief, a case of NS is defined as a previously healthy 3 to 18 years old child who developed repetitive episodes of involuntary drops of the head to the chest on two or more occasions. They also have to present at least one other criterium: neurological abnormalities, clustering in space and time with other cases, triggering by food and/or cold weather, stunting or wasting, delayed sexual or physical development or psychiatric symptoms. A probable case is a case in which the nodding episode was not documented by a trained healthcare worker, videotaped or shown by EEG/EMG.

A case of Nakalanga Syndrome was defined as a person with a combination of stunted growth, delayed or absent external signs of secondary sexual development, and mental impairment, often associated with epileptic seizures and facial dysmorphia [21].

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2.3. Study procedures

After written informed consent was obtained, participants or their guardians were interviewed in Swahili (*Kiswahili*) language about their sociodemographic and clinical characteristics, such as ethnicity, epilepsy (*kifafa, Kiswahili*) status, head nodding history (*amesinzia kichwa, Kiswahili*) and ivermectin intake, and underwent a physical examination by a medical doctor (DB). The latter included detecting palpable onchocercal nodules (onchocercomata) and dermatological lesions. The mothers of the participants present were also interviewed and examined for the presence of onchocercal nodules and dermatological lesions after signing informed consent.

Blood samples were examined to identify *O. volvulus* and *M. perstans* infections, where a finger prick of blood was obtained from all cases and controls to make a dried blood spot and thick and thin films. Samples were collected, air-dried and stored at 4ºC until handled for further analysis. The procedure for *O. volvulus* detection involved the presence and concentration of IgG4 antibodies recognising the parasite Ov16 antigen in a dried blood spot by ELISA using the commercially available Standard Diagnostic Ov16 IgG4 ELISA kits and following its manufacturer instructions. In turn, the procedure for *M. perstans* detections encompassed staining the dried films using Giemsa and examining them for microfilariae under a microscope using a high-power objective lens.

Skin snips were not obtained for *O. volvulus* detection as nearly all study participants took ivermectin during CDTI, and the latter has been shown to mask the association between onchocerciasis and epilepsy [23].

2.4. Statistical analysis

The sociodemographic and clinical characteristics of cases and controls were described with median and interquartile range (IQR) for continuous variables and number and frequency for categorical data. Differences in sociodemographic variables between cases and controls were studied using the chi-square test for categorical variables and the t-test for continuous variables. Clinical variables of cases and controls, such as *M. perstans* infection and *O. volvulus* exposure status, were assessed by a logistic regression model adjusted for age, sex and village and odds ratios (OR) and 95% confidence intervals (95% CI) were presented. Variables with frequencies < 5% were compared using Fisher's exact test. Two-tailed p-values of <0.05 were considered statistically significant.

Analyses were performed using R and R Studio (4.2.1 and 2022.06.23 versions, respectively).

3. Results

3.1. Sociodemographic and clinical characteristics of the study population

The mean age of the study population was 28.3 (IQR 22.0-34.0) years, with 125 (51%) females and 120 (49%) males (Table 1). Cases and controls had similar sex, age and village distributions; most were ethnically Wapogoro (97.3% cases and 98.5% controls). Nearly all participants reported taking ivermectin in the past.

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Table 1. — Sociodemographic characteristics between epilepsy cases and controls.

Variable	Cases n = 113	Controls n = 132	p-value
Male, n (%)	56 (49.6)	57 (43.2)	0.39
Age, median (IQR)	28.0 (22.0-35.0)	27.0 (21.0-33.3)	0.64
Village type, n (%)			0.92
Rural[a]	78 (69.0%)	93 (70.4%)	
Suburban[b]	35 (31.0%)	39 (29.6)	
Ivermectin intake, n (%)	105 (92.9)	126 (95.5)	0.57

n — Number; % — Percentage frequency; IQR—Interquartile range. [a] — Mzelezi, Mdindo, Sali, Msogezi, Ebuyu, Euga, Isyaga and Mgolo. [b] — Isongo and Vigoi.

3.2. Characteristics of the epilepsy cases

Forty-three (38.1%) cases met the criteria of probable NS (Table 2). The median age of seizure onset was ten years. Most persons with epilepsy were under antiseizure treatment, usually phenobarbital (67 cases, 79.8%).

Table 2. — Epilepsy and treatment characteristics of persons with epilepsy.

Variable	Cases n = 113
Probably Nodding Syndrome, n (%)	43 (38.1%)
Nakalanga Syndrome, n (%)	5 (4.4%)
Age of seizure onset, median (IQR)	10.0 (8.0-13-0)
Antiseizure treatment, n (%)	84 (74.3%)

n — Number; % — Percentage frequency; IQR—Interquartile range.

3.3. Mansonella perstans infection and Onchocerca volvulus exposure status

M. perstans was not found in any of the cases and controls (Table 3). Onchocercal nodules in mothers was positively associated with epilepsy ($p < 0.05$). Ov16 prevalence was also associated with epilepsy including probable NS. Onchocercal skin manifestations were only found in epilepsy and NS cases.

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Table 3. — *M. perstans* and *O. volvulus* prevalence in all epilepsy cases, probable Nodding Syndrome cases and controls.

	Epilepsy cases (n = 113)	**pNS cases** (n = 43)	**Controls** (n = 132)	**OR all epilepsy cases** (95% CI)[b]	**OR pNS cases** (95% CI)[b]	**p-value** (Epilepsy cases / pNS)
M. perstans blood smear (positive)	0.0% (0 out 113)	0.0% (0 out 43)	0.0% (0 out of 132)	1	1	-
O. volvulus ELISA (positive)	75.0% (78 out of 104)	84.6% (33 out of 39)	60.0% (72 out of 120)	2.1 (1.1-4.1)	4.8 (1.7-16.0)	0.02 / 0.006[b]
Onchocercal nodules (1 or more)	17.7% (20 out of 113)	16.3% (7 out of 43)	11.4% (15 out of 132)	1.8 (0.8-4.0)	1.4 (0.4-4.3)	0.16 / 0.57[b]
Onchocercal nodules of mothers (1 or more)	42.9% (30 out of 70)	38.2% (13 out of 34)	38.8% (19 out of 49)	2.7 (1.1-7.2)	2.0 (0.6-6.4)	0.04 / 0.25[b]
Onchocercal skin manifestations[a] (1 or more)	6.2% (7 out of 113)	14.0% (6 out of 43)	0.0% (0 out of 132)	-	-	<0.001 / 0.003[c]

n — Number; pNS – Probable Nodding Syndrome; OR – Odds ratio; 95% CI – 95% Confidence interval. [a] — Leopard skin; dry, thickened, wrinkled skin; papular rash. [b] — Binomial logistic regression model controlled for the village, age and sex. [c] — Fisher's exact test was used to account for the rare frequency.

4. Discussion

To identify the cause of NS, a common risk factor must be identified in all areas where NS has been reported. So far, the only risk factor present in all those areas is onchocerciasis. We explored the possible association between *M. perstans* and NS in Mahenge, where NS and Nakalanga Syndrome have been known to occur since 1960. We also aimed to confirm the association between onchocerciasis and NS.

Overall, none of the 113 epilepsy cases (43 with probable NS) and 132 controls were diagnosed with an *M. perstans* infection. Therefore, *M. perstans* is likely not endemic in Mahenge and should no longer be considered a risk factor for NS in the area. In contrast, Ov16 seroprevalence (OR 2.1, 95% CI: 1.1-4.1) and onchocercal skin manifestations (p-value < 0.001) were positively associated with cases of epilepsy, including probable NS (OR 4.8, 95% CI: 1.7-16.0, and p-value = 0.003, respectively). Also, onchocercal nodules in the mothers of participants were positively associated with epilepsy (OR 2.7, 95% CI: 1.1-7.2). These findings confirm the association between onchocerciasis and epilepsy.

Only 16.3% of the epilepsy cases and 11.4% of controls presented onchocercal nodules. This low prevalence was expected given the more than 20 years of distributing ivermectin to control onchocerciasis. The lack of association between the onchocercal nodules prevalence of participants and epilepsy could also be related to the low sample size and the difficulty of palpating a low number of nodules. Indeed, more nodules were observed among the mothers of persons with epilepsy compared to control mothers. In onchocerciasis-endemic areas, the number of nodules increases with age and, therefore, may have been easier to palpate in the mothers [24]. This finding among the mothers suggests that parasitic tolerance transmitted by the *O. volvulus*-infected mothers may have played a role in developing epilepsy in their children [25]. Such children, already after a first nodule, might develop a very high microfilarial load at a very young age putting them at risk of developing epilepsy. Once they developed epilepsy, they might have avoided going to the river because of the risk of onchocerciasis, as the population has been made aware of

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the association between onchocerciasis and epilepsy and that blackflies spread the former. Therefore, the cases may develop fewer or no additional nodules, and their single initial nodule may have been missed during palpation.

Ultimately, nodule palpitation is a less reliable methodology in settings with onchocerciasis control and younger populations, as they usually have fewer nodules to be detected [24]. A study in an onchocerciasis hyperendemic focus in Cameroon before the implementation of CDTI found a positive association between onchocercal nodules and cases of epilepsy [26]. In contrast, similarly to our findings, a study in an onchocerciasis hyperendemic area in northern Uganda under CDTI found a lack of association between NS and onchocercal nodules but a strong association with a positive onchocerciasis serology [27].

Onchocercal skin manifestations were more prevalent in cases than controls. However, these skin lesions were mainly chronic lesions because of a past *O. volvulus* infection (e.g. leopard skin; dry, thickened, wrinkled skin). The median age of the cases of epilepsy was 28.0 years (IQR 22.0-35.0), while the median age of onset of epilepsy was 10.0 (IQR 8.0-13.0), the latter as expected for OAE. This age difference of 18 years supports the statement that the onchocerciasis burden in Mahenge has recently decreased.

Regarding *M. perstans*, there is no recent information on its epidemiology in Tanzania. Previous research has identified the parasite between Lakes Tanganyika and Lake Victoria, particularly in areas with banana plantations and abundant rainfall, as the *Culicoides* vector can grow on decaying banana trees [29]. To our knowledge, no *M. perstans* studies have been conducted in Mahenge. The presence of the *Culicoides* vector in the area can be inferred from a report on the Schmallenberg virus in sheep and goats, also spread by this vector [29]. Our study shows the absence of *M. perstans* in Mahenge, which may be due to the unique ecology of the area, with a lower temperature than mainland Tanzania, higher altitude and perhaps less competent or anthropophilic *Culicoides* spp. It is very unlikely that *M. perstans* was in Mahenge before, as no control was done against the parasite, and CDTI has little to no impact on the parasite [30,31] and does not reduce its prevalence [32].

Given the geographic distribution of *M. perstans* in sub-Saharan Africa, a main causal role with NS is very unlikely. Indeed, *M. perstans* infections are widespread in sub-Saharan Africa and are present in many areas where NS has never been reported. Furthermore, the distribution of *M. perstans* infections cannot explain the localisation of NS in villages and households close to rapid-flowing rivers and *O. volvulus* breeding sites [33,34]. In Northern Uganda, in the area where a NS epidemic appeared around the year 2000 [35], the prevalence of *M. perstans* infection was very low and much lower than in other parts of Uganda where NS has never been reported [36]. Moreover, in a cohort study in an onchocerciasis-endemic area in Cameroon, the relative risks of children developing epilepsy depended on the *O. volvulus* microfilarial density in a dose-response way, but no association was found between the presence of *Loa loa* or *M. perstans* microfilaremia and the development of epilepsy [37]. NS and Nakalanga Syndrome were previously reported in an onchocerciasis and *M. perstans* co-endemic area in Western Uganda. However, while *M. perstans* is still endemic in the area, NS and Nakalanga Syndrome ceased to appear when *O. volvulus* transmission was eliminated by CDTI and vector control [8].

How to explain the association between an *M. perstans* infection and NS in South Sudan? One possibility could be that children with NS in South Sudan nearly never sleep under insecticide-impregnated bed nets together with other children because the local community believes that NS is transmissible through contact [38]. Therefore, children with NS are often isolated from the rest of the family and are not allowed to eat from the same plate with other children or to sleep with them in the same room. The midges that transmit *M. perstans* are most active at night [39]. Midges are very small and hence are considered to pass through bed nets [40]. However, insecticide-treated nets have been shown to offer protection to horses from *Culicoides*, the *M. perstans* vector, in the event of an African horse sickness virus epidemic [41]. Nonetheless, as impregnated bed nets were

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not distributed in 2001 – 2002 in Mundri, bed nets are unlikely to explain the observed association between NS and *M. perstans* infection in the study by Tumwine *et al.* [8].

Factors associated with poverty also have the potential to explain an association between *M. perstans* infection and NS. Indeed, a study on Bioko Island, Equatorial Guinea, showed that people belonging to the lowest socioeconomic quintile were five times more likely to be *M. perstans* infected [42]. Children with NS and their families also belong to the poorest and most vulnerable groups in their communities [43].

The vector *Culicoides* presence is more likely associated with aquatic environments, banana and plantain stems [44]. Families with children with NS generally live close to rapid-flowing rivers [45] and blackfly breeding sites, the vector of *O. volvulus* [46]. However, these sites are not known to be particular sites where *Culicoides* bite, possibly explaining why *M. perstans* infection is absent from many sites with NS prevalence.

Farming could be another explanation for the association between an *M. perstans* infection and NS. In Maridi, South Sudan, belonging to a farming family was found to be a risk factor for NS [47]. In a study in Nigeria, the prevalence of mansonellosis was significantly higher among rural dwellers (34.6%) than among urban dwellers (22.5%), and among farmers (59.8%) than in civil servants (7.6%) ($p < 0.05$) [48]. Moreover, farming is also a risk factor for onchocerciasis, as many fertile lands are close to blackflies breeding sites and biting rates are high [47].

The higher prevalence of onchocercal nodules in mothers of NS cases may suggest that an *O. volvulus* infected mother could be a risk factor for developing epilepsy. However, this may also be because both the child and the mother were living in a place where they were both heavily exposed to bites of *O. volvulus* infected blackflies. To determine the role of the mother we also should have examined the father for the presence of *O. volvulus* infection.

In a case-control study in Northern Uganda, preterm birth was found to be a risk factor for NS [49]. As preterm birth could be related to an *O. volvulus* infection of the pregnant mother [50], it was suggested that an *O. volvulus* infection of the mothers could cause a "parasitic tolerance" in their offspring. This tolerance can induce a higher load of *O. volvulus* microfilarial infection when the children are exposed to *O. volvulus*-infected blackflies than if the mothers were free of infection during pregnancy [51]. This high-level *O. volvulus* infection in young children was documented as a risk factor in onchocerciasis endemic areas in Cameroon for developing epilepsy [37,52]. It should be investigated whether an *O. volvulus* infection of mothers can also induce "parasitic tolerance" to an *M. perstans* infection. For instance, concomitant infection with *O. volvulus* and *M. perstans* was common in rural villages of southern Cameroon, while the opposite was seen with *Loa loa* [53], the latter possibly due to distinct vector ecologies [54]. Another study in central Cameroon found a positive association between *O. volvulus* and *L. loa* infections [54]. Hence, concomitant filarial infections may increase the tolerance of the human host to these parasites. Further studies could be conducted to explore this hypothesis among the different filariae.

We also need to mention the limitations of our study. First, controls were chosen among volunteers without epilepsy of the same age as the cases. However, as they were not chosen at random, it may be that children with visible onchocerciasis skin lesions did not participate as controls. Second, nearly all participants have taken ivermectin (94.3%). Therefore, no strong association between onchocerciasis and epilepsy could be documented. Third, the association found between anti-*O. volvulus* antibodies and NS cases could have been stronger if a more sensitive test than Ov16 ELISA had been used, such as the *O. volvulus* luciferase immunoprecipitation system (LIPS) assay. In northern Uganda, ELISA and LIPS detected, respectively, 66.7% and 94.9% *O. volvulus* seropositivity in NS cases and 31.8% and 48.8% in healthy controls [27]. Fourth, a diagnostic test with higher sensitivity could have been used to assess *M. perstans* infection status, such as PCR [55]. However, as all cases and controls were negative for *M. perstans* infection and the blood smear has an estimated sensitivity of 78% [55], the parasite is likely absent from the region or present in very low levels.

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5. Conclusions

M. perstans is likely not endemic in Mahenge and, therefore, cannot play a co-factor role in developing NS in the area. To elucidate the pathogenesis of NS, a first step is to identify a common risk factor that is present at all sites where this condition is reported. Therefore, based on our findings in Mahenge, *M. perstans* should no longer be considered a potential main and sole cause of NS. However our study can not exclude that M. perstans could be a co-causal factor for NS in an M. perstans endemic area. Clearly, more research about *M. perstans* is needed to determine its ecology, pathogenicity, and link with other infections, including onchocerciasis.

Onchocerciasis remains the most likely cause of NS and other forms of epilepsy meeting the criteria of OAE. However, the mechanism of how the *O. volvulus* parasite may cause epilepsy remains to be identified. This could be because *O. volvulus* microfilariae may occasionally pass the blood-brain barrier in children with a high microfilarial load [52] or through indirect means, for instance, an immunological reaction induced by the parasite or perhaps even through a still-to-discover pathogen within the parasite microbiome.

In the meantime, onchocerciasis elimination efforts must be strengthened in endemic regions with a high incidence and prevalence of epilepsy. In addition, communities living in these areas should be explained that the clustering of epilepsy in certain villages and families is linked to the common exposure to *O. volvulus*-infected blackflies and that children with NS and other forms of epilepsy should not be isolated and should sleep under insecticide-impregnated bed nets like other children.

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Informed Consent Statement: The study goals and procedures were explained to all participants in the language of their choice, and signed- or thumb-printed informed consent was obtained from participants, parents or caregivers, and assent was additionally obtained from adolescents (aged 12–18 years).

Data Availability Statement: The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

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