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Systemic and cerebrospinal fluid immune and complement activation in Ugandan children and adolescents with long-standing nodding syndrome: a case-control study

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Abstract

Objective: Nodding syndrome is a poorly understood epileptic encephalopathy characterized by a unique seizure type— head nodding— and associated with *Onchocerca volvulus* infection. We hypothesized that altered immune activation in the cerebrospinal fluid (CSF) and plasma of children with nodding syndrome may yield insights into the pathophysiology and progression of this seizure disorder.

Method: We conducted a case-control study of 154 children (8 years or older) with long-standing nodding syndrome and 154 healthy age-matched community controls in 3 districts of northern Uganda affected by nodding syndrome. Control CSF samples were obtained from Ugandan children in remission from haematological malignancy during routine follow-up. Markers of immune activation and inflammation (cytokines and chemokines) and complement activation (C5a) were measured in plasma and CSF using ELISA or Multiplex Luminex assays. *O. volvulus* infection was assessed by serology for anti-Ov16 IgG levels.

Results: The mean (SD) age of the population was 15.1 (SD 1.9) years and the mean duration of nodding syndrome from diagnosis to enrolment was 8.3 (SD 2.7) years. Majority with nodding syndrome had been exposed to *O. volvulus* 147/154 (95.4%) compared to community children 86/154 (55.8%), OR 17.04 (95% CI 7.33, 45.58), $p<0.001$. C5a was elevated in CSF of children with nodding syndrome compared to controls, ($p<0.0001$). The levels of other CSF markers tested were comparable between cases and controls after adjusting for multiple comparisons. Children with nodding syndrome had lower plasma levels of IL10, APRIL, CCL5 (RANTES), CCL2, CXCL13, MMP-9 compared to community controls ($p<0.05$ for all; multiple comparisons). Plasma CRP was elevated in children with nodding syndrome compared to community children and correlated with disease severity.

Significance: Nodding syndrome is associated with exposure to *O. volvulus*. Compared to controls, children with long-standing symptoms of nodding syndrome show evidence of complement activation in CSF and altered immune activation in plasma.

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Key points box (3-5) Please ensure each bullet point is no longer than 140 characters.

1. Long-standing nodding syndrome may be associated with complement activation in CSF.
2. The pathogenesis of chronic nodding syndrome may involve systemic and CSF immune alterations.
3. *Onchocerca volvulus* infection is associated with nodding syndrome, but its role in disease pathogenesis remains inconclusive.

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Introduction

Nodding syndrome is a poorly understood severe neurological disorder characterized by a unique seizure manifesting as repeated head nods and complicated by other generalized seizures, motor, neuropsychiatric, and cognitive impairments[1,2]. In addition, undernutrition, delayed development of sexual characteristics and stunting is observed in a proportion of affected children. Nodding syndrome was initially reported in southern Tanzania, followed by South Sudan and later in northern Uganda[3–5]. More recently cases have been reported in central Africa[6]. Nodding syndrome occurs in previously normal children between the ages of 3-18 years, it is chronic, shows progression in some, and frequently occurs in epidemics with clusters of cases observed in geographically distinct regions[7,8]. Clinical and electrophysiological studies suggest nodding syndrome may be an epileptic encephalopathy[9].

The aetiology of nodding syndrome remains unknown. It has been proposed that nodding syndrome may be linked to a nutritional, genetic, or an unknown infectious agent [10,11]. However, several epidemiological studies have demonstrated a consistent relationship with *O. volvulus* infection leading to the hypothesis that *O. volvulus* may underlie the pathogenesis of nodding syndrome[5,12–14]. Confounding this hypothesis is the fact that *O. volvulus* is observed across Africa and in parts of Latin America[15,16], whereas nodding syndrome is largely confined to east and central Africa [17]. In addition, a proportion of healthy community controls typically have evidence of *O. volvulus* infection. Despite this, recent evidence suggests a link between seizure related disorders and *O. volvulus* leading to the study of a spectrum of syndromes - Onchocerca associated epilepsies (OAE). The mechanism by which *O. volvulus* infection may led to nodding syndrome and/or the spectrum of disorder labelled as OAEs' remains unclear. One school of thought has suggested that *O. volvulus* may be a secondary opportunistic infection unrelated to the aetiology of nodding syndrome[18]. It has also been hypothesized that antibodies to *O. volvulus* or its symbiont *Wolbachia* may cross-react with neuronal proteins leading to neuro-inflammation and nodding syndrome [13]. This hypothesis is supported by studies showing auto-antibodies against actin-binding protein leiomodin-1[19], and the voltage-gated potassium channel complex[13] in the cerebrospinal fluid (CSF) of patients with nodding syndrome. A recent post-mortem study of brains from patients with nodding syndrome demonstrated evidence of past ventriculitis, meningitis and gliotic lesions suggesting nodding syndrome may be a post-infectious encephalopathy[20].

We hypothesized that nodding syndrome is a neuro-inflammatory disorder associated with *O. volvulus* infection and systemic changes in immune and complement activation. To evaluate this, we measured markers of immune and complement activation in the cerebrospinal fluid and plasma of Ugandan children and adolescents with chronic nodding syndrome on anti-epileptic drug therapy.

Methods

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Design

This was an exploratory case-control study of neuro- and peripheral inflammation in children and adolescents with a long-standing diagnosis of nodding syndrome compared to frequency and age matched controls. Cerebrospinal fluid (CSF) and plasma concentrations of a selected panel of cytokines, chemokines, and complement factors, were assayed.

Setting

The study was conducted in the nodding syndrome affected districts of Kitgum, Pader and Lamwo in northern Uganda. **(Figure 1)**. This region suffers high levels of poverty and is still recovering from a two-decade old Lord's Resistance Army war against the Government of Uganda. Subsistence farming is the major economic activity. The age-specific prevalence of nodding syndrome in the affected age group in these districts was approximately 6.8 (95% CI: 5.9-7.7) per 1,000[4]. Study participants were screened for eligibility at the nodding syndrome treatment centres within the respective districts[13]. Those enrolled were hospitalized in Kitgum General hospital (KGH) where all study procedures were conducted. KGH is the largest epilepsy and nodding syndrome treatment centre in the region.

Ethical approval

Ethical approval was obtained from Makerere University School of Medicine Research and Ethics Committee (SOMREC) and University of Oxford Tropical Medicine Research Ethics Committee (OXTREC). Regulatory approvals were provided by Uganda National Council for Science and Technology (UNCST). Written informed consent was obtained from the primary caregiver of all participants enrolled in the study. In the event a caregiver was unable to read and write, consent was obtained using an independent witness and the caregiver provided a fingerprint. Assent was obtained from all children, except from those that were severely cognitively impaired as judged by a trained psychologist. All consent forms are stored at the Centre of Tropical Neurosciences (CTN) for future reference.

Participants

The study recruited 154 children and adolescents with nodding syndrome receiving standard symptomatic care at the dedicated treatment centres and 154 healthy community controls from neighbouring homes in the same communities. All nodding syndrome cases were 8 years or older and diagnosis was according to WHO criteria[21]: head nodding on two or more occasions, occurring in clusters at a frequency of 5-20/minute; observed by a trained health worker; onset between 3-18 years; plus any one of the following: a) triggered by food or cold weather; b) presence of other seizures or neurological abnormalities and cognitive decline; and c) clustering in space or time. The community controls were frequency matched based on prior defined age groups 6-9, 10-14 and 15-18 years. Control CSF was obtained from fifteen well Ugandan children in

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remission from a haematological malignancy as part of their routine follow-up at the Uganda Cancer Institute in Mulago hospital located in Kampala, Uganda.

Clinical history and examination

On enrolment, all participants had a detailed history taken to characterize the burden and type of seizures experienced in the previous month and a detailed physical exam including evaluations for neurologic, cognitive and psychiatric disorders. Finally, following standard aseptic collection methods, EDTA-anticoagulated blood and CSF by lumbar puncture were obtained. Plasma was separated by centrifugation and all samples stored at -20 °C prior to transfer on dry ice to Kampala for long-term storage at -80 °C until testing. All samples were shipped on dry ice and sample integrity was confirmed on arrival. Exposure to *O. volvulus* was measured at the laboratory of parasitic research at the National Institute of Health, by evaluating levels of anti-OV-16 IgG in plasma as previously described [22]. A sample was considered sero-positive for *O. volvulus* when anti-OV-16 IgG signal-to-noise ratio was above a cut-off of 2. The cut-off was determined previously using receiver operator characteristic curves using 'other helminth' control samples[22]. The presence of active *O. volvulus* infection was determined by microscopy of saline solutions in which skin snips obtained from the iliac crest of all participants were incubated following standard microscopy protocols.

As part of National Onchocerciasis control programs, all children in the region receive bi-annual ivermectin at a standard dose of 150 ug/kg. All nodding syndrome participants also received routine care including nutritional supplementation and physical therapy as necessary for rehabilitation, and symptomatic treatment of seizures with sodium valproate (dose rationalised between 10 – 35 mg/kg/day depending on seizure frequency).

A validated disease staging system was adapted to classify nodding syndrome participants into 3 categories: mild, moderate, and severe disease [2,7]. Cases were defined to have mild disease if they only had head nodding in the absence of convulsive seizures (tonic, clonic, myoclonic, absence, tonic-clonic) or other disabilities. Moderately severe disease included children with head nodding and convulsive seizures: tonic, clonic, tonic-clonic, myoclonic or absence seizures. Severe disease included cases with convulsive seizures and: i) focal neurological deficits (e.g. impaired motor function); ii) recognizable cognitive impairment, behavioural disorders or psychiatric disorders; iii) severe malnutrition (severe stunting [height-for-age z score < -3] or severe wasting [BMI-for-age z score< -3]); iv) physical deformities including kyphosis, limb, and pectus deformities; v) severe disability with limited independent mobility.

Inflammatory marker quantification

Markers of immune and complement activation were measured in plasma and CSF by enzyme-linked immunoassays (ELISAs) or custom MagPix Luminex assay. Markers were selected based on previous associations with neuroinflammation in other neurological disorders[23,24]. Markers measured by ELISA were:

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C-reactive protein (CRP, dilution: 1:20,000 plasma, undiluted CSF), matrix metallopeptidase-9 (MMP-9, dilution: 1:2 plasma, undiluted CSF) and chemokine (C-C motif) ligand 5 (CCL5) and regulated on activation, normal T cell expressed and secreted [RANTES]; 1:2 plasma, undiluted CSF) using commercially available kits (DuoSet® ELISA Development System, R&D Systems, Minneapolis, MN, USA). Plasma was tested in duplicate and CSF tested in single replicate due to limited sample volume. Assays were performed according to the manufacturer's instructions except for overnight sample incubation at 4°C to increase assay sensitivity. Neopterin assays were performed using a competitive ELISA (DRG Instruments, GmbH, Germany) following the manufacturer's instructions. A custom-developed multiplexed fluorescent magnetic bead-based immunoassay (R & D Systems, Minneapolis, USA) was used to measure the remaining markers (CXCL10, CXCL13, CXCL9, CCL2, APRIL, BAFF, IL6, IL4, IL13, IL10, TNFα, INFγ, C5/C5a) according to the manufacturer's instructions. Plasma samples were tested using a 2-fold dilution, and CSF samples were tested undiluted.

Statistical Methods

Epi Info™ version 7.1.5, R studio version 3.6.3, and GraphPad Prism version 6.05 for windows were used. Proportions were compared using Pearson's Chi-Square test. For continuous data, the means of normally distributed data were compared using the Student's t-test as appropriate. In case this was not normally distributed, data were analysed using the Mann-Whitney U test and presented as medians (interquartile range, IQR). The relationship between markers of immune activation in CSF and seizure burden was evaluated using a Spearman rank correlation test. The normal range for CSF markers was estimated using the 95th centile of control values to establish the upper and lower limits of normal range. A multivariable logistic regression analysis was performed to evaluate plasma inflammatory markers associated with nodding syndrome adjusting for demographics. As *P. falciparum* is associated with systemic inflammatory responses and there were differences in the prevalence of *P. falciparum* infections between groups, the analysis was stratified by *P. falciparum* status. Multicollinearity in models was assessed using the variance inflation factor (VIF) and confirming that is was < 10.

Results

General characteristics

The mean age of the population was 15.1 (SD 1.9) years and 45.1% were female. The age and sex distribution between groups were similar **(Table 1)**. The geographic location of the nodding syndrome cases, and community controls is shown in **Figure 1**. The CSF controls were enrolled from Kampala in central Uganda. In children with nodding syndrome, the average time from nodding syndrome diagnosis to enrolment in the study was 8.3 (SD 2.7) years, and 62/154 (40.3%) of participants were seizure free in the previous 30 days. The major other seizure type reported was mainly generalized tonic-clonic seizures, reported in 83/154 (53.8%) cases. (**Table 1**). A total of 31/154 (20.1%) had mild, 34/154 (22.1%) had

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moderately severe and 89/154 (57.8%) had severe disease. **(Table S1).** The mean age was 15.5 (SD 1.7), and 85/154 (55.2%) of the cases were male. A total of 9/154 (5.8%) nodding syndrome cases were severely stunted compared 6/154 (3.9%) community controls p=0.59, while 35/154 (22.7%) cases were severely wasted compared to 17/154 (11.0%) community controls p=0.009.

Although none of the children were acutely ill at the time of enrolment, asymptomatic malaria was common occurring in 72.7% (112/154) of participants with nodding syndrome and 54.5% (84/154) of community controls with an odds ratio (OR) of 2.2 (95% CI 1.34, 3.68, *p*=0.001). Among children with nodding syndrome there were no differences in the prevalence of malaria among children with mild, moderate or severe disease, (*p* trend = 0.88). One child with nodding syndrome was HIV infected, while none of the community children or cancer survivors tested positive for HIV infection. The majority of cases of nodding syndrome were seropositive for OV 147/154 (95.4%) compared to community controls 86/154 (55.8%), OR 17.04 (95% CI 7.33, 45.58, p<0.0001). In addition, the cases had higher relative levels of anti-OV-16 IgG, compared to the community controls, the median signal-to-noise (S/N) ratio was 27.0 (IQR, 7.6-46.3) in cases compared to 2.40 (IQR, 1.2-10.3) in community controls (p<0.0001) (**Figure 2**). There was a trend to an increased level of anti-OV-16 IgG with disease severity with increasing median [IQR] across mild (12.07 [3.45-49.31]), moderate (26.6 [9.4-26.6]) and severe (31.4 [9.5-48.9]) signal-to-noise ratio disease although this was not significant (*p* trend=0.06). By skin snip microscopy, active *O. volvulus* infection was observed in 13/154 (8.4%) cases, (the microfilaria [MF] load ranged from 1-88 MF per skin snip), and only 2/154 (1.3%) among community controls (MF range 1-16 per skin snip). Children with nodding syndrome had increased odds of active *O. volvulus* infection with an OR of 7.0 (95% CI 1.53, 64.70, p=0.006) compared to community controls.

Immune and complement activation in CSF of nodding syndrome

To investigate pathways of central nervous system inflammation in nodding syndrome, levels of immune and complement activation were measured in CSF of children with nodding syndrome and CSF controls (Ugandan children in remission for a haematological malignancy). Compared to the normal range established in the CSF of CSF controls recovered from haematological malignancy, complement factor 5 (C5/C5a) was elevated in all cases of nodding syndrome compared to CSF controls (p <0.0001) (**Figure 3, Table S2**). C-reactive protein (CRP), another marker of complement activation was elevated in 56 (36.3%) of cases relative to the population normal derived from CSF controls; however, there was no significant difference in CRP levels between cases and controls with an OR of 1.44 (95% CI 0.38, 5.32) (**Figure 3, Table 2**). None of the other markers of immune activation were above or below the normal limits determined in greater than 5% of nodding syndrome cases (**Table S3**), and levels were comparable between groups when adjusting for multiple comparisons. Levels of CXCL9, CCL5 (RANTES), IL13, IL6, TNFα, MMP-9 and INFγ were undetectable in both cases and controls (**Figure 3)**.

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Next, we evaluated whether there were differences in the median levels of CSF markers of immune and complement activation in nodding syndrome cases by disease severity (**Table 2**). No differences in markers of immune or complement activation by disease severity were observed according to analysis using a non-parametric test for trend. Among nodding syndrome cases there was no correlation between markers of immune or complement activation and malaria, seizure burden, dose of sodium valproate (mg/kg/day), OV-16 exposure (S/N) and duration with disease (years). **(Figure S1).**

Systemic immune activation in nodding syndrome compared to community controls

To investigate whether nodding syndrome is associated with systemic changes in inflammatory responses, plasma levels of markers of immune or complement activation were measured in nodding syndrome cases and compared to healthy community controls. In plasma, using the clinically recognized cut off for elevated CRP levels (≥ 10 mg/L), CRP was elevated in 55/154 (33.7%) cases compared to 48/154 (31.1%) controls (p= 0.46). The median CRP levels was 6.45 (2.43-12.19) mg/L in cases compared to 4.34 (1.45-13.35) mg/L in the healthy controls, OR of 1.22 (95% CI 0.83, 1.80). Among nodding syndrome cases there was a stepwise increase in median CRP levels with disease severity (*p* trend =0.008). **(Table 2).** Furthermore, CSF and plasma CRP levels were strongly correlated (r=0.7, p>0.0001) **(Figure S2).**

Inflammatory markers IL10, APRIL, CCL5 (RANTES), CCL2, CXCL13, MMP-9 were significantly lower among cases compared to community controls after adjusting for multiple comparisons. There was no significant difference in the level of C5/C5a, IL4, BAFF, or CXCL9 between cases and community controls. (**Figure 2)**. In multivariable models, elevated levels of CRP, BAFF and IL4 were independently associated with nodding syndrome, while CCL2, IL10, APRIL, and MMP-9 were lower in children with nodding syndrome. When stratifying by *P. falciparum* infection, elevated BAFF and downregulated CCL2 and IL10 were independently associated with nodding syndrome in children who were malaria-positive and malaria negative (**Figure 4**).

Among nodding syndrome cases there was no correlation between plasma markers of immune or complement activation and seizure burden, dose of sodium valproate (mg/kg/day), OV-16 exposure (S/N), duration of illness (years). **(Figure S1).** Furthermore, cytokine levels were not significantly affected by *O. volvulus* infection or nutritional status (Table S4, S5).

Discussion

This study aimed to examine pathways of immune and complement activation in children with chronic nodding syndrome. We observed evidence of complement system activation in the CSF of children with nodding syndrome with elevated levels of CRP and C5/C5a. The findings suggest a possible role of central nervous system complement activation in the pathogenesis of chronic nodding syndrome. There was evidence of altered immune activation in children with chronic nodding syndrome with several markers of

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immune activation lower in children with nodding syndrome compared to community children. Further investigation of the complement system and immune dysregulation is required, particularly in children with acute nodding syndrome.

Inflammation is associated with epileptic and neurological disorders and has been shown to exacerbate disease progression[25,26]. In the context of nodding syndrome, a recent post-mortem study identified gliosis and features of past ventriculitis and/or meningitis in 80% of cases studied suggesting nodding syndrome maybe a post-infectious encephalopathy or autoimmune disorder[20]. Although auto-reactive antibodies have been observed in independent case-control studies, no conclusive evidence of neuroinflammation has been demonstrated. In the present study, despite elevated CRP levels in CSF of one-third of cases and a significant elevation of complement factor C5/C5a in CSF, there is limited evidence of immune activation in both the CSF and peripheral circulation of the cytokines and chemokines tested. Regardless, the observation of elevated CRP and of complement activation in CSF are suggestive of ongoing innate immune responses within the CSF of children with nodding syndrome. Additional studies to understand pathways of complement activation and regulation in samples from children with both acute and chronic nodding syndrome, and comparison with CSF from children with other chronic neurodegenerative disorders may help elucidate the role of the complement system in nodding syndrome pathogenesis.

It is unclear why the levels of APRIL, IL10, IL13, CXCL10, CXCL13, and CCL2 were reduced in plasma of children with nodding syndrome compared to controls, but similar decreases in levels of Th-2 type responses have been observed in individuals with prolonged occult *O. volvulus* infection[27]. Furthermore, children affected with nodding syndrome are more likely to have a history of chronic malnutrition or other infections which may further alter their immune profiles. Interestingly, in all multivariable models nodding syndrome was associated with higher plasma levels of BAFF, even after stratifying for malaria infection, which is consistent with observations in autoimmune disorders such as systemic lupus erythematosus (SLE) [28] and Sjögren's syndrome [29]. These results support an immunological component to the pathophysiology of nodding syndrome.

The mean duration of nodding syndrome in this study was 8.3 years. During this timeframe children were treated using a standard treatment plan aimed at symptomatic care[33]. This included long-term use of the antiepileptic sodium valproate for seizure control [21]. Sodium valproate is documented to have anti-inflammatory properties and may have further modulated immune responses [30–32]. These may explain the absence of significant immune dysregulation among children receiving treatment [33]. Furthermore all children in the region received ivermectin biannually, which has been shown to transiently impact cytokine levels[34]. These may have affected the level of immune activation leading to normalization of responses over time. Finally, the region is endemic to several infectious diseases which may have confounded inflammatory levels in both cases and controls[35–37]. Although the study examined for the effect of malaria, the present

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study did not systematically evaluate other infections, including helminths that have immunomodulatory properties[38].

To the best of our knowledge, this is the first study to demonstrate an association between complement activation and nodding syndrome. Several cells of the nervous system (astrocytes, neurons, endothelial cells and oligodendrocytes) express complement proteins and receptors[39]. Complement activation has been well described in other neurological conditions, including: epilepsy (e.g. post-viral epilepsy, CASPR2-associated encephalitis and Rasmussen's syndrome)[40,41], major psychiatric disorders (e.g. major depressive disorder and schizophrenia)[42], and neurological disorders (e.g. Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease and parkinsonism)[43]. Complement associated proteins have been implicated in several neuro-degenerative disorders, and C5/C5a has been associated with neuronal death *in vitro* [43,44]. In the present study, peripheral elevation of complement was not observed suggesting interictal synthesis of C5a may occur in nodding syndrome as reported in Guillain-Barré syndrome[45]. Multiple causes of complement dysregulation have been demonstrated including autoantibodies, damaged cells, exposed myelin antigens, pathogens, trauma or anoxia[39,40,43]. The precise mechanism by which this dysregulation may occur or result in nodding syndrome disease pathology is unclear. We speculate that complement activation may be secondary to an underlying etiological factor resulting in interictal complement synthesis.

It has been suggested that nodding syndrome may be a post-viral disorder analogous to measles-associated SSPE based on clinical and neuropathological overlaps[18]. Therefore in a similar fashion to viral encephalitis[46], and septic encephalopathy[47], invasion of CSF by a neurotropic viral infection may trigger complement activation. In line with this observation, early evidence demonstrated complement activation in measles infections[48]. Nodding syndrome has been liked strongly to *O. volvulus* however, invasion of CSF by *O. volvulus* has not been demonstrated. Despite this, it is possible that CSF invasion by onchocerca metabolites may occur resulting in immune and or complement activation. *O. volvulus* has been shown to activate the complement cascade however it can also bind human complement factor H which regulates complement activation by cleaving C3b into an inactive form averting continued activation[27]. A breakdown in this regulation within the CSF of children with nodding syndrome may have adverse effects. Finally previously identified auto-antibodies[13,19] may be neurotoxic through a complement antibody-mediated mechanism.

The study had limitations. Children with nodding syndrome were assessed on average 8 years after their initial diagnosis and following treatment. Children had also been on medications for a number of years (including bi-annual treatment of *O. volvulus* infection) that could modulate immune responses and affect conclusions drawn. Therefore, prospective enrolment of a cohort of children with new-onset nodding syndrome will be important to extend and validate these findings. The CSF controls used in this study were Ugandan children in remission for a haematological malignancy. As it is unethical to collect CSF from

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healthy community children, we enrolled controls without evidence of infection or active disease undergoing lumbar puncture as part of follow-up care for prior haematological malignancy. These patients may have had substantial difference in nutritional status and history of infection compared to children with nodding syndrome. While we cannot definitively state that the levels of immune activation in the controls are representative of a healthy population, they are consistent with levels reported in the literature[23].

Strengths of this study include the enrolment of a large population of children with nodding syndrome and inclusion of controls from the same community. This population is well characterized and data on seizure history, disease staging, and medication use were systematically collected which enabled comparisons between pathways of interest and disease severity.

Conclusion

This study demonstrates evidence of complement activation in the cerebrospinal fluid of Ugandan children with nodding syndrome. However, in this population with chronic nodding syndrome, receiving symptomatic therapy of whom approximately two-fifths had attained good seizure control, there was no evidence of systemic immune activation. Future studies are needed to evaluate the spectrum of complement proteins and inflammation in incident cases in order to design therapies that can be used in the acute phase of disease to mitigate disease progression and improve long-term outcomes in children with nodding syndrome.

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Disclosure of conflicts of interest

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None of the authors has any conflict of interest to disclose

Ethical publication statement

We confirm that we have read the Journals position on issues involved in ethical publication and affirm that this report is consistent with those guidelines

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Tables

Table 1: Study participant demographics and infections

Variable	Nodding syndrome (n=154)	Community Controls (n=154)	CSF Controls (n=15)	P value
Demographics				
Age, years, mean (SD)	15.5 (1.7)	14.6 (2.1)	12.3 (1.5)	<0.0001*
Male, % (n/N)	55.2% (85/154)	54.5% (84/154)	46.7% (7/15)	0.81**
Height for age z scores, median (IQR)	-1.11 (1.81-0.36)	-0.8 (-1.62-0.01)	NA	0.01#
Severe stunting, % (n/N) (z score<-3)	5.8% (9/154)	3.9% (6/154)	NA	0.59**
BMI for age z scores mean (SD)	-2.02 (1.47)	1.65 (1.15)	NA	0.01##
Severe wasting, % (n/N) (z score<-3)	22.7% (35/154)	11% (17/154)	NA	0.009**
MUAC, cm, mean (SD)	22.16 (3.33)	21.76 (2.84)	NA	0.26##
Infection				
Malaria smear positive, % (n/N)	72.7% (112/154)	54.5% (84/154)	0% (0/15)	<0.0001**
HIV infection, % (n/N)	0.6% (1/154)	0% (0/154)	0% (0/154)	0.14**
Seropositive OV16, % (n/N)	95.4% (147/154)	55.2% (86/154)	NA	<0.0001**
Active *O. volvulus* infection, % (n/N)	8.4% (13/154)	1.3% (2/154)	NA	0.006**

Definitions *ANOVA, **χ2 test., # Mann-Whitney U test, ## unpaired Student's t-test, NA, data not available/applicable

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Table 2: Table comparing markers of immune activation in nodding syndrome case with mild, moderate and severe disease

	Plasma				CSF			
Marker	**Mild disease (n=31)**	**Moderate disease (n=34)**	**Severe disease (n=89)**	**P trend**	**Mild disease (n=31)**	**Moderate disease (n=34)**	**Severe disease (n=89)**	**P trend**
Immune activation								
CXCL10, pg/ml	68.32 (50.59, 121.2)	64.85 (44.25, 86.81)	71.47 (43.5, 106.7)	0.84	112.0 (64.4, 172.70)	103.6 (78.96, 214.7)	82.22 (55.06, 143.6)	0.08
CCL2, pg/ml	38.28 (22.01, 63.76)	37.72 (21.24, 80.45)	34.56 (21.24, 63.76)	0.42	256.3 (221.1, 396.1)	244.2 (176.1, 459.4)	237.5 (166.9, 390.7)	0.36
CXCL13, pg/ml	44.73 (24.31, 70.09)	47.31 (25.29, 87.5)	34.8 (25.13, 66.83	0.73	18.96 (15.43, 24.36)	18.96 (15.43, 23.5)	18.96 (14.48, 22.64)	0.50
CXCL9, pg/ml	399.7 (399.7, 878.5)	399.7 (399.7, 923.3)	399.7 (399.7, 619.5)	0.32	ND	ND	ND	-
CCL5(RANTES) ng/ml	2.13 (1.94, 2.59)	2.19 (1.80, 2.62)	2.24 (1.60, 2.82)	0.88	ND	ND	ND	-
IL10, pg/ml	38.18 (29.8, 59.98)	41.25 (33.99, 56.33)	38.18 (20.79, 58.77)	0.14	29.17 (24.59, 33.14)	26.89 (24.05, 38.65)	29.17 (24.39, 38.35)	0.19
IL4, pg/ml	2.42 (2.42, 4.93)	2.42 (2.42, 4.63)	2.42 (2.42, 9.87)	0.21	11.27 (8.02, 13.68)	11.38 (8.54, 13.64)	9.65 (7.23, 12.17)	0.99
IL13, pg/ml	245.2 (245.2, 554.2)	245.2 (245.2, 554.2)	245.2 (245.2, 357)	0.36	ND	ND	ND	-
APRIL, pg/ml	149.7 (97.65, 256.8)	184 (110.7, 267.3)	163.3 (91.48, 230.9)	0.87	45.66 (32.64, 60.87)	41.29 (26.45, 53.42)	36.99 (26.43, 50.08)	0.06
BAFF, pg/ml	664.0 (570.1, 891.9)	643.8 (553.4, 852.9)	647.5 (568.5, 769.5)	0.71	73.65 (58.85, 96.54)	63.41 (49.72, 92.05)	59.58 (47.98, 85.4)	0.07
MMP-9, ng/ml	0.87 (0.65, 1.53)	1.16 (0.74, 1.52)	1.05 (0.71, 1.50)	0.71	ND	ND	ND	-
Neopterin, mmol/L	Not measured	Not measured	Not measured	-	2.268 (1.23, 3.90)	2.715 (0.95, 4)	2.84(1.43, 4.0)	0.53
Complement activation								
C5/C5a, ng/ml	18.06 (11.54, 29.87)	23.45 (16.69, 31.51)	16.89 (11.33 29.50)	0.47	14.90 (9.53, 21.31)	14.68 (8.73, 18.96)	11.99 (7.79, 19.37)	0.14
CRP**,** mg/L	4.11 (0.88 – 8.26)	5.58 (2.15, 22.48)	7.71 (4.02, 17.59)	0.008	0.0029 (0.001, 0.003)	0.0024 (0.001, 0.003)	0.00214 (0.0008, 0.003)	0.33

Data presented as median (IQR) and analysed using a non-parametric test for trend.

Abbreviations: CXCL10 – C-X-C motif chemokine 10/ Interferon gamma-induced protein 10, CCL2 – chemokine (C-C motif) ligand 2, CXCL13 – chemokine (C-X-C motif) ligand 13, CXCL9 – Chemokine (C-X-C motif) ligand 9, CCL5(RANTES) – Chemokine (C-C motif) Ligand 5 (regulated on activation, normal T cell expressed and secreted), IL10 – Interleukin 10, IL4 – Interleukin 4, IL13 – Interleukin 13, April – A proliferation-inducing ligand, BAFF – B lymphocyte stimulator, MMP-9 – Matrix metallopeptidase 9, CRP – C -reactive protein, C5/C5a – Complement component, CSF – Cerebral spinal fluid , ND – Not detected

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Figure Legends

Figure 1: Study area in northern Uganda

Figure 2: Plasma markers of immune activation in nodding syndrome and community controls. Box plots showing plasma levels of markers of immune activation in cases with nodding syndrome (Nodding syndrome, n=154, red) and healthy community controls (HC, n=154, blue). Box plots represent median and IQR with whiskers denoting the 5% and 95% percentiles data compared using Mann-Whitney U test where *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$, ****$p \leq 0.00001$. After adjusting for multiple comparisons, IL10, April, RANTES, CCL2, CXCL13, MMP-9 and OV-16 remained significant

Figure 3: Cerebral spinal fluid inflammatory markers in patients with nodding syndrome; Box plots showing CSF levels of markers of immune activation in cases of nodding syndrome (NS, n=154, red) compared to CSF controls (CC, n=15, blue). Box plots represent median and IQR with whiskers denoting the 5% and 95% percentiles data compared using Mann-Whitney U test where *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$, ****$p \leq 0.00001$. After adjusting for multiple for multiple comparisons only C5/C5a remained significant.

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Figure 4: Forest plot depicting the relationship between plasma markers of inflammation in children with nodding syndrome and community children. Forest plots show odds ratio (95% CI) from a multivariable logistic regression model based on a one-unit change in log 10 transformed plasma biomarker with nodding syndrome cases as the dependent variable. **(A)** Model with all participants (NS =154 and CC = 154). **(B)** Model in *P. falciparum* positive individuals (NS=112, CC=84). **(C)** Model in *P. falciparum* negative participants (NS=42, CC= 70). Adjusted models included participant age, sex, BMI-for–age z score, *O. volvulus* seropositivity, and *P. falciparum* status (in combined model). Plasma markers upregulated in nodding syndrome are shown in red, markers with no change are shown in grey and markers down regulated are depicted in blue. Markers significant following adjustment for multiple comparisons are indicated with an asterisk. Markers are ordered by odds ratio from highest to lowest within each plot.

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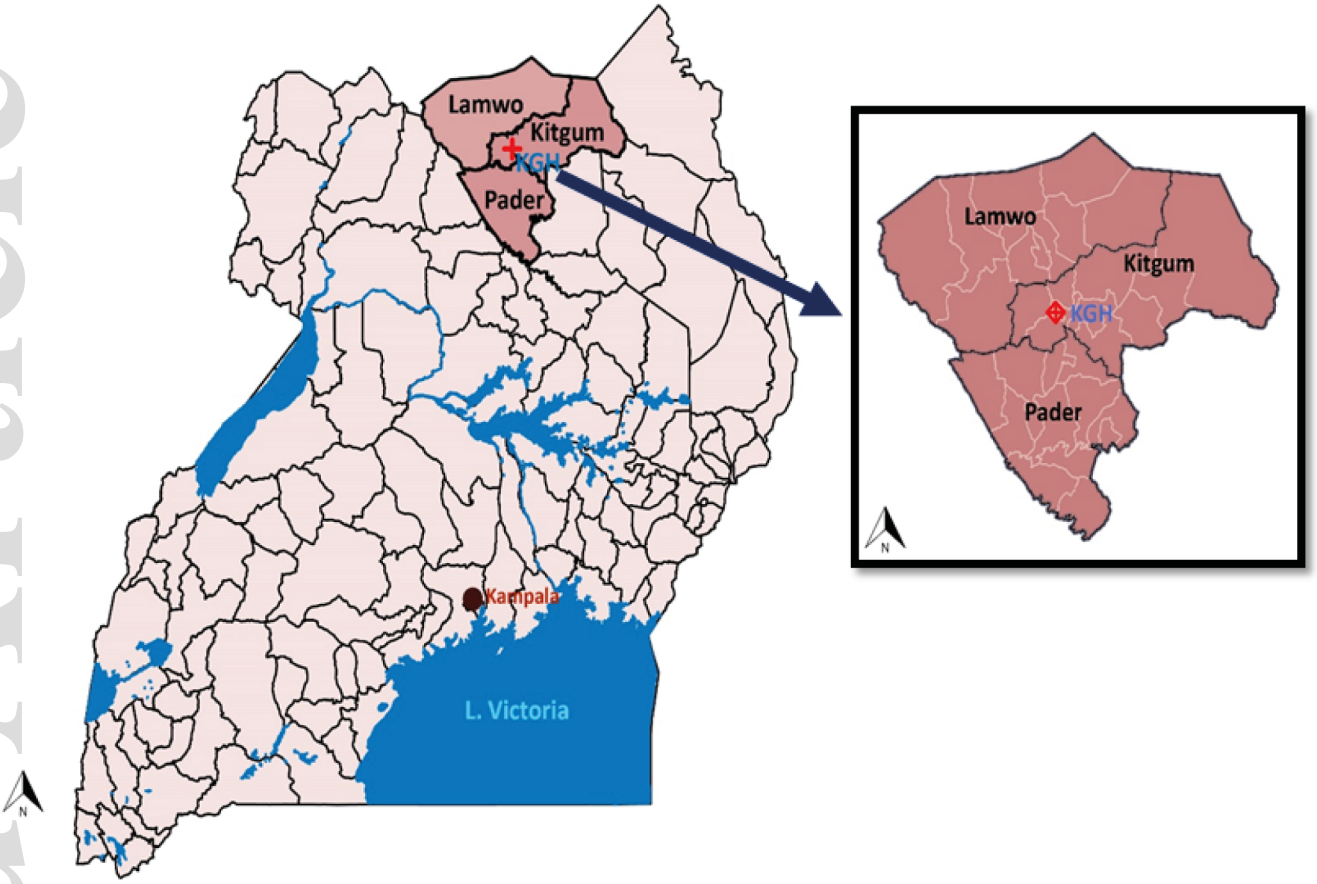

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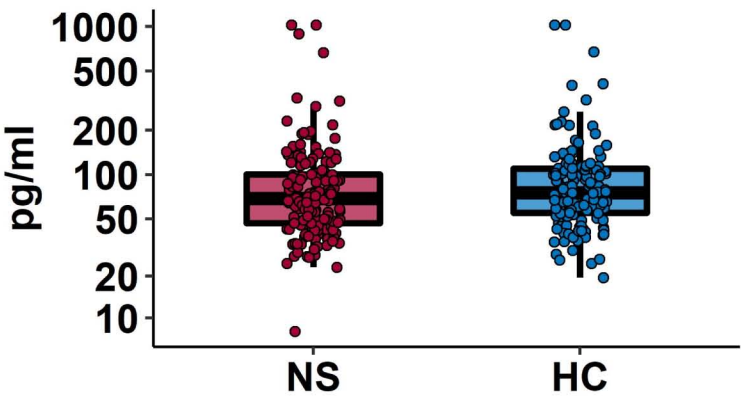

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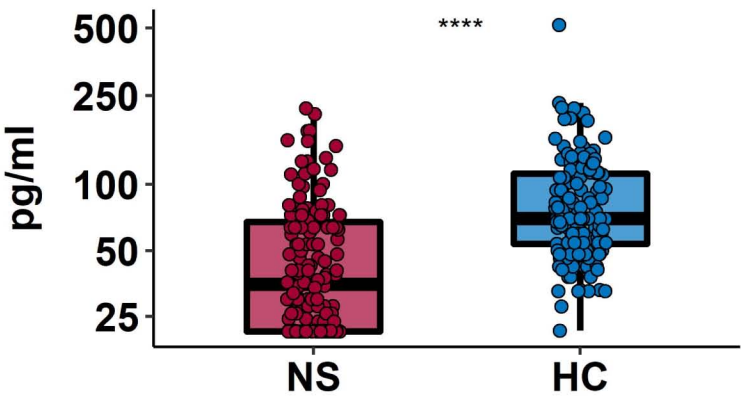

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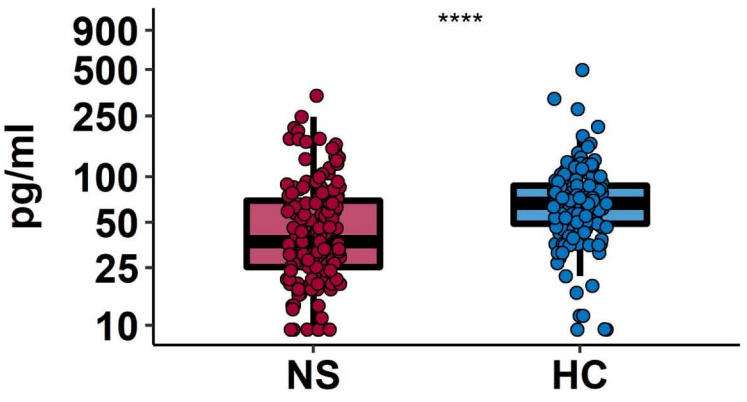

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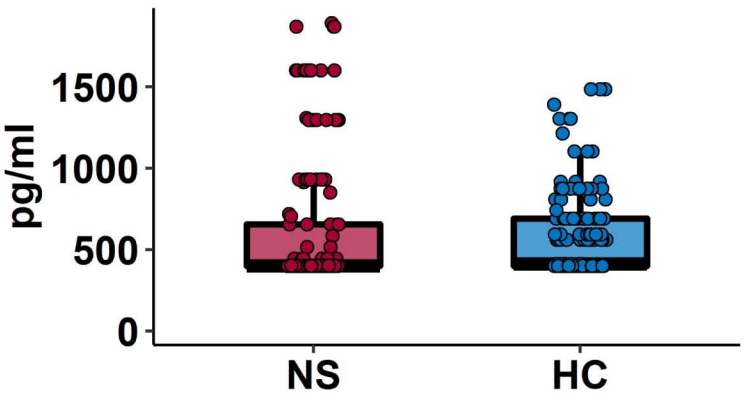

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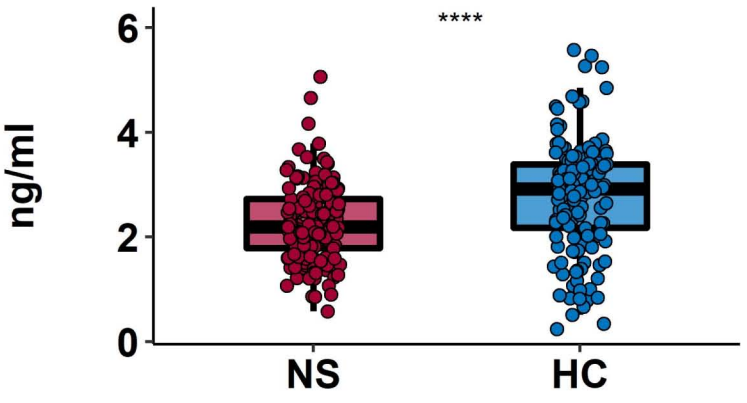

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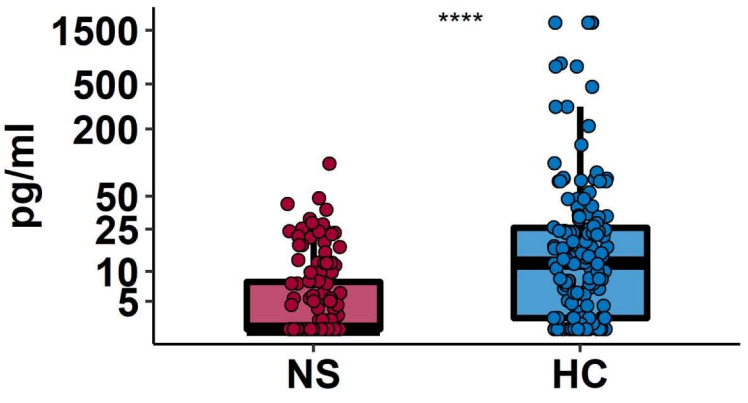

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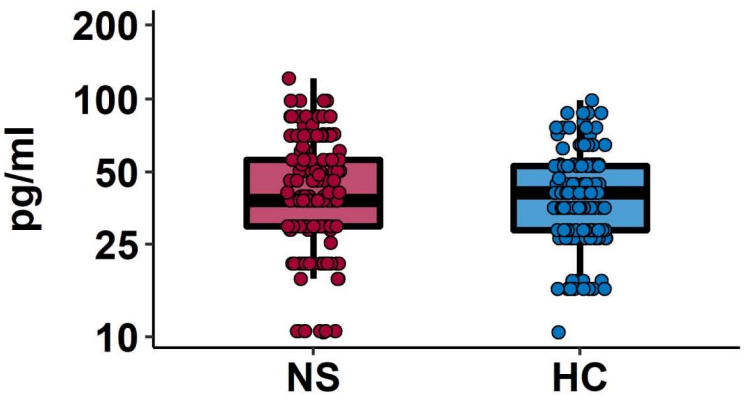

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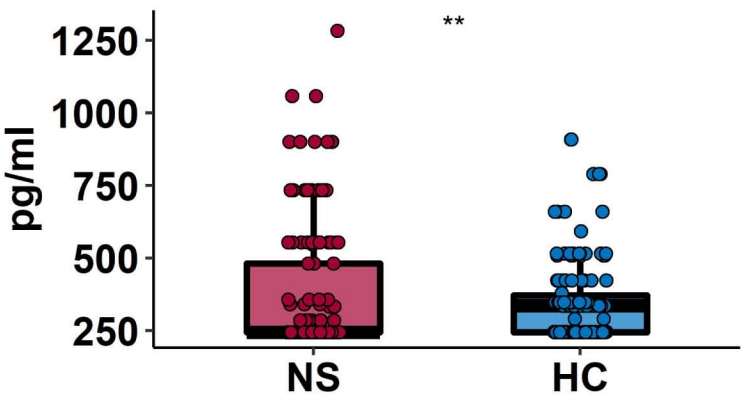

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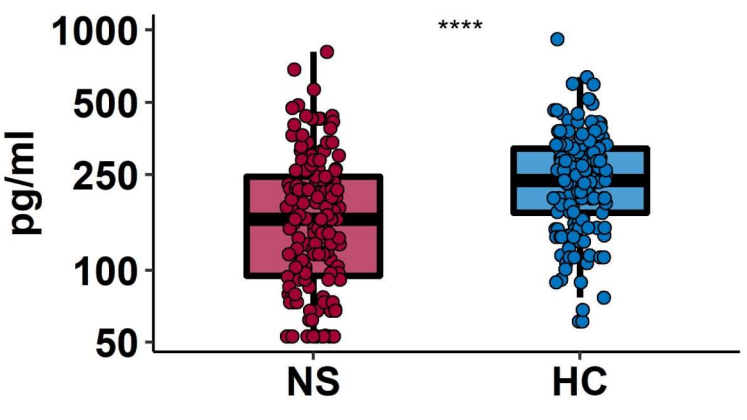

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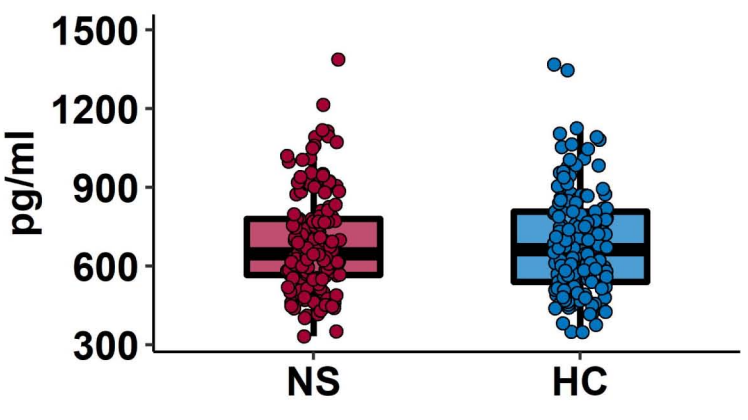

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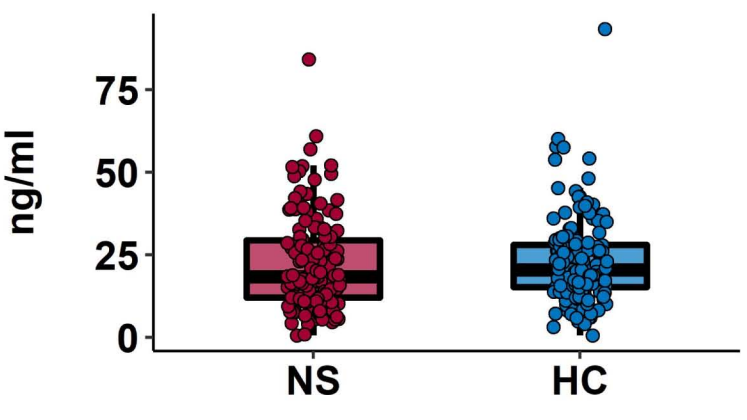

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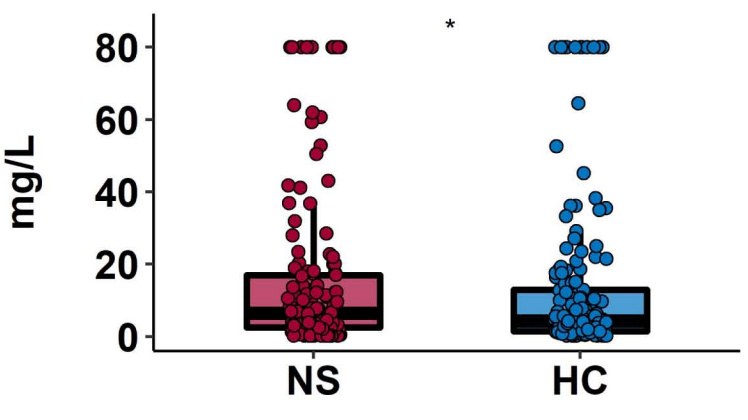

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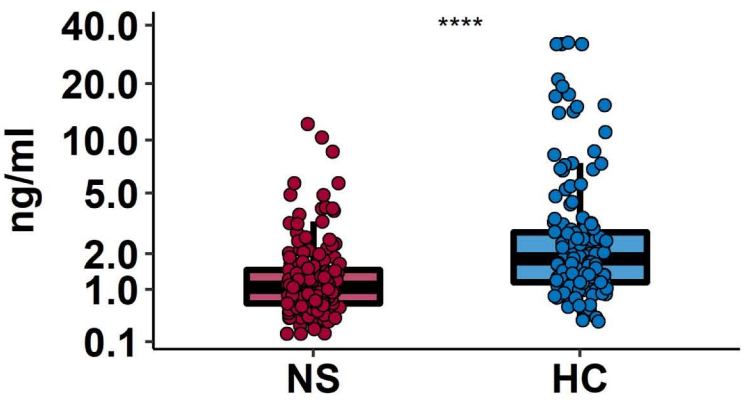

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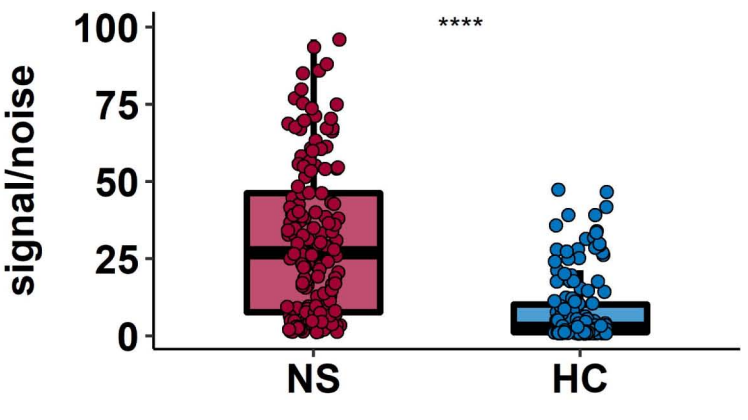

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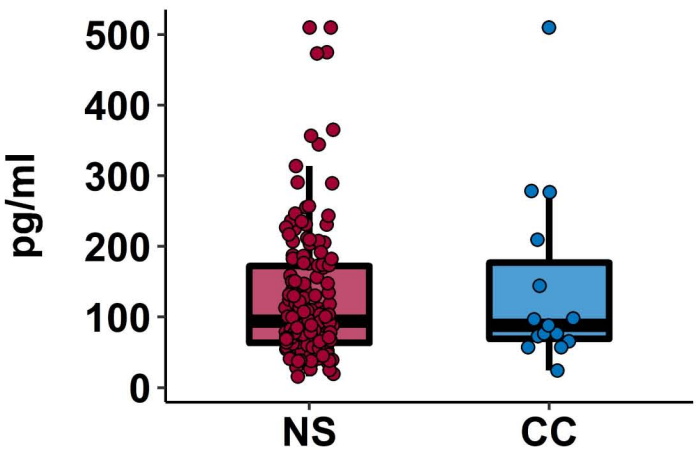
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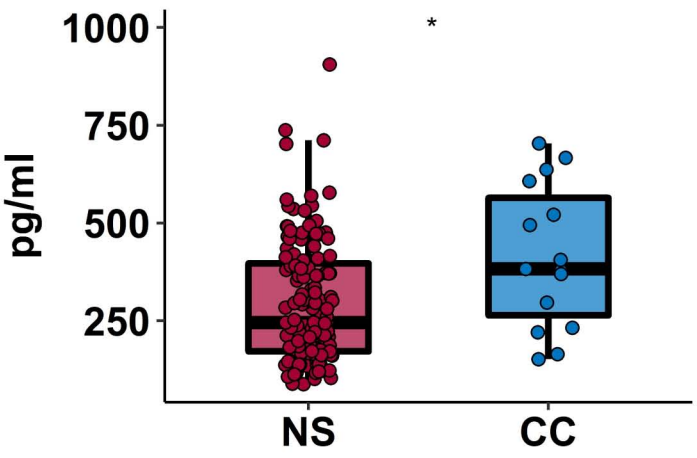
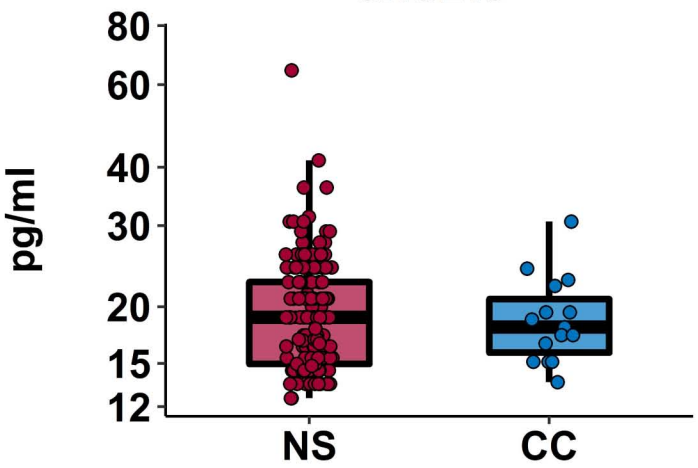
CC

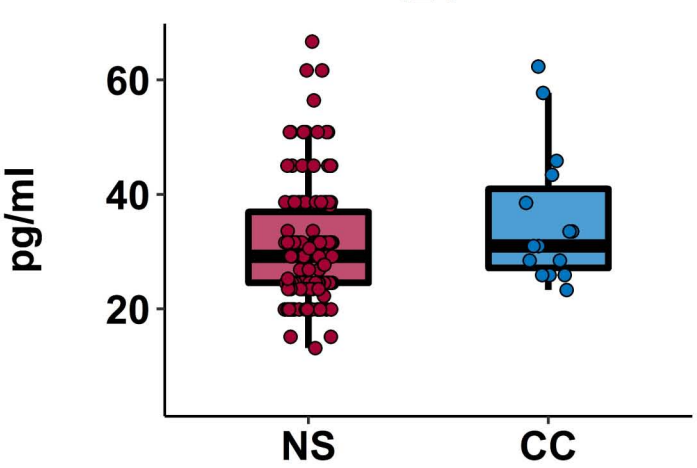
B


CCL2
*
pg/ml
1000
750
500
250
NS
CC

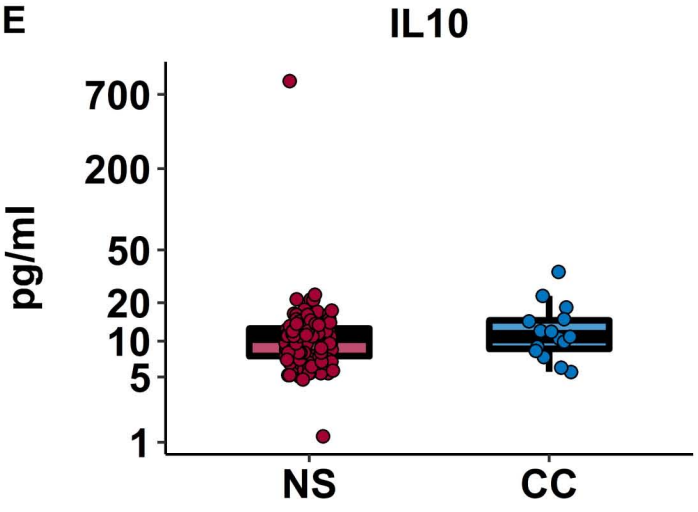
C


CXCL13
pg/ml
80
60
40
30
20
15
12
NS
CC

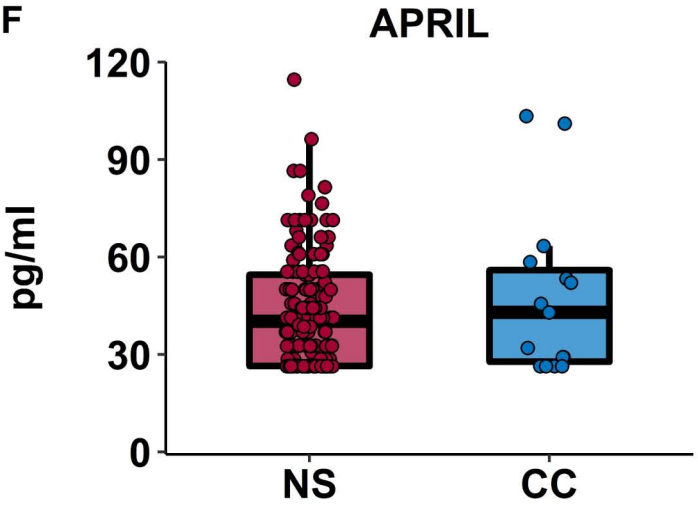
D


IL4
pg/ml
60
40
20
NS
CC

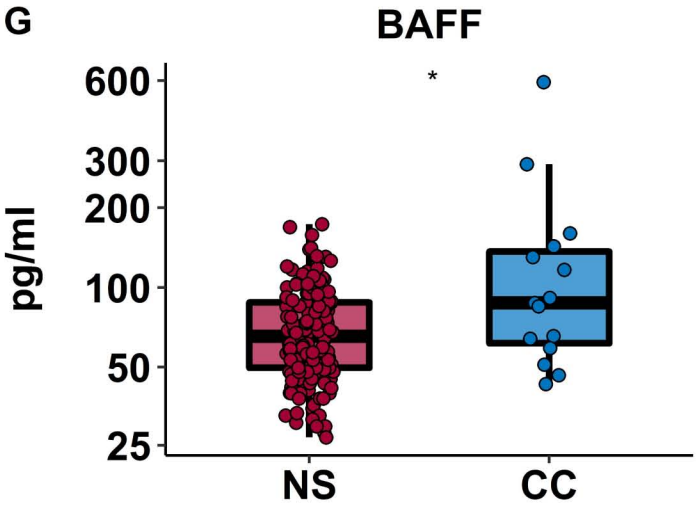
E


IL10
pg/ml
700
200
50
20
10
5
1
NS
CC

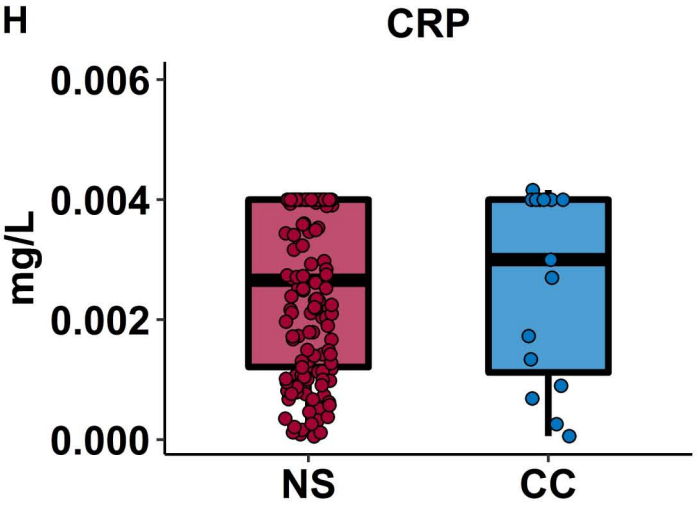
F


APRIL
pg/ml
120
90
60
30
0
NS
CC

G


BAFF
*
pg/ml
600
300
200
100
50
25
NS
CC

H


CRP
mg/L
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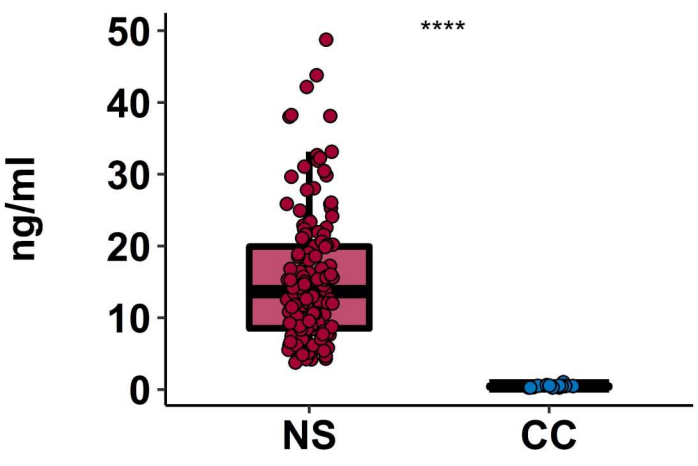
0.004
0.002
0.000
NS
CC

I


C5/C5a
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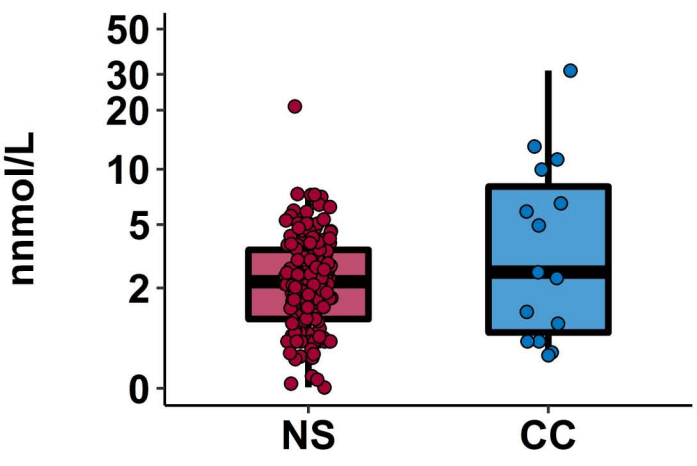
ng/ml
50
40
30
20
10
0
NS
CC

J


Neopterin
nmol/L
50
30
20
10
5
2
0
NS
CC

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