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Serum levels of neurofilament light chain and glial fibrillary acidic protein correlate with disease severity in patients with West Nile virus infection

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ABSTRACT

West Nile virus (WNV) is a neurotropic mosquito-borne orthoflavivirus, representing a relevant public health threat. Identification of biomarkers that would predict the course of WNV infection is of interest for the early identification of patients at risk and for supporting decisions on therapeutic interventions. In this study, serum levels of glial fibrillary acidic protein (sGFAP) and neurofilament light chain (sNfL), which are markers of brain tissue damage and inflammation, were analysed in 103 subjects with laboratory-confirmed WNV infection, comprising 13 asymptomatic blood donors, 23 with WN fever (WNF), 50 with encephalitis/meningoencephalitis (E/ME) and 17 with acute flaccid paralysis (AFP). In addition, 55 WNV-negative subjects with fever, encephalitis or healthy asymptomatic were included as controls. Age-adjusted levels of both sNfL and sGFAP were significantly higher in patients with neuroinvasive disease than in those with fever or asymptomatic (both WNV-positive and WNV-negative), suggesting a broad association of these biomarkers with systemic inflammation and brain injury resulting from infection. In WNV patients, the combined analysis of sNfL and sGFAP early after symptom onset allowed discrimination between neuroinvasive disease and fever with 67.2% sensitivity and 91.3% specificity, but not between E/ME and AFP. Furthermore, high levels of sNfL and sGFAP were significantly associated with prolonged hospital stay, intensive care unit admission and the occurrence of death or severe sequelae. Detection of WNV RNA in CSF was associated with increased sGFAP. In conclusion, our study indicates the potential utility of sNfL and sGFAP as biomarkers of WNV disease severity and adverse outcome.

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Introduction

West Nile virus (WNV) is a neurotropic orthoflavivirus, included as prototype pathogen in the WHO list of pathogens with high epidemic and PHEIC (public health emergency of international concern) risk [1]. In the enzootic cycle, WNV is transmitted among birds by *Culex* spp. mosquitoes, while humans and other mammals are incidental dead-end hosts. During the last 30 years, the virus has spread globally, causing every year thousands of human cases of infection, especially in Europe and North America. Most WNV infections in humans are asymptomatic; approximately 20–30% develop influenza-like illness, defined as West Nile fever (WNF), while less than 1% of infected individuals develop West Nile neuroinvasive disease (WNND), characterized by encephalitis, meningitis, acute flaccid paralysis, or polyradiculoneuritis. In WNND patients, mortality ranges from 10% to 20% and severe sequelae persist in 20–40% of survivors. Old age, male sex, immunodeficiency, hypertension, diabetes and other comorbidities have been identified as risk factors for WNND [2].

Identification of biomarkers that would predict the course of WNV disease is of great interest for the early identification of patients at risk and for supporting decisions on therapeutic interventions. In this regard, high serum levels of inflammatory cytokines and chemokines [3], the presence of autoantibodies neutralizing type I interferon (IFN) in serum [4], and a signature of dysregulated sphingolipid metabolism in serum [5] have been identified in patients with WNND. In addition, elevated levels of markers of neural damage and inflammation have been detected in the cerebrospinal fluid (CSF) of patients with

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WNND, such as amyloid-β and glial fibrillary acidic protein (GFAP) [3,6]. In the CNS, the virus can infect and replicate in neurons, astrocytes, and microglial cells, causing neuronal cell death and neuroinflammation, with activation of astrocytes and microglia cells and production of inflammatory cytokines and chemokines [7].

The development of ultra-sensitive assays, such as single-molecule array (Simoa®) technology, allows to noninvasively quantify biomarkers of neural damage in blood. Several studies applying these highly sensitive techniques showed that serum neurofilament light chain (sNfL) and sGFAP are valuable prognostic biomarkers in a variety of neurological conditions, including traumatic brain injury and inflammatory CNS diseases [8,9]. Neurofilaments are intermediate filaments that are exclusively and abundantly expressed in neurons. They are released into the CSF and blood following axonal damage in neurodegenerative, inflammatory, vascular and traumatic diseases, and are considered highly specific markers of neuronal cell damage [8]. A variance, GFAP is an intermediate filament of astrocytes and is considered a biomarker of glial activation and blood–brain barrier dysfunction [10]. Serum levels of GFAP and NfL are increased in patients with brain tissue damage and inflammation, like traumatic brain injury, multiple sclerosis, neuromyelitis optica spectrum disorder, and Alzheimer diseases [9,11–13], correlate with adverse outcome in patients with stroke [14] and COVID-19 [15], and predict cognitive decline in patients with neurodegenerative disease [16]. On the basis of these and other findings establishing sNfL and sGFAP as biomarkers of neuroaxonal and glial injury, respectively [8,9], in this study we investigated whether the levels of NfL and GFAP were elevated in the serum of patients with WNV infection and whether they correlated with disease severity and clinical outcome.

Methods

Study design and patient description

In 2022, out of 1750 subjects with suspected acute WNV infection referred for testing to the Reference Laboratory at Padova University Hospital, 531 had confirmation of WNV infection based on the presence of at least one of the following laboratory criteria: WNV isolation from serum, urine, CSF or other biological specimens; detection of viral RNA in blood, urine, CSF, or other biological specimens; detection of WNV-specific IgM antibody response in CSF; high WNV IgM antibody titre and detection of WNV IgG antibodies in serum and confirmation by neutralization assays [17]. Among confirmed WNV cases, 103 subjects aged ≥18 years, referred to Verona or Padova University Hospitals and providing consent to participate in the study, were included in the present analysis. Subjects were classified, according to the worse observed clinical presentation of WNV infection, in the following groups: 23 cases of WNF, 50 cases of encephalitis or meningoencephalitis (WNV E/ME), 17 cases of acute flaccid paralysis or polyradiculoneuritis (AFP), and 13 asymptomatic WNV infections (2 females and 11 males; median age 53; range 36–67 years) detected in blood donors screened by WNV nucleic acid amplification test (WNV Asympt). As control groups, we included 16 patients with symptoms similar to those observed in WNF (Fever; 6 females and 10 males; median age 50, range 18–76 years) and 15 with encephalitis or meningoencephalitis (E/ME; 8 females and 7 males; median age 70 years, range 18–88 years), in whom WNV infection was not confirmed by laboratory testing. Finally, we included a control group of 24 healthy asymptomatic subjects (Asympt; 7 females and 17 males; median age 32, range 21–58 years) recruited among those performing routine laboratory screening tests including WNV testing and in whom WNV and other arbovirus infections were not confirmed. All study subjects provided written informed consent and the study was revised and approved by the local ethics committee (Approval No. 1757/CESC Verona).

Laboratory diagnosis of WNV infection

For WNV RNA detection, total nucleic acids were purified from 200 µl of whole blood, plasma, urine, saliva, or CSF by using a MagNA Pure 96 System (Roche Diagnostics, Basel, Switzerland) and were amplified by two in-house real-time RT–PCR methods, which allowed the discrimination between WNV lineage 1 (WNV-1) and WNV-2 [18,19]. Real-time RT–PCR assays were carried on using one-step real-time RT–PCR kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and run on an ABI 7900HT Sequence Detection System or a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific). In addition, the cobas® WNV Test on a cobas® 6800 System (Roche Diagnostics) was used to detect WNV RNA in 1000 µl of plasma samples. This test is highly sensitive but cannot discriminate between WNV-1 and WNV-2. For the identification of the WNV lineage in WNV RNA positive samples, we used a broad-range RT–PCR targeting the NS5 region of orthoflaviviruses [20], followed by cycle sequencing. Testing for other vector-borne viruses (Usutu virus, Toscana virus, tick-borne encephalitis virus, TBEV, dengue virus, Zika virus and chikungunya virus) was included in the differential diagnosis, as reported [21]. The presence of WNV IgM and IgG antibodies in serum and CSF was determined by commercial ELISA kits (Euroimmun, Lübeck, Germany). Serum samples with

positive results were further tested for confirmation by plaque reduction neutralization test against WNV and microneutralization assay against the antigenically related USUV, as reported [22].

sGFAP and sNfL measurements

Serum samples were collected within two weeks from the onset of symptoms (or index blood donation for blood donors) and stored at −80 °C until testing for sGFAP and sNfL. Concentrations of sGFAP and sNfL were measured in duplicate in a blinded fashion using the ultrasensitive single molecule array (SiMoA) technology with the Neurology 2-plex B assay in SR-X immunoassay analyser (Quanterix, Boston, Massachusetts, USA), as previously described [23]. Analyses were performed at the Neuropathology and Neuroimmunology Laboratory, University of Verona, Italy, according to manufacturer's instructions. Since the levels of both sGFAP and sNfL increase with age, we calculated age-adjusted values as the difference between measured biomarkers and reference values. Specifically, we considered the age-specific reference values determined by Cooper et al. [24] from the analysis of N = 900 specimens obtained from Statistics Canada Biobank participants, aged 3 to 79 years, and calculated the relative difference between each measured biomarker and the median reference values for each year of age. In addition, we considered the lower and upper limits of the reference interval values, defined by the 5th and 95th percentiles, as references to determine if the measured sNfL and sGFA*P* values were below or above the reference intervals.

Statistical analysis

Descriptive statistics were performed using median (interquartile ranges [IQR]) for continuous variables and percentages for categorical variables. Group comparisons were assessed using nonparametric tests (Fisher's exact test, Mann–Whitney test, and Kruskal–Wallis test), as appropriate. The correlation between sGFAP and sNfL levels was investigated by calculating Pearson correlation coefficient. Logistic regression analysis and receiver-operating characteristic (ROC) curve analysis were performed to verify the discriminative power of age-adjusted sGFAP and sNfL in differentiating WNF and WNND groups and WNV-infected patients according to outcome parameters. The performance of a composite of both biomarkers in prognosticating WNV infection was investigated by multiple logistic regression analysis, categorizing patients according to high and low levels for each biomarker, using the cut-off values identified by ROC curve analysis. Associations between biomarker values and clinical characteristics and outcome parameters were assessed by univariate analysis and by multivariate linear regression models using each age-normalized biomarker value as a dependent variable, and age, sex, clinical diagnosis, number of days between symptom onset or index blood donation and serum sampling for testing, WNV lineage, detection of WNV RNA in blood and CSF, occurrence of death or sequelae, length of hospitalization, and ICU hospitalization as independent variables. The F test was used to assess how each multivariate linear regression model fitted the data. Statistical analyses and graphs were generated using GraphPad Prism 10.1.2; p values < 0.05 were considered statistically significant.

Results

Demographic, clinical and virological characteristics of patients with WNV infection

A summary of demographics, clinical presentation, outcomes and virological data of patients with symptomatic WNV infection is reported in Table 1. There were 29 (32%) females and 61 males (68%), with no significant difference in sex distribution among disease groups, i.e. WNF, WNV ME/E, and AFP. Patients with WNV ME/E were significantly older than patients with WNF or AFP. Hypertension and cardiovascular disease were reported more frequently by WNV ME/E patients than by WNF patients, while cancer, autoimmune disease, recent hospitalization for COVID-19 and chronic pulmonary disease were reported more frequently by patients with AFP than by WNV ME/E patients (Table 1). The median time from symptoms onset to hospitalization/diagnosis was similar in all patients' groups, ranging from 5 to 6 days. Patients with WNF reported more frequently headache, rash, arthralgia and myalgia than those with ME/E or AFP. The length of hospitalization, the rate of patients who were admitted to intensive care units (ICU), mortality, and the occurrence of long-term sequelae were significantly higher in AFP patients than in WNV E/ME (Table 1).

During the large WNV outbreak that occurred in 2022 in the Veneto Region, Italy, two viral strains co-circulated, i.e. an endemic WNV-2 strain and a newly introduced WNV-1 strain [25]. Epidemiological investigation suggested that patients with WNV-1 infection had a higher risk to develop WNND than those with WNV-2 infection [26]. In the present study, comparison among patients with WNF, ME/E and AFP showed a significant association between the presence of WNV-1 infection and the occurrence of AFP (Table 1). At variance, detection of WNV RNA in CSF, which indicates WNV replication in

Table 1. Demographic, clinical and virological findings in patients with WNV infection.

Variable	WNF		ME/E		AFP		
	no.	% or IQR	no.	% or IQR	no.	% or IQR	*P* value (group comparisons)*
All patients	23	25.5	50	55.6	17	18.9	
Demographic paramenters							
Female	8	34.8	18	36.0	3	18.0	NS
Male	15	65.2	32	64.0	14	82.0	
Age, median years	60	[47.0–76.0]	77.5	[71.5–84.3]	70	[57.5–81.5]	**0.0002** (WNF vs E); **0.0432** (E vs AFP)
Other clinical conditions							
Diabetes	2	8.7	7	14.0	6	35.3	NS
Hypertension	5	21.7	33	66.0	9	52.9	**0.0008** (WNF vs E)
Cardiovascular disease	3	13.0	23	46.0	7	41.2	**0.0081** (WNF vs E)
Metabolic syndrome	4	21.1	7	14.0	3	17.6	NS
Cancer	1	4.4	7	14.0	7	41.2	**0.0061** (WNF vs AFP); **0.0340** (E vs AFP)
Immunosuppressive therapy	5	21.7	7	14.0	6	35.3	NS
Autoimmune disease	0	0.0	4	8.0	4	23.5	**0.0260** (F vs AFP)
COVID-19	5	21.7	3	6.0	8	47.1	**0.0004** (E vs AFP)
Chronic pulmonary disease	0	0.0	3	6.0	5	21.7	**0.0094** (F vs AFP); **0.0211** (E vs AFP)
Symptoms							
Median days since onset	5	[3.0–8.0]	5	[3.0–8.3]	6	[5.0–9.5]	NS
Fever	23	100.0	47	94.0	17	100.0	NS
Asthenia	13	56.5	26	52.0	10	58.8	NS
Headache	18	78.2	21	42.0	2	11.8	**0.0053** (F vs E); **< 0.0001** (F vs AFP); **0.0366** (E vs AFP)
Rash	10	43.5	7	14.0	3	17.6	**0.0148** (F vs E)
Arthralgia	12	52.2	9	18.0	1	5.9	**0.0048** (F vs E); **0.0023** (F vs AFP)
Myalgia	10	43.5	7	14.0	2	11.8	**0.0148** (F vs E); **0.0408** (F vs AFP)
Gastrointestinal symptoms	3	13.0	10	20.0	6	35.3	NS
Meningeal symptoms	NA		20	40.0	5	29.4	NS
Confusion	NA		8	16.0	3	17.4	NS
Coma	NA		6	12.0	2	11.8	NS
Paralysis	NA		0	0.0	17	100.0	**<0.0001** (E vs AFP)
Psychomotor slowing	NA		11	22.0	4	23.5	**0.0210** (E vs AFP)
Dizziness	NA		3	6.0	1	5.9	NS
Outcome							
Lengh of hospitalization, median days	1	[1–5]	14	[7–35]	64	[31–160]	**<0.0001** (WNF vs E; E vs AFP)
ICU hospitalization	NA		12	24.0	13	76.5	**0.0003** (E vs AFP)
Death	NA		3	6.0	5	29.4	**0.0200** (E vs AFP)
Neurological sequelae	NA		11	23.4	9	75.0	**0.0420** (E vs AFP)
Virologial data							
WNV-1	13	56.5	26	52.0	14	82.4	**0.0048** (F vs AFP); **0.0013** (E vs AFP)
WNV-2	9	39.1	20	40.0	0	0.0	
WNV lineage not determined	1	4.3	4	8.0	3	17.6	
WNV RNA in CSF	NA		16	48.0	7	54.0	NS
WNV RNA in CSF negative	NA		17	52.0	6	46.0	

Note: WNF: West Nile fever; ME/E: meningoencephalitis/encepahlits: AFP: acute flaccid paralysis. CSF: cerebrospinal fluid; IQR: interqurtile range.
*Comparisons between groups were made by Fisher's exact test for categorical variables and by Mann-Whitney test for continous variables.

the brain, was not significantly associated with the severity of clinical presentation in patients with WNND.

High sGFAP and sNfL in patients with WNND

To assess whether sGFAP and sNfL could be candidate serum biomarkers of the severity of WNV infection, we evaluated if sNfL and sGFAP, measured at the time of hospital admission (i.e. within two weeks from symptoms onset) or at the time of index blood donation, correlated with the clinical presentation of WNV infection. Since sNfL and sGFAP levels are associated with age, the difference between measured sNfL and sGFAP levels and median reference values for each year of age [24] were calculated and used for statistical analyses. Within each group of WNV-infected subjects, age-adjusteded sGFAP and sNfL levels showed no significant association with age or sex. Considering the 95th percentiles of year-specific reference values as the upper limit, sGFAP levels measured early after onset were elevated in 9 (70%) blood donors who remained asymptomatic (WNV Asympt), in 16 (83%) patients with WNF, in 42 (84%) classified as WNV E/ME, and 14 (82%) patients who developed AFP. Likewise, one (8%) WNV Asympt, 16 (70%) WNF, 31 (62%) WNV ME/E and 16 (94%) AFP had sNfL above age-adjusted reference values. Among healthy WNV-negative control subjects (Asympt), 11 (46%) and one (4%), respectively, had sGFAP and sNfL above the upper reference values. Comparison of age-adjusted levels of sNfL and sGFAP among WNV groups showed that both sNfL and sGFAP levels were significantly higher in patients with WNND than in those with WNF and in WNV Asympt (Figure 1(a)). However, within the group of patients with WNND, no significant differences of sNfL and sGFAP levels were observed between WNV E/ME and AFP groups (Figure 1(b)). ROC curve analysis demonstrated that both sGFAP and sNfL could discriminate between WNF and

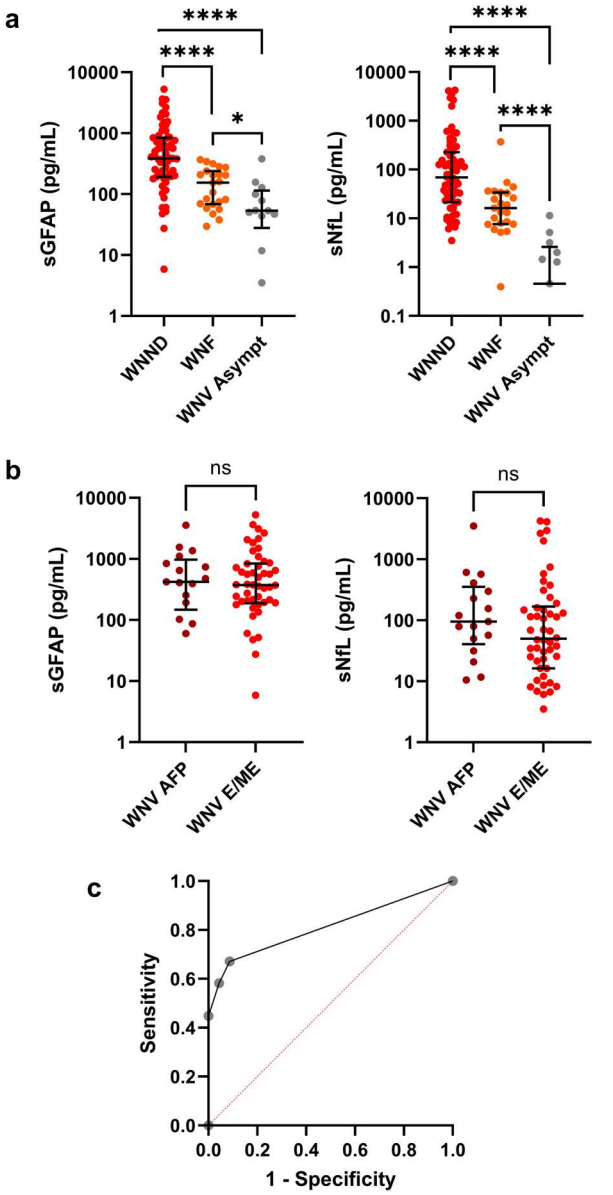

Figure 1. Association between age-adjusted sGFAP and sNfL levels and severity of disease in WNV-infected patients (a,b). Comparisons between groups were done by Mann–Whitney test. WNND: West Nile neuroinvasive disease; WNF: West Nile fever; AFP: Acute flaccid paralysis; E/ME: encephalitis/meningoencephalitis. (c) Multiple logistic regression analysis of the performance of composite sGFAP and sNfL (with cut-offs of 344.0 and 55.5 pg/mL, respectively) in prognosticating WNND vs WNF. ****$p < 0.0001$; ns: p not significant.

WNND with age-adjusted cut-offs of 344.0 and 55.5 pg/mL, respectively, with high specificity (95.7% and 95.7%, respectively) but relatively low sensitivity (58.2% and 53.7%, respectively) (Table 2). Multiple logistic regression analysis showed that testing for both sGFAP and sNfL improved sensitivity (67.2%) and slightly decreased specificity (91.30%) in discriminating between WNF and WNND (AUROC 0.81; 95% CI, 0.72–0.90; $p < 0.0001$) (Table 2 and Figure 1(c)). Like for the single biomarkers, the combination of sGFAP and sNfL did not allow distinguishing between E/ME and AFP. Overall, a positive correlation was found between the two parameters sGFAP and sNfL after Log transformation (Pearson r 0.77; CI 95% 0.67–0.84; $p < 0.0001$).

Elevated sGFAP and sNfL levels correlate with worse clinical outcomes in patients with WNV infection

To assess whether sGFAP and sNfL could predict outcome in WNV-infected patients, we investigated if serum values of these analytes at the time of hospital admission correlated with the following clinical outcomes: ICU admission, length of hospital stay, and death or severe neurological sequalae at discharge. As shown in Figure 2, age-adjusted levels of both sGFAP and sNfL were significantly higher in patients admitted to ICU, in those with a hospital stay longer than 15 days or who died, and in those who died during hospitalization or had severe sequelae at the time of discharge. A significant association between age-adjusted sGFAP levels and the occurrence of death or sequelae ($p = 0.0004$), hospitalization in ICU ($p = 0.0004$), and hospital stay longer than 15 days ($p = 0.0001$) was confirmed by multivariate linear regression analysis. A significant association was also confirmed by multivariate analysis between age-adjusted sNfL levels and outcome parameters: death or sequelae ($p = 0.0009$), ICU admission ($p = 0.0013$), and long hospital stay ($p = 0.0028$). ROC curve analysis showed that sGFAP and sNfL could predict outcome parameters with good sensitivity and specificity; the combination of the two biomarkers improved test performance in the discrimination between clinical outcomes (Table 2).

Serum GFAP and NfL levels according to virological parameters

To assess if infection with the new WNV-1 strain, which was associated with increased risk of AFP, was also associated with higher levels of neural biomarkers, we compared age-adjusted sGFAP and sNfL levels between patients infected with WNV-1 and WNV-2. This analysis did not find any differences between the two viral lineages both when considering all WNV patients and when analysing the subgroup of patients with WNND (Figure 3).

The presence of a high WNV load in blood has been suggested to elevate the risk of neuroinvasion due to the increased probability of crossing the damaged blood–brain barrier and the heightened secretion of WNV nonstructural protein 1, which promotes brain endothelial cell dysfunction [27]. Thus, we compared age-adjusted sNfL and sGFAP levels in patients with detectable WNV RNA in blood or in CSF at the time of diagnosis and those with undetectable viral RNA. This analysis showed that patients

Table 2. Associations between serum GFAP and NfL values and outcomes in patients with WNV infection.

	AUROC	95% CI	*P* value	Cut-off (pg/mL)	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	Likelihood ratio
WNND vs WNF							
sGFAP	0.68	0.57–0.80	<0.0063	>344.0	55.6 [43.3–67.2]	81.5 [63.3–91.8]	3.00
sNfL	0.69	0.60–0.80	<0.0053	>55.5	50.8 [38.8–62.7]	81.5 [63.3–91.8]	2.74
sGFAP + sNfL	0.81	0.73–0.80	<0.0001	>344.0 and/or >55.5	67.1	91.3	
Death or neurological sequelae							
sGFAP	0.87	0.80–0.95	<0.0001	> 378.2	85.7 [68.5–94.3]	85.3 [75.6–91.6]	5.84
sNfL	0.87	0.80–0.93	<0.0001	> 49.0	82.1 [66.6–85.3]	77.33 [66.7–85.3]	3.62
sGFAP + sNfL	0.88	0.81–0.96	<0.0001	>378.2 and/or 49.0	85.7	85.4	
ICU admission							
sGFAP	0.79	0.69–0.90	<0.0001	>378.2	76.0 [56.6–88.5]	79.5 [69.3–87.8]	3.70
sNfL	0.84	0.75–0. 93	<0.0001	>49.0	84.0 [63.4–93.6]	83.1 [71.5–90.5]	3.45
sGFAP + NfL	0.84	0.85–0.93	<0.0001	>378.2 and/or >49.0	92.0	65.0	
Hospitalization ≥15 days or death							
sGFAP	0.78	0.69–0.87	<0.0001	>344.0	68.2 [53.4–80.0]	81.4 [69.6–89.3]	3.66
sNfL	0.82	0.75–0.93	<0.0001	>49.0	68.2 [53.4–80.0]	83.1 [71.5–90.5]	4.02
sGFAP + sNfL	0.80	0.71–0.89	<0.0001	>344.0 and/or >49.0	77.3	79.7	

Note: AUROC: Area under the ROC curve.

with detectable WNV RNA in CSF had significantly higher sGFAP levels than those with undetectable viral RNA (Figure 4).

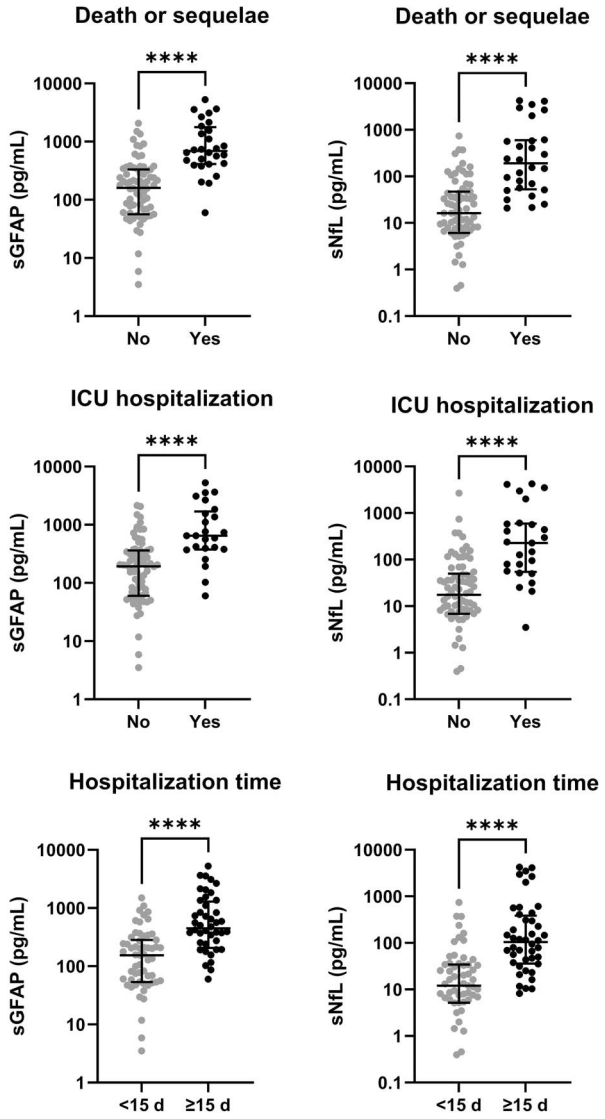

Figure 2. Association between age-adjusted sGFAP and sNfL levels and outcome parameters in patients with WNV infection. Comparisons between groups were done by Mann–Whitney test. ICU: intensive care unit. ****$p < 0.0001$.

Serum GFAP and NfL levels in WNV-negative control subjects

To evaluate if increased sGFAP and sNfL were specific of WNV infection or a common event in patients with febrile illness or meningoencephalitis, we evaluated the levels of both biomarkers in a subgroup of patients referred to our Institution for fever or encephalitis/meningoencephalitis of suspected viral aetiology, in whom WNV infection and other arboviral infections

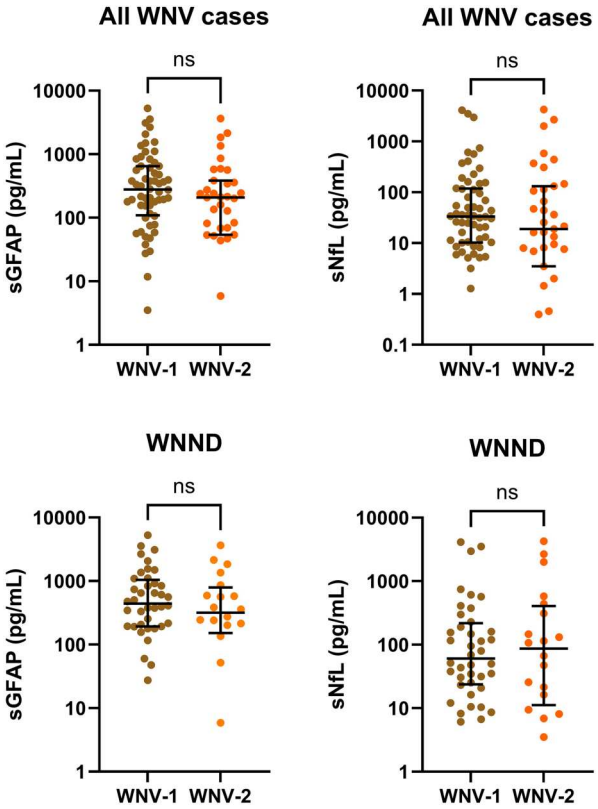

Figure 3. Serum levels of age-adjusted GFAP and NfL according to the lineage of the infecting WNV in all the patients with WNV infection and in the subgroups of patients with neuroinvasive disease (WNND). Comparisons between groups were done by Mann–Whitney test. WNV-1: WNV lineage 1; WNV-2: WNV lineage 2. ns: *p* not significant.

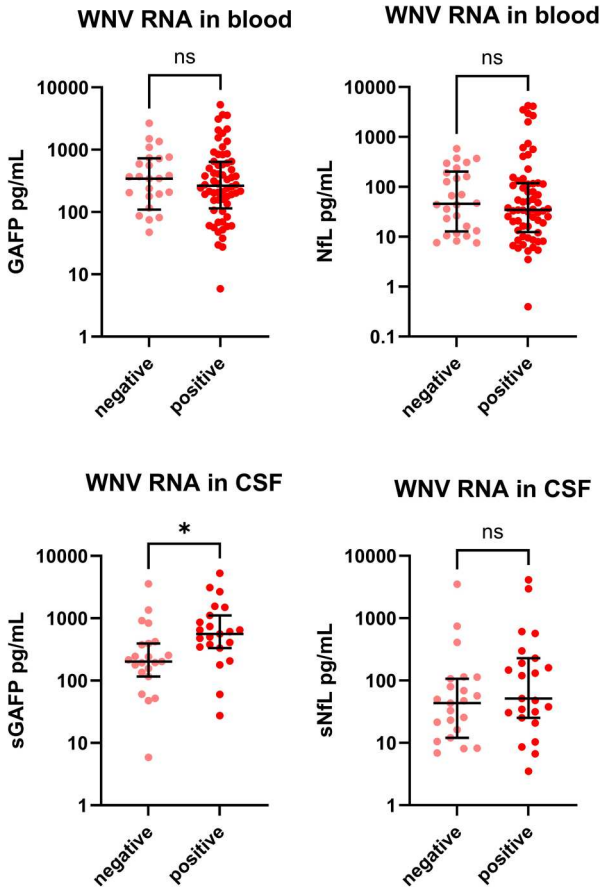

Figure 4. Serum levels of age-adjusted GFAP and NfL in WNV patients according to the detection of WNV RNA in blood and CSF. Comparisons between groups were done by Mann–Whitney test. CSF: cerebrospinal fluid. *$p < 0.05$; ns: p not significant.

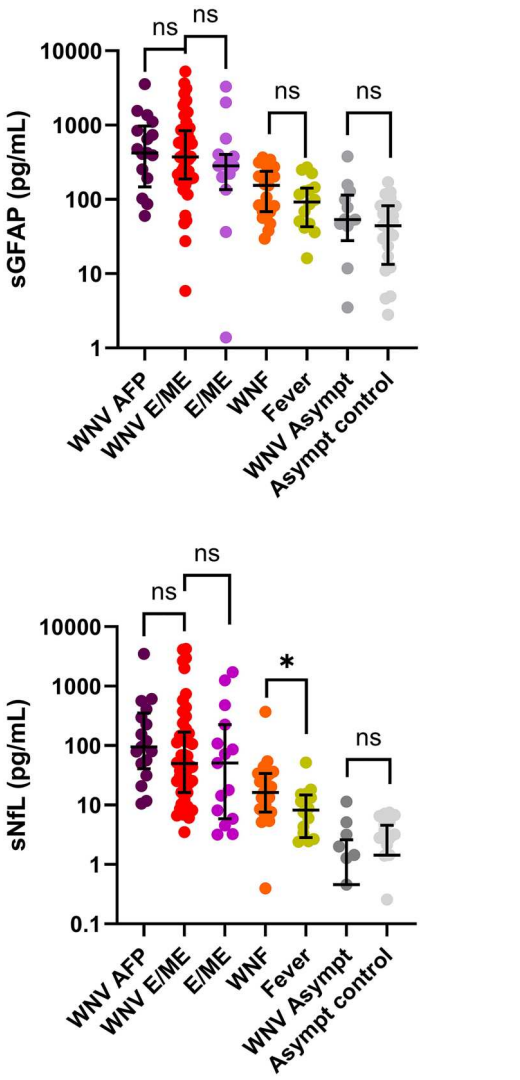

Figure 5. Serum levels of age-adjusted GFAP and NfL in all study subjects grouped according to WNV infection and clinical presentation. Comparisons between groups were done by Mann–Whitney test. AFP: Acute flaccid paralysis; E/ME: encephalitis/meningoencephalitis; WNF: West Nile fever; Asympt: asymptomatic. ns: p not significant.

were not confirmed by virological testing, and in a group of healthy individuals undergoing routine testing, with negative results for WNV. Results showed that age-adjusted levels of sGFAP and sNfL were not significantly different from those of the corresponding groups of WNV-infected individuals, with the exception of the higher levels of sNfL in patients with WNF than in those with WNV-unrelated fever (Figure 5). These results indicate that these biomarkers are not specific of WNND, but may be increased in other conditions of systemic inflammation and brain injury resulting from viral or bacterial infections, as also demonstrated by recent studies [28,29].

Discussion

Our retrospective cohort study aimed to assess the potential of sNfL and sGFAP, which are biomarkers of neuroaxonal and astrocyte damage, respectively, as markers for the severity of WNV infection and predictors of disease outcome. We observed a significant association between the severity of WNV infection and sNfL and sGFAP values. Specifically, early after symptom onset, both biomarkers were significantly higher is serum of patients who developed WNND than those with WNF, suggesting their potential utility in prognostic assessments. Indeed, their combined analysis allowed to discriminate between WNF and WNND with 67.2% sensitivity and 91.3% specificity. In addition, elevated levels of sNfL and sGFAP predicted ICU admission, prolonged hospital stay, and the occurrence of death or severe sequelae. Interestingly, sGFAP levels were significantly higher in the serum of WNND patients with detectable WNV RNA in CSF.

Besides discrimination of patients with WNND from those with WNF, our findings underscore the clinical significance of sNfL and sGFAP as potential early indicators of severe outcomes in WNV-infected patients. The correlation of these biomarkers with clinical parameters such as ICU admission, length of hospital stay, and mortality emphasizes their potential prognostic value, like in the cases of

neurodegenerative diseases, for which both sGFAP and sNfL has been demonstrated to be reliable markers predicting progressive neurodegeneration [16].

Few previous reports analysed GFAP and NfL values in patients with WNV infection. In an exploratory study in a group of 24 patients with WNV infection, [6], Petzold et al. showed that GFAP levels in CSF were significantly higher in individuals with WNV infection than in the control group with nonspecific symptoms, but did not investigate any correlation with disease severity [6]. In agreement with the results of our study, Constant et al. [3] found increased levels of markers of neuronal damage and neuroinflammation (MIF, NCAM1, TDP-43, and YKL-40, GFAP, GDNF, KLK6, and BDNF) in the sera of patients with WNND, but not in those with WNF, and serum biomarkers could not discriminate between the severity of neurological conditions, i.e. meningitis and encephalitis [3]. Notably, serum and CSF levels of neurofilament heavy chain (NF-H) were also analysed, but no differences were found between WNV patients and healthy controls [3]. At variance, a study by Veje et al. in patients with encephalitis caused TBEV, another neurotropic orthoflavivirus, showed that values of GFAP and NfL in CSF and in serum correlated with the severity of disease and with the occurrence of paralysis [30]. Other markers of brain damage were investigated by Fraisier et al., who evaluated serum levels of high-mobility group box-1 (HMGB1) and peroxiredoxin-6 (PRDX6) in WNV-infected patients and in non-infected healthy individuals [31]. These proteins, which are released from necrotic brain cells [32], were previously identified by proteomic screening as upregulated in brains from WNV-infected mice [33]. Results showed that serum HMGB1 concentrations were significantly higher in WNV-infected patients than in healthy controls and in WNND than in WNF patients, while, unexpectedly, serum PRDX6 concentrations were lower in WNV patients than in healthy controls [31]. ROC curve analysis estimated the sensitivity and specificity of serum HMGB1 in discriminating between WNV infection and healthy controls as 59.2% and 100%, respectively [31]. However, none of the above mentioned studies [3,30,31] reported the sensitivity and specificity of biomarkers for discrimination between WNND and WNF, nor investigated the association between biomarker concentrations and outcome parameters, such as death or neurological sequelae. However, taken together, these studies and our results indicate that serum markers of neuroinflammation and neural damage are promising diagnostic and prognostic biomarkers of WNV disease severity and outcome, which warrant further investigation and validation in prospective studies.

In the CNS, WNV primary targets neurons, but can also infect astrocytes that produce pro-inflammatory cytokines leading to impaired neurogenesis [34]. Post-mortem analyses in humans localized the virus to the hippocampus, cerebellum, basal ganglia, thalamus, midbrain, and pons, where it was associated with neuronal cell death, reactive astrocytosis, and inflammatory cell infiltration [35]. Outside of the brain, WNV has been detected in the spinal cord, dorsal root ganglia, and peripheral motor neurons [36].

In our study, increased levels of sGFAP and sNfL were also observed in several patients with WNF, suggesting that neural injury, either by direct infection or indirectly as a consequence of inflammation, might occur also in patients without clear neurological manifestations. In this regard, in nonhuman primate models, WNV is detectable in brain tissues (cerebellum, hippocampus) even in the absence of signs and symptoms of infection [37,38], while, in humans, neurological sequelae have been also reported among patients with WNF [39,40]. Mechanistic studies are warranted to improve our understanding of the pathophysiology of WNV-associated neurological complications.

Notably, elevated levels of sGFAP and sNfL were observed also in patients with suspected viral encephalitis/meningoencephalitis, and, at a lesser extent, in those with febrile illness, but in whom WNV infection was not confirmed. Thus, increased sGFAP and sNfL values should not be considered specific of WNV infection, but indicators of several conditions leading to neural injury, including viral infections [28,29].

Our study presents some limitations: the retrospective design did not allow to measure biomarkers at defined time points after symptom onset; the results were not validated in a prospective cohort study; potential confounders, such as body mass index which has an inverse association with BMI [41], were not included in regression models to correct for biases. Further research is thus required to elucidate the causative relationship and explore temporal patterns of biomarker expression throughout the course of WNV infection.

In conclusion, the results of our study indicate the potential of serum NfL and GFAP as biomarkers for predicting the severity of WNV infection and outcome and suggest a more broad potential application for other infections involving the CNS.

Author contributions

Conceptualization: AD, MP, SF, LB. Investigation: All authors. Funding: LB; Writing: original draft preparation: LB. Writing – review and editing: AD, MP, SM, DA, SF, LB.

Disclosure statement

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