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Can biomarkers of oxidative stress in serum predict disease severity in West Nile virus infection? A pilot study

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Abstract: West Nile virus (WNV) can cause asymptomatic infection in man, result in self-limiting febrile illness, or lead to severe West Nile Neuroinvasive disease (WNND).

We conducted a pilot study to compare selected biomarkers of oxidative stress in sera of viremic West Nile virus patients and asymptomatic infected blood donors to investigate their potential as predictors of disease severity.

We found that Total Oxidant Status was elevated in WNND and in uncomplicated WNV infections (median 9.05 (IQR 8.37 to 9.74) and 7.14 (7.03 to 7.25) $\mu\text{mol H}_2\text{O}_2$ equiv./L, respectively) compared to asymptomatic infections (0.11 (0.07 to 0.19) $\mu\text{mol H}_2\text{O}_2$ equiv./L) ($p = 0.048$). MDA levels showed a similar trend to TOS, but differences were not significant at $\alpha=0.05$. Total antioxidant status did not differ significantly between different disease severity groups.

Oxidative stress appears to be associated with more severe disease in WNV infected patients. Our preliminary findings warrant prospective studies to investigate the correlation of oxidative stress with clinical outcomes and severity of WNV infection.

Keywords: Oxidative stress; West Nile virus; West Nile Fever; West Nile Neuroinvasive disease; Flaviviridae; biomarkers

1. Introduction

West Nile virus (WNV) is a single-stranded, positive sense RNA virus part of the genus *Flaviviridae*. It is one of multiple arboviruses of growing public health importance [1]. It is transmitted in bird populations by *Culex* mosquitoes, and several larger mammals, including humans, are considered dead-end hosts. WNV is endemic in the United States and an increasing public health threat across Europe and other continents, mainly due to climate change and global travel [1,2].

Human infections are frequently asymptomatic but lead to West Nile Fever (WNF) in 20% of infected patients, and in approximately 1% of infections to more serious pathologies like meningitis, encephalitis, or flaccid paralysis [1,3]. Neurological presentations, commonly referred to as West Nile Neuroinvasive Disease (WNND) have fatality rates of approximately 10% [1,4]. Many WNND survivors report significant neurological sequelae that may persist for years after acute infection, including abnormal reflexes, muscle weakness, chronic fatigue and hearing and sensory loss [1,4]. Age is the main risk factor for severe WNV infection, and WNND incidence increases 1.5-fold for each decade [3].

Our understanding of the pathogenesis, predictors of disease severity and neurological sequelae of neuronal injury in WNV infection remains incomplete. Previous studies implicated oxidative stress (OS), an imbalance between antioxidative defense mechanisms and production of cell-damaging reactive oxygen species (ROS), in Flavivirus pathogenesis [5–7]. Several studies have observed an increase in ROS in dengue-infected patients, by comparing patients' OS markers to healthy controls [6,7]. However, data about OS in WNV infected patients is scarce. We hypothesized that OS plays an important role in the pathogenesis and severity of WNV disease [8,9]. In this pilot study we evaluated selected OS biomarkers on a panel of stored human sera from patients with West Nile virus infection of varying severity.

2. Materials and Methods

The study protocol was approved by the relevant ethics committees (see ethics statement) and informed consent was provided by all participants. We obtained WNV-RNA qRT-PCR positive serum samples from blood donors and patients with varying disease severity, *i.e.*, asymptomatic (AS), WNF or WNND, at Sheba Medical Center, Tel Hashomer, Israel, during the 2020 transmission season. The qRT-PCR used primers and probes that targeted the envelope and capsid protein coding regions and the 5' untranslated region, as described elsewhere [10,11]. Disease severity was assessed by the attending physicians. Patients were classified as WNF when febrile (temperature $\geq 38^{\circ}\text{C}$), presenting with headache, rash or myalgia, and no signs of WNND. Patients were classified as WNND when they presented with encephalitis (altered mental status lasting ≥ 24 h with no alternative cause identified, generalized or partial seizures, new onset of focal neurologic findings, MRI abnormalities suggestive of encephalitis), meningitis (fever and headache AND/OR signs of meningeal irritation), or flaccid paralysis (as acute onset of progressive limb weakness over 48 hours AND without sensory abnormalities). AS viremic individuals were identified among blood donors during routine safety evaluation. Samples were collected at one time point only, handled, frozen and stored on-site until shipment to and analysis in the NatuRA lab in Antwerp in 2021. All OS markers were analyzed using commercially available kits, per manufacturer's instructions: Total Oxidant Status (TOS) and Total Antioxidant Status (TAS) using colorimetric kits (RelAssay Diagnostics, Şehitkamil, Turkey), malondialdehyde (MDA) was quantified using ELISA (My Biosource, San Diego, USA).

SPSS Statistics (IBM; Armonk, USA) was used for statistical analyses. The association of disease severity and markers of OS was analyzed using Kruskal-Wallis tests. Bonferroni's correction for multiple testing was implemented where appropriate. All hypotheses were tested at significance level $\alpha=0.05$.

3. Results

Eight patients were recruited, six of which (75%) were female. Cycle-threshold values (Ct-values) for WNV RNA by qRT-PCR ranged from 32.63 to 41.25. Patients were categorized based on disease severity: four were asymptomatic (age range 18 to 59 years old), two had WNF (aged 23 and 43 years old) and two developed WNND (11 and 84 years old). Patient characteristics, WNV viral loads and serum concentrations of OS markers are shown in Table 1.

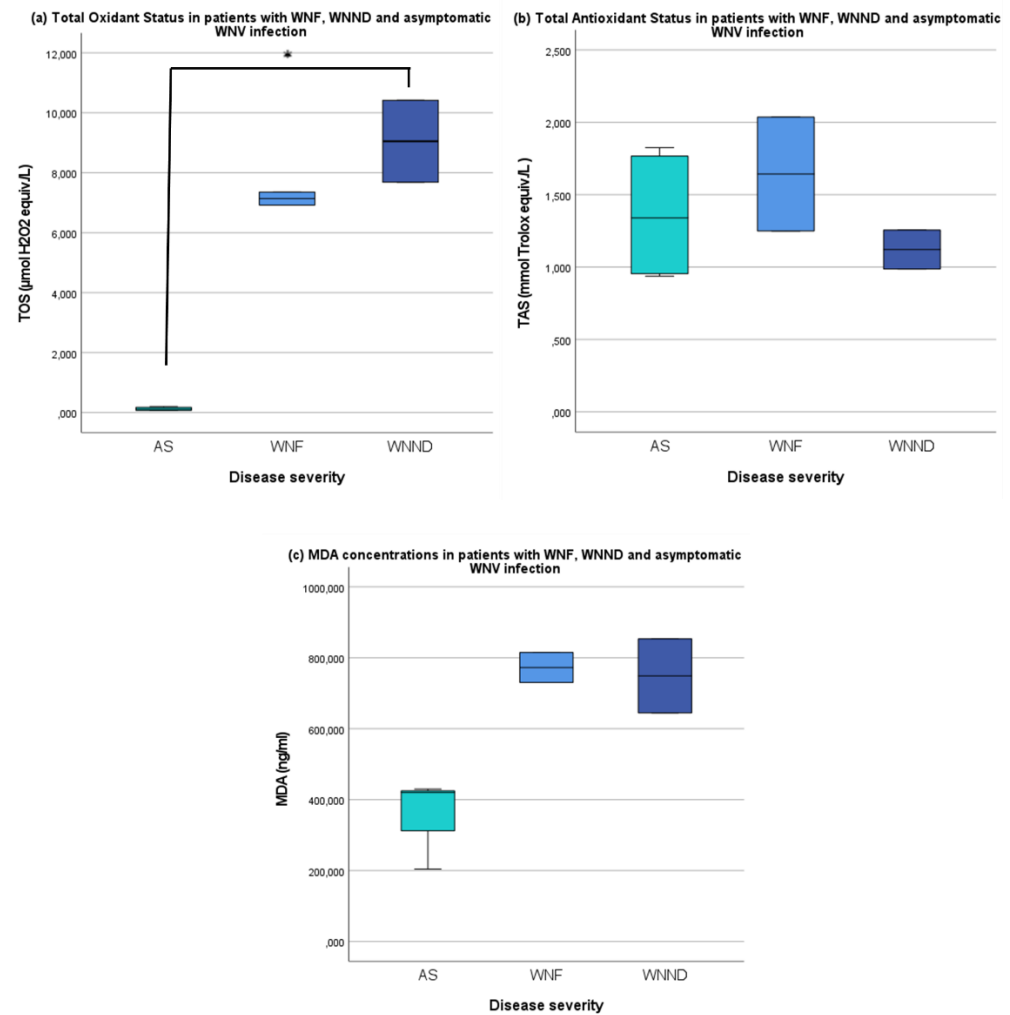
Table 1. Serum concentrations of markers of oxidative stress in 8 WNV-infected patients from Israel.

Patient	Age	Sex	WNV (Ct-value)	disease severity	TOS (μmol H2O2 equiv./L)	TAS (mmol Trolox equiv./L)	MDA (ng/mL)
1	84	female	32.63	WNND	10.42	1.26	644.44
2	11	female	41.25	WNND	7.69	0.99	853.63
3	23	female	38.38	WNF	7.36	2.04	730.30
4	43	female	33.13	WNF	6.92	1.25	814.99
5	59	female	36.64	AS	0.07	1.71	204.19
6	21	female	35.29	AS	0.07	0.97	^a
7	18	male	35.58	AS	0.20	1.83	420.26
8	20	male	37.50	AS	0.15	0.94	429.76

Legend. WNV = West Nile virus, Ct-value = cycle threshold value, WNND = West Nile Neuroinvasive Disease, WNF = West Nile Fever, AS = asymptomatic, TOS = Total Oxidant Status, H2O2 = hydrogen peroxide, TAS = Total Antioxidant Status, MDA = malondialdehyde, ^a = measurement omitted due to spurious results. Each value for TOS, TAS and MDA is the mean of 2 measurements.

TOS in the WNND and WNF patients was significantly higher than in AS; 9.05 (8.37 to 9.74) (median, IQR) and 7.14 (7.03 to 7.25) and 0.11 (0.07 to 0.19) μmol H2O2 equiv./L, respectively (p = 0.048) (Figure 1a). Median TAS was 1.12 (1.05 to 1.19), 1.64 (1.45 to 1.84) and 1.34 (0.95 to 1.80) mmol Trolox equiv./L, respectively (Figure 1b). TAS was slightly lower in the two WNND patients, but differences were less pronounced than with TOS or MDA. MDA concentrations were 749.04 (696.74 to 801.33) ng/mL in WNND, 772.65 (751.47 to 793.82) ng/mL in WNF and 420.26 (312.23 to 425.01) ng/mL in the AS group. While MDA levels showed a similar trend to TOS, differences were not statistically significant (p = 0.105) (Figure 1c).

Figure 1. Boxplots of TOS (a), TAS (b) and MDA levels (c) in WNV-infected patients from Israel, per disease severity subgroup



Legend. (a) TOS (median) 9.1, IQR [8.4- 9.7], 7.1 [7.0- 7.3] and 0.1 [0.1- 0.12] $\mu\text{mol H}_2\text{O}_2$ equiv./L in WNND, WNF and AS groups, respectively. TOS was significantly higher in the WNND and WNF groups ($p = 0.048$) compared to AS. (b) TAS (median) 1.1 [1.1- 1.2], 1.6 [1.5- 1.8] and 1.3 [1.0- 1.8] mmol Trolox equiv./L, respectively. TAS was lower in the WNND patients (not significant). (c) MDA concentrations were higher in WNND (median) 749.0 ng/mL [696.7-801.3] and in WNF 772.7 [751.5- 793.8] ng/mL than in AS (420.3 [312.2- 425.0] ng/mL. (not significant, $p = 0.105$).

AS = asymptomatic, WNF = West Nile Fever, WNND = West Nile Neuroinvasive Disease, TOS = Total Oxidant Status, H_2O_2 = hydrogen peroxide, MDA = malondialdehyde, TAS = Total Antioxidant Status. * = $p < 0.05$ (Kruskal-Wallis test)

4. Discussion

We observed a significant increase of TOS in WNF and WNND, compared to AS. The increased TOS, a summary parameter of the total oxidative capacity in a sample, suggests that oxidation is more prominent in WNF and WNND than in AS [12]. TOS did not discriminate between WNF and WNND in our pilot study, possibly because of the small sample size. However, the marked difference in TOS between symptomatic WNV disease and AS individuals calls for a detailed assessment of the redox status in WNV infections, and an investigation of more specific biomarkers than TOS as a potential predictor of severe WNV disease.

Whereas TOS does not necessarily reflect oxidative damage (OD) to the host, MDA, the most widely studied end-product of lipid peroxidation, is a highly relevant marker of OD in neuronal toxicity studies [7,13]. The observed histopathology of the brain in WNND resembles that of neurodegenerative diseases, where brain cell damage results from lipid

peroxidation [9,14]. This similarity may indicate that WNV-induced OS is an important contributor to the observed brain injury in WNND. We observed higher MDA levels in symptomatic patients, suggesting that lipid peroxidation was increased, although not significant at $\alpha=0.05$. Therefore, our pilot study does not allow us to conclude that disease severity is positively correlated with the extent of OD to the host.

Studies of more specific biomarkers of OS than TOS in dengue virus (another member of the *Flavivirus* genus) infections, detected significant differences in subgroups with varying disease severities. Protein carbonylation (PCO) was higher in dengue hemorrhagic fever (DHF) than in dengue fever in two studies by Soundravally *et al.* [15,16]. Patra *et al.* found higher intracellular ROS levels in erythrocytes of severely ill dengue patients than in patients with mild disease [17].

TAS levels did not follow the same distinctive trends as TOS and MDA, as shown in Figure 1. In other studies, TAS was significantly lower in patients with DHF and dengue shock syndrome, compared to uncomplicated dengue, but only later in the course of infection (day 5 and 7 after symptom onset) [18]. While TAS summarizes the total capacity of the antioxidant defense against ROS, TAS levels are more difficult to interpret because of a variety of confounders, such as dietary antioxidant intake and lifestyle [12]. Our results may indicate that the antioxidant capacity across different groups is comparable, but the investigation into the relationship between TAS and disease severity requires further study. Prospective, controlled studies are needed to establish if there is a correlation between the age-related declining efficiency of endogenous antioxidant systems and increased susceptibility to WNV-induced oxidative stress [19,20].

Because we studied single samples only, no changes of OS biomarkers over time could be established within the different severity subgroups. Longitudinal follow-up of WNV-infected patients is needed to study the intra-host kinetics of these biomarkers. Only markers that differentiate between patients at risk for WNND and patients with mild disease in the early stages of infection are likely to have diagnostic utility for clinical management.

Important limitations apply to our study. First, the small sample size limited statistical analysis. Second, while all samples were stored at -80°C immediately after being received in the lab, OS marker measurements are susceptible to pre-analytical variations, and we cannot exclude this to have occurred in our pilot study. Third, variations in the duration of infection at the time of sampling could account for the observed differences in biomarker assays between individual patients. Nonetheless, all patients in the acute, viremic stage of WNV infection with viral loads in a similar range (Ct-values 32.6-41.3). This duration could affect biomarker measurements. Still, we think these pilot results warrant further research into using more selective biomarkers for OS as potential predictors, reflecting its role in the neurological symptoms seen in WNF/WNND, and to see whether this OS could lie at the base of the observed pathology.

5. Conclusions

In our pilot study, we observed higher TOS-levels in WNF and WNND patients than in asymptomatic WNV-infected controls. Prospective studies are warranted to investigate the association of increased lipid peroxidation and declining total antioxidant capacity with disease severity in WNV infections. We are planning an adequately powered prospective controlled cohort study to evaluate the clinical relevance of an extensive panel of peripheral OS biomarkers as predictors of disease severity in patients with WNV and related *Flavivirus* infections. In the projected study, we will also look at intra-host kinetics of the selected OS biomarkers at pre-specified timepoints since symptom onset. The identification of OS biomarkers as predictors of disease severity and long-term outcomes of WNND, may assist physicians in the clinical management of patients with WNV infection.

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Data Availability Statement: All data generated or analyzed during this study are included in this published article.

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References

1. Petersen, L.; Brault, A.; Nasci, R. West Nile Virus: Review of the Literature. *Jama* **2013**, *310*, 308–315, doi:10.1001/jama.2013.8042.
2. Rizzoli, A.; Jiménez-Clavero, M.; Barzon, L.; Cordioli, P.; Figuerola, J.; Koraka, P.; Martina, B.; Moreno, A.; Nowotny, N.; Pardigon, N.; et al. The Challenge of West Nile Virus in Europe: Knowledge Gaps and Research Priorities. *Euro surveillance* **2015**, *20*, 21135.
3. Habarugira, G.; Suen, W.W.; Hobson-Peters, J.; Hall, R.A.; Bielefeldt-Ohmann, H. West Nile Virus: An Update on Pathobiology, Epidemiology, Diagnostics, Control and “One Health” Implications. *Pathogens* **2020**, *9*, 1–51, doi:10.3390/pathogens9070589.
4. Weatherhead, J.E.; Miller, V.E.; Garcia, M.N.; Hasbun, R.; Salazar, L.; Dimachkie, M.M.; Murray, K.O. Long-Term Neurological Outcomes in West Nile Virus-Infected Patients: An Observational Study. *Am J Trop Med Hyg* **2015**, *92*, 1006–1012, doi:10.4269/ajtmh.14-0616.
5. Zhang, Z.; Rong, L.; Li, Y.-P. Flaviviridae Viruses and Oxidative Stress: Implications for Viral Pathogenesis. *Oxid Med Cell Longev* **2019**, *2019*, 1409582, doi:10.1155/2019/1409582.
6. Gil, L.; Martínez, G.; Tápanes, R.; Castro, O.; González, D.; Bernardo, L.; Vázquez, S.; Kourí, G.; Guzmán, M.G. Oxidative Stress in Adult Dengue Patients. *Am J Trop Med Hyg* **2004**, *71*, 652–657, doi:10.4269/ajtmh.2004.71.652.
7. Soundravally, R.; Hoti, S.L.; Patil, S.A.; Cleetus, C.C.; Zachariah, B.; Kadhiravan, T.; Narayanan, P.; Kumar, B.A. Association between Proinflammatory Cytokines and Lipid Peroxidation in Patients with Severe Dengue Disease around Defervescence. *International Journal of Infectious Diseases* **2014**, *18*, 68–72, doi:10.1016/j.ijid.2013.09.022.
8. Koh, W.-L.; Ng, M.-L. Molecular Mechanisms of West Nile Virus Pathogenesis in Brain Cells. *Emerging Infectious Diseases* **2005**, *11*, 629–632, doi:10.3201/eid1104.041076.
9. Dimitrion, P.; Zuo, L.; Zhou, T.; Ziegler, A.C.; Liu, Z. Oxidative Stress in Neurodegenerative Diseases: From Molecular Mechanisms to Clinical Applications. *Oxidative Medicine and Cellular Longevity* **2017**, *2017*, 1–11, doi:10.1155/2017/2525967.

10. Lanciotti, R.S.; Kerst, A.J.; Nasci, R.S.; Godsey, M.S.; Mitchell, C.J.; Savage, H.M.; Komar, N.; Panella, N.A.; Allen, B.C.; Volpe, K.E.; et al. Rapid Detection of West Nile Virus from Human Clinical Specimens, Field-Collected Mosquitoes, and Avian Samples by a TaqMan Reverse Transcriptase-PCR Assay. *J Clin Microbiol* **2000**, *38*, 4066–4071, doi:10.1128/JCM.38.11.4066-4071.2000.
11. Linke, S.; Ellerbrok, H.; Niedrig, M.; Nitsche, A.; Pauli, G. Detection of West Nile Virus Lineages 1 and 2 by Real-Time PCR. *Journal of Virological Methods* **2007**, *146*, 355–358, doi:10.1016/j.jviromet.2007.05.021.
12. Wu, R.; Feng, J.; Yang, Y.; Dai, C.; Lu, A.; Li, J.; Liao, Y.; Xiang, M.; Huang, Q.; Wang, D.; et al. Significance of Serum Total Oxidant/Antioxidant Status in Patients with Colorectal Cancer. *PLoS ONE* **2017**, *12*, doi:10.1371/journal.pone.0170003.
13. Katerji, M.; Filippova, M.; Duerksen-Hughes, P. Approaches and Methods to Measure Oxidative Stress in Clinical Samples: Research Applications in the Cancer Field. *Oxidative Medicine and Cellular Longevity* **2019**, *2019*, doi:10.1155/2019/1279250.
14. Grubaugh, N.D.; Massey, A.; Shives, K.D.; Stenglein, M.D.; Ebel, G.D.; Beckham, J.D. West Nile Virus Population Structure, Injury, and Interferon-Stimulated Gene Expression in the Brain from a Fatal Case of Encephalitis. *Open Forum Infectious Diseases* **2016**, *3*, doi:10.1093/ofid/ofv182.
15. Soundravally, R.; Sankar, P.; Bobby, Z.; Hoti, S.L. Oxidative Stress in Severe Dengue Viral Infection: Association of Thrombocytopenia with Lipid Peroxidation. *Platelets* **2008**, *19*, 447–454, doi:10.1080/09537100802155284.
16. Rajendiran, S.; Lakshamanappa, H.S.; Zachariah, B.; Nambiar, S. Desialylation of Plasma Proteins in Severe Dengue Infection: Possible Role of Oxidative Stress. *Am J Trop Med Hyg* **2008**, *79*, 372–377.
17. Patra, G.; Saha, B.; Mukhopadhyay, S. High Titres of IgM-Bound Circulating Immune Complexes and Erythrocytic Oxidative Damage Are Indicators of Dengue Severity. *Clin Exp Immunol* **2019**, *198*, 251–260, doi:10.1111/cei.13346.
18. Soundravally, R.; Sankar, P.; Bobby, Z.; Hoti, S.L. Oxidative Stress in Severe Dengue Viral Infection: Association of Thrombocytopenia with Lipid Peroxidation. *Platelets* **2008**, *19*, 447–454, doi:10.1080/09537100802155284.
19. Montgomery, R.R. Age-Related Alterations in Immune Responses to West Nile Virus Infection. *Clinical and Experimental Immunology* **2017**, *187*, 26–34, doi:10.1111/cei.12863.
20. Tan, B.L.; Norhaizan, M.E.; Liew, W.P.P.; Rahman, H.S. Antioxidant and Oxidative Stress: A Mutual Interplay in Age-Related Diseases. *Frontiers in Pharmacology* **2018**, *9*, 1–28, doi:10.3389/fphar.2018.01162.