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ORIGINAL ARTICLE

# Assessing the role of Ndel1 oligopeptidase activity in congenital Zika syndrome: Potential predictor of congenital syndrome endophenotype and treatment response

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## Abstract

Maternal infections are among the main risk factors for cognitive impairments in the offspring. Zika virus (ZIKV) can be transmitted vertically, causing a set of heterogeneous birth defects, such as microcephaly, ventriculomegaly and corpus callosum dysgenesis. Nuclear distribution element like-1 (Ndel1) oligopeptidase controls crucial aspects of cerebral cortex development underlying cortical malformations. Here, we examine Ndel1 activity in an animal model for ZIKV infection, which was associated with deregulated corticogenesis. We observed here a reduction in Ndel1 activity in the forebrain associated with the congenital syndrome induced by ZIKV isolates, in an in utero and postnatal injections of different inoculum doses in mice models. In addition, we observed a strong correlation between Ndel1 activity and brain size of animals infected by ZIKV, suggesting the potential of this measure as a biomarker for microcephaly. More importantly, the increase of interferon (IFN)-beta signaling, which was used to rescue the ZIKV infection outcomes, also recovered Ndel1 activity to levels similar to those of uninfected healthy control mice, but with no influence on Ndel1 activity in uninfected healthy control animals. Taken together, we demonstrate for the first time here an association of corticogenesis impairments determined by ZIKV infection and the modulation of Ndel1 activity. Although further studies are still necessary to clarify the possible role(s) of Ndel1 activity in the molecular mechanism(s) underlying the congenital syndrome induced by ZIKV, we suggest here the potential of monitoring the Ndel1 activity to predict this pathological condition at early stages of embryos or offspring development, during while the currently employed methods

**Abbreviations:** CMV, cytomegalovirus; CZS, congenital zika virus syndrome; DISC1, *Disrupted-in-Schizophrenia 1*; FBS, fetal bovine serum; LIS1, lissencephaly 1; NDE1, nuclear distribution factor E-homolog 1; Ndel1, nuclear distribution element-like 1; ReLAMC, Latin American Network of Congenital Malformations; ZIKV, zika virus.

Raissa R. Christoff and João V. Nani co-authors contributed equally to this work.

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are unable to detect impaired corticogenesis leading to microcephaly. Ndel1 activity may also be possibly used to follow up the positive response to the treatment, such as that employing the IFN-beta that is able to rescue the ZIKV-induced brain injury.

**KEYWORDS**
congenital zika virus syndrome, microcephaly, Ndel1, oligopeptidase, zika virus

## 1 | INTRODUCTION

A collection of diverse etiologies can culminate in microcephaly or in other brain damages during pregnancy, including those triggered by infections, such as toxoplasmosis, rubeola, cytomegalovirus, herpes simplex, as well as the zika virus (ZIKV). Although ZIKV was first discovered in 1947 (in Uganda, Africa), little attention was paid to this specific virus until ZIKV has attracted a global audience during the Summer Olympics Game hosted in Brazil in 2016, because of the relatively high incidence of Congenital Zika Syndrome (CZS) that encompass several birth defects including microcephaly (Vue and Tang, 2021). The Latin American Network of Congenital Malformations (ReLAMC) was established in 2017, following the Summer Olympics Game hosted in Brazil in 2016, to provide accurate surveillance of congenital anomalies. Since then, the prevalence of microcephaly in Brazil increased from 0.6 per 10 000 (95% CI 0.5–0.6) in 2010–2014 to 5.8 (95% CI 5.6–6.1) in 2015, and 8.0 (95% CI 7.6–8.3) in 2016, showing a clear increase in the incidence of microcephaly and birth defects among women giving birth in Brazil, and with an abrupt decrease only in 2017 (Morris et al., 2021). The economic burden of CZS over 10 years was reported to be high, with incremental economic burden in Brazil of about US$69.4 million and US$129.0 million from the household and government perspective, respectively (Fernandes et al., 2022).

ZIKV is a flavivirus that can be primarily transmitted by the mosquito vector or to a lesser extent by sexual transmission. After crossing the placental barrier, ZIKV predominantly affects neuronal progenitor cells (van der Linden Jr. et al., 2022). Moreover, ZIKV can disrupt the centrosome organization leading to mitotic abnormalities, which alters neural progenitor differentiation and can lead to cell cycle arrest, increased apoptosis and inhibition of neural progenitor cell differentiation. Together, these abnormalities ultimately result in microcephaly (Vue and Tang, 2021). In prenatal ZIKV infection rat model, adult offspring that were prenatally infected with ZIKV failed to develop a normal interferon (IFN) response to a viral immune challenge, resulting in lasting consequences that could significantly impact the health of offspring later in life (Sherer et al., 2021). The immune evasion by ZIKV involves non-structural proteins from virus, which modulate IFN signaling and production, and it can be critically strengthened by subverting the host immunity (Estévez-Herrera et al., 2021).

Neurotropic viral pathogens can also evoke inflammatory responses (Lima et al., 2019), and the maternal inflammatory responses to these neurotrophic pathogens play a significant role in negatively affecting neurodevelopment, increasing the risk of various neurodevelopmental disorders, including microcephaly, schizophrenia, autism spectrum disorder, cerebral palsy, epilepsy and among others (Ganguli & Chavali, 2021). Hereupon, we evaluate in this work a biochemical biomarker, namely Ndel1 (nuclear distribution element-like 1), which was demonstrated to play crucial roles in neuronal differentiation, neuron migration and brain formation during embryogenesis (Bradshaw & Hayashi, 2017; Hayashi et al., 2015).

Ndel1 has unique features which include a dual role as an enzyme (oligopeptidase activity) and as a ligand protein important for the formation of complexes with several other cytosolic cytoskeleton proteins, which were demonstrated to be involved in the neurite outgrowth, neuronal precursor proliferation and differentiation, neuronal migration and brain formation (Bradshaw & Hayashi, 2017; Hayashi et al., 2010). Indeed, nuclear distribution factor E-homolog 1 (NDE1), lissencephaly 1 (LIS1), *Disrupted-in-Schizophrenia* 1 (DISC1), as well as Ndel1 participate together in processes essential for neurodevelopment (Nani et al., 2021). Ndel1 is also involved in the regulation of microtubule organization, while only NDE1 was found substantially enriched at the centrosome during mitosis, showing parallel but distinct roles for NDE1 and Ndel1 with impacts on neurodevelopmental disorders and psychiatric illnesses (Bradshaw & Hayashi, 2017). Additionally, mutations in the *NDE1* gene were previously associated with severe cases of microcephaly (Lipka et al., 2013; Paciorkowski et al., 2013), and experiments with animal models have demonstrated that conditional knockout of *NDE1* or *Ndel1* inhibits postmitotic neuronal migration (Doobin et al., 2016). Interestingly, Ndel1 oligopeptidase activity is competitively inhibited by its interaction with DISC1 protein and, in physiological condition, about 98% of Ndel1 present in brain are forming complexes with DISC1 and/or other proteins, such as LIS1 and NDE1 (Hayashi et al., 2005). We could also demonstrate a significantly lower Ndel1 activity in antipsychotic-naïve first episode psychosis individuals and in medicated patients with chronic schizophrenia, suggesting Ndel1 enzyme activity as a potential biomarker of early stages and/or of treatment resistance in schizophrenia (Dal Mas, Carvalho, et al., 2019; Gadelha et al., 2013; Rodríguez et al., 2020).

DISC1 was initially associated with the risk to develop schizophrenia, and the interaction of Ndel1 and DISC1 was demonstrated to underlie the neurite outgrowth and brain formation, reinforcing the neurodevelopmental hypothesis of schizophrenia. Moreover, a transgenic animal model over-expressing a full-length non-mutated human

DISC1 was used to demonstrate an association between the neurodevelopmental impairments and dysfunctional dopamine responses (Bader et al., 2016; Dahoun et al., 2017; Trossbach et al., 2016). Studies with this transgenic rat model allowed us to state that decreased Ndel1 activity reflects both a trait (neurodevelopmental phenotype) and a state (amphetamine-induced dopamine release) features, suggesting a role for decreased Ndel1 oligopeptidase activity both for the developing (the neurodevelopmental phenotype) and for the adult (interaction with dopaminergic responses) brain, unifying the neurodevelopmental features with dysfunctional dopamine responses in vivo (Nani, Fonseca, et al., 2020). Interestingly, there are no reports suggesting the inflammatory impairments in this specific animal model, as well as we did not observe any sign of neuroinflammation in a study employing the positron emission tomography (PET) and the radiopharmaceutical $^{11}$C-PK11195 (data not published), which is a selective ligand for the 18 kDa translocator protein expressed in the outer mitochondrial membrane of activated microglia and macrophages (Chauveau et al., 2008), although the clear demonstration of impaired neuronal migration association with lower Ndel1 activity (Nani, Fonseca, et al., 2020).

As ZIKV infection during pregnancy is linked to birth defects, most notably to microcephaly, which is associated with neurodevelopmental delays, we have employed in this work an animal model for ZIKV infection in mice to evaluate the potential use of Ndel1 oligopeptidase activity as a surrogate biomarker for CZS.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

The animals employed in this study were Swiss mice embryos at 13.5 embryonic days (E13.5) or newborn mice (day 0), of either sex from institutional facility (CCS—UFRJ). Pregnant Swiss mice were maintained in standard animal housing in 21 × 32 × 20 cm cages (4 mice per cage) with food and water ad libitum and circadian cycles of 12 h of light/dark each, housed in the Animal Care Facility of the Microbiology Institute of the Federal University of Rio de Janeiro. One male and two females were housed together for mating for 24 h (6–8 weeks old). Pregnancy was confirmed through observation of the postcoital vaginal plug, and embryos were considered E0. Pregnant females were euthanized by cervical dislocation after anesthesia. Embryos and pups were euthanized by decapitation. Groups were treated and assessed in an arbitrary order. Experiments were performed in the morning. Animals' general well-being was access by using a numerical score to classify the condition of the animal through the following parameters: body weight, general appearance, posture, behavior/activity and breathing pattern. Animals were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA. The Ethical Committee of the Universidade Federal do Rio de Janeiro (UFRJ) approved this study under protocol #A06/22-153-19, and the Universidade Federal de São Paulo (UNIFESP/EPM) approved this study under CEUA No 4337070322. No randomization or blinding methods were used for the animals.

### 2.2 | ZIKV propagation and titration

A Brazilian ZIKV strain (Recife/Brazil, ZIKV PE/243, GenBank accession number: KX197192.1) was used in the experiments. The virus was propagated in C6/36 cells. Cells were inoculated with ZIKV at a multiplicity of infection (MOI) of 0.1 and incubated at 28°C for 1 h. Next, the inoculum was removed and replaced with growth media supplemented with 2% fetal bovine serum (FBS), and cultured for further 5 days. The conditioned medium was harvested, centrifuged at 300 *g* and sterile-filtered to remove cells and cellular debris. Virus stocks were stored at −80°C. ZIKV titers were determined by plaque assay performed on Vero cells, as previously described (Contreras & Arumugaswami, 2016; Garcez et al., 2016). As a negative control, the conditioned medium of uninfected C6/36 (prepared exactly as performed for viral propagation) was used to MOCK-infect cells.

### 2.3 | ZIKV infection

For embryonic infection, pregnant dams were anesthetized with a dosage of 100 mg/kg ketamine and 8 mg/kg xylazine by intraperitoneal (IP) route. After loss of foot reflex, the uterus was exposed, so that the virus could be injected into the lateral ventricle of mouse brains at embryonic day E13.5. Embryonic brains were either MOCK- or ZIKV-infected with 30 plaque forming unit (PFU) (in a volume of 1.5 μL). After surgery, mice were placed on a heating pad until recovery of anesthesia. Brains were collected at E18 for analyses (Figure 1).

Postnatally, mice were also infected by intracerebroventricular (ICV) route. At postnatal day 0 (P0), mice were injected with 30 PFU of ZIKV or MOCK after anesthesia induced by hypothermia. Brains were collected at P3, P8 and P12. Mice were also injected at P3 with $10^4$ PFU and harvested at P3. In addition, P0 mice were injected by IP route with 100 PFU of ZIKV or MOCK and harvested at P7.

### 2.4 | Viral detection

Embryonic brains were weighted and mechanically homogenized in a T-10 basic Ultra-Turrax instrument (IKA) in 500 μL of PBS. Brain tissue homogenates were centrifuged at 3400 *g* for 5 min, and the supernatant was collected. RNA was extracted from the embryonic brain tissue homogenates using the RNeasy Plus Mini Kit (Qiagen), following the recommendations of the manufacturer. To quantify viral RNA, we used One Step TaqMan RT-qPCR (Thermo Fisher Scientific) on a 7500 Real-Time PCR System (Applied Biosystems, RRID:SCR_018051), with primers and probe as previously described

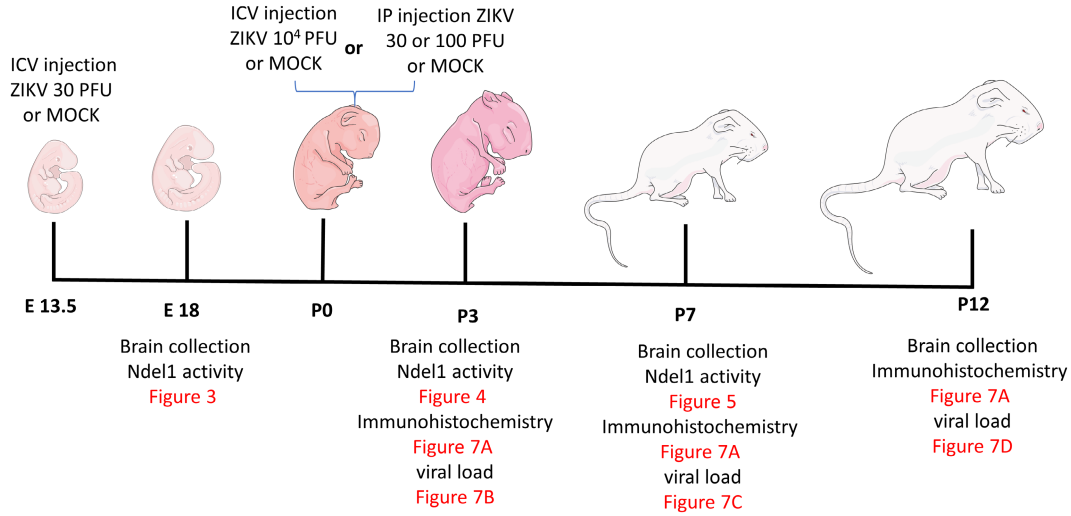



**FIGURE 1** Schematic representation indicating the MOCK or ZIKV injections in mice by intracerebroventricular (ICV) or intraperitoneal (IP) routes. Animals received 30, 100 or $10^4$ PFU of ZIKV, and the brains were collected for Ndel1 activity measures or for immunohistochemistry analysis as presented in the indicated figures. The viral load was also evaluated by RT-qPCR.

(Lanciotti et al., 2008). The ZIKV quantification was expressed as ZIKV RNA copies per *g* of tissue.

## 2.5 | IFN treatment

P0 mice were infected with 30 PFU via ICV and 2-, 4- and 6-days post infection (dpi), pups were treated with 10 μg/mL interferon-beta (IFNb, R&D SYSTEMS number 8234-MB) in a volume of 1 μL injected in the lateral ventricle (Figure 2).

No exclusion criteria were predetermined. Infected animals that died before brain collection were excluded and not analyzed. A total of six infected animals died before the last brain collection at P12. For embryonic infection, a total of four embryos died and were not collected.

## 2.6 | Immunohistochemistry

Postnatal brains were fixed with 4% paraformaldehyde and cut coronally at 70 μm at vibratome (Vibratome VT1000S Leica Microsystems, RRID:SCR_016495) and stored in 0.01% PBS. After standard antigenic retrieval, coronal sections were permeabilized with 0.1% Triton X-100 (Sigma–Aldrich) and incubated with 3% bovine serum albumin (Sigma–Aldrich) for 2 h. Next, rabbit anti-Caspase3 1:300 (Cell signaling 9664); anti-SATB2 (Abcam Cat# ab34735, RRID:AB_2301417) and mouse anti-NS1 primary antibodies were incubated overnight. Then, samples were washed with PBS and incubated with secondary antibodies: goat anti-rabbit Alexa Fluor 488 1:500 AP132JA4 (Millipore Corporation) and goat anti-mouse Alexa 546 1:500 (Millipore Cat# AP192SA6, RRID:AB_2687879). Nuclei were stained with DAPI (0.5 mg/mL) for 20 min. Images were acquired with a TCS SP8 confocal microscope (Leica) with an oil immersion 40× objective of high-numerical apertures. Analyses of the acquired images were carried out using Fiji software (RRID:SCR_002285).

## 2.7 | Brain dissection and tissue homogenate preparation

In embryonic infected mice, brains were harvested at E18, and the forebrain was dissected and collected for further analysis. Also, for P0 mice infected via ICV route, the forebrain region was dissected and collected. For mice injected via IP, the whole brain was collected by the group in the Federal University of Rio de Janeiro (UFRJ). These samples were identified with a code and stored at −80°C until the shipment in dry ice to the Federal University of São Paulo (UNIFESP), where the Ndel1 activity was measured by different experimenters who were blinded to the samples. The brain samples were weighted and defrost, before the preparation of the homogenates following the previously described procedure (Nani, Fonseca, et al., 2020). The identification of the samples was opened to the experimenter responsible for the Ndel1 activity only after the enzyme activity measurements were concluded.

## 2.8 | Ndel1 enzyme activity measurements

Ndel1 enzyme activity was measured essentially as described (Nani, Fonseca, et al., 2020). The fluorescence resonance energy transfer (FRET) substrate (Abz-GFSPFRQ-EDDnp) was employed, and the increase of fluorescence as a result of the cleavage of the substrate by the Ndel1 oligopeptidase was measured in F-7000 fluorimeter (Hitachi Ltd.). The concentration of protein was determined by employing the Bradford Protein Assay (BioRad) essentially as indicated by the manufacturer, with measurements at 595 nm. The bovine

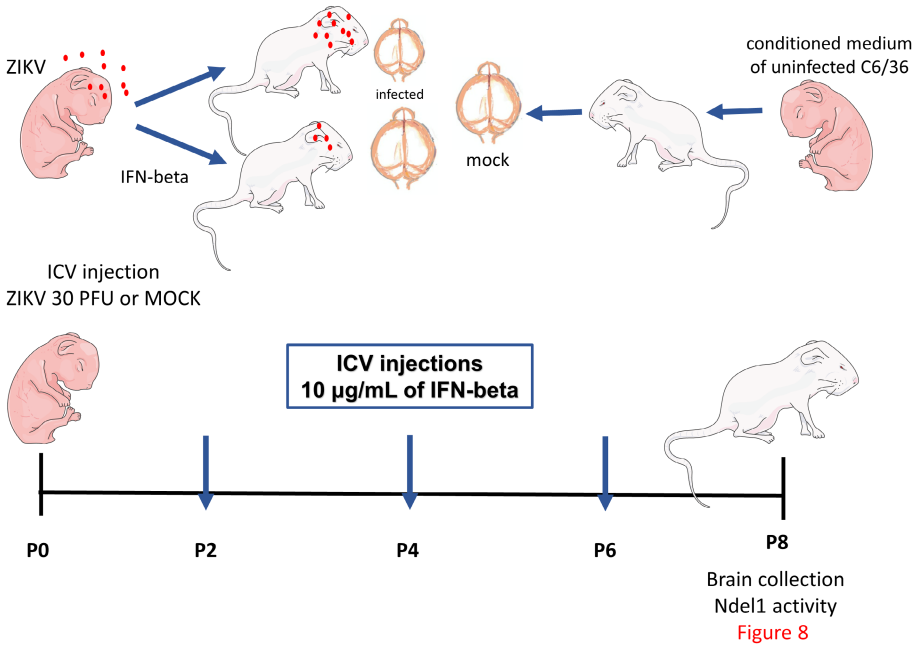



FIGURE 2 Schematic figure showing the treatment protocol following the MOCK or ZIKV injections in mice by intracerebroventricular (ICV) route. Animals received 30 PFU of ZIKV or MOCK, and then received three doses of 10 µg/mL interferon-beta (IFNb) each as indicated. The brains of the animals were collected for Ndel1 activity measurements at the end of the treatment.

serum albumin was used as control for the calibration curve preparation. The Ndel1 specific activity was determined by employing the polyclonal antibody against Ndel1 to confirm the Ndel1 specific activity, as previously described (Hayashi et al., 2000).

## 2.9 | Statistical analysis

The total number of 64 animals was used in this work, with a minimum sample size of five animals per group, which was estimated based on previous studies measuring Ndel1 activity in different animal models (Nani, Fonseca, et al., 2020; Nani, Lee, et al., 2020). All distributions were verified by the Shapiro–Wilk test, and homogeneity for Levenne's test. Data analysis was performed using JAMOVI version 2.39. Parametric tests (Student-*t* test, ANOVA and Spearman's correlation) were used according to the distribution of variables, and Welch correction was used for variables that did not suit homogeneity. *Z*-score transformation method was used for identification of outliers (±2 standard deviation). Effect size was determined as $g_{hedge}$ to overcome the upward bias when estimate effect size in small samples ($N < 20$) (McCormack & McLeod, 2008). For ANOVA analysis, partial eta square was determined. Graphs of Ndel1 activity values and correlations were prepared using GraphPad Prism version 7.0.

# 3 | RESULTS

First, to examine the Ndel1 activity in a mouse model of Congenital Zika Syndrome (CZS), we injected in utero 30 plaque forming units (PFU) of ZIKV or vehicle (MOCK) directly into the forebrain

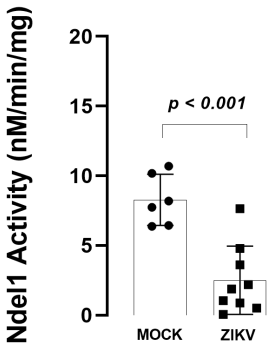



FIGURE 3 Ndel1 oligopeptidase activity in the forebrain collected at E18 from MOCK and ZIKV infected mice by intracerebroventricular (ICV) route at E13 with 30 PFU. Animals received 30 PFU of ZIKV (■) or vehicle (MOCK) (•) by ICV route at E13, and the brains were collected at E18. The Ndel1 activity was determined in samples of homogenates freshly prepared from the forebrain region, and the data are presented as nM/min/mg of total protein. Differences were considered significant for values of $p \leq 0.05$, for Student *t*-test.

of embryos at the neurogenesis peak (E13), and the embryos were harvested 5 days after injection (at E18). A significant lower Ndel1 activity was observed in forebrain region of ZIKV infected E18 mice embryos (2.59 ± 2.61 nM/min/mg) compared to those from MOCK group animals (8.27 ± 1.83 nM/min/mg) ($t = 4.55$, $p < 0.001$, $df = 13$) (Figure 3). A strong effect size was estimated for Ndel1 activity modulation following the infection by this route ($g_{hedges} = 2.46$), suggesting significative decreases in Ndel1 activity following ZIKV infection during the forebrain's neurogenesis stage. To investigate whether Ndel1 activity is affected during a differentiation stage of forebrain

development, animals were also injected by intracerebroventricular (ICV) route with $10^4$ PFU of ZIKV or vehicle (MOCK) at P0, and then harvested at P3.

Following injection by ICV route with $10^4$ PFU of ZIKV at P0, a significant difference between the infected (3.12 ± 1.12 nM/min/mg) and MOCK group animals (7.72 ± 3.17 nM/min/mg) was also observed at P3 ($t = 3.317$, df = 10, $p = 0.007$), therefore, showing a significant lower Ndel1 activity following ZIKV infection and with a strong size effect ($g_{hedges} = 1.93$) (Figure 4a). Interestingly, a moderate correlation was also observed between Ndel1 activity and the brain size, as estimated by considering the whole tissue weight ($r = 0.637$, $p = 0.034$) (Figure 4b).

Now, aiming to verify if Ndel1 activity was also modulated by lower loads of ZIKV in postnatal infection, animals at P0 were injected by intraperitoneal (IP) route with 100 PFU or vehicle (MOCK), and then harvested at P7, showing a significant lower Ndel1 in infected animals (4.81 ± 2.08 nM/min/mg) compared with MOCK group animals (10.7 ± 3.66 nM/min/mg). This lower Ndel1 activity was statistically significant ($t = 3.11$, $p = 0.014$, df = 8), and with a strong estimated size effect ($g_{hedges} = 0.91$) (Figure 5a).

More importantly, a significant and strong correlation were also observed between the Ndel1 activity and the whole brain size collected at P7 from the animals injected at P0 with 100 PFU ZIKV by IP route ($r = 0.818$, $p = 0.004$; Figure 5b).

A significant decrease in Ndel1 activity was also observed for the ZIKV group ($t = 5.67$, df = 33.7, $p < 0.001$) when all ZIKV and MOCK animals prepared with different virus loads, development stage and infection route were compared together (Figure 6a). In

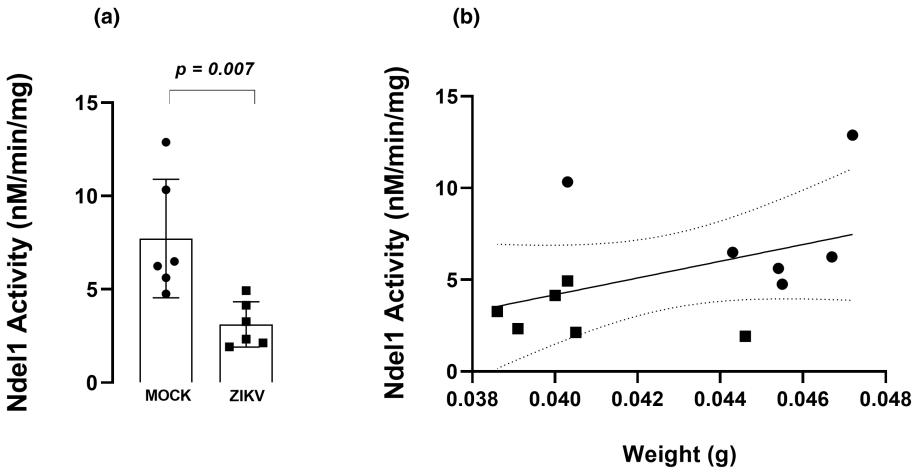



FIGURE 4 Ndel1 oligopeptidase activity in forebrain collected at P3 from MOCK and ZIVK infected mice by intracerebroventricular (ICV) route in mice at P0 with $10^4$ PFU. ZIKV ($10^4$ PFU) (■) or vehicle (MOCK, •) was injected by ICV route in mice at P0, and the brains were collected at P3. (a) Ndel1 activity was determined in sample homogenates freshly prepared, and the data are presented as nM/min/mg of total protein. (b) The weight of the brains (g) was also determined, and these values were plotted against the Ndel1 activity. Differences were considered significant for values of $p \leq 0.05$, for Student *t*-test and Spearman correlation.

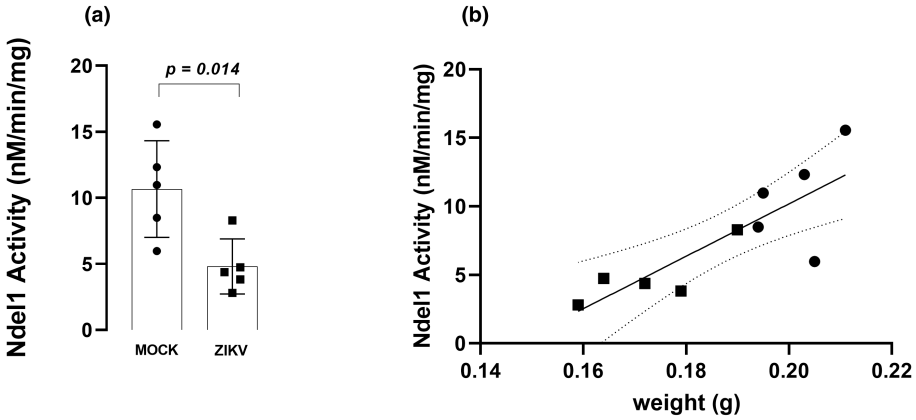
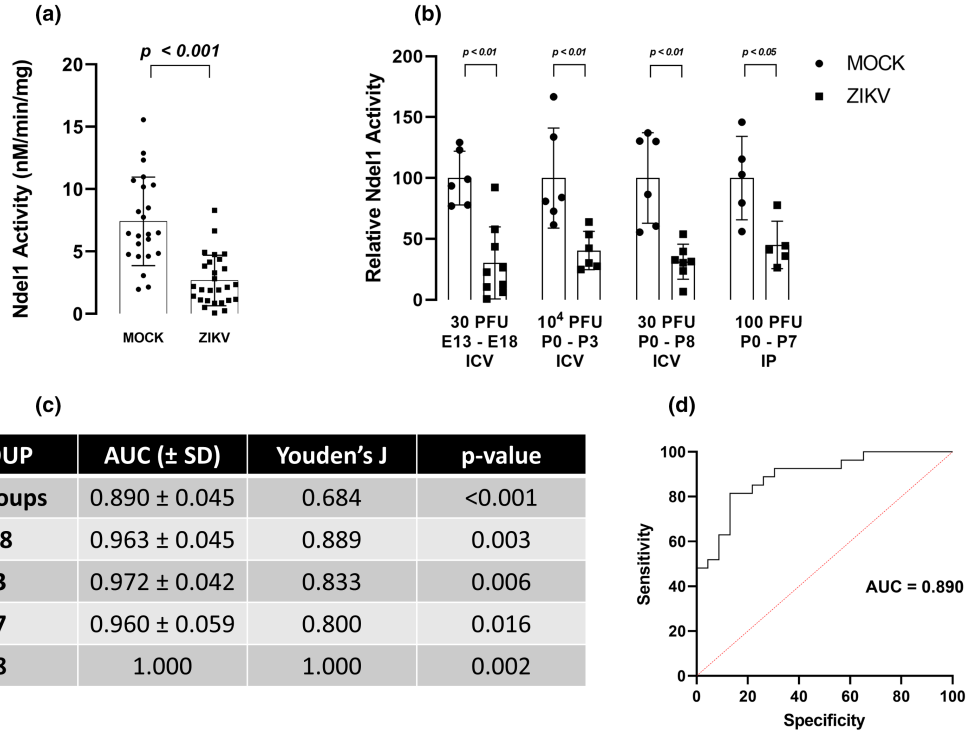



FIGURE 5 Ndel1 oligopeptidase activity in the whole brain collected at P7 from MOCK and Zika virus (ZIVK)-infected mice by intraperitoneal (IP) route in mice at P0 with 100 PFU. ZIKV (100 PFU) (■) or vehicle (MOCK •) was injected by intraperitoneal (IP) route in mice at P0, and the brains were collected at P7. (a) Ndel1 activity was determined in sample homogenates freshly prepared, and the data are presented as nM/min/mg of total protein. (b) The weight of the brain (g) was also determined, and these values were plotted against the Ndel1 activity. Differences were considered significant for values of $p \leq 0.05$, for Student *t*-test and Spearman correlation.




| GROUP | AUC (± SD) | Youden's J | p-value |
|---|---|---|---|
| All groups | 0.890 ± 0.045 | 0.684 | <0.001 |
| E18 | 0.963 ± 0.045 | 0.889 | 0.003 |
| P3 | 0.972 ± 0.042 | 0.833 | 0.006 |
| P7 | 0.960 ± 0.059 | 0.800 | 0.016 |
| P8 | 1.000 | 1.000 | 0.002 |

**FIGURE 6** Ndel1 oligopeptidase activity in the brain of MOCK- and ZIKV-infected mice by different routes, at different developmental stages and inoculum doses. (a) Animals received 30, 100 or $10^4$ PFU of ZIKV (■) or vehicle (MOCK, •) by intracerebroventricular (ICV) or intraperitoneal (IP) routes at E13 or P0, and the brain of the animals was collected at E18 or at P3-8. (b) Lower Ndel1 activity of all infected animals independent of age or viral load relative to MOCK controls. (c) Values of area under de curve (AUC) of receiving operating characteristic (ROC) curve for each developmental stage and inoculum doses evaluated here. (d) ROC curve with AUC value for Ndel1 activity of all samples from infected animals independent of age or viral load relative to MOCK controls. Ndel1 activity was determined in sample homogenates freshly prepared from the forebrain region as described in Methods, and the data are presented as nM/min/mg of total protein normalized relative to the average Ndel1 activity of each respective MOCK group (considered as 100% of activity). The differences were considered significant for values of $p \leq 0.05$, for two-way ANOVA.

addition, the combination of all these data allowed us to perform a comparative analysis after the normalization of the data considering the mean value of each MOCK group as 100%, leading us to observe that all groups infected by ZIKV exhibited lower Ndel1 activity with large size effect ($\eta^2 p = 0.591$) compared to MOCK control group (two-way ANOVA $F(1, 42) = 60.66$, $p < 0.001$), employing *post-hoc* analysis by Sidak's multiple comparisons test and thus showing no significant effect of inoculum dose, administration route or animal age/development stage for the decreases in Ndel1 activity following the ZIKV infection ($F(3, 42) = 0.189$, $p = 0.9031$; Figure 6b). The area under the curve (AUC) for the Receiver Operating Characteristic (ROC) curve for the diagnostic ability of a binary classifier system considering Ndel1 enzyme activity and ZIKV/MOCK status as outcome was determined as $0.963 \pm 0.045$; $0.972 \pm 0.042$; $0.960 \pm 0.059$; and 1, for ZIKV infected animals at E18 (ICV injection with 30 PFU at E13); P3 (ICV injection with $10^4$ PFU at P0); P7 (ICV injection with 30 PFU at P0) and P8 (IP injection with 100 PFU at P0), respectively (Figure 6c). Interestingly, we also generated a ROC curve to better estimate the usefulness of this measurement to differentiate ZIKV/MOCK status, for all these samples analyzed together, independent of mice age, viral load, injection route or animal age at infection, considering ZIKV/MOCK status, and an AUC of $0.890 \pm 0.045$ was determined for the diagnostic ability considering a binary classifier system (Figure 6d). In addition, the Youden J index, which is a measure of diagnostic accuracy that takes into account both sensitivity and specificity, was calculated for each analysis and yielded values greater than 0.650, with *p*-value lower than 0.05, indicating a strong discriminatory power of Ndel1 enzyme activity as a biomarker for ZIKV infection at any stage or condition analyzed here.

The cerebral cortex of animals infected with 30 PFU of ZIKV by ICV route at P0 was immunostained for the apoptotic marker cleaved caspase-3 (CASP3+) and an anti-ZIKV non-structural protein 1 (anti-NS1), at three different time points. At P3, there was no apparent immunostaining in the cerebral cortex of infected animals for either CASP3+ or NS1. However, after 7 days post-infection, CASP3+/NS1+ were present in the cerebral cortex, with higher expression observed at P12. The viral load was quantified using RT-qPCR in animals infected by ICV (30 and $10^4$ PFU) or IP route (100 PFU). In all cases, an increase in viral replication was observed over time in the embryonic brain of animals infected at P0 and harvested at P3, P7 or P12 (Figure 7). Interestingly, animals at P3 that still did not exhibit staining for the viral protein NS1 or cell death also had lower Ndel1 activity, supporting the idea of using this enzyme activity as

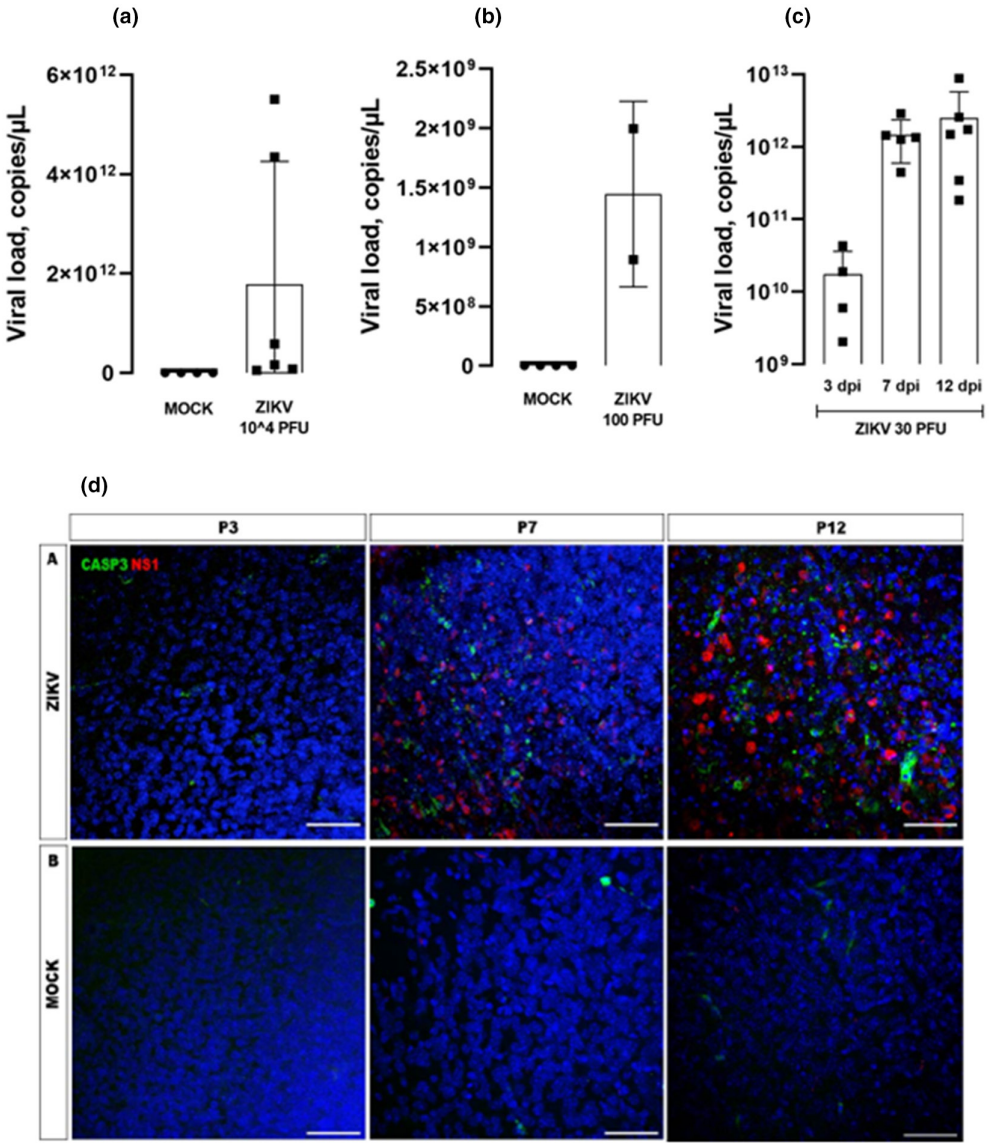



**FIGURE 7** Progression of ZIKV infection. Animals were infected at P0 with 30 PFU of ZIKV or MOCK by intracerebroventricular (ICV) and harvested at P3, P7 and P12. Viral load was quantified by RT-qPCR on brain of animals (a) infected at P0 with $10^4$ by ICV and harvest at P3; (b) infected with 30 PFU at P0 by ICV and harvest at P3, P7 and P12; (c) infected at P0 with 100 PFU by intraperitoneal (IP) and harvest at P7; (d) Immunofluorescence labelling for NS1 (red), active-caspase3 (CASP3) (green), and DAPI staining (blue) (a) ZIKV-infected and (b) MOCK cerebral cortex. Scale bar = 50 µm.

an early biomarker for predicting impaired neuronal formation and/or increased risk of neurodevelopmental disorders because of virus infection.

Finally, to examine whether Ndel1 activity is correlated with ZIKV infection and with consequent increased risk of neurodevelopmental disorders, we compared all infected groups after a type 1 interferon beta (INFβ) treatment, which was described to reduce ZIKV activity (Krenn et al., 2021). The animals were then infected with 30 PFU of ZIKV by ICV route at P0, and the MOCK+IFNβ and ZIKV+IFNβ groups received the administration of a single dose of 1 µL of IFNβ (10 µg/mL), by ICV route, at P2, P4 and P6, in a total of three doses per treated animal, as previously described (Krenn et al., 2021). A significant difference was also observed between the treated and untreated groups ($F(3,\ 11.4) = 13.7$, $p < 0.001$, $\eta^2 p = 0.455$), and as expected, Ndel1 oligopeptidase activity was lower in ZIKV infected group ($1.11 \pm 0.51$ nM/min/mg) if compared to animals from MOCK ($3.54 \pm 1.34$ nM/min/mg) or MOCK+IFNβ ($3.16 \pm 1.07$ nM/min/mg) groups, with a $p$ value of 0.019 and 0.016, respectively, as determined by a post hoc Games-Howell. More importantly, Ndel1 activity was restored by the treatment of animals infected with ZIKV with IFNβ ($4.54 \pm 1.66$ nM/min/mg), with a value of $p = 0.002$, and with a strong effect size ($g_{hedges} = 2.64$) compared with ZIKV-infected group, as determined by a post hoc Games-Howell (Figure 8a). IFNβ treatment reduced viral load in ZIKV-infected mice (Figure 8b). SATB2 is a transcriptional factor expressed in upper-layer cortical neurons and is required for corticogenesis. Immunohistotochemistry analysis showed a reduction of SATB2+ neurons in the upper layer of cortex of ZIKV-infected

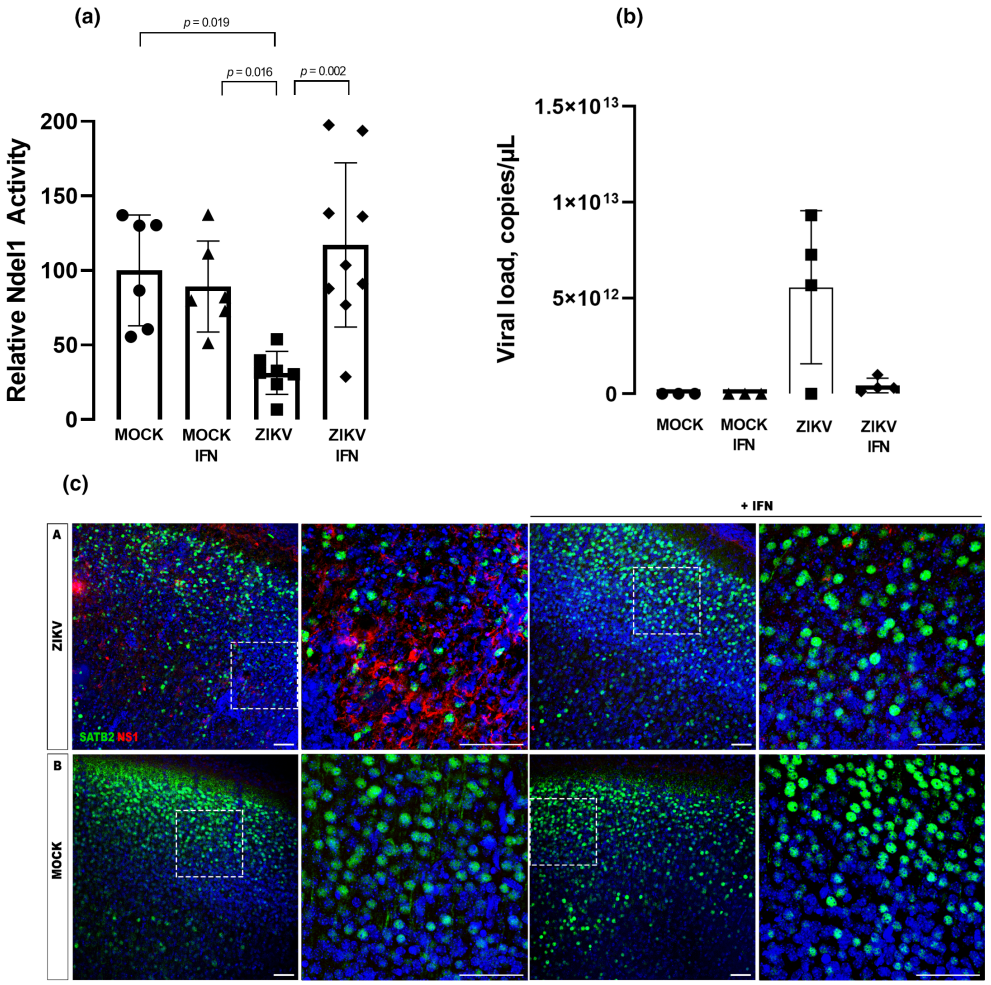



FIGURE 8 Ndel1 oligopeptidase activity in the forebrain of MOCK and Zika virus (ZIKV)-infected mice by intracerebroventricular (ICV) route following the treatment with IFN-beta. Animals received 30 PFU of ZIKV (■) or vehicle (•) by ICV at P0, and the animals were then treated with IFN-beta (♦) at P2, P4 and P6 or the vehicle for INF-beta (▲) by ICV route. (a) The brains were collected at P8 and the Ndel1 activity was determined in sample homogenates freshly prepared from the forebrain region as described in Methods. Data are presented as nM/min/mg of total protein normalized relative to the average Ndel1 activity of each respective MOCK group (considered as 100% of activity). The differences were considered significant for values of $p \leq 0.05$, for one-way ANOVA. (b) Viral load was determined by RT-qPCR as described in the Methods section. (c) Immunofluorescence labelling for NS1 (red), SATB2 (green) and DAPI staining (blue). On (a) ZIKV and ZIKV+ IFN and (b) MOCK and MOCK + IFN cerebral cortex. Scale bar = 50 µm.

animals that was also restored after IFNβ treatment. Moreover, IFNβ treatment alone did not interfere in the expression of this marker (Figure 8c).

## 4 | DISCUSSION

The association of virus infections during pregnancy and anomalies in the offspring's fetal neurodevelopment are widely studied, not only as a result of earlier impacts observed at birth, such as the microcephaly determined by Zika virus (ZIKV) infection (Guardado et al., 2022) but also because of the increased risk in neuropsychiatric outcomes in later stages of life (Fung et al., 2022). Despite the epidemiological and experimental studies showing the association between these apparently non-related events (Ganguli & Chavali, 2021; Simões e Silva et al., 2016), the cellular and molecular mechanisms underlying these effects are still unclear. Further studies, aiming to better understand these mechanisms, are clearly crucial to create new strategies to prevent and treat the outcomes following the infections by the already known pathogens, such as the ZIKV, or by emerging pathogens as the SARS-CoV2 (Hessami et al., 2022). Although several efforts to identify repositioning or investigational drugs for ZIKV infection treatment (Giovannoni et al., 2020; Li et al., 2019; Sariyer et al., 2019; Viveiros Rosa et al., 2020), there is no good biomarkers for the early diagnosis of congenital ZIKV infection syndrome (CZS) that can lead to microcephaly and which diagnosis is still limited to the measurement of the brain size by ultrasonography or tomography, or by the imprecise measurements of the circumference of the baby's skull after birth (Forster et al., 2020; Gullo et al., 2022).

Previously, we have demonstrated a lower Ndel1 activity in plasma of patients with chronic schizophrenia compared with healthy

controls (Gadelha et al., 2013), in addition to an even lower Ndel1 activity in treatment resistant compared to non-resistant schizophrenia patients (Gadelha et al., 2013). The resistance to the treatment with antipsychotic has been associated with more pronounced deficits in corticostriatal connectivity in schizophrenia (Molent et al., 2019; Shin et al., 2022). Interestingly, we also observed lower Ndel1 activity in several brain regions (prefrontal cortex, striatum and nucleus accumbens) of a transgenic animal model for the study of schizophrenia overexpressing the full-length human non-mutant *Disrupted-in-Schizophrenia* 1 (DISC1), exhibiting abnormal dopaminergic signaling and defective neuronal positioning, which is characteristic of impaired cell migration (Nani, Fonseca, et al., 2020). Interestingly, DISC1 is a competitive inhibitor of Ndel1, and we have demonstrated that 98% of Ndel1 in brain is complexed with DISC1 and, therefore, displaying no enzyme activity. In other words, only 2% of Ndel1 protein is in non-complexed monomeric form, which is responsible for almost all oligopeptidase activity measured in brain homogenates (Hayashi et al., 2005). So forth, attempts to measure the RNA or protein expression levels were demonstrated to be of low value to monitor the modulation of Ndel1 in these pathological conditions.

Ndel1 activity was also shown to be crucial for neurite outgrowth (Hayashi et al., 2010; Kamiya et al., 2006), and this deficit was rescued by overexpressing the enzymatically active wild-type Ndel1 but not by the catalytically dead mutant Ndel1C273A (Hayashi et al., 2010), suggesting the importance of Ndel1 oligopeptidase activity in neuron maturation. Although the generation of Ndel1-null knockout mice resulted in perinatal lethality, the conditional knockout was demonstrated to display severe alterations in neuronal migration (Sasaki et al., 2005), deficits in the neocortical and hippocampal formation (Youn et al., 2009), postnatal disruption in forebrain excitatory neurons, spatial learning and memory deficits, seizures and with shortened lifespan (Gavrilovici et al., 2021), leading us to hypothesize Ndel1 activity could be a biomarker of impaired neuronal migration. Although neuronal migration impairment associated to the risk of developing schizophrenia and other mental disorders may suggest a possible decrease in size or thickness of certain brain regions of patients, neuroimaging data for identifying individuals with schizophrenia diagnosis still show inconsistent results and have limited ability to support the diagnosis of this mental disorder in clinical practice (Sun et al., 2023). On the other hand, the potential power of measuring Ndel1 activity to support the diagnosis of schizophrenia and/or the clinical symptom amelioration of subjects in first episode of psychosis was demonstrated (Dal Mas, Nani, et al., 2019; Gadelha et al., 2013).

All these findings strengthen the critical role of Ndel1 activity for the correct neurodevelopment, which are also in good agreement with our present data showing a moderate to strong correlation between lower Ndel1 activity and reduced size of brain of mice infected with different loads of ZIKV, that is, $10^4$ PFU (Figure 2) or 100 PFU (Figure 3), and injected at different developmental stages of the animal and by diverse administration routes. Interestingly, a previous study has also suggested the association of Ndel1, as well as of NDE1, with microcephaly because of their roles in the cell cycle progression and postmitotic neuronal migration (Doobin et al., 2016), although we are the first to demonstrate the modulation of Ndel1 activity by the ZIKV infection in an in vivo animal model.

Taken together, we demonstrate here that the Ndel1 activity is lower in the brain of animal model for CZS, infected under different conditions, such as varying the injection route (IP or ICV), inoculum dose (30, 100 or $10^4$ PFU/animal) and/or developmental stage/age of animal (with injections in embryos at E13 or post-natal P0, followed by analysis at E18 or post-natal P3-P8, respectively). These infection routes and viral load were chosen based on each model's severity outcome and feasibility, as reviewed by Christoff and Garcez (2021). For instance, ZIKV infections with higher titters lead to a lower survival rate in a dose-dependent manner (Zhang et al., 2019). Also, ZIKV infections performed earlier in development have a more severe outcome (Xavier-Neto et al., 2017). Therefore, lower viral titters and later conditions were used to prolong the survival of infected animals (Cui et al., 2017). In addition, wild-type animals do not transmit ZIKV efficiently vertically, so the ICV route of ZIKV infection results in a more severe outcome than IP (Dick, 1952). We have also demonstrated that under these conditions (employing the described injection route, inoculum dose and/or at these specific animal developmental stage/age), the effect size of the Ndel1 activity decreases because of the ZIKV infection was not significantly different (Figure 6). The area under the curve (AUC) of receiving operating characteristic (ROC) curve combines the information of the true positive rate and the true negative rate, and an AUC, which is a measure of the overall discriminative power for using Ndel1 enzyme activity for differentiating ZIKV/MOCK status, value of 0.890 was determined after considering all Ndel1 activity measurements performed in this work. In general, an AUC of 0.8 to 0.9 is considered excellent, and more than 0.9 is considered outstanding regarding the ability to diagnose (Mandrekar, 2010). In addition, we could demonstrate that the decrease in Ndel1 activity could be observed even before the viral protein NS1 or cell death could be detected, while it also remained low along the infection progression (Figure 6), suggesting the power of measuring Ndel1 activity aiming for an early detection of viral infection and CZS prediction.

More interestingly, we also demonstrate here that the treatment with type 1 interferon beta (IFNβ), which is a well-known immunomodulator capable of decreasing inflammation and of rescuing the CZS phenotype ultimately characterized by the microcephaly (Bulstrode et al., 2022) restores the Ndel1 activity to the levels of the uninfected healthy control mice (Figure 8). Interestingly, productive ZIKV infection in human developing brain and glioblastoma (GBM, which is an adult brain cancer) primary tissue explants, both contain SOX2+ neural progenitors, proved that human developing brain is uniformly vulnerable to ZIKV infection while GBM was more refractory, which correlated with an innate immune expression signature (Bulstrode et al., 2022). Moreover, GBM-derived CD11b+microglia/macrophages were necessary and sufficient to protect neuronal progenitors against ZIKV infection in a non-cell autonomous manner, as CD11b+ conditioned medium and IFNβ treatment rendered human developing brain progenitor lines and explants refractory to ZIKV (Bulstrode et al., 2022). Also, IFNβ treatment demonstrated a

neuroprotective effect in brain organoids infected by ZIKV by rescuing organoids growth and size and also reverting the transcriptional changes caused by ZIKV infection (Krenn et al., 2021). Therefore, as IFNβ treatment is able to block the ZIKV infection progress, we hypothesize here that the restoration of Ndel1 activity in IFNβ treated animals infected with ZIKV may be signaling a neuroprotective effect of this treatment in our present experimental model.

The IFN system was described as a key mechanism for the host defense (Ngono & Shresta, 2018). The maternal immune activation (MIA) hypothesis proposes that perturbations in the inflammatory system caused by a cytokine storm can affect the fetal neurodevelopment, and this was observed in different flavivirus infection, including ZIKV (Ngono & Shresta, 2018; Sosa-Acosta et al., 2022). ZIKV infection caused profound downregulation of the transcriptional activity of genes that may underly tissue morphology, neurological development, metabolism, cell signaling and inflammation, possibly explaining the interplay between the MIA and ZIKV replication (Creisher et al., 2022). MIA is also vastly studied in the context of neuropsychiatric disorders, including schizophrenia or autism, as demonstrated not only by epidemiological studies (Estes & McAllister, 2016) but also by studies with animal models with MIA induced mainly with a synthetic RNA that mimic a viral genetic material (polyinosinic:polycytidylic (poly(I:C))) and/or bacterial (lipopolysaccharide (LPS)) derived agents, ultimately leading to offspring exhibiting a wide range of endophenotypes associated to several psychiatric disorders (Estes & McAllister, 2016). The possible interplay between MIA induced psychiatric disorders risk and CSZ will be further explored by us in future works specifically designed to clarify the possible role(s) of Ndel1 in these pathologies.

Interestingly, ZIKV targets various cells in the brain, including radial glial cells, neural progenitor cells (NPCs), astrocytes, microglial and glioblastoma stem cells, affecting these brain cells by different mechanisms, such as apoptosis and cell cycle dysregulation, which may induce neurological complications and neuroimmunopathogenesis (Komarasamy et al., 2022), possibly causing the depletion of neural progenitors in the cortical layer of the brain (Ihunwo et al., 2022). Indeed, cell-intrinsic innate immune responses to ZIKV infection profoundly shape neuronal transcriptional profiles by inducing changes to neurologic gene expression associated with psychiatric disorders (Kung et al., 2022). Noteworthy, we have previously observed a correlation between a lower Ndel1 activity and inflammatory marker interleukin-4 (IL-4) levels in patients with chronic schizophrenia (Nani et al., 2022). Intriguingly, IL-4 plays key roles in the generation and regulation of cytokine storms during virus infections (Yuan et al., 2021). Lower Ndel1 was also observed in patients with bipolar disorder (Dal Mas, Carvalho, et al., 2019), which is another psychiatric disorder in which the immune-inflammatory response system as a result of the viral infections, such as the human cytomegalovirus, contribute to an immune-risk phenotype (Maes et al., 2021). Therefore, the evaluation of possible involvement of inflammatory pathways in Ndel1 activity modulation also needs attention, as well as the possible modulation of Ndel1 activity by different virus with potential to induce MIA, although the transgenic animal model overexpressing DISC1 shows lower Ndel1 activity but with no signs of inflammation (Nani, Fonseca, et al., 2020).

However, this study has potential limitations. As a resut of significant decreases in the cases of microcephaly, we could not have access to freshly collected blood or amniotic liquid/fluid from pregnant patients with CZS risk. And biological samples collected during and soon after the Summer Olympics Game hosted in Brazil in 2016, when unusual high incidence of CSZ was reported, may now be too old to be assessed for biochemical measurements of Ndel1 enzyme activity, and as we know there is no more reported cases of CSZ in Brazil, since 2017. In addition, because of technical limitations, it was not possible to collect enough blood samples from the infected mice embryos or neonatal mice to evaluate the Ndel1 activity. However, we have already demonstrated that the validity of evaluating Ndel1 activity in the blood of patients with schizophrenia and other mental disorders, by measuring the Ndel1 activity in blood samples from several patients and healthy controls, as well as by concomitantly measuring this oligopeptidase activity in blood serum and several brain regions of different animal models for schizophrenia studies (Nani et al., 2019; Nani, Fonseca, et al., 2020; Nani, Lee, et al., 2020), to show that the Ndel1 activity determined in the brain of ZIKV-infected mice is expected to show correspondence to the activity of this enzyme measured in the peripheral blood of the same animals.

Therefore, we believe that Ndel1 activity could be a potential biomarker for the early diagnosis of CZS and/or for the follow-up of treatment efficacy, although further studies are still necessary to confirm if this activity is also similar between the blood and brain in ZKV-infected animal models and especially in patients with CZS.

## 5 | CONCLUSION

We could demonstrate for the first time here that the Ndel1 activity is decreased in the brain of animal models for congenital ZIKV-infection syndrome (CZS), following the infection under different conditions, such as varying the injection route (namely, in utero intraperitoneal and/or intracerebroventricular routes), inoculum dose (30, 100 or $10^4$ PFU/animal) and/or at different developmental stage/age of host animal (with injections in embryos at E13 or post-natal P0, followed by analysis at E18 or post-natal P3–P8, respectively) showing an AUC for ROC curve of 0.890, which is suggestive of an excellent power for diagnosis. In addition, the decrease in Ndel1 activity showed a good and linear correlation with the infected animal brain size. More importantly, we have also demonstrated that the decrease in Ndel1 activity could be observed even before the viral protein NS1 or cell death could be detected, while it also remained low along the infection progression, suggesting together that Ndel1 activity is a potential good biomarker for the early diagnosis of CZS and/or also for the follow-up of treatment efficacy.

### AUTHOR CONTRIBUTIONS

P.P.G., J.V.N. and M.A.F.H. conceived the study. R.R.C., G.L., T.R., A.D.R., L.M.H. and A.T. performed the experiments with ZIKV. G.L.

and J.V.N. performed the homogenate preparation and Ndel1 activity measurements. V.K. contributed with the INF-beta treatment approach. J.V.N., R.R.C., P.P.G. and M.A.F.H. performed data analysis. R.R.C., P.P.G., J.V.N. and M.A.F.H. wrote and revised the manuscript. All authors approved the publication of this manuscript.

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All experiments were conducted in compliance with the ARRIVE guidelines.

## PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jnc.15918.

## DATA AVAILABILITY STATEMENT

Data is available on request from the authors.

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