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Zika Virus in Extracellular Vesicles: Insights from Integrated Proteomic and Metabolomic Dependent Regulation of B Cell and PI3K/AKT/mTOR Signaling Pathway

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Abstract: (1) Background; Cell-released extracellular vesicles (EVs) acting as 'metabolically and proteolytically active machines,' show potential in metabolomic and proteomic analysis of serum EVs. Despite diverse challenges, post-isolation omics characterization EVs offers crucial insights for effective analysis; (2) Methods: The research, involved children with Congenital Zika Syndrome, utilizing mass spectrometry for proteomics and GC-MS for metabolite identification. Vesicles were isolated using Izon qEV columns, quantified, and characterized by NTA and TEM. Data analysis employed Cytoescape/String and MetaboAnalyst, revealing variations in metabolic and proteomic profiles among groups through PCA and volcano plots. Proteins and Metabolite set enrichment analysis provided biologically meaningful patterns to enriched metabolites; (3) Results: Using molecular exclusion chromatography, the EVs were characterized, revealing size variations. Protein analysis identified 13 significantly altered proteins, including upregulated (e.g., AOM8Q6 - IGLC7) and downregulated (e.g., Q8TD86 - CALML6) ones. Metabolite analysis indicated involvement in the PI3K-AKT-mTOR pathway and suggested a role in Angiotensin inhibition in CZS+. Upstream of mTOR, Akt is the central signaling molecule in the PI3K pathway and plays critical roles in brain development as well as synaptic plasticity important for Zika Virus. The study provides insights into molecular mechanisms associated with CZS; (4) Conclusions: The study pinpointed valuable possible biomarkers, specifically proteins and metabolites, in Zika virus (ZIKV) infection. It stresses the necessity for broader investigations with advanced techniques to uncover molecular targets, potentially advancing pharmacological strategies.

Keywords: Zika infection; extracellular; vesicles; proteome; metabolome; network

1. Introduction

The infection of ZIKV (ZIKV) during gestation carries a risk of serious injury, including the loss of fetal tissue, microcephaly, and other congenital Zika-related brain malformations [1]. Additionally, severe neurological conditions like encephalopathies, meningoencephalitis, myelitis, uveitis, Guillain-Barré syndrome, and severe thrombocytopenia have been associated with ZIKV infection [2,3]. The causes of the severe forms of Zika are still unknown, as of this moment, there are no effective vaccines or specific treatments for the virus available. The increase in prevalence and range

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of ZIKV is attributed to the virulence of the circulating strains, the vulnerable populations, and the spread of its vectors.

The mosquitoes Aedes aegypti and Aedes albopictus are primarily responsible for transferring various Flaviviruses to humans, these viruses include Zika and dengue (DENV) [4,5]. The female mosquitoes become infected with the virus while consuming a host that is infected with it, this results in the virus's replication in the gut. Later, the virus was transferred to the salivary glands, where it was released into the saliva. The transmission to a new host is completed during the following feeding process [6]. Researchers described the documented increase in the viral infection of mammalian cells because of the exposure of these cells to the extract of mosquitoes's salivary glands. This was observed in the context of a DENV infection in humans. However, the specific mechanisms involved in the transmission of Flaviviruses from vector to host are still not fully understood [7].

EV are cell-secreted enveloped particles that are predominantly 50–200 nm in diameter and are released from the plasma membrane and endosomal compartments [8]. In human hosts, ZIKV primarily infects monocytes, macrophages, endothelial cells, and neurons. The process of cell differentiation or activation causes the release of EVs from the cell's exterior (also known as exosomes or microvesicles) [9]. Metabolites and proteins are both integral to biological importance and participate in various cellular processes [10,11]. The evaluation of metabolomes and proteomes becomes crucial, this is because it provides information about potential dysregulations, similar to the way EVs are used to investigate cellular behavior. EVs have the intriguing ability to function as metabolically and proteolytically active machines, as evidenced by the metabolomic analysis of serum samples in conjunction with hepatocytes' EVs [12]. While EVs have a bright future in metabolomics and proteomics, the field is still in the early stages of development, specifically about the diverse classes of EVs and their inherent diversity [13].

Mainly in the field of EV we note in the literature that PI3K inhibitors that target the stroma and focus on immune modulation, we refer the reader to Okkenhaug et al. 2016 [14] and Vanhaesebroeck et al. 2022 [14]. For more information on past and future PI3K inhibitors, we refer the reader to Castel et al. 2021 [14]. In addition, although our manuscript shows an overview of all upregulated proteins and metabolites, mostly all observed PI3K-driven precursors, for a specific perspective on the relevance of the PI3K pathway in ZIKV which have been extensively [15]. Furthermore, since this paper does not discuss in detail the role of the PI3K pathway in metabolism, we direct the reader to Vasan and Cantley 2022 [16] for this specific topic.

The characterization of omics following the isolation procedure via SEC has a significant impact on the scientific community as a whole and is of crucial importance to the research community specifically. As a result, we focus on the identity and intricate analysis we employed to facilitate additional advances in this field. Despite the inherent complexity, the metabolomic analysis of an isolated EV sample is a new approach. Employing established methods of metabolite/ protein extraction and analysis has been successful in producing large amounts of data and addressing these issues.

2. Materials and Methods

2.1. Ethical Aspects

The research adhered to the criteria outlined in the Helsinki conference. Before commencement, it underwent scrutiny and gained approval from the Research Ethics Committee at the Federal University of Maranhão Hospital and Medicine School of Jundiaí (CAAE: 86696618.7.0000.5467). All actions followed the acknowledgment and endorsement expressed through the informed consent form.

2.2. Study Design

The research involved a prospective cohort of children residing in the State of Maranhão, located in northeastern Brazil. It was a collaborative effort with the Reference Center for Neurodevelopment, Assistance, and Rehabilitation of Children (NINAR). Additional data for control children in this

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study were primarily collected at the Infectious Pediatric Laboratory of the Medicine School of Jundiaí in Jundiaí. A blood sample (5 mL) was obtained from each child. Microcephaly, in this study, is defined as a head-circumference measurement of less than two standard deviations (SDs) below the average [17,18]. The study included a total of 14 children with confirmed Congenital Zika Syndrome (CZS+) and 15 children with confirmed Congenital Zika Syndrome (CZS-). Serum from were fractionated by Izon qEV size-exclusion SEC resulting in five fractions of 500 µL. The F3 fractions from 14 children with confirmed Congenital Zika Syndrome (CZS+) and 15 children with confirmed Congenital Zika Syndrome (CZS-) were analyzed by mass spectrometry techniques and metabolomic analysis. We subsequently carried out the characterization of isolated EV fractions by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) was used. The protocols for isolation, characterization and spectrometry analyzes adhered to the methodology outlined by Pontes, et. al, 2020 [19]

2.3. Vesicle Isolation and Purification

To isolate vesicles, 500 µL of serum samples were placed onto Izon qEV size-exclusion chromatography columns that were pre-conditioned with PBS buffer (Izon Science, UK) [20,21]. The elution process was carried out using PBS buffer as per the manufacturer's guidelines. The columns were filled with a resin featuring a pore size of around 75 nm, a bed volume of 10 mL, an inner tube diameter of 15.6 mm, and a void volume of 3.0 ± 0.25 mL. Before use, the columns were pre-loaded with PBS containing 0.05% sodium azide. Following the exclusion of the column's void volume (3 mL), three fractions were collected from each sample, each consisting of 500 µL.

2.4. EV Sample Preparation for MS-Based Proteomics Analysis

EV (F3) suspended in phosphate-buffered saline (PBS) containing protease inhibitors (obtained from Sigma Aldrich, Brazil) and stored at −80°C for six months were reconstituted in a lysis buffer (comprising 7 M urea, 2 M thiourea, 10 mM dithiothreitol (DTT), and 0.01% Triton X-100) at room temperature for a duration of 2 hours. Subsequently, the lysis buffer was eliminated using Amicon® Ultra 0.5 3KD (Millipore), and protein quantification was confirmed using NanoDrop One (Thermo Scientific, United States). To assess protein banding patterns, a 10% SDS-Page gel analysis was conducted with 10 µg of bovine serum albumin (BSA) serving as the standard protein sample. The gel electrophoresis ran at 50 v for 4.5 hours. Following the run, the gel was detached from the glass plates and immersed in a fixative solution (composed of 50% methanol and 7% acetic acid) for one hour. Subsequently, it underwent a washing step with Milli-Q water and was left overnight in GelCode Blue stain.

For the EV samples (10 µg), incubation was carried out with RapiGest™ SF Surfactant (Waters, United States) for 15 minutes at 80°C, and 100 mM dithiothreitol (DTT) for 30 minutes. Alkylation followed, utilizing 300 mM iodoacetamide (IAA) for 30 minutes in the dark. The samples then underwent digestion with trypsin (at a ratio of 1:100 w/w trypsin: protein) overnight at room temperature. Finally, the peptides were acidified using 1% v/v formic acid to halt the trypsin digestion process and lyophilized before desalting. Resuspension of the samples took place in 0.1% v/v trifluoroacetic acid (TFA), and desalting was achieved using ZipTipC18 Pipette Tip with C18 Resin (Sigma Aldrich, Brazil) following the provided manufacturer's instructions.

The processing parameters encompassed specific modifications, such as carbamidomethylation of cysteine, treated as a fixed amino acid modification. Variable modifications included methionine oxidation and N-terminal acetylation. Trypsin served as the proteolytic enzyme, allowing for a maximum of 2 potential cleavage errors. Peptide and fragment ion mass shift tolerances were set at 20 ppm and 0.05 Da, respectively. Peptide and protein identification adhered to a maximum false positive rate (FDR) of 1%, requiring at least one single peptide for protein identification. Adapted from the protocol Pontes, et. al, 2020 [19]

Bioinformatic analysis relied on information from UniProt and *Cytoscape* and *String* (https://cytoscape.org/ and https://string-db.org/). The mass spectrometry proteomics data (Table S1-raw proteomics data).

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2.5. EV Identification of Metabolites with GC-MS

EV (F3) suspended in phosphate-buffered saline (PBS) of metabolites was carried out in accordance with the methodology outlined by Hoffman et al., incorporating minor adjustments [22]. Subsequently, extraction took place in microtubes, utilizing 200 mg of fungal macerate to which 1 mL of an ice-cold extraction solution comprising methanol:chloroform:water in a ratio of 6:2:2 was added. After rigorous vortexing of these extraction microtubes, they were subjected to ultrasonic low-temperature bath treatment at 20 Hz s-1 for 15 minutes. The ensuing step involved centrifugation (Eppendorf, Germany) at 4°C for 10 minutes at 14,000 rpm. The resulting supernatant underwent filtration using a 0.22 µm Whatman® filter (Merck, Germany) and was then transferred to a chromatographic vial for lyophilization (Thermo Fischer Scientific, MA, USA) until complete desiccation. The lyophilized samples were subsequently reconstituted in 200 µL of the extraction solution and portioned for utilization in GC-MS and LC-MS/MS analyses. Each extract aliquot received 10 µL of a 1 mg. mL-1 solution of the isotopically labeled compounds succinic acid (D4, 98%—DLM 584–5), myristic acid (1, 2, 3–13C3, 99%—CLM 3665– 0.5), and palmitic acid (1, 2, 3, 4 –13C4) as external standards. The samples were once again lyophilized for subsequent derivatization using 30 µL of a 15 mg·mL-1 solution of methoxyamine and pyridine for 16 hours at room temperature. The silylation of the samples occurred promptly at room temperature for 1 hour by introducing 30 µL of MSTFA(N-methyl-trimethylsilyl-trifluoroacetamide) with 1% TMCS (trimethylchlorosilane). Finally, 30 µL of heptane containing 15 ng. g-1 of methylesterase was introduced.

Data derived from GC-MS underwent processing using ChromaTOF 4.32 software, involving baseline correction, deconvolution, retention index (RI), retention time correction (RT), identification, and alignment of peaks. Identification of metabolites employed the NIST library version 11, with only metabolites scoring 700 or above being considered. The intensity of each metabolite was normalized by the total ion count (TIC) of each sample. The analysis of samples using GC-MS adhered to the methodology outlined by Budzinski et al. [23], with minor modifications. At this stage, a series of n-alkanes (C12 –C40) was utilized to calculate the sample retention index [24]. One microliter of each derivatized sample was automatically injected in splitless mode by a CTC Combi Pal Xt Duo autosampler (CTC Analytics AG, Switzerland) into an Agilent 7890A gas chromatographer. The chromatographer featured two fused-silica capillary columns; one column with a 20 m x 0.18 mm chemically bonded with 0.18 µm DB-5 film (Agilent) stationary phase, and the other column with 0.9 m x 0.10 mm chemically bonded with 0.10 µm RX-T 17 film (Restek) stationary phase. The injection temperature was 280°C, with a purge flow of 20 mL min-1. The gas flow through the column was 1 mL min-1, and the column temperature was maintained at 70°C for 2 min, followed by an increase of 15°C min-1 until reaching 320°C, and then held at this temperature for 4 min. The column effluent was introduced into the GC x GC/TOF-MS Pegasus 4D ion source (Leco Corporation, St. Joseph, MI, USA) at a temperature of 250°C. Ions were generated by a 70 eV electron beam at an ionization current of 20 mA and 20 spectra·s-1, recorded in the range of 45–800 m/z. The detector voltage was set at 1500 V.

Statistical analyses were executed using the MetaboAnalyst 4.0 online software (accessible at http://www.metaboanalyst.ca/MetaboAnalyst/) [25]. A log2 transformation and Pareto scaling were implemented for data normalization. Analysis of variance (ANOVA) and principal component analysis (PCA) were applied to identify variations in the metabolic profiles among the different groups. Metabolite set enrichment analysis (MSEA) was conducted to explore and attribute biologically meaningful patterns to specific groups of significantly enriched metabolites.

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3. Results

3.1. EV Characterization

To isolate EVs, the collected sera underwent fractionation using a molecular exclusion chromatography column, and the resulting isolates were preserved. Subsequently, quantification (Figure 1 - A), nanoparticle tracking (Figure 1 - B), and transmission electron microscopy analysis (Figure 1 - C) were performed on the isolates. The sizes of the isolated extra-cellular vesicles ranged from 154.9 nm for the control child pool (CZS-) to 185.4 nm for the affected child pool (CZS+) [26–29]

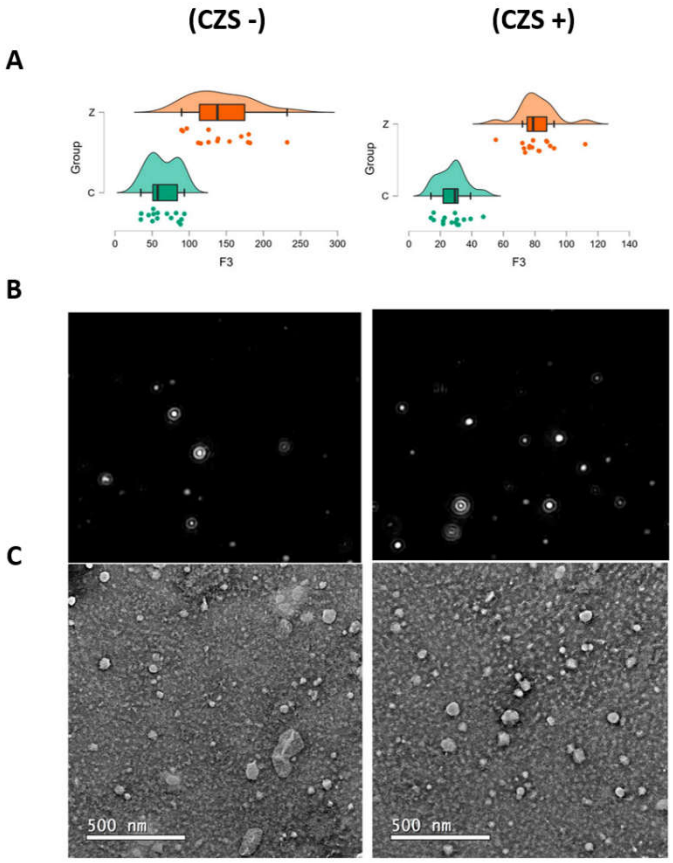

Figure 1. Analysis for EV Characterization: A) Results of quantification by NanoDrop One, B) Nanoparticle tracking analysis and C) transmission electron microscopy of extracellular vesicles isolated in the children's serum.

3.2. Proteomic Analysis

The differentially expressed proteins observed by mass spectrometry were compared to their obtained Fold change values (Figure 2) and interaction network (Figure 3). While, in the *String* software we were able to characterize the functionality and through its plugin for *Cytoscape* we were able to obtain a network for positive and negative proteins, compared to the control.

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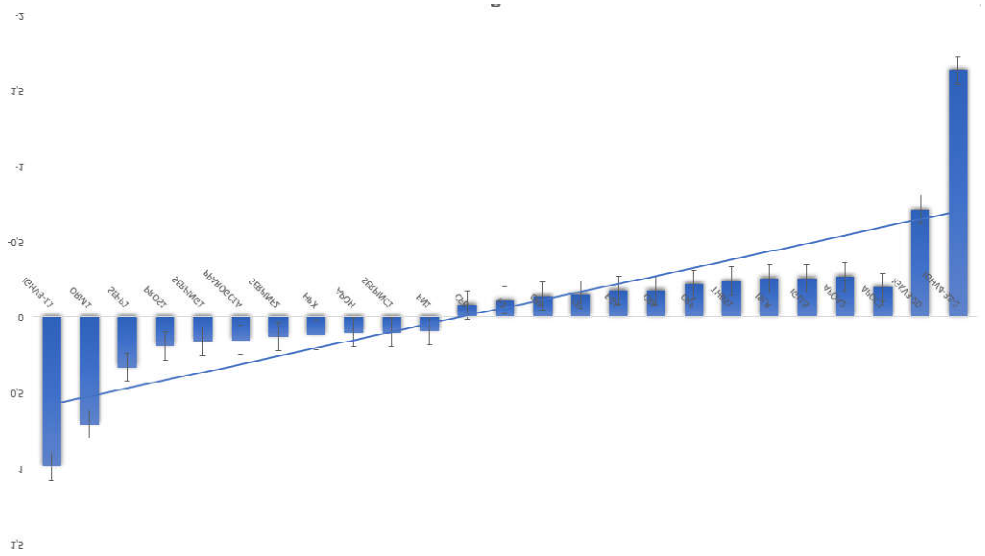

Figure 2. Fold change of proteins identified by mass spectrometry that showed positive and negative regulation.

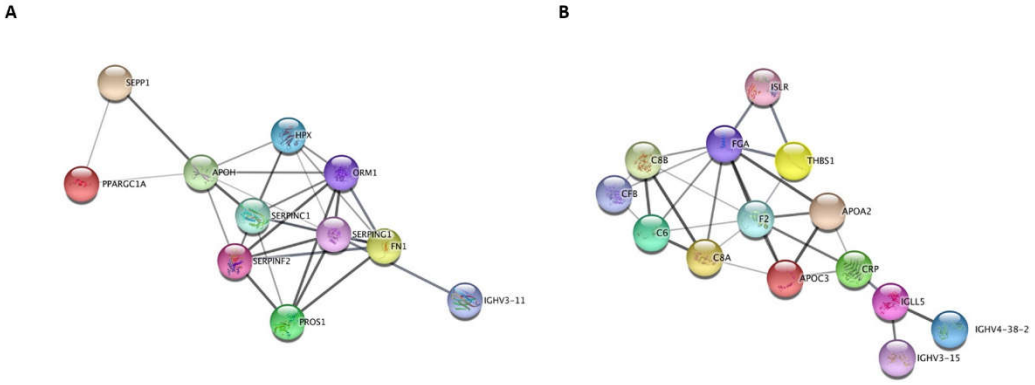

Figure 3. Circles represent proteins, while straight lines represent interactions between different proteins. Stronger associations are represented by thicker lines. A) Interaction network between proteins identified by mass spectrometry that showed positive regulation, compared to the control. B) Interaction network between proteins identified by mass spectrometry that showed negative regulation, compared to the control. This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3.1. Proteomics and Metabolomics

The Principal Component Analysis (PCA) data obtained from protein analyses (Figure 4 - A) reveal elevated values exclusively for the maximum variance. Conversely, when considering successive variances, the highest values are evident in the PCA derived from metabolite analyses (Figure 4 - B), utilizing the same samples of EVs obtained from both CZS- and CZS+ children. In our research, we identified 13 proteins (depicted as red circles) that exhibited at least a twofold alteration and statistical significance ($p \leq 0.05$) in extracellular vesicle (EV) samples isolated from the serum of children with congenital Zika syndrome (CZS+) (Figure 5 - A). Notably, our investigation revealed an association between the up-regulation of extracellular vesicular proteins (AOM8Q6 - IGLC7, P35219 - CA8, Q6NW40 - RGMB, Q9Y6OO - CSAD, P62820 - RAB1A, and Q92764 - KRT35) and the down-regulation of others (Q8TD86 - CALML6, POO734, Q96GR2 - ACSBG1, Q8N461 - FBXL16), suggesting a crucial role of the complement cascade in children affected by CZS (Figure 5 - B) [30–32]. In light of this, we can highlight the presence of upregulated EVs metabolites, predominantly

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involved in the PI3K-AKT-mTOR pathway. These include Alanylalanine, NN'-dimethyl-N'-methoxycarbonyl-methyl ester, Phthalic acid di 3-methylphenyl ester, Phthalic acid decyl 2-ethylhexyl ester, D-Lyxose, Diuoropropanodiamide, and Manose, among others. Conversely, there are downregulated metabolites such as Phenylpropanolamine, Methyl-5-mercaptotetrazole, Glycerol monostearate succinic acid ester 3-chlorophenyl 3-phenylprop-2-en-1-yl, 5-Oxoproline, D-Glucitol, Phthalic acid decyl 2-ethylhexyl ester, 3-Methylbenzamidoxine, 1-Propenol, and Alanine N-methyl-N-methoxycarbonyl hexyl ester. These findings suggest a significant role in the inhibition of Angiotensin in CZS+ children. Studies on viral infections such as DENV and H5N1 have explored the inhibition of ACE [33–36] and kinases involved in the PI3K-AKT-mTOR pathway [37–39]. Literature data suggests that cellular locations may determine functions as follows [40–42]: 1) Extracellular - Cell shrinkage causes the cytosol to push vesicles towards the perinuclear region with "addressing labels" for swift transport; 2) Plasma Membrane – membrane ligand specificity and recognition of PAMPs; 3) Intracellular - leading to the formation of multivesicular bodies and actively participating in intracellular signaling pathways; and 4) Nucleus – intracellular signaling and modulation of the immune response (Figure 6).

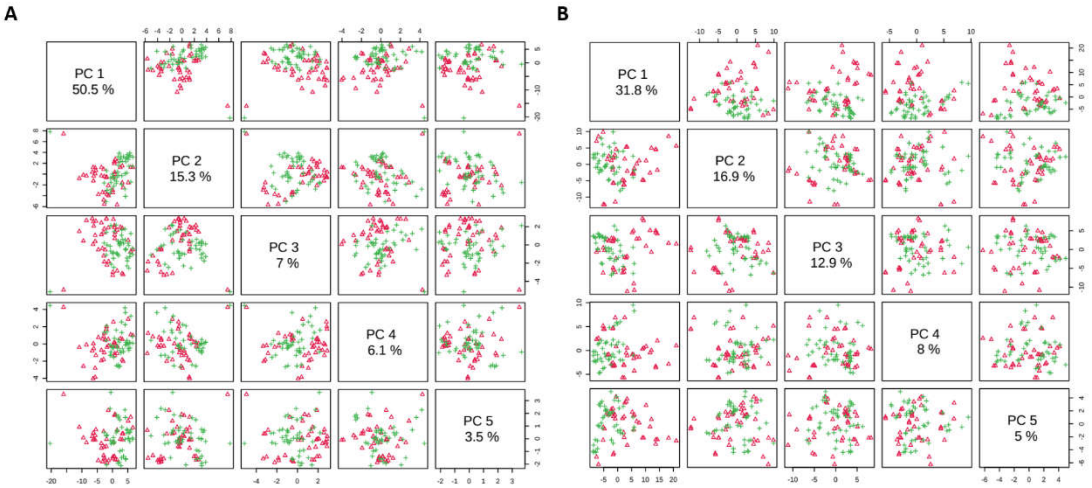

Figure 4. Data analysis by volcano plot using the MetaboAnalyst 4.0 software. A) Analysis of main components of the analyzed data set obtained from proteins; B) Principal component analysis of the analyzed dataset obtained from metabolites. In both images, the red components represent the EV samples from CZS+ children and the green components represent the EV samples from CZS- children.

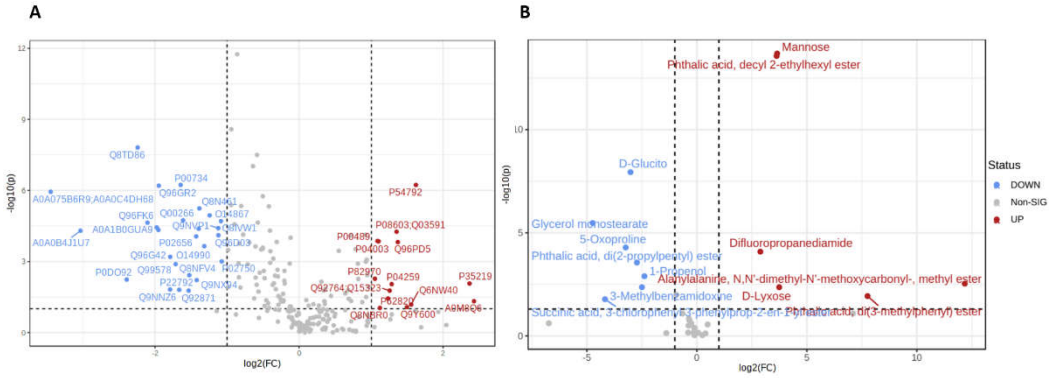

Figure 5. Data analysis by volcano plot using the MetaboAnalyst 4.0 software. A) Volcano plot of the abundance of proteins identified by proteomics. B) Volcano plot of the abundance of metabolites identified by the metabolomics platform. In both graphs, the numbers and letters represent the code

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for each protein identified and analyzed. Red circles represent features above (UP) the threshold for fold change and t-test threshold ($p \leq 0.05$) compared to control samples. Blue circles represent features below (DOWN) the fold change threshold and t-test threshold ($p \leq 0.05$) compared to ZIKV samples. Both values are log transformed.

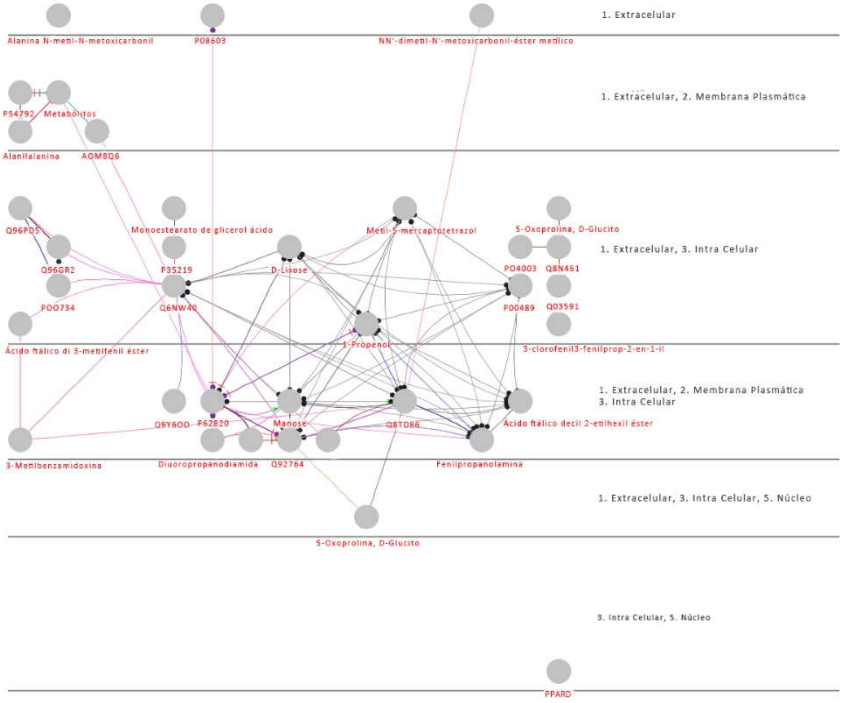

Figure 6. Part of the image from the analysis of categories from the public domain database obtained in this study of serum EVs from CZS+ children.

4. Discussion

The cellular communication of EVs influences various physiological and pathological processes, including immune responses [43,44], cancer [45–47], and cardiovascular diseases [47]. Additionally, it plays a role in delivering bacterial virulence factors to the host [48–50] and modulating inflammatory and immune responses in fetal-maternal communication [51,52]. This communication occurs among different cell types, but when it involves the participation of immune system cells or molecules secreted by them, it gains greater prominence in the literature due to its immunomodulatory capacity. The discovery that EVs act as vehicles for the transfer of membrane, cytosolic proteins, lipids, and proteins between cells strongly suggests their contribution as communicators in cellular communication. When secreted by immune system cells, they exhibit immunomodulatory functions, inducing both suppression and activation of immune responses [53,54].

We have the PROS1 protein (positive regulation), which is described in the literature as vitamin K-dependent, serving as a cofactor for CRP-mediated cleavage (negative regulation) of factors Va and VIIIa on the surface of phospholipids in the presence of Ca^{2+} [55–57]. Additionally, the serpins SERPINC1, SERPING1, SERPINF2 (all with positive regulation), in turn, inhibit and block the activity of thrombin, fIXa, fXa, fXIIa, kallikrein, and plasmin [58]. Thus, compromising the activity of platelet procoagulant.

In summary, HPX (positive regulation) is produced in the brain [59–61] and has a higher affinity for binding to the heme group, potentially reducing the regulation of Angiotensin II Type 1 receptor [62]. The heme prosthetic group imparts a characteristic color to these proteins, consisting of an organic part and a ferrous iron atom in the ferrous state [63,64]. Its function is to capture proteins with the heme group released or lost during hemoglobin recycling, thereby protecting the body from

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oxidative damage that heme groups could induce [65]. The circulating level of HPX is associated with the prognosis in patients with septic shock [66–68].

The analyses of protein-protein interaction in EVs, as revealed by the String software, provided us with two network maps (Figure 3) and insights into the functionality of proteins obtained through mass spectrometry. In proteins exhibiting positive regulation, processes such as leukocyte migration, cytokine secretion, acute inflammatory response, and platelet degradation were observed [69]. On the other hand, proteins identified by mass spectrometry with negative regulation were associated with processes including regulation of protein activation cascade, reverse cholesterol transport, phagocytosis, negative regulation of lipid localization, B cell receptor signaling pathway, and adaptive immune response. Another crucial aspect in the functional evaluation of EVs is to consider their ability to activate the adaptive immune response [70].

Interestingly, similar findings have been reported previously in patients with an increased production of interferon alpha (IFN-α) by plasmacytoid dendritic cells [71]. This was observed through their interaction with EV originating from a hepatocarcinoma cell line infected with the hepatitis C virus (HCV) [72,73]. This result underscores a defense strategy of the organism, activating the innate immune response of the host [74,75]. Furthermore, the proteins with the codes Q03591 (CFHR1 abbreviation), P00489 (PYGM abbreviation), and PO4003 (C4BPA abbreviation) originating from the serum isolate of EV-affected children were also identified as positively regulated in HIV-1 [76] and SARS-CoV-2 [77,78] infections. EV derived from infected cells can transport viral regulatory proteins without being recognized by the immune system due to the lipid bilayer coating from host cells, functioning as a Trojan horse [79–82].

However, there are additional proteins identified in this study as potential biomarkers for serum EVs in children affected by SCZ that have already been described in the literature, exhibiting new functionalities such as immunomodulation and increased infectivity. Some of them include protein code P54792 (abbreviated as DVL-1), described with positive regulation in cellular susceptibility to HIV infection [83,84]; protein code P82970 (abbreviated as HMGN5), known for increasing apoptosis in CD4+ T lymphocytes, which may contribute to the depletion of these cells in the pathogenesis of Acquired Immunodeficiency Syndrome [85–88].

The protein with the code P08603 (CFH) enhances cellular lysis by B cell. The surface of viruses often has dense, repetitive structures that promote multivalent interactions with B-cell receptors and influence the functional characteristics of antibodies [89–91]. Early in pregnancy, B cells are stimulated to produce IL-10, which promotes the survival of mature B cells and reduces the frequency of naive B cells. This study examined the contribution of maternal plasma IgM to ZIKV neutralization, and the results suggest that IgM is effective within the first 3 months of infection [92].

On the other hand, this intravesicular protein has also been described as increased in the survival of the cytotoxic T cell lineage dependent on IL-2 [93–95]. Although, the extravesicular protein with the code Q96PD5 (abbreviated as PGLYRP2) plays an active role in stimulating the production of pro-inflammatory cytokines by cells through the activation of the NFkB pathway [96,97]. The NFkB pathway stands out in EVs due to its wide range of actions and the fact that various extracellular proteins are integrated into the dynamics of its activation [98,99]. In the analysis of metabolites, we observed six metabolites (red circles) with at least a two-fold alteration and a significant value ($p \leq 0.05$) in samples of EVs isolated from the serum of CZS+ children (Figure 5 - B).

Furthermore, the most promising metabolites as potential biomarkers are Manose and Decyl 2-ethylhexyl phthalate acid ester, which are part of the activation of the Renin-Angiotensin System pathway. This pathway is directly involved in the uptake and excretion of sodium and potassium, possessing ions that promote vasoconstriction and regulation of blood pressure, respectively [100]. However, their importance in the viral infection process has only recently been observed, as demonstrated by a study in which rats infected with DENV were treated with losartan or enalapril. In this case, the treatment reduced DENV absorption by macrophages, indicating that the activation pathways of the RAS may be associated with the severity of the infection [101,102]. Therefore, the potential biomarkers elucidated in our study reveal that these species may also be linked to the control of the immune response to ZIKV infection.

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The other four biomarkers found in serum-derived EVs from CZS+ children (Alanilalanine, NN'-dimethyl-N'-methoxycarbonylmethyl ester, Phthalic acid di 3-methylphenyl ester, D-Lyxose, and Diuoropropanodiamide) contribute to supporting the role of the immune response to infection [103]. These metabolites, when combined, are responsible for activating the PI3K-AKT-mTOR pathway [104,105]. Akt is upstream of mTOR and is fundamental in the PI3K pathway, playing important roles in brain development and synaptic plasticity. Non-functional Akt mutations cause microcephaly, while activating mutations cause megalencephaly. Expression of dominant negative Akt blocks neurogenesis in human foetuses in vitro. Neurogenesis, the process of forming neurons from neural stem cells or neural progenitor cells, is most active during prenatal development and is critical for brain growth. Genetic defects in neurogenesis and cell migration can lead to neurological disorders such as microcephaly[106]. Cell signalling pathways, especially the PI3K-Akt-mTOR pathway, are critical for neurogenesis, migration and neuronal maturation. Activating mutations in this pathway can lead to brain overdevelopment syndrome, while inhibition of this pathway results in microcephaly. Akt is a key molecule in the PI3K pathway, essential for brain development and synaptic plasticity. Mutations in Akt can cause microcephaly or megalencephaly [107]. The PI3K-Akt-mTOR pathway can be hijacked by human pathogens for replication, but a causal relationship between ZIKV infection and inhibition of this pathway has not been established to date [107,108]. However, despite the common outcomes of ZIKV infection and inhibition of the PI3K-Akt-mTOR pathway, no causal association between the two has been reported yet.

To conclude this data analysis, we will highlight the localization of both proteins and metabolites. Considering that the vesicle's location can shed light on its classification and transport function, we will perform this identification to distinguish between structural and non-structural proteins (Figure 6). While both the activation of SRA by metabolites and the activation and regulation of the complement by proteins contribute to higher concentrations of biomarkers, an additional factor appears to decrease selected biomarkers in the process of ZIKV infection by vesicles [109–111] (Melo et al., 2017).

After analyzing their location, we observed the presence of two non-structural proteins, CSAD and KRT35, which inhibit the AKT-mTOR signaling pathway [112–114]. This inhibition leads to the accumulation of intermediate metabolites and precursors involved in the PI3K-AKT-mTOR pathway signaling. As the virus inhibits the SRA signaling, other metabolites and proteins persist due to viral infection, maintaining positive modulation over PI3K [115–117]. Therefore, ZIKV infection induced alterations in different signaling pathways, resulting in the overexpression of certain metabolites. Our findings suggest that it is possible to study the mechanism of viral infection by directly analyzing serum EV from infected children.

5. Conclusions

Akt-mTOR signaling is essential for neurogenesis by human NSCs as well as the induction of autophagy. Specifically, phosphorylation of Akt on mTOR is required for its full kinase activity, and subsequently, Akt-mediated phosphorylation of mTOR on PI3K is essential for keeping autophagy in check. Our results show that ZIKV replication led to suppression of Akt phosphorylation, which subsequently led to reduced mTOR phosphorylation. Further screening of key proteins and metabolites in EV in individual ZIKV viruses revealed that PI3K/AKT/mTOR signaling pathway expression detectably reduced Akt phosphorylation under normal conditions. All possible biomarkers were carefully chosen and validated through rigorous statistical analysis, aligning with prior research that focused on proteins and metabolites implicated in the infectious process. Nevertheless, it is imperative to conduct further investigations involving a larger cohort of children, employing advanced sample extraction technologies and specific markers. This will enable a comprehensive understanding of the overexpression of specific metabolites. For instance, the introduction of quantitative NMR techniques has enhanced precision in quantifying minor components within a mixture. Thus, the proteomic and metabolomic knowledge regarding the EVs in the same samples, viewed uniquely in the context of human ZIKV infection, significantly contributes to our understanding of the disease's physiopathological aspects. It sheds light on

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molecular targets of the cellular immune response to infection and viral replication. This not only forms the basis for new developments in pharmacology for diverse therapies but also provides insights into the management of ZIKV infection.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. The datasets generated by this study will be available upon reasonable request to the authors.

Author Contributions: Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used Conceptualization, L.G.P. and A.C.N; methodology, L.A.B. and P.V.S.P; software, L.N.G and R.C.S; validation, R.C.S, S.M.S.N, L.N.G, and L.G.P; formal analysis, L.A.B. and R.C.S; investigation, S.M.S.N and S.D.P; resources, L.A.B and L.G.P ; data curation, R.C.S, S.M.S.N, L.N.G, A.C.N and L.G.P ; writing—original draft preparation, L.G.P and A.C.N; writing—review and editing, L.G.P., S.D.P, P.V.S.P, L.N.G, A.C.N and SDP; visualization, L.N.G, P.V.S.P, R.C.S, L.A.B. and L.G.P; supervision, L.G.P, A.C.N and SDP; project administration, A.C.N and SDP; funding acquisition, A.C.N. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Universidade de São Paulo (protocol code CAAE: 86696618.7.0000.5467 and date of approval 02/14/2019).

Data Availability Statement: The datasets generated by this study will be available upon reasonable request to the authors.

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