Newborns with Zika virus-associated microcephaly exhibit marked systemic inflammatory imbalance

Caian L. Vinhaes[1,2,3,*], María B. Arriaga[1,2,4*], Breno L. de Almeida[1], João V. Oliveira[1], Cleiton S. Santos[1], Juan I. Calcagno[5] , Tereza X. Carvalho[5], Marta Giovanetti[6,7], Luiz Carlos J. Alcantara[6,7], Isadora C. de Siqueira[1#], Bruno B. Andrade[1,2,3,4,8,9,10#],

[1]Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador 40296-710, Brazil

[2]Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) Initiative, Salvador 40210-320, Brazil

[3]Curso de Medicina, Faculdade de Tecnologia e Ciências (UniFTC), Salvador 40290-150, Brazil

[4]Faculdade de Medicina, Universidade Federal da Bahia, Salvador 40110-100, Brazil

[5]Maternidade de Referência Prof. José Maria de Magalhães Netto-Secretaria de Saúde do Estado da Bahia (SESAB), Salvador 40320-350, Brazil

[6]Laboratório de Flavivírus, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro 21040-900, Brazil

[7]Laboratório de Genética Celular e Molecular, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte 31270-901, Brazil

[8]Universidade Salvador (UNIFACS), Laureate Universities, Salvador 41720-200, Brazil

[9]Wellcome Centre for Infectious Disease Research in Africa, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town 7925, South Africa

[10]Escola Bahiana de Medicina e Saúde Pública (EBMSP), Salvador 40290-000, Brazil

*****C.L.V. and M.B.A. equally contributed to the work

[#]B.B.A. and I.C.S. equally contributed to the work

Correspondence: B. B. Andrade, MD, PhD, Laboratório de Inflamação e Biomarcadores, Instituto Gonçalo Moniz, Fiocruz Bahia, Salvador 40296-710, Bahia, Brazil; bruno.andrade@fiocruz.br

summary of the article's main point: The recent Brazilian epidemic of zika virus in Brazil and its associated microcephaly emerges as a critical public health challenge. Here, we performed immune activation profiling of newborns with zika-associated microcephaly and demonstrated an inflammatory imbalance that hallmarks this condition.

ABSTRACT

Background: Zika Virus (ZIKV) is an emergent flavivirus initially considered a benign and self-limited exanthematic illness. In 2015, a new epidemic emerged in northeastern of Brazil with increased incidence of a previously rare clinical outcome, microcephaly, in newborns from mothers who were infected during pregnancy. Little is known about the immunopathogenesis of ZIKV-associated microcephaly. Understanding the inflammatory profile and degree of inflammation of persons affected with such condition is an important step towards development of innovative therapeutic strategies.

Methods: A case-control study compared plasma levels of several inflammatory biomarkers from newborns with ZIKV-microcephaly, asymptomatic ZKV infection or uninfected controls. Plasma biomarkers were assessed using Luminex. A series of multidimensional analysis was performed to characterize the systemic immune activation profile of the clinical groups.

Results: We identified an inflammatory signature associated with ZIKV-microcephaly that suggested an increased inflammation. Network analysis suggested that ZIKV-microcephaly is associated with imbalanced immune activation and inflammation. The cephalic perimeter was inversely proportional with the degree of inflammatory perturbation. Furthermore, a combination of plasma inflammatory biomarkers could discriminate ZIKV with microcephaly from those with ZIKV without microcephaly or uninfected neonates.

Conclusions: An intense inflammatory imbalance which is proportional to the disease severity hallmarks ZIKV-microcephaly.

Key words: Zika Virus, newborns, microcephaly, inflammation

INTRODUCTION

Zika Virus (ZIKV) is an emergent flavivirus that was first detected in Uganda in 1947 [1] and associated with outbreaks in Asia and the Pacific areas [2-4]. In May 2015, the first case of ZIKV was confirmed in northeastern Brazil [5, 6] and has rapidly spread throughout South, Central America and the Caribbean [7], affecting thousands of people in the Americas between 2015-2016 [7]. Brazil, with 440,000-1.3 million of affected people, has had the highest number of reported ZIKV cases worldwide [8].

Initially, human ZIKV infection was considered a benign and self-limited exanthematic illness[9]. However, at the end of 2015, an inexplicable and unexpected increase in number of cases of newborns with microcephaly was notified in northeastern Brazil which was found later to be associated with congenital Zika infection (CZI) [10, 11], culminating with declaration of state of public health emergency in the country.

Microcephaly is defined as a head circumference of at least two standard deviations below-average for gestational age and gender [12]. It is known that microcephaly is one of manifestations of CZI, which is characterized by a set of birth defects and deficiencies associated with viral neuropathogenesis [13] . The CZI clinical spectrum could be range of less severe neurologic abnormalities to neurodevelopment delays in normocephalic. Other documented findings include hearing and ocular abnormalities [14], arthrogryposis, dysphagia and brain sequels, such as calcifications and ventriculomegaly [13].

The role of immune response to ZIKV in pregnant women and their babies remains poorly understood. A recent analysis suggested that ZIKV-microcephaly is linked to increases in concentration of inflammatory markers [15]. Nevertheless, it is not yet known whether systemic immunological mechanisms are involved in the clinical outcome of intrauterine infection caused by ZIKV and the interaction between inflammation and microcephaly development. Herein, we aimed to investigate the degree of inflammatory imbalance linked to ZIKV-microcephaly by comparing the expression of several biomarkers of inflammation in peripheral blood of ZIKV newborns with or without microcephaly and well as uninfected controls. The findings elucidate immune pathways possibly associated with microcephaly.

MATERIALS AND METHODS

Ethics Statement

Written informed consent was obtained from legal guardians of all newborns, and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of the Gonçalo Muniz Institute, Oswaldo Cruz Foundation (protocol no. 1.935.854/2016).

Study design

Participants were recruited from a previous neonatal surveillance for CZI [16, 17] in 2016, in a public maternity hospital in Salvador, Northeast Brazil, that was strongly affected by the microcephaly outbreak. We enrolled a total of 50 participants: 14 ZIKV-exposed normocephalic newborns, 22 newborns with ZIKV-associated microcephaly and 14 healthy newborns from mothers not exposed to ZIKV.

Clinical and epidemiological data of newborns were obtained through interviews with mothers and review of medical records. Data storage and management was performed using the REDCap 6.18.1 (Vanderbilt University, Nashville, TN).

The newborns enrolled were classified according to the International Fetal and Newborn Growth Consortium for the 21st Century charts. Microcephaly was defined as a head circumference below two standard deviations from the mean for sex and gestational age and a normal head circumference would be one whose measurement was within two standard deviations from the mean [13]. Biological samples were obtained from umbilical cord blood collected at birth. EDTA plasma was obtained by centrifugation and stored at -80ºC. Serological and molecular diagnosis for ZIKV was performed according to previously described [16, 17]. CZI was defined as newborns whose serological testing (anti-ZIKV IgM) or a qualitative RT-PCR assay for ZIKV was positive. Health controls had negative serological and molecular results for ZIKV.

Immunoassays

We evaluated a panel of 26 soluble markers to examine inflammation and immune activation. The cytokines interleukin (IL)-1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL10, IL-12p40, IL-12p70, IL-15, IL-17A, interferon (IFN)-α2, IFN-γ, tissue necrosis factor (TNF)-α, granulocyte colony-stimulating factor (GCSF), granulocyte macrophage colony-stimulating factor (GMCSF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1/CCL2), interferon gamma induced protein/chemokine (C-X-C motif) ligand 10 (IP-10/CXCL10), macrophage inflammatory protein-1 alpha (MIP-1α/CCL3), MIP-1 beta (MIP-1β/CCL4) and eotaxin (CCL11) (Millipore, Boston, MA) were measured in duplicate samples using a Luminex-FLEXMAP3D 348-well plate reader (Austin, TX). The quality control of each analyte included: the standard concentrations needed to be within 80%-110% of their expected values; the accepted coefficient of variation between replicated samples was ≤10%. Values that were under the limit of detection were inputted as zero or .01 (to be log2-transformed in heatmaps). No values above the upper limit of detection were obtained.

Data analysis

Median values with interquartile ranges (IQR) were used as measures of central tendency and dispersion. The Kruskal–Wallis test with the Dunn's multiple-comparison were used to compare continuous variables and the Pearson's chi-square was used to compare variables showed as percentage. The Spearman rank test was used to identify associations between different cytokines or between the degree of molecular perturbation of each biomarker and the cephalic perimeter. In the network analysis based on Spearman correlations, nodes represent each given marker and lines represent statistically significant correlations (correlation coefficient [rho] >±.5 and P<.05).

Hierarchical cluster analyses (Ward's method) of log10-transformed and z-score normalized data were employed to depict the overall expression profile of indicated biomarkers in the study groups. Dendrograms represent Euclidean distance. Venn diagrams were used to illustrate differentially expressed markers. All comparisons were pre-specified and two-tailed. Differences with P-values <.05 after Holm-Bonferroni's adjustment for multiple comparisons were considered statistically significant.

The molecular degree of perturbation (MDP) was calculated to infer the level of inflammatory imbalance associated with ZIKV. This method has been used and detailed previously [18, 19].

Healthy controls were defined as the “reference” group, and the average level and standard deviation of this reference group were calculated for the plasma concentrations of each marker. The MDP score of an individual marker in a given sample “s” was defined by taking the difference in concentration level in sample “s” from the average of the marker in reference group divided by the corresponding standard deviation. The MDP score represents the number of standard deviations from the reference. The statistical analyses were performed using JMP 14.0 (SAS, Cary, NC) and Gephi (version 0.9.2).

RESULTS

A total of 50 participants were enrolled, 14 normocephalic newborns exposed to ZIKV, 22 newborns with ZIKV-microcephaly and 14 healthy newborns without ZIKV infection. Newborns with ZIKV-microcephaly were similar to those did not developed such clinical presentation and controls with regard to gestational age at delivery, weight and size at birth (Table 1). There was a high frequency of female individuals in the groups of newborns with ZIKV infection with microcephaly (63.3%) or asymptomatic (57.1%), whereas male individuals were predominant in the control group (71.4%) (Table 1).

Inflammatory imbalance in ZIKV-infected newborns

To further evaluate inflammatory profiles in patients with ZIKV, we examined the overall expression of several soluble proteins in cord blood of study participants stratified according to the presence of ZIKV and microcephaly (Table 1). Unsupervised hierarchical cluster analysis of biomarker values identified three main groups of individuals (Figure 1A). While the first cluster included all groups of study participants, with notable tendency of increased expression levels of IL-12p40, IL-12p70, IL-3, IL-4 and EGF, the second cluster showed only those that developed microcephaly and the third was basically composed by healthy controls. Of note, individuals that presented microcephaly showed a tendency of increase in levels of IFN-γ, IFN-α2, CXCL-10, IL-1α, TNF-α, CCL3 and CCL4 (Figure 1A). Furthermore, we employed a fold-difference analysis and identified that concentrations of only IFN-γ, IFN-α2 and CXCL10 were statistically significant between ZIKV and HC groups, whereas microcephalic patients exhibited several markers with significant differences, such as IFN-γ, IFN-α2, CXCL10, IL-1α, TNF-α, CCL3, CCL4, IL-15, IL-10, IL-6, IL-1RA and IL-8. Curiously, the same analysis when performed between patients with only ZIKV infection and those that developed microcephaly, we identified some similarities with the finding described for comparison between microcephaly vs. HC, with the addition of CCL2 (Figure 1A). Furthermore, we designed a Venn diagram to summarize the differences between the study groups. As expected, ZIKV infection led to increases in cytokine expression levels when compared to HC group independent on presence of microcephaly (Figure 1B). Of note, concentrations of 4 markers were substantially higher in both groups of ZIKV patients whereas 12 markers were elevated uniquely in those with ZIKV-microcephaly compared to controls (Figure 1B).

ZIKV-microcephaly leads to consistent alterations in correlations between plasma concentrations of inflammatory biomarkers

Inflammation is a coordinated process that occurs with synchronized changes in production of inflammatory molecules and cells. To understanding these intricate relationships, we have previously used a method based on network analysis employing Spearman correlation matrices to identify the dynamicity, degree and quality of inflammation in several diseases [18-21]. Here, we extended this approach to infer ZIKV pathophysiology and found that networks from the distinct clinical groups displayed differences in complexity and quality of statistical interactions between the inflammatory markers (Figure 2). The highest density of significant correlations was found in the group of ZIKV microcephaly whereas the lowest values were detected in those with ZIKV without this condition (Figure 2). Regardless of the clinical groups, most of the significant correlation were positive, meaning that increases in concentrations of a given marker were mostly followed by rises in levels of other inflammatory molecules. The few negative correlations found included TNF-α vs. IL-12p70 and IL-8 vs. CCL11 in healthy controls (Figure 2A) whereas IFN-γ vs. IL-1β and GCSF vs. CXCL10 were observed in ZIKV without microcephaly (Figure 2B). An increased number of negative correlations were found in the group of neonates with ZIKV microcephaly (Figure 2C).

The network analysis highlighted that in healthy controls, GMCSF was the most highly connected marker, followed by IL-17A, IL-1RA, IL-1β, IL-8, and IL-10 (Figure 2A). In ZIKV without microcephaly, IL-1β was the most relevant marker, with positive correlations with EGF, GMSCF, IFN-α2, and negative interactions with IFN-γ. Furthermore,IL-15 was also a significant node in the network of ZIKV without microcephaly, exhibiting positive interactions with CCL2, IL-2, IL-8, IL-12p70 and IL-17A (Figure 2B) a third significant node detected in this groups was IFN-α2, which displayed only positive correlations with IL-2, IL-4, IL-17A, IL-1β and IL-15. In patients with ZIKV microcephaly, our analysis indicated that IL-7, IL-15 and GMCSF were the most highly connected markers. Curiously, in this latter group, we found a number of negative interactions between pro inflammatory markers such as IL-1α vs EGF, IL-6 vs IL-12p70, TNF-α vs IL-3, and IFN-γ vs EGF (Figure 2C). Thus, these results argue that ZIKV microcephaly is associated with several alterations in cytokine correlations, including increases in number of negative interactions.

ZIKV infection is associated with increased systemic molecular degree of inflammatory perturbation

To deep the analyses of systemic inflammatory activity, we used a molecular degree of perturbation (MDP) (Figure 3A), a new statistical approach proposed recently by us to evaluate the degree and intensity of inflammatory disturbance in patients with a variety of infectious diseases [18, 19, 22]. As expected, individuals from the healthy control group exhibited diminished MDP values, whereas patients with ZIKV microcephaly exhibited substantial increase in MDP score values compared to controls and those with ZIKV without microcephaly (Figure 3B). In addition, MDP values of patients with ZIKV without microcephaly were also significantly higher than those from uninfected controls (Figure 3B).

Considering the microcephaly diagnosis is based on cephalic perimeter, we performed a correlation analysis to identify possible relationships between the cephalic perimeter measurement values and overall MDP expression as well as the perturbation values for each individual biomarker. This approach was designed to test if the severity of microcephaly was proportional to increases in inflammatory perturbation. Our findings revealed that the measures of cephalic perimeter were inversely correlated with molecular perturbation values of several of inflammatory parameters (Figure 4). Furthermore, when the clinical groups were tested individually, a dramatic decrease in statistically significant correlations were detected. Interestingly, the cephalic perimeter values exhibited different correlations with inflammatory markers depending on the clinical group. In healthy neonates, the only significant finding was a positive correlation between anti-viral cytokine IFN-α2 and cephalic perimeter (Figure 4). In those with ZIKV without microcephaly, another single significant correlation was found, but now with EGF. Noteworthy, in ZIKV-microcephaly, the cephalic perimeter was inversely correlated with both IFN-α2 and IL17A. Our findings suggest that the changes in cephalic perimeter are associated with specific and gradual modification in the degree of inflammatory perturbation in ZIKV infected neonates.

Plasma concentrations of inflammatory biomarkers can distinguish ZIKV infection with or without microcephaly from uninfected controls

We finally tested whether simultaneous measurements of the plasma biomarkers of inflammation could be used to distinguish the clinical groups. Given that we observed a distinct correlation profile between the concentrations of the biomarkers (Figure 2), we employed a discriminant model using canonical correlation analysis, which takes into account: (i) number,

(ii) strength and (iii) parameters involved in correlations between the biomarker concentrations[19]. We found that ZIKV infection with or without microcephaly could be completely distinguished from healthy controls (area under the curve [AUC] of the receiver operator characteristics [ROC] curve = 1.0 [100% accuracy], P<0.0001) (Figure 5A). In addition, among neonates with ZIKV infection, those presenting with microcephaly could also be distinguished from the infected individuals who did not have such condition (AUC=1.0, P<0.0001) (Figure 5A). Moreover, we plotted the canonical coefficient scored of each biomarker included in the model to assess the top markers responsible for the discrimination between groups. In the comparison between ZIKV without microcephaly and uninfected controls, HC we identified IFN-α2, CXCL10, IL-1RA, IL-2, IL-1β, IL-17 and CCL2 as the top markers in our model (Figure 5B). When the groups of neonates with ZIKV microcephaly and controls, IL-6, CXCL10, IFN-γ, IL-4 and IL-10 contributed the most for the discrimination (Figure 5B). Furthermore, when ZIKV groups were compared directly, CXCL10, IL-1α, VEGF and IL-8 were identified as the top canonical markers. These findings suggest that the infection with ZIKV and occurrence of microcephaly lead to unique disturbances in systemic inflammation and immune activation profile that hallmark this condition.

DISCUSSION

In the present study, we performed a detailed investigation of the immunological profile in blood of newborns with CZI. Our exploratory results delineated an inflammatory biosignature that could distinguish ZIKV neonates from uninfected controls and also hallmarked infected patients who presented with microcephaly. Such signature included biomarkers derived from both innate and adaptive immune responses and the findings presented here advanced the field by describing intricate relationships between these cytokines and growth factors. Recent studies have described a cytokine storm in *Flavivirus* infection model, with the profile of the immune activation imbalance being associated with the phase of the viral infection [23, 24]. Hence, in acute phase of infection, a higher expression of several biomarkers, as IL-1β, IL-2, IL-4, IL-6 and IL-10, and also CCL3 and VEGF was found [23]. In our investigation, newborns with ZIKV but without microcephaly exhibited specific increases of concentrations of IFN-γ, IFN-α2 and CXCL10, which are biomarkers from the IFN pathway and infer antiviral immunity [25-27]. The involvement of IFN pathways in ZIKV infection has been studied in both human [25] and experimental murine models [28]. Thus, ZIKV infection may cause substantial activation of IFN-related genes that leads to substantial increases in circulating levels of the cytokines described here. Of note, markers derived from the arachidonic acid were reported to hallmark ZIKV-microcephaly [15]. The interplay between IFN pathways and lipid mediators is well described in other infectious diseases such as tuberculosis, in which the balance between such players dictates disease outcomes [29]. Curiously, when neonates with ZIKV-microcephaly were tested, we found that, in addition to high levels of the IFN-related cytokines described above, unique increases in several markers, including CCL3, IL-6 and IL-10, which have been previously described to be induced by ZIKV infected placental macrophages [26]. Thus, we propose that ZIKV infection resulting in microcephaly may be caused by disturbances in IFN pathway linked other myeloid activation signals that results in this clinical outcome. Further studies are warranted to specifically address this hypothesis.

The inflammatory pathways controlling the development of microcephaly remains unclear, and the magnitude of the influence of immune activation in such process is not yet known. Several studies have been tried to explain the pathophysiology of this outcome in ZIKV patients and reported that the presence of infection leads to an altered regulation of genes associated with immune response, cell cycle, differentiation and apoptosis in neural progenitor cells (NPCs) [30, 31]. These modifications are then associated with reduced cell growth which culminates in neurological malformations such as microcephaly [31]. Despite this elucidation, the interplay between cytokines, chemokines and microcephaly development remains unclear.

Understanding the specific immune mechanisms linked to microcephaly may aid development of potential interventions that could either prevent of minimize the effects of ZIKV infection and microcephaly. Our results reveled that not only the concentration values of inflammatory markers are altered in ZIKV microcephaly, but also the relationships between them, which was read by our network analyses.

Using correlation matrices, we demonstrated that the cephalic perimeter values displayed unique statistical relationships with plasma biomarkers depending on the ZIKV infection status and occurrence of microcephaly. Our network analysis showed that the presence of ZIKV infection leads important differences in connectivity profile, involving the number of correlations, direction of the association as well as the main markers representing the most significant nodes. Indeed, the group of ZIKV microcephaly displayed a network with increased density of connections and changes in strength and directionality of the relationships, including appearance of several negative correlations. Similar analyses performed by our group in a number of clinical scenarios, such as tuberculosis [18, 19], HIV [32], malaria [33], and leishmaniasis [34], suggest that changes in the correlation network profile indicates alteration of the systemic inflammatory status. In this setting, increased connectivity usually infers augmented inflammation whereas decreased number of significant correlations compared to baseline control group may indicate uncoupling of the inflammatory response. We hypothesize that ZIKV infection itself leads to an immune homeostatic disturbance associated with uncoupling of the systemic inflammation whereas development of microcephaly may be due to a probable secondary factor that then intensifies the coordination of the inflammatory processes which is demonstrated by increased number of significant connections.

Another important contribution of our study was the assessment of the molecular degree of inflammatory perturbation and its association with the cephalic perimeter of neonates infected with ZIKV. To our knowledge, no previous study has estimated the global inflammatory disturbance in blood of ZIKV neonates. We demonstrated that ZIKV infection is indeed linked to overall higher inflammatory perturbance, which was shown to be even more significant in the presence of microcephaly. We extended these observations to show that, in the entire study population, decreases in the cephalic perimeter measures was directly associated with increases in perturbance of several inflammatory biomarkers. In addition, microcephaly led to unique inverse relationships between cephalic perimeter values and degree of perturbance of key inflammatory cytokines such as IFN-α2 and IL-17A. IFN-α2 is known to be critical to promote antiviral immune responses [25] whereas IL-17A has also been described to play

significant role in pathogenesis of several lung-associated viral infection [35]. Whether manipulation of these two important cytokines in experimental models results in alleviation of the pathological immune activation observed in ZIKV microcephaly is a potential topic of interest for further investigations.

Of note, the discriminant analyses based on a canonical model described here identified candidate biomarkers likely responsible for the distinction between uninfected neonates and ZIKV infection with or without microcephaly. Hence, this approach indicated that, compared to uninfected neonates, in the group of ZIKV without microcephaly, IFN-α2, CXCL10 and IL-1β were the most significant markers with discriminatory power, whereas in the presence of microcephaly, IL-6, CXCL10 and IL-4 were the top parameters implicated in the discrimination. This finding suggests that the immune activation profile detected in ZIKV infection was so substantially different that could distinguish infection and microcephaly with high degree of accuracy. It is possible that the top markers identified play significant role in the pathogenesis of the clinical outcomes described here. It is reasonable to hypothesize that upon exposure to ZIKV during pregnancy, the degree of inflammatory disturbance may directly cause immune-mediated organic dysfunction that promotes development of microcephaly. Such hyper-inflammation could alter permeability of the blood-brain barrier [36]. In the central nervous system, ZIKV infection could then drive tissue damage mediated by cytotoxicity [15, 37] leading to microcephaly. This hypothesis requires direct testing in future mechanistic studies.

Our study has some limitations. We performed a cross-sectional investigation and examined samples obtained from a single timepoint, which preclude us from making conclusions about the dynamicity of inflammatory process before birth. The number of individuals investigated in this study was relatively small, but the groups were carefully matched to reduce the influence of potential confounding factors. Regardless of such limitations, our finds extend the current information about ZIKV infection and immunological chances in human model, with delineation of immune profile and inflammatory changes and its association with microcephaly.

NOTES

Acknowledgments: The authors thank the study participants and their legal guardians.

Disclaimer: The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Financial support: The study was supported by the intramural research program from Fundação Oswaldo Cruz, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; award number: 443875/2018-9 and 425380/2016) and Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB; award number: PPSUS/BA-FAPESB003/2017/SESAB/CNPq/MS 5125/2017). LCJA, MG and BLA were supported by CNPq/Ministério da Ciência, Tecnologia e Inovação (MCTI), Departamento de Ciência e Tecnologia (DECIT) / Secretaria de Ciência, Tecnologia e Insumos Estratégicos (SCTIE) / Ministério da Saúde (440685/2016-8) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (88887.130716/2016-00). LCJA and ICS were supported by the European Union's Horizon 2020 Programme through ZIKAlliance (grant no. PRES-005- FEX-17-4-2-33). The work from BBA was supported by the National Institutes of Health (U01AI115940) and by CNPq (senior fellowship). MBA received a fellowship from the FAPESB. CLV is a scientific initiation fellow from CNPq.

Potential conflicts of interest: All authors: No reported conflicts of interest.

REFERENCES

1. Dick GW, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. Trans R Soc Trop Med Hyg **1952**; 46(5): 509-20.
2. Duffy MR, Chen TH, Hancock WT, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med **2009**; 360(24): 2536-43.
3. Cao-Lormeau VM, Roche C, Teissier A, et al. Zika virus, French polynesia, South pacific, 2013. Emerg Infect Dis **2014**; 20(6): 1085-6.
4. Roth A, Mercier A, Lepers C, et al. Concurrent outbreaks of dengue, chikungunya and Zika virus infections - an unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012-2014. Euro Surveill **2014**; 19(41).
5. Campos GS, Bandeira AC, Sardi SI. Zika Virus Outbreak, Bahia, Brazil. Emerg Infect Dis **2015**; 21(10): 1885-6.
6. Zanluca C, Melo VC, Mosimann AL, Santos GI, Santos CN, Luz K. First report of autochthonous transmission of Zika virus in Brazil. Mem Inst Oswaldo Cruz **2015**; 110(4): 569-72.
7. Pan American Health Organization WHO. Epidemiological Update: Neurological syndrome, congenital anomalies and Zika virus infection.: 17 January, **2016**.
8. Saúde MdSSdVe. Protocolo de Vigilância e resposta à ocorrência de microcefalia relacionada à infecção pelo vírus Zika. **2015**.
9. Heang V, Yasuda CY, Sovann L, et al. Zika virus infection, Cambodia, 2010. Emerg Infect Dis **2012**; 18(2): 349-51.
10. Mlakar J, Korva M, Tul N, et al. Zika Virus Associated with Microcephaly. N Engl J Med **2016**; 374(10): 951-8.
11. de Araujo TVB, Rodrigues LC, de Alencar Ximenes RA, et al. Association between Zika virus infection and microcephaly in Brazil, January to May, 2016: preliminary report of a case-control study. Lancet Infect Dis **2016**; 16(12): 1356-63.
12. World Health Organization (WHO) NCoBDaDDftUSCfDCaPC, International Clearinghouse for Birth Defects Surveillance and Research (ICBDSR). Birth defects surveillance: a manual for programme managers. **2014**.
13. Moore CA, Staples JE, Dobyns WB, et al. Characterizing the Pattern of Anomalies in Congenital Zika Syndrome for Pediatric Clinicians. JAMA Pediatr **2017**; 171(3): 288-95.
14. van der Linden V, Pessoa A, Dobyns W, et al. Description of 13 Infants Born During October 2015-January 2016 With Congenital Zika Virus Infection Without Microcephaly at Birth - Brazil. MMWR Morb Mortal Wkly Rep **2016**; 65(47): 1343-8.
15. de Oliveira DN, Lima EO, Melo C, et al. Inflammation markers in the saliva of infants born from Zika-infected mothers: exploring potential mechanisms of microcephaly during fetal development. Sci Rep **2019**; 9(1): 13606.
16. Faical AV, de Oliveira JC, Oliveira JVV, et al. Neurodevelopmental delay in normocephalic children with in utero exposure to Zika virus. BMJ Paediatr Open **2019**; 3(1): e000486.
17. Duarte AO, Oliveira JV, Carvalho TCX, et al. Maternal and congenital infections arising from Zika, dengue and Chikungunya arboviruses in Salvador, Brazil. Trans R Soc Trop Med Hyg **2020**; 114(3): 222-5.
18. Oliveira-de-Souza D, Vinhaes CL, Arriaga MB, et al. Molecular degree of perturbation of plasma inflammatory markers associated with tuberculosis reveals distinct disease profiles between Indian and Chinese populations. Sci Rep **2019**; 9(1): 8002.
19. Vinhaes CL, Oliveira-de-Souza D, Silveira-Mattos PS, et al. Changes in inflammatory protein and lipid mediator profiles persist after antitubercular treatment of pulmonary and extrapulmonary tuberculosis: A prospective cohort study. Cytokine **2019**; 123: 154759.

20. Fernandes CD, Arriaga MB, Costa MCM, et al. Host Inflammatory Biomarkers of Disease Severity in Pediatric Community-Acquired Pneumonia: A Systematic Review and Meta-analysis. Open Forum Infect Dis **2019**; 6(12): ofz520.
21. Cruz LAB, Moraes MOA, Queiroga-Barros MR, Fukutani KF, Barral-Netto M, Andrade BB. Chronic hepatitis B virus infection drives changes in systemic immune activation profile in patients coinfected with Plasmodium vivax malaria. PLoS Negl Trop Dis **2019**; 13(6): e0007535.
22. Prada-Medina CA, Fukutani KF, Pavan Kumar N, et al. Systems Immunology of Diabetes-Tuberculosis Comorbidity Reveals Signatures of Disease Complications. Sci Rep **2017**; 7(1): 1999.
23. Tappe D, Perez-Giron JV, Zammarchi L, et al. Cytokine kinetics of Zika virus-infected patients from acute to reconvalescent phase. Med Microbiol Immunol **2016**; 205(3): 269-73.
24. Routhu NK, Byrareddy SN. Host-Virus Interaction of ZIKA Virus in Modulating Disease Pathogenesis. J Neuroimmune Pharmacol **2017**; 12(2): 219-32.
25. Bayer A, Lennemann NJ, Ouyang Y, et al. Type III Interferons Produced by Human Placental Trophoblasts Confer Protection against Zika Virus Infection. Cell Host Microbe **2016**; 19(5): 705-12.
26. Quicke KM, Bowen JR, Johnson EL, et al. Zika Virus Infects Human Placental Macrophages. Cell Host Microbe **2016**; 20(1): 83-90.
27. Pierson TC, Graham BS. Zika Virus: Immunity and Vaccine Development. Cell **2016**; 167(3): 625-31.
28. Yockey LJ, Jurado KA, Arora N, et al. Type I interferons instigate fetal demise after Zika virus infection. Sci Immunol **2018**; 3(19).
29. Mayer-Barber KD, Andrade BB, Oland SD, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. Nature **2014**; 511(7507): 99-103.
30. Faizan MI, Abdullah M, Ali S, Naqvi IH, Ahmed A, Parveen S. Zika Virus-Induced Microcephaly and Its Possible Molecular Mechanism. Intervirology **2016**; 59(3): 152-8.
31. Tang H, Hammack C, Ogden SC, et al. Zika Virus Infects Human Cortical Neural Progenitors and Attenuates Their Growth. Cell Stem Cell **2016**; 18(5): 587-90.
32. Manion M, Andrade BB, DerSimonian R, et al. Country of residence is associated with distinct inflammatory biomarker signatures in HIV-infected patients. J Virus Erad **2017**; 3(1): 24-33.
33. Mendonca VR, Queiroz AT, Lopes FM, Andrade BB, Barral-Netto M. Networking the host immune response in Plasmodium vivax malaria. Malar J **2013**; 12: 69.
34. Araujo-Santos T, Andrade BB, Gil-Santana L, et al. Anti-parasite therapy drives changes in human visceral leishmaniasis-associated inflammatory balance. Sci Rep **2017**; 7(1): 4334.
35. Mebratu YA, Tesfaigzi Y. IL-17 Plays a Role in Respiratory Syncytial Virus-induced Lung Inflammation and Emphysema in Elastase and LPS-injured Mice. Am J Respir Cell Mol Biol **2018**; 58(6): 717-26.
36. Neal JW. Flaviviruses are neurotropic, but how do they invade the CNS? J Infect **2014**; 69(3): 203-15.
37. Wen Z, Song H, Ming GL. How does Zika virus cause microcephaly? Genes Dev **2017**; 31(9): 849-61.

Figure 1. Inflammatory imbalance in Zika virus infection. Concentrations of several markers of inflammation were assessed in plasma samples from a cohort of 50 children. Data were log10-transformed. **(A)** Hierarchical cluster analysis using z-scored values of each parameter (Ward's method) was employed to depict the overall biomarker expression profile in the study. Fold differences were calculated and statistically significant differences are highlighted in colored bars. **(B)** Venn Diagram describes the markers which values were statistically different between ZIKV vs ZIKV+MC. HC=Healthy control, ZIKV=Zika virus, MC=Microcephaly.

Figure 2. Zika virus microcephaly leads to consistent alterations in correlations between plasma concentrations of inflammatory biomarkers. Network analysis of the biomarker correlation matrices was performed with bootstrap (100x). Significant correlations ($p < 0.05$) and Spearman rank (rho)> 0.3 are shown. Each node represents a different parameter. The size of each circle (node) is proportional to the number of significant correlations involving such node. Connecting lines represent the Spearman rank coefficient (rho) values. Red color infers positive correlation whereas blue color denotes negative correlations. Color maps on the right of each network denote the number of significant correlations per parameter (node) per clinical group. HC=Healthy control, ZIKV=Zika virus, MC=Microcephaly.

Figure 3. ZIKV infection is associated with increased systemic molecular degree of inflammatory perturbation. (A) Histograms show the single sample molecular degree of perturbation (MDP) score values relative to each study group as indicated in Y axis. **(B)** Box plots represent the distribution of the MDP between study groups. Values were compared between control, Zika virus and zika virus microcephaly groups using the Kruskal-Wallis test. HC=Healthy control, ZIKV=Zika virus, MC=Microcephaly.

Figure 4. Associations between cephalic perimeter and plasma markers and MDP value in study groups. Spearman correlation analysis was used to test association between cephalic perimeter and plasma markers and overall inflammatory profile assessed by the molecular degree of perturbation (MDP) in all study participants (left panel), healthy control (HC) group, patients only with Zika infection (ZIKV) and patients that present Zika virus and microcephalia (ZIKV+MC). Bars represent the Spearman rank (rho) values. Colored bars indicate statistically significant correlations ($p < 0.05$) after adjustment for multiple measurements. Highlighting color infers significant correlations.

Figure 5. Plasma concentrations of inflammatory biomarkers can distinguish ZIKV infection with or without microcephaly from uninfected controls. (A) In an exploratory approach, a sparse canonical correlation analysis (sCCA) was employed to test whether experimental groups could be distinguished based on the overall expression profile of all the markers measured. **(B)** Canonical coefficient scores were calculated to identify the biomarkers responsible for the difference between groups in the sCCA model. HC=Healthy control, ZIKV=Zika virus, MC=Microcephaly

46 **Table 1. Characteristics of study participants.**

Characteristics /cytokines	All (N=50)	Healthy Control (n=14)	ZIKV with microcephaly (n=22)	ZIKV without microcephaly (n=14)	p-value
Female – no. (%)	26 (52)	4 (28.6)	14 (63.6)	8 (57,1)	0.54
Gestational age (week)	39 (38-40)	38 (37-39)	39 (38-40)	39 (38-40)	0.32
Prematurity birth – no. (%)	10 (20)	4 (28.6)	4 (18.2)	2 (14.3)	0.87
Head circumference (cm)	32 (31-34)	32.5 (32-34.5)	31 (30-31)	34 (33-35.5)	**<0.01**
Weight at birth (g)	2850 (2328-4130)	2813 (2393-2930)	2705 (2209-2929)	2973 (2755-3540)	0.08
Size at birth (cm)	46.0 (45.0-47.5)	50.0 (45.0-47.6)	46.0 (42.0-48.2)	47.0 (46.0-49.5)	0.29
CCL2	497.85 (304.57-1214.09)	475.81 (325.04-533.63)	1050.17 (377.49-1494.95)	301.68 (179.63-934.69)	**0.01**
CCL3	9.26 (2.82-22.45)	3.97 (0-10.77)	20.18 (7.38-80)	7.85 (3.2-13.62)	**0.01**
CCL4	28.42 (20.12-39.6)	21.86 (19.08-28.22)	42.06 (26.15-59.2)	28.22 (20.63-30.19)	**0.01**
CCL11	95.45 (66.29-114.58)	95.45 (46.07-122.04)	101.6 (71.12-117.63)	78.69 (66.29-101.76)	0.22
CXCL10	239.25 (161.91-410.13)	131.34 (80.47-152.45)	517.71 (378.55-631.44)	202.55 (185.87-235.08)	**<0.01**
EGF	69.96 (31.81-133.89)	83.98 (41.92-115.13)	54.84 (29.1-109.63)	112.73 (31.81-196.72)	0.27
GCSF	188.28 (105.22-313.06)	161.42 (100.33-243.39)	289.62 (141.32-368.29)	163.76 (97.88-227.44)	0.10
GMCSF	29.47 (20.73-39.9)	24.64 (10.64-37.08)	34.25 (20.73-49.2)	27.07 (22.69-38.02)	0.35
IFN-α2	90.37 (19.36-127.41)	11.95 (9.91-13.12)	101.98 (82.35-141.14)	114.32 (95.6-136.62)	**<0.01**
IFN-γ	15.57 (8-31.27)	3.88 (2.65-7.04)	31.63 (25.05-51.13)	11.38 (8.93-15.37)	**<0.01**
IL-1RA	29.84 (10.26-65.32)	9.35 (3.95-23.25)	46.51 (23.25-79.91)	33.64 (12.1-47.95)	**0.01**
IL-1α	327.86 (223.75-524.41)	192.07 (126.62-206.74)	519.18 (397.8-605.29)	303.7 (245.63-321.5)	**<0.01**
IL-1β	1.63 (0.47-2.8)	1.63 (0.73-2.58)	1.58 (0.47-2.58)	1.8 (0.18-3.33)	0.95
IL-2	2.57 (1.88-4.18)	2.4 (0.97-2.9)	2.81 (1.88-4.18)	3.3 (2.23-4.49)	0.20

IL-3	0.71 (0.16-1.51)	0.93 (0.64-1.79)	0.56 (0-1.65)	0.86 (0.16-1.37)	0.52
IL-4	9.47 (5.39-21.1)	11.45 (5.39-21.1)	9.47 (3.28-21.1)	11.45 (5.39-24.87)	0.91
IL-6	9.4 (3.79-22.42)	4.92 (0-12.42)	20.92 (7.01-33.49)	3.94 (2.05-14.96)	**<0.01**
IL-7	10.86 (7.52-13.47)	10.04 (7.52-11.51)	9.88 (5.41-14.84)	10.99 (8.15-12.99)	0.65
IL-8	12.9 (6.8-23.23)	6.25 (3.42-13.01)	16.82 (10.99-27.85)	14.5 (8.26-23.23)	0.01
IL-10	16.2 (9.22-30.01)	9.22 (2.67-11.68)	28.91 (16.82-55.95)	12.85 (9.58-19.24)	**<0.01**
IL-12p40	6.5 (0-17.34)	0 (0-12.79)	7.68 (0-38.48)	6.5 (0-13.73)	0.16
IL-12p70	1.38 (0-7.26)	0 (0-4.8)	0 (0-6.08)	2.36 (0-8.37)	0.26
IL-15	8.38 (5.25-13.77)	5.64 (4-6.4)	12.28 (7.32-20.31)	9.16 (4.84-12.04)	**0.01**
IL-17A	3.69 (0.67-7.42)	2.77 (0-4.49)	4.3 (0-7.71)	4.06 (1.63-9.62)	0.31
TNF-α	28.47 (7.38-72.38)	5.71 (4.66-31.7)	66.93 (21.83-130.72)	23.26 (7.88-56.03)	**<0.01**
VEGF	154.36 (100.09-227.18)	118.2 (89.16-185.53)	176.75 (110.62-252.75)	147.34 (89.16-214.98)	0.12

Data represent in median and interquartile range or frequency (percentage). Plasma markers concentrations are in pg/mL. Data were compared between the clinical groups using the Kruskal-Wallis test (continuous variables) or the Pearson's qui-square test (for data on frequency).

Abbreviations: CCL: Chemokine (C-C Motif) Ligand; CXCL: Chemokine (C-X-C Motif) Ligand; EGF: Epidermal Growth Factor; GCSF: Granulocyte Colony Stimulating Factor; GMCSF: Granulocyte-Macrophage Colony Stimulating; IFN: Interferon; IL: Interleukin; TNF: Tumor Necrosis Factor; VEGF: Vascular Endothelial Growth Factor.

Figure 1

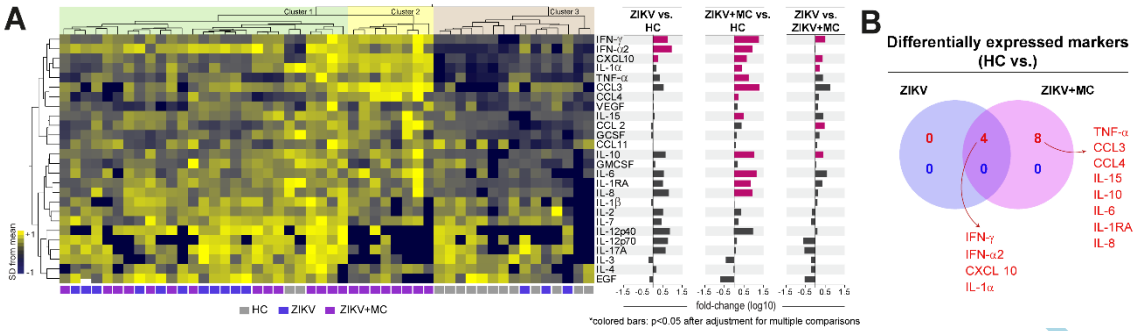

Figure 2

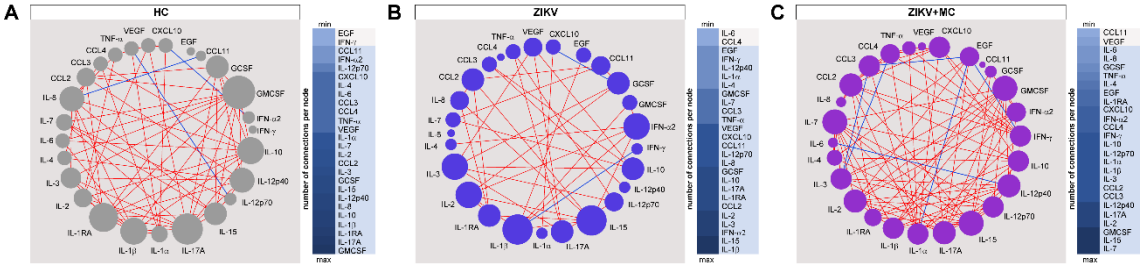

Figure 3

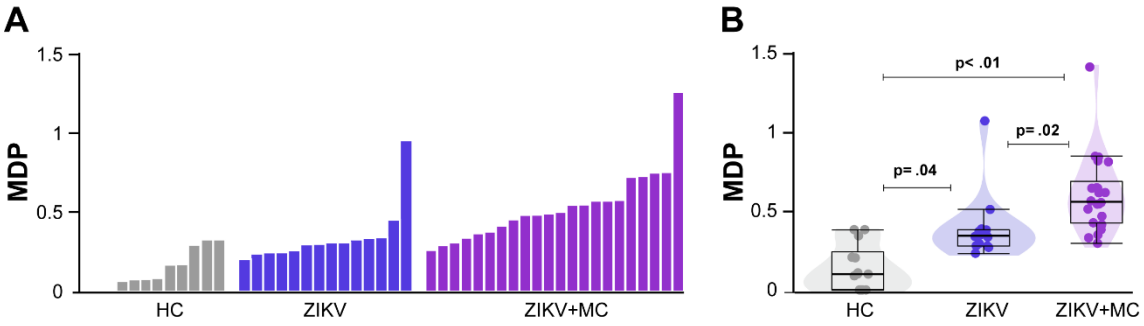

Figure 4

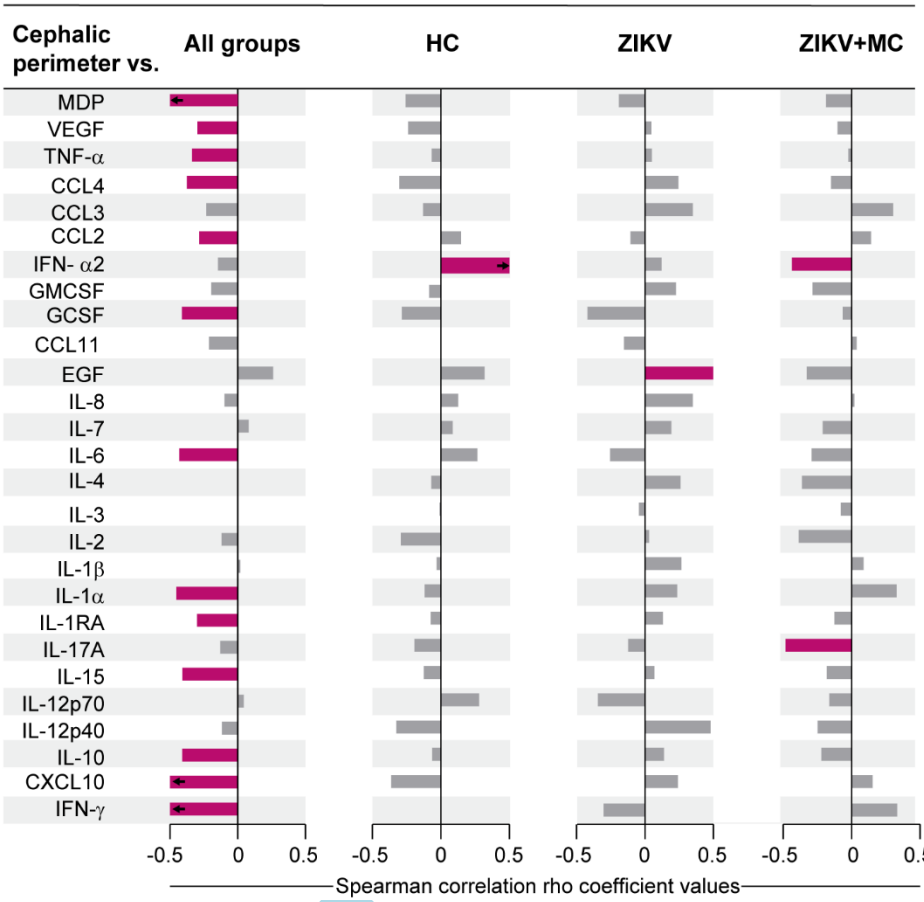

Figure 5